# Frost tolerant eucalyptus clones : an overview of micropropagation tools and wood and pulp quality

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## Abstract

Eucalyptus development is drastically limited in Europe (except Portugal and Spain) due to cold sensitivity. To face this sensitivity, a large program of genetic improvement was thus undertaken by FCBA (formerly AFOCEL) in the seventies to select fast growing species and provenances adapted to French southern climatic conditions. Among 20 temperate species that have been tested, *E. viminalis*, *E. nitens*, *E. gunnii*, *E. dalrympleana* and *E.gundal* (*E. gunnii* x *E. dalrympleana*) exhibited the best performances, the latter hybrid species cumulating high vigour from *E. dalrympleana* and high frost tolerance from *E. gunnii*. The first part of this presentation is an overview of the recent progress carried out on the clonal propagation of selected genotypes with either high frost tolerance (up to –18°C, mainly *E. gunnii* clones) or moderate frost tolerance (up to –12°C) but improved growth rate (mainly *E. gundal* clones: +20% compared to *E. gunnii* clones). In particular, some aspects of micropropagation in combination with cryopreservation methods of active buds developed by the FCBA Biotechnology Laboratory in order to accelerate the production of high quality, juvenile mother plants suitable for industrial production of cuttings for 2 to 3 years are discussed. In the second part of the presentation, the wood, fibre and pulp quality of some selected clones of *E. gunnii* and *E. gundal* are presented and compared to the reference eucalypt in Southern Europe, *E. globulus*. Despite lower pulp yield, frozen-temperature clones showed better beatability, and higher breaking length and burst index, but lower bulk than *E. globulus*.

**Keywords:** Eucalyptus, frost tolerance, cold temperature, biotechnology, Eucalyptus gunnii, Eucalyptus gundal, Eucalyptus dalrympleana, Eucalyptus globules, micropropagation, cryopreservation, kraft cooking, pulp yield, refining, bulk, burst index, breaking length.

## Introduction

Eucalyptus is attractive for rapid production (short rotations) of considerable woody biomass together with low requirements for soil quality. It is the most planted angiosperm tree worldwide owing to wood properties that properly fit the pulp and paper industry requirements. Among 600 different eucalyptus species known around the world, 30 to 40 present a real economic importance. Among them, *E. globulus, E. camaldulensis, E. Grandis, E. saligna* and *E. urophylla* and their hybrids are surely the most important ones for pulp and paper production.

However, other lesser known eucalyptus species arouse the interest of pulp industry and are the subject of attention of many academic and industrial research groups worldwise. Thus, 13 eucalyptus species were compared by Clark et Hicks [2] : *E. badjensis, E. benthamii, E. globulus ssp. bicostata, E. kartzoffiana, E. macarthurii, E. nitens, E. smithii, E. viminalis, E. dunnii, E. cloeziana, Corymbia maculata, E. pilularis et E. occidentalis*.

Based on the results, the authors concluded that the most promising among these eucalyptus species for the pulp production were *Corymbia maculata, E. occidentalis* then *E. dunnii,* while the two less adapted species were *E. benthamii* and *E. kartzoffiana*.

In Europe, except for Portugal and Spain, eucalyptus development is drastically limited by cold sensitivity. A large program of genetic improvement was thus undertaken by AFOCEL (which became FCBA in 2007 after merging with CTBA) in France since 1972 to select fast growing species and provenances adapted to French southern climatic conditions.

Among 20 temperate species that have been tested, *E. viminalis*, *E. nitens*, *E. gunnii*, *E. dalrympleana* and *E.gundal* (*E. gunnii* x *E. dalrympleana*) exhibited the best performances, the latter hybrid species cumulating high vigour from *E. dalrympleana* and high frost tolerance from *E. gunnii*. Industrial application was however clearly hampered by intraspecific variability, especially within provenance.

