CHAPTER 13

PROPAGATION OF SELECTED *PINUS* GENOTYPES REGARDLESS OF AGE

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1. INTRODUCTION

Most of pine trees do not reproduce naturally from sprouts. Reproduction from artificially rooted propagules of pines has been proved as a successful reproduction method in several countries. This leads us to two different ways to obtain artificial reproduction. Hedging method is used for mass-producing large number of symmetrical and straight cuttings and for maintaining the juvenile nature of propagules (Libby et al., 1972). It has been widely used in breeding programs of plant species for preservation and multiplication of the desired genotypes to establish seed orchards. In this technique we include micrografting which is useful to rejuvenate adult material (Fraga et al., 2002b). Plantlet regeneration from embryos and cotyledons is another approach (Aitken-Christie et al., 1981), it has a great potential for forest tree

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multiplication compared to other alternative techniques, such as cutting propagation. In this chapter we describe protocols for in vitro micropropagation of selected *Pinus* species like radiata pine (*Pinus radiata* D. Don), austrian pine (*Pinus nigra* Arn.) and caribbean pine (*Pinus caribaea* Mor).

2. *IN VITRO* APPROACHES

Micropropagation is an attractive alternative for the multiplication of selected genotypes. Taking into account the actual barrier that exists in this area for achieving industrial plant production in the following sections we will pay special attention to micropropagation techniques, considering the available information from previous reports and also our own experience with juvenile and mature radiata, austrian and caribbean pines (Rodríguez, 1990; Fraga et al., 2002a,b, 2003; Uribe et al., 2004; Rodríguez et al., 2005b).

A successful micropropagation technique is strongly influenced by the genotype and chronological/physiological age of the donor plant (Rodríguez et al., 2004a,b,c, 2005b). This work is presented in three sections: I) Morphogenesis from embryos and seedlings (Figures 1 and 2a), II) Micropropagation from trees younger than 5 years (Figure 2b), and III) Micropropagation from mature trees (Figure 2c–e). Thereby, the extent of currently available information (Horgan, 1987; Smith, 1997; Fraga et al., 2002a,b; Diego et al., 2004; Rodríguez et al., 2004c) would be of great help to overcome the limitations facing *in vitro* plant propagation.

2.1. Explants and Sterilization

Explant selection depends on the final aim of the program. The selection of different explants has shown significant influence on shoot production (Aitken-Christie et al., 1981).

Embryos and cotyledons. To proceed, imbibe seeds in running tap water for 24 h and then stratify in plastic bags for 2 days at 4°C. Seeds are surface sterilized in 5% (v/v) sodium hypochlorite and then washed in sterile water. For embryo culture, dissect embryos from seeds under sterile conditions, or excise cotyledons after aseptically germination (Villalobos & Engelmann, 1995).

There is another similar protocol, place seeds for 30 min in 3% sodium hypochlorite solution containing 0.05% (w/v) of Tween® 20 followed by five rinses with sterile water. Once the seed coat is removed the megagametophyte is re-sterilized for 15 min in 15% bleach and for 5 min in hydrogen peroxide (Muriithi et al., 1993). We have proved that mild treatments, 7.5% sodium hypochlorite (v/v) 45 min or ethanol 50% (v/v) 90 min, followed by three rinses in sterile water and after removing seed coats, also work efficiently.

Mature tissues. This type of tissues is difficult to sterilize, and propagation of adult trees can be initiated with different tissues. Apical buds or healthy branch tips are usually collected from vigorous trees, but best responses are obtained when caulinar

terminal portions are taken just prior to active growth manifestation. Any plant material is superficially sterilized dipping into 70% (v/v) ethanol for 15 seconds, washed with sterile water, dipped into a solution of 5% sodium hypochlorite plus 0.05% (w/v) Tween® 20 for 15 min, and then washed four times with sterile water in which they were kept until used. On the other hand, mature tissues from field have a higher contamination degree and even endogenous contamination. Protocols based on fungicide–bactericide mixture are used to remove it. Use cuttings 2 cm long from caribbean and radiata selected trees cleaned in laminar flow chamber, stirring continuously in 70% ethanol for 30 seconds and wash with distilled water. Then submerge them in a solution of Benomyl (6 g L^{-1}) plus rifampicin (25 mg L^{-1}) infiltrated with a vacuum pump, wash with distilled water and immerse in sodium hypoclorite 2.5% (v/v) plus Tween® 20 0.05% (w/v) solution for 15 min. Finally wash four times with sterile water and put under *in vitro* culture. A mixture of Captan–Benlate in agitation is also employed (Prehn et al., 2003). Another highly effective, but contaminating disinfectant solution is $HgCl_2$ (0.1–1.0%) for 2–10 min (Gupta & Durzan, 1985).