After 2 severe winters (1985 and 1986) with serious frost damages on most ongoing experimental trials, the breeding strategy was reoriented towards clonal propagation via cuttings of selected genotypes with either high frost tolerance (up to –18°C, mainly *E. gunnii* clones) or moderate frost tolerance (up to –12°C) but improved growth rate (mainly *E. gundal* clones: +20% compared to *E. gunnii* clones). After field-testing of more than 600 clones, several *E. gunnii* and *E. gundal* elite clones have been proposed to the French paper industry for pre-development, one of them (*E. gunnii*, clone 870634 “cagire”) being registered in the official French species and variety catalogue (N°CPOV: 15092) and deployed in commercial plantations since 1996.

Plant regeneration from *E. gunnii* and *E. gundal* clones is still a limiting step because of clone recalcitrance or physiological ageing and maturation of mother plants resulting in non-optimal rooting ability and subsequent poor cuttings behaviour.

In this contribution we reported on the recent use of micropropagation in combination with cryopreservation methods of active buds developed by the FCBA Biotechnology lab in order to accelerate the production of high quality, juvenile mother plants suitable for industrial production of cuttings for 2 to 3 years. In the second part of the paper, the wood, fibre and pulp quality of some selected clones of *E. gunnii* and *E. gundal* are presented and compared to the reference eucalypt in Southern Europe, *E. globulus*.

# Experimental

## Micropropagation

The different steps of the micropropagation of frost-tolerant eucalyptus clones are summarized in Figure 1.

*In vitro* cultures freshly initiated or reactivated from cryopreserved meristems in alginate beads [3] (A) of 4 *E. gunnii* (870634, 890063, 890001, 890073) and 2 *E. gundal* elite clones (821366, 821290) were biweekly amplified (light room with a 16 h photoperiod, 25°C) using a modified Murashige and Skoog (MS) medium supplemented with growth regulators and sucrose, and gelled with Bacto-agar (Difco).

The Petri dishes containing 6 subculture units (B), each composed of a clump of 3 shoots were used. Root induction occurred for 7-10 days in the darkroom using a modified Knop medium, followed by a 4-weeks-expression step under light on the same medium without growth regulator.

Acclimatization of rooted microcuttings with minimal height of 2 cm was then performed in the greenhouse (16 h photoperiod) with 100% relative humidity for 10-15 days (C).

Final enumeration of plant was done 2 months after acclimatization (20 cm plantlets, D) at the time of delivery to our customer. *In vitro* plantlets were then managed as mother plants outdoor (E) to produce cuttings.

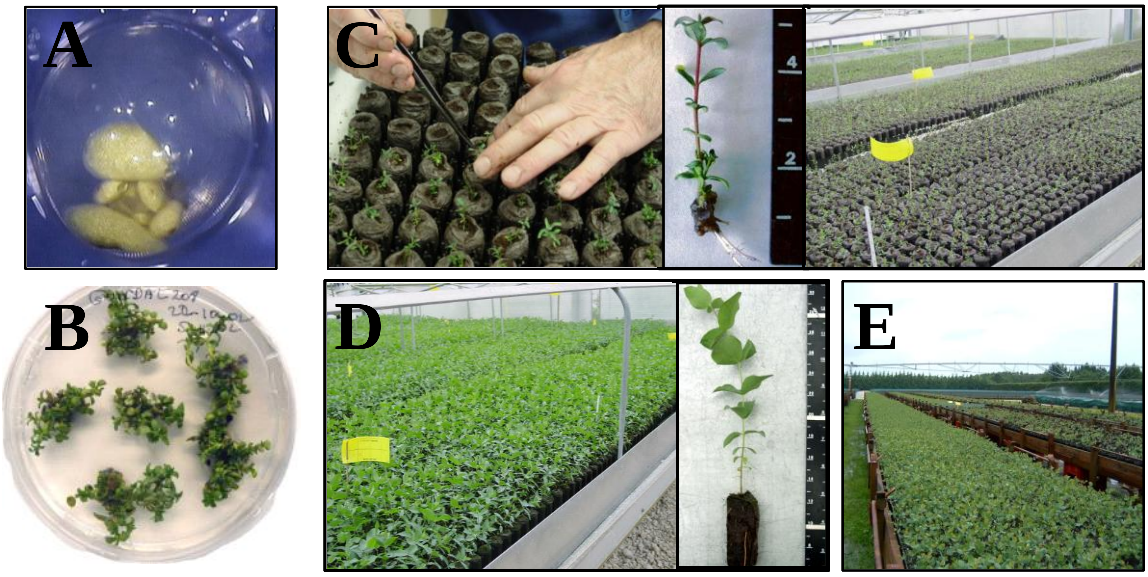


Figure 1 – Different steps of the micropropagation of frost tolerant clones of Eucalyptus at FCBA.

## Wood and pulp quality

The performances of two *E. gunnii* clones (870634 and 890063) and two *E. gundal* clones (821366 and 850645) were evaluated and compared to *E. globulus* of similar same age and soil quality originated from Spain. Ten 1-m logs per clone were 5 trees from 2 different sites (Longages in Haute Garonne, 9 years old at harvesting time; Moupas in Gers, 7 years old at harvesting time) were sampled, chipped and carefully mixed in order to produce a homogenous lot per clone. The different wood lots were cooked using the following conditions in an oil-heated, multi-digestor, rotatory system in triplicates:

- Wood mass : 200 g sec
- Wood/liquor ratio : 4
- Alcali (expressed as NaOH): 18, 20, 21 et 22
- Sulfidity: 32
- Cooking profile : room temperature to 170°C in 90 min plus 90 min of plateau at 170 °C

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ISO standards were used for the evaluation of pulp properties.

# Results and Discussion

## Production of high quality micropropagated mother plants

*E. gunnii* (870634, 890063, 890001, 890073) and *E. gundal* elite clones (821366, 821290) selected for industrial plantations in southern France (frost tolerance) were micropropagated in the frame of commercial mid-scale productions (up to 15 000 plants in one or several batches). Figure 2 presents the results obtained for the number of short produced in vitro, the number of rooted shoots acclimatized and the number of high quality mother plants delivered to the customer. Detailed numbers are given in Table 1.

The number of high quality mother plants delivered to our customers hit the target objective in the case of 3 highly responsive clones (870634, 821366, 890073) exhibiting 1.9-3.6 shoots per subculture unit, 64-77% acclimatization rate of rooted shoots as well as 59-73% survival rate after two months in the greenhouse. In contrast, 3 clones (890063, 890001, 821290) were more difficult to micropropagate owing to low acclimatization (25-43%) and related overall survival rate of rooted shoots (14-36%). The rooted cuttings production potential of micropropagated mother plants from E. gunnii clones estimated during their 2nd growth year (2 collections rounds, 2 months apart) is quite high (20- 67, maximum potential).

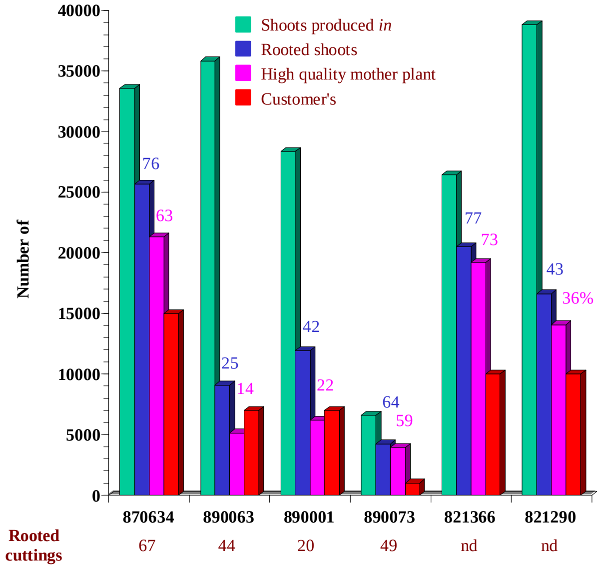


Figure 2 – Performance of 4 E. gunnii (870634, 890063, 890001, 890073) and 2 E. gundal frost tolerant elite clones (821366, 821290) selected for industrial plantations in southern France and micropropagated in the frame of commercial mid-scale productions (up to 15 000 plants).