Micrografting. Apical portion of macroblasts from the selected trees are used as scion source. They are sealed with Parafilm® to avoid drying and stored at 4°C for a maximum of 40 days until to be used. Just before sterilization needles are removed and brachyblasts are kept to prevent basal oxidation. Scions composed of basal parts of needles containing an axillary bud (≈40 mm) are sterilized by dipping into 70% ethanol for 30 s in sterile conditions; washed with sterile water, dipped into a 2.5% sodium hypochlorite plus 0.05% (w/v) Tween® 20 solution for 15 min, and finally washed four consecutively times with sterile water.

2.2. Media Composition for Organ Culture

There are several culture media used for pine *in vitro* culture, like modified Schenk and Hildebrandt (SH) (Schenk & Hildebrandt, 1972) or Wolter & Skoog (WS) basal medium (Wolter & Skoog, 1966). Most used media have been developed as combination of QL medium (Quoirin & LePoivre, 1977), MS medium (Murahsige & Skoog, 1962) and different growth regulators for each kind of treatment, like benzylaminopurine (BAP), thidiazuron (TDZ), metatopolin (mT), indolebutyric acid (IBA) or naphtaleneacetic acid (NAA) in *P. nigra*, *P. caribaea* and *P. radiata* (Table 1).

In all cases, media are solidified with agar (0.8%) or Gelrite® (0.5%), pH is adjusted to 5.8 and media are sterilized for 20 min to pressure conditions of 1 Kg cm^{-2} at 120°C. Explants are maintained in sterile conditions at 25 ± 2°C, 70–80 μmol m^{-2} s^{-1} light intensity and 16:8 (day/light) photoperiod.

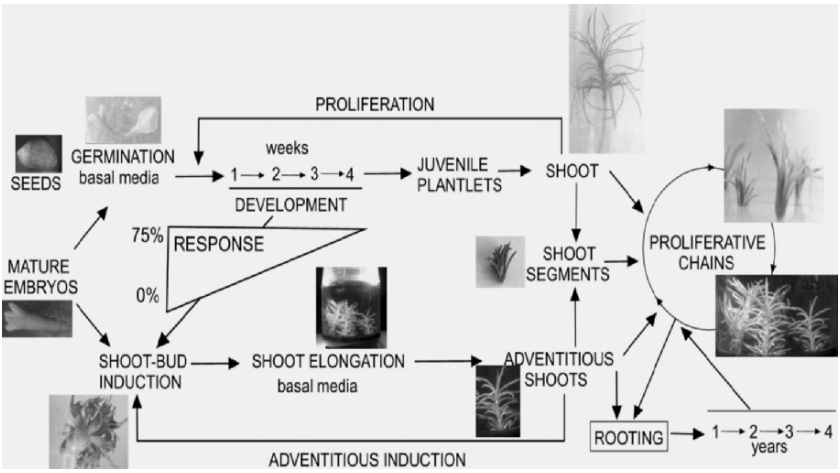

Figure 1. *In vitro amplification of embryogenic and juvenile P. radiata, P. nigra and P. caribaea genotypes.*

Table 1. *Composition of the culture media for P. radiata, P. nigra and P. caribaea.*

Medium	Elongation	Multiplication		Micrografting
Composition	QLP	QLP_1	QLP_2	QLS1
Macroelements	QL	QL	QL	1/3 QL
Microelements	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS
Iron	MS	MS	MS	MS
Active Charcoal (3 g L^{-1})	+	–	+	–
BAP µM	–	4.44	8.87	22.19
IBA µM	–	0.49	0.49	–
NAA µM	–	–	–	2.69
Sucrose (30g L^{-1})	+	+	+	+