Table 1 - High quality mother plant production using micropropagation for 4 E. gunnii (870634, 890063, 890001, 890073) and 2 E. gundal frost tolerant elite clones (821366, 821290).

| Clone (batch) | Shoots produced | Shoots per subculture unit (Petri dishes) | Rooted shoots acclimatized (%) | High quality mother plants delivered (%) | Objective (% of customer demand target) |
|---|---|---|---|---|---|
| 870634 (1)<br>870634 (2) | 21356<br>12235 | 2.4 (1483)<br>3.6 (566) | 15077 (70.6)<br>10590 (86.5) | 13429 (62.9)<br>7877 (64.4) | 10 000 (134)<br>5000 (157) |
| 890063 (1)<br>890063 (2) | 16688<br>19145 | 3.4 (818)<br>4.5 (709) | 3692 (22.1)<br>5386 (28.1) | 2021 (12.1)<br>3116 (16.3) | 2000 (101)<br>5000 (62) |
| 890001 (1)<br>890001 (2) | 8316<br>20040 | 1.7 (815)<br>4.4 (759) | 2587 (31.1)<br>9335 (46.6) | 940 (11.3)<br>5254 (26.2) | 2000 (47)<br>5000 (105) |
| 890073 | 6610 | 1.9 (580) | 4245 (64.2) | 3936 (59.5) | 1000 (393) |
| 821366 | 26432 | 2.8 (1570) | 20500 (77.6) | 19208 (72.7) | 10 000 (192) |
| 821290 | 38836 | 3.2 (1990) | 16630 (42.8) | 14058 (36.2) | 10 000 (140) |

*Cuttings rooting and quality as a function of mother plant origin for the clone 870634*

Micropropagation of mother plants resulted in significant increase in cuttings quality. In the case of clone 870634 (Table 2), a 16 % enhancement of rooting rate was observed with clear indicators of increased juvenility of sampled shoots (lower number of axillary buds/plant, ovoid vs. oblong leaves, improved growth rate). As a consequence, a 25% reduction in the cost price of the reforestation plantlet could be achieved. The annual cuttings production potential of micropropagated mother plants from *E. gunnii* clones estimated during their 2$^{nd}$ growth year (2 collections rounds, 2 months apart) is quite high (20-67, Table 3).

Table 2 - Cuttings rooting and quality as a function of mother plant origin (clone 870634 “Cagire”)

| **Mother plant origin** | **Shoots sampled within 6 months** | ***Rooting rate of shoots (%)*** | **Quality traits of cuttings (year 1)** | | |
|---|---|---|---|---|---|
| | | | **Mean number of auxillary buds/plant** | **Mean length/width ratio of leaves (%)** | **Mean initial growth (mm)** |
| Cuttings | 63838 | 68 | 2.5 | 1.78 | 16 |
| Microcuttings | 7731 | 84 | 2.1 | 1.65 | 19 |

Table 3 - Cuttings production potential of 2-year-old micropropagated mother plants (n = 10 plants)

| ***E. gunnii* clone (n = 10 plants)** | **Shoots available per plant (standard deviation)** | **Maximum rooting rate of shoots (%)** | **Cuttings production potential per year** | |
|---|---|---|---|---|
| | | | **per mother plant** | **managing 10 000 mother plants (equivalent ha, 1250 plantlets/ha)** |
| 870634 | 80 (12) | 84 | 67 | 670 000 (536) |
| 890001 | 38 (8) | 52 | 20 | 200 000 (160) |
| 890063 | 54 (10) | 82 | 44 | 440 000 (352) |
| 890073 | 66 (11) | 74 | 49 | 490 000 (392) |

## Clone identification and conformity

Molecular tools based on microsatellite variation analysis at 7 loci (capillary electrophoresis on automatic ABI310 platform) were developed to certify clone identity and estimate conformity of micropropagated material. In the example of Figure 3 all 4 clones were identified by alleles of specific sizes (green) in homo- (890001, 821366) or heterozygote states (870634, 821290) based on the microsatellite locus Embra 8 [1].

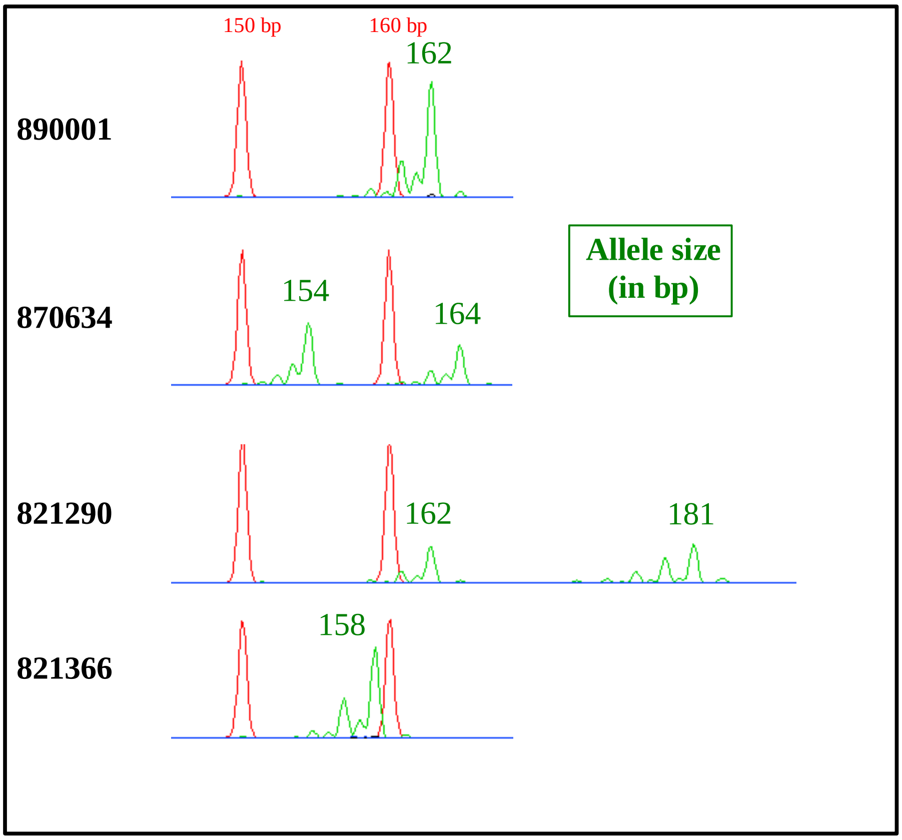


Figure 3 - Molecular marker identification of 4 clones by the alleles of specific sizes (green) in homo- (890001, 821366) or heterozygote states (870634, 821290). In red: molecular weight marker based on the microsatellite locus Embra 8 by Brondani *et al.* [1]).

## Kraft pulping and pulp properties of frost-tolerant eucalyptus clones

Pulp yield and residual effective alkali relationships with Kappa number obtained for the 2 *E. gunni* and *E. gundal* clones are presented in Figures 4 and 5. *E. globulus* data cooked in the same conditions are also presented. Due to the chemical composition, *E. globulus* gives at least 10 % of pulp yield higher than the frost-tolerant eucalyptus clones. Differences of up to 4 % are also observed between *E. gundal* and *E. gunnii* with advantage to the first ones. Moreover, although pulp yield is lower, *E. gundal* is deslignified to the same range of Kappa number as *E. globulus*, while the performances of *E. gunnii* is poorer, especially for the cooking conditions using low alkalinity liquor. Indeed, when an effective alkalinity of 18 % is used, pulps presenting Kappa number around 22-24 are obtained for *E. globulus* and *E. gundal*, while those obtained from *E. gunnii* using the same liquor have Kappa number higher than 30 (37 for the clone 870634). Alkali consumption explains part of the results. The residual effective alkali observed for *E. globulus* is much higher for all the cooking conditions, demonstrating a weak consumption, while the alkali is not enough for the cooking for most for the frost-tolerant clones, especially the *E. gunnii* ones. Only for the highest alkali concentrations, a residual is observed for *E. gundal* clones. The chemical composition of the eucalyptus clones partially explains these differences. *E. globulus* present very low lignin content compared to the *E. gundal* and *E. gunni* clones.