+: Presence; –: Absence.
QLP: Shoot induction medium; QLP_1 and QLP_2: Shoot multiplication medium 1 and 2; QLS1: Stimulation medium; QL: Quoirin & LePoivre medium; MS: Murashige & Skoog medium; BAP: Benzylaminopurine; IBA: Indolebutyric acid; NAA: Naphthaleneacetic acid

2.3. In Vitro Morphogenesis and Micropropagation from Embryos and Seedlings

Although culture of isolated cotyledons could be an interesting source of new shoots in *P. radiata*, better results are being obtained when whole mature embryos are placed upside down, with cotyledons immersed in the induction medium. QLP has been used as basal medium (Rodríguez et al., 2005b) as shoot induction medium for embryos. QLP supplemented with cytokinins like 22.19 μM BAP, 9.08 μM TDZ or 8.88 μM mT has been usually used.

Liquid medium with same composition is being used for multiplying embryos by temporary immersion systems, with an immersion period of 5 min each 6 h. These systems open the possibility of scaling-up results, achieving 80% of caulogenesis with 10–14 shoot-buds per cotyledon. When embryos or excised cotyledons are first cultured on basal medium, morphogenic capacity declines and reaches to non-responsive situation after few weeks of development on basal media (Figure 1).

2.4. In Vitro Vegetative Amplification of Juvenile and Mature Selected Genotypes

The success of *Pinus* micropropagation is highly dependent on original explant genotype and physiological status of donor plant. Therefore, it is very difficult to generalize and to compare shoot production in terms of yield and quality.

Shoots, and adventitious shoots from embryos and seedlings, can be directly included to *in vitro* multiplication culture (Figure 2a). Selected progenies not older than 5 years are able to establish *in vitro* (Figure 2b). Older plant materials remain unresponsive and die shortly before culture establishment. Explants taken from adult trees must be first reinvigorated (Figure 2c–e) before establishment of *in vitro* cultures.

The continuous cultures of shoots, in shoot multiplication QLP_1 and QLP_2 media containing cytokinins, result in limited elongation and unsuccessful rooting. Therefore, in order to establish a highly efficient shoot production chain, it is recommended to use media sequence QLP_2–QLP_1–QLP, associated to a decreasing level of plant growth regulators.

3. MICROGRAFTING, REACTIVATION AND MICROPROPAGATION OF MATURE TREES

Direct *in vitro* establishment of mature explants is not possible in general terms without a previous reinvigoration. Classical *ex vitro* reinvigoration techniques, such as intensive pruning or cascade macrografting, can be used to reinvigorate adult materials (Figure 2c–e).