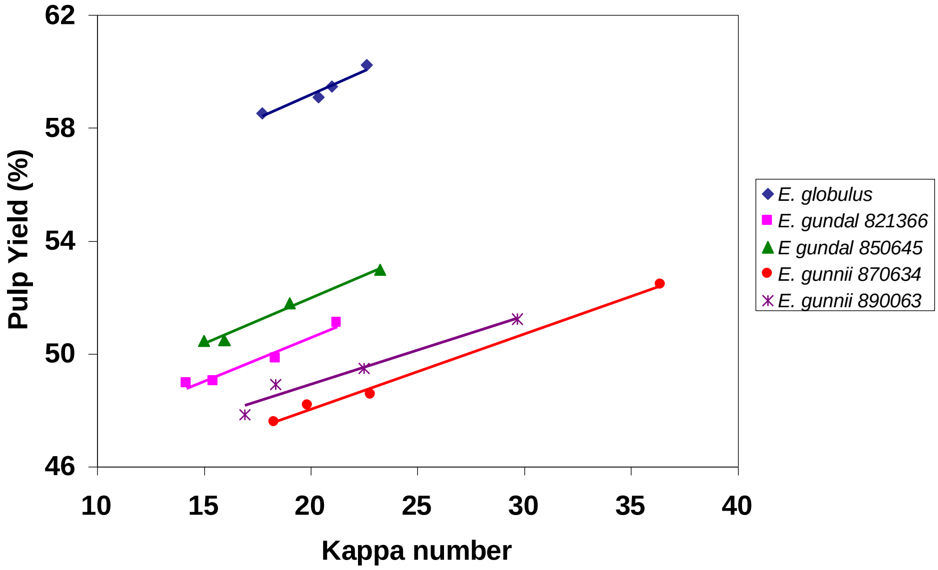


Figure 4 – Pulp yield – kappa number relationships for the frost tolerant clones compared to *E. globulus*.

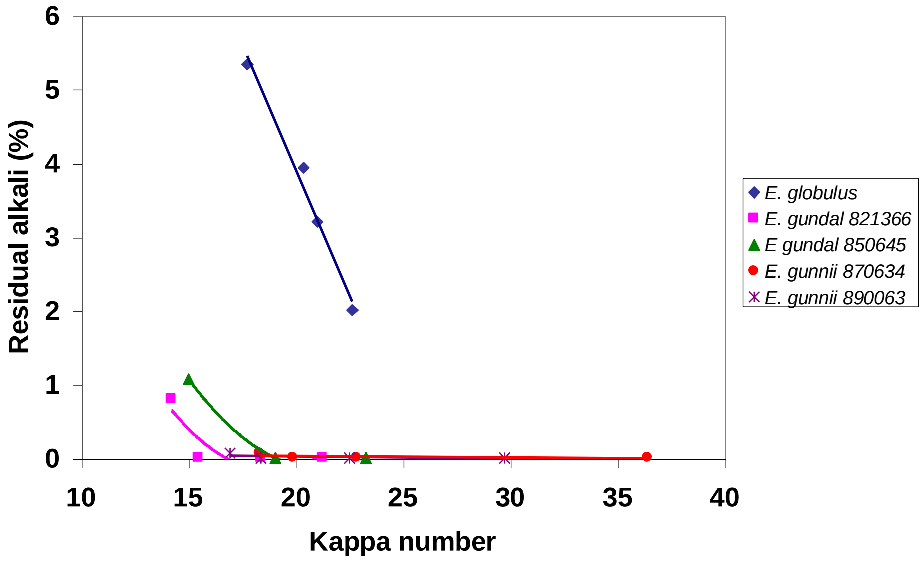


Figure 5 – Residual effective alkali – kappa number relationships for the frost tolerant clones compared to *E. globulus*.

The beating behaviour of the different clones using a laboratory scale refiner is shown in Figure 6. A clear difference is observed between the frost-tolerant clones and the reference *E. globulus*. This latter is much harder to refine than the *E. gundal* and *E. gunnii* clones. To achieve the same refining level (35°SR for example), *E. globulus* needs at least 3 times more revolutions. Although the number of revolution at laboratory scale beating cannot be directly transposed to disk refiner conditions, it is expected that the refining specific energy consumption of the frost-tolerant clones is lower than the *E. globulus*. Paper handsheets (80 g/m²) were produced and their main physical properties measured (Figure 7).

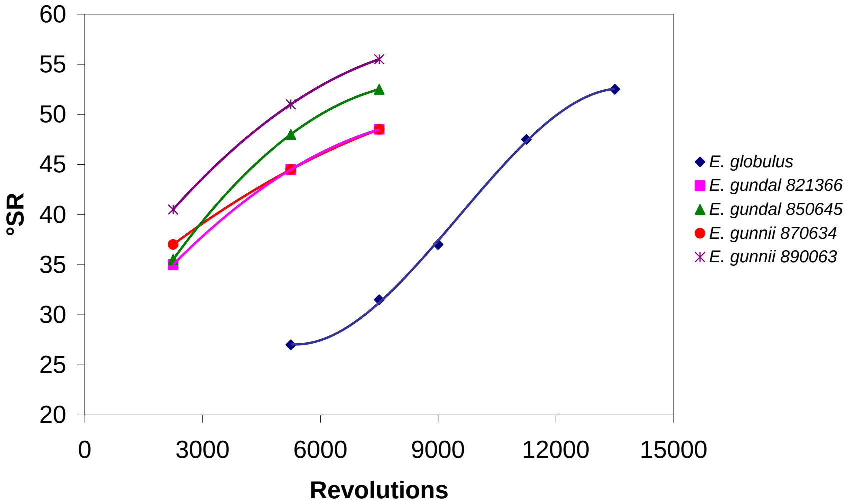


Figure 6 – Drainage index evolution of pulps according to the number of revolution of laboratory scale refiner for the frost tolerant clones compared to *E. globulus*.