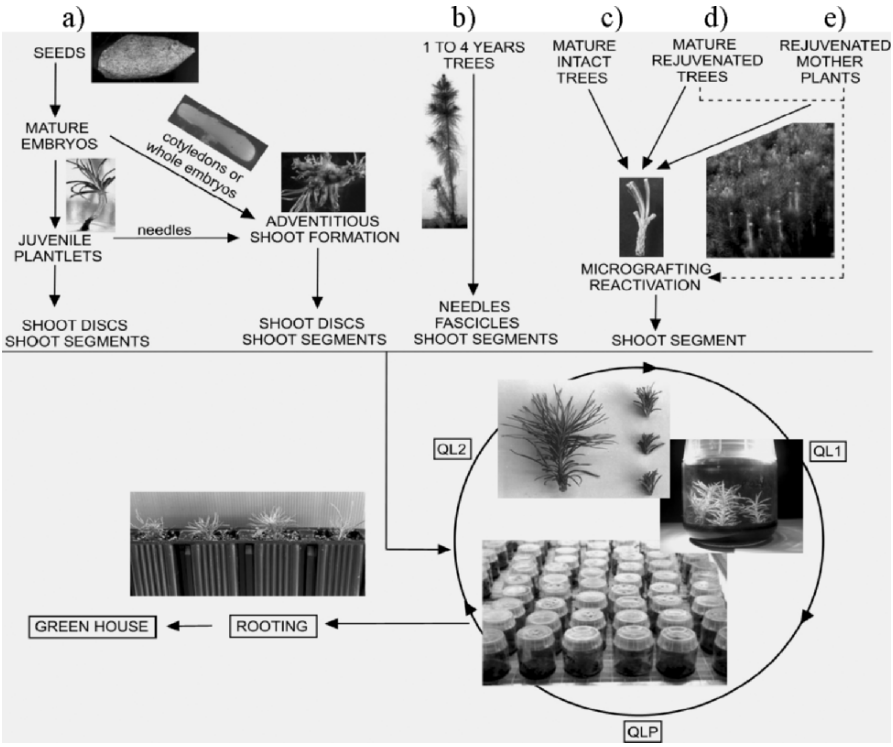

Figure 2. *Propagation of P. radiata, P. nigra and P. caribaea genotypes independent of age: (a) morphogenesis from embryos and seedlings, (b) micropropagation from trees younger than 5 years, (c), (d) and (e) micropropagation from mature trees through micrografting, or directly from rejuvenated trees or mother plants.*

Scions are obtained from surface sterilizing selected needles (Figure 3a) and cutting its basal part V-shaped (Figure 3b) which allows better scion–rootstock contact. A cut is made underneath cotyledons of 1-month-old seedlings to obtain the rootstock. In its apical part a 3 to 5 mm long incision is made to insert an scion later on (Figure 3c). Rootstock–Scion systems (Figure 3d) are cultured first for 20 days in stimulation medium QLS1. Culture conditions are 25°C ± 2°C, 16 h photoperiod at 70–80 µmol m^{-2} s^{-1}. After this period, if the scion is established (being green, non-necrotic showing an interphase callus between rootstock and scion), the micrografts are transferred to a QLP culture medium for 40 days. Fully developed scions (Figure 3e) can be directly propagated as juvenile tissue in most of the cases. The proliferation responses develop through consecutive transfers in the following medium sequence: $QLP_2 \rightarrow QLP$ or $QLP_1 \rightarrow QLP$ being of 25 days each one.

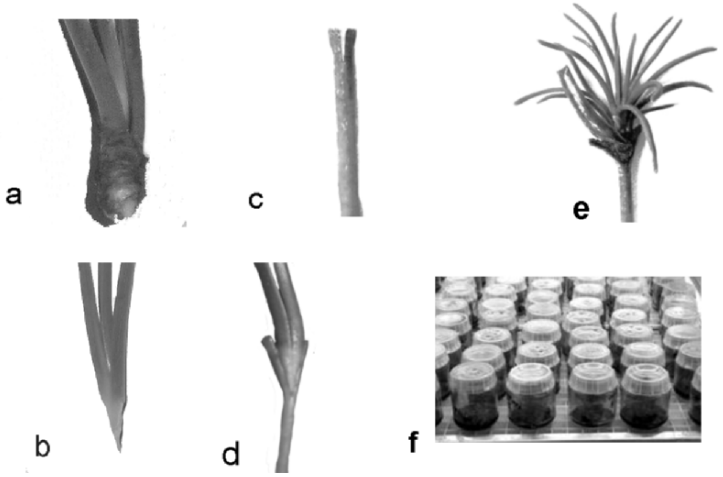

Figure 3. *Phases of micrografting in P. radiata and P. nigra: (a) needles taken from terminal branches were surface sterilized as described before, and then a V cut was made (b) which permits its insertion in a rootstock with a 3–5 mm cleft (c) Rootstock–scion systems (d) were cultured for 60 days till scion was fully developed (e), at this point scions can be used to start proliferative chains (f).*

Micrograft development takes place along three phases: after 10 days of culture, an interphase callus between scion–rootstock initiates (establishment). In the following days, vascular tissue between scion and rootstock develops (consolidation) and a subsequent outgrowth of the bud may occur (development). Micrografting has been tested both in juvenile progenies and in different mature trees, obtaining different responses in each case (Figure 4).

Except for explants coming from plant material with limited proliferation ability, the rest of the plant material gradually increased their proliferation capacity. Results of multiplication obtained after 3 consecutive cycles established effective cycloclonal chains and microshoot rooting.

4. ROOTING AND MICROPLANT ESTABLISHMENT

Auxin pulse treatment. There are some protocols in several pine species that use high concentration of IBA. It is possible the use 250 μM IBA for 24 h (Rodríguez et al., 2005a) or 100 μM IBA for 2, 3, 4, 5, 6 h followed by a transfer to a mixture of peat and vermiculite (Muriithi et al., 1993).