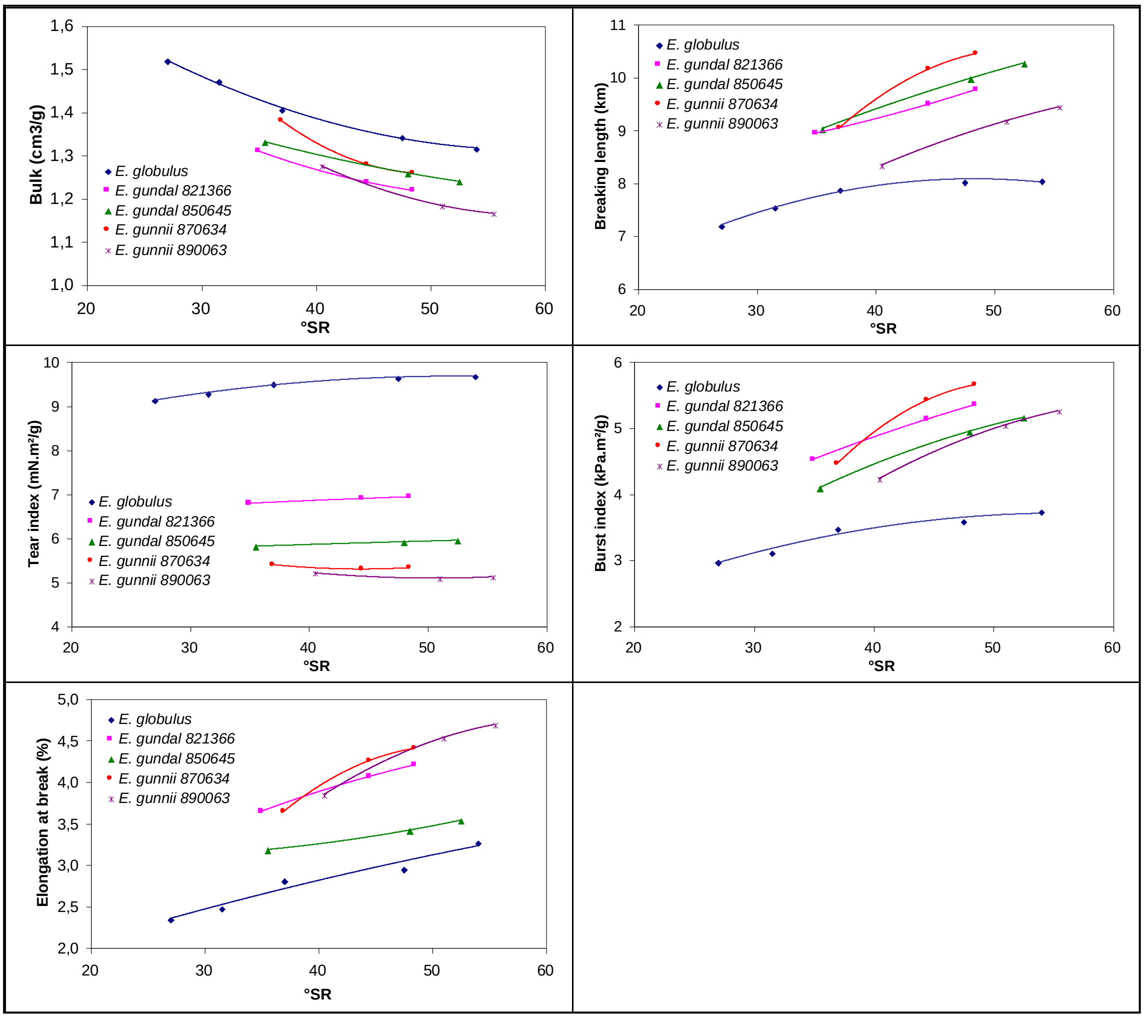


Figure 7 – Main physical properties as function of drainage index of the pulps produced from frost tolerant clones compared to *E. globulus*.

*E. globulus* are 0.05 to 0.10 $cm^3/g$ higher in bulk than the frost-tolerant clones. In opposition, traction (breaking length) and burst indexes are considerably higher for *E. gundal* and *E. gunnii* clones. Between these two later, there is no clear trend of differences.

Roughly speaking, *E. gunnii* clone 870634 gives the highest values of traction and burst indexes at higher °SR than the others clones, while the other *E. gunnii* clone (890063) presents the lowest ones. *E. gundal* clones are in between. For tear index, the trends are practically the opposite, as usually observed for hardwoods, with *E. globulus* presenting very high values, followed by *E. gundal* and *E. gunnii*. Concerning the elongation at break, *E. globulus* handsheets present much lower values than the other clones, what explains their low resistance to traction. Curiously, one of the frost-tolerant clones in terms of traction (*E. gundal* 850645) presents a quite low value of elongation at break, not far from *E. globulus* and completely separated from the group formed by the other three clones.

## Conclusions

Frost-tolerant *E. gunnii* and *E. gundal* clones selected for industrial plantations in southern France were successfully micropropagated at commercial mid-scale production levels (15 000 plants). The number of high quality mother plants produced hit the target objective for 3 highly responsive clones (870634, 821366, 890073) exhibiting 1.9-3.6 shoots per subculture unit, 64-77% acclimatization rate of rooted shoots as well as 59-73% survival rate after two months in the greenhouse. Some difficulties were observed for 3 other clones (890063, 890001, 821290) with lower rate of acclimatization (25-43%) and related overall survival rate of rooted shoots (14-36%).

In terms of pulp quality, these frost-tolerant clones present considerably lower pulp yield when compared to *E. globulus* due to huge difference in chemical composition. However, the pulps refine very well and the traction/burst properties are by far better than those obtained for *E. globulus* used here as the reference for the European market of pulp producers.

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