Low plant growth regulator concentration. Lower concentrations of IBA and NAA must be used in most cases because they are present for a long time in the plant and may inhibit root development, IAA may be preferred because it is more labile (De Klerk, 2002). In this sense there are some protocols which use low plant growth regulator concentration; use 4.94 μM IBA and 0.54 μM NAA in a medium

containing 1% agar for 10 days and a transfer of the explants for root induction to a mixture of peat: perlite: vermiculite: sand in the ratio of 1:2:2:1 (Bergmann & Stomp, 1994). It is also possible to use 8.2 μM IBA and 5.4 μM NAA for 5 days in 5% agar medium, transferring explants to a ½ LP medium reducing sucrose concentration to 10 g L^{-1} which facilitates rooting process (Prehn et al., 2003). Also a last protocol uses a 5 day period in agar medium containing 11.42 μM IBA and 2.69 μM NAA prior to shoot establishment into a peat: pumice substrate, which improves rooting to 86%. In all cases *P. radiata*, *P. nigra* and *P. caribaea* rooted plants were observed after 4–6 weeks.

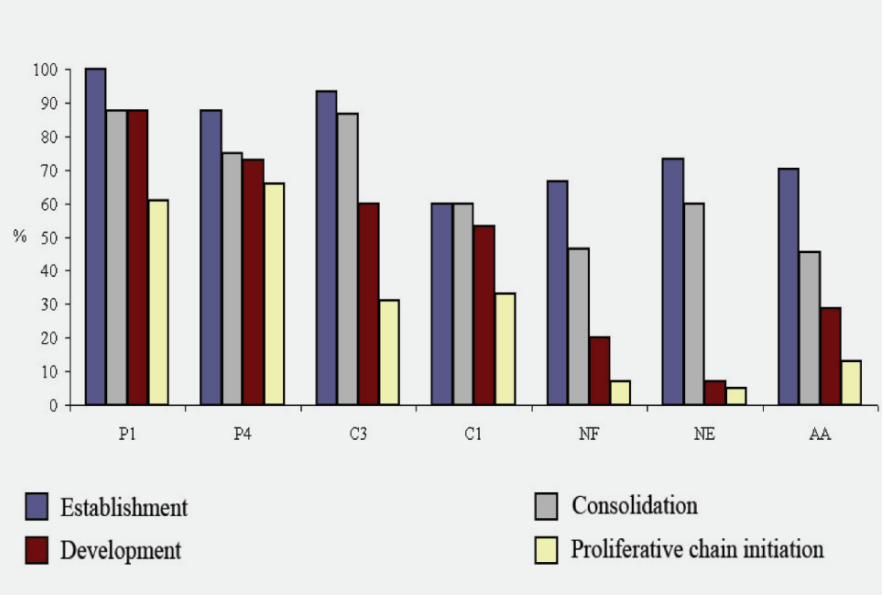

Figure 4. *Quantification of micrografting establishment responses and scion outgrowth after homomicrografting in different-origin P. radiata materials: Juvenile progenies (J1, J4); mature trees of the same age and origin but subjected to different reinvigoration treatments based in one (C1) or to three (C3) macrograftings; mature trees with different morphogenic potential as limited rooting ability (NE) or not ability to flower (NF); adult field trees older than 30 years (AA).*

Bacteria and rooting. Roots can also be induced employing *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*. This is done either in the presence of IBA, which increases root response, or without it (Bergmann et al., 1997; Li & Leung, 2003). The rooting response depends on the age and origin (direct or indirect caulogenesis) of the plant material, but it is also affected by the proliferation medium. Best rooting induction response was achieved with 1-year-old microshoots (P1). The best culture media for rooting was: (QLP_2–QLP_1–QLP). Several microshoot reinvigoration treatments showed proportional root induction.

5. CONCLUSION

In vitro Pinus organogenesis is feasible for several purposes from adventitious organ induction to scaling-up plant multiplication. All desirable responses are restricted by age barrier, which makes difficult reversion to undifferentiated status. Micrografting on juvenile rootstocks is an ideal reinvigoration technique, and it is more effective when donor plant is previously pruned or grafted. Temporary Immersion System facilitates microshoot response, increasing its efficiency. Rooting depends on the quality of microshoots, and basal or low content of plant growth regulators in the culture media greatly enhance microplant production.

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