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**Original article**

# Factors involved in *Pinus radiata* D. Don. micrografting

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**Abstract** – A series of micrografting conditions using needle fascicles from trees of different ages as scions have been evaluated for *Pinus radiata* D. Don. to increase success of in vitro propagation. Micrografting success depended on the quality of the graft process as well as age, location and development stage of the scion and tree age. 11-month-old scions, taken in January from terminal portions of basal branches show the best micrografting-induced response. Responsiveness of scions decreases with the donor tree age, although this could be overcome by optimising micrografting conditions.

**reinvigoration / micrografting / maturation / vegetative propagation / *Pinus radiata* / in vitro culture**

**Résumé – Facteurs impliqués dans le micro-greffage de *Pinus radiata* D. Don.** Différentes conditions de micro-greffage, utilisant comme greffons des brachyblastes provenant d'arbres d'âges différents, ont été comparées afin d'évaluer les possibilités d'améliorer la propagation in vitro de *Pinus radiata*. Le succès du micro-greffage dépend tout autant de la qualité du processus de greffage que de l'âge, de la localisation et du stade de développement du greffon, ou que de l'âge de l'arbre. Des greffons de 11 mois prélevés en janvier sur la portion terminale de branches de la base de l'arbre donnent les meilleures réponses au micro-greffage. Cette réponse diminue avec l'âge de l'arbre sur lequel ils sont prélevés, bien que ceci puisse en partie être surmonté en optimisant les conditions du micro-greffage.

**vigueur / micro-greffage / maturation / multiplication végétative / *Pinus radiata* / culture in vitro**

**Abbreviations**
BA: benzyladenine
IBA: indolebutyric acid
MS: Murashige and Skoog culture medium
NAA: naphtalenacetic acid
QL: Quoirin and Lepoivre culture medium
QLP: elongation culture medium
QLS1: stimulation culture medium
QLY: high proliferation culture medium
QL1: proliferation culture medium.

## 1. INTRODUCTION

Maximizing gains from genetic improvement programs in forestry requires propagation of genotypes. Unfortunately, the maturation and ageing processes which affect the expression of additive and non-additive desirable characteristics, also hinders the exploitation of trees by traditional methods and biotechnological techniques

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since morphogenic competence is generally lost. Practical benefits from vegetative multiplication are possible when effective methodologies that allow the multiplication of mature trees are available.

Mature conifer trees are generally cloned in vivo by grafting whereas propagation of juvenile individuals is done via rooted cuttings [1, 16]. Unless scions or cuttings are taken from very juvenile plants of specific clones, the explants recovered generally retain undesirable characteristics of the mature state, such as reduced growth and increased plagiotropism [7]. Traditional methods of vegetative propagation have not been very successful in the Pinaceae, and particularly in *Pinus radiata* [18]. The success declines during the juvenile-mature phase change.

Reinvigoration of explants from mature selections that have lost their vegetative propagation ability could allow in vitro establishment of mature radiata pine. Although in vitro multiplication of radiata pine was previously reviewed [18], no study of effects of serial propagation on propagation success and in vitro establishment of mature radiata pine material through micrografting has been published, unlike in other Pinaceae such as larch [5].

Micrografting is used for both practical applications and basic research [9, 12]. It has becoming an acceptable methodology for the cloning of several mature species, as *Sequoiadendron giganteum* [11], *Pinus pinaster* [4] and *Pinus nigra* [14].

The practical interest of micrografting mature selections onto juvenile rootstocks arises from the potential of this technique to facilitate in vitro establishment and, therefore, cloning of selected mature materials [6, 8].

Although the advantages of this technique are clear, micrografting is a very complex procedure because different factors contribute to the final success. Manipulation of scions, physiological state and scion age were studied. This provides a basis for the definition of optimal conditions for micrografting *Pinus radiata* and so, for the in vitro establishment of selected mature material.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

Different genotypes of *Pinus radiata* D. Don. were used from the genetic improvement program developed by the Environmental Research Centre NEIKER (Vitoria, Spain).

One-year-old (P1) and four-year-old (P4) plants from controlled pollinated seeds (68 of "Iurre" × 40 of "Orozko") were tested as juvenile trees.

Four types of mature trees were used: C1, grafted from a 30-year-old selected tree (clone 7); C3, three consecutive grafts from C1; NF, grafted from a 32-year-old selected tree (clone 32) and NR, grafted from a 30-year-old selected tree (clone 45). In all cases, 1-year-old seedlings were used as rootstocks. Chronological age of the treated trees when collection was 8-year-old except C3 that was 3-year-old. Also a series of non-treated trees at age varying between 15 and 40-year-old were used (AA).

### 2.2. Micrografting technique

Micrografts were carried out as indicated (*figures 1a–f*) by apical grafting of needle fascicle scions to microshoot rootstocks. To prepare the scions, the needle sheath was removed and the needle was cut just above needle base (*figures 1a, b*). After 2 slanted cuts of 3 mm in the basal portion (*figure 1c*), the scion was inserted inside a cut (3 mm) in the apical part of the rootstock (*figures 1d, e*). Contact among the surfaces of the rootstock-scion was assured by elastic silicone rings (*figure 1f*).

### 2.3. Rootstocks

*Pinus radiata* microshoots (25–30 mm length) isolated from in vitro proliferation series started from young seedlings were used as rootstock. Multiplication of microshoots was as previously reported [17].

### 2.4. Scion collection types and factors analysed

Terminal parts of the shoots were taken from the selected trees, sealed with Parafilm® to avoid drying and stored at 4 ºC for a maximum of 40 days until tested. Just prior to sterilisation, needles were removed and the brachyblasts were kept to avoid dehydration.

Isolated needles prepared as indicated were used as scions. For the evaluation of the tree age, scions collected in January from all the selected trees were used.

The evaluation of the scion chronological and physiological age was developed using isolated needles of trees in three stages of maturation: b1, b11 and b13. The index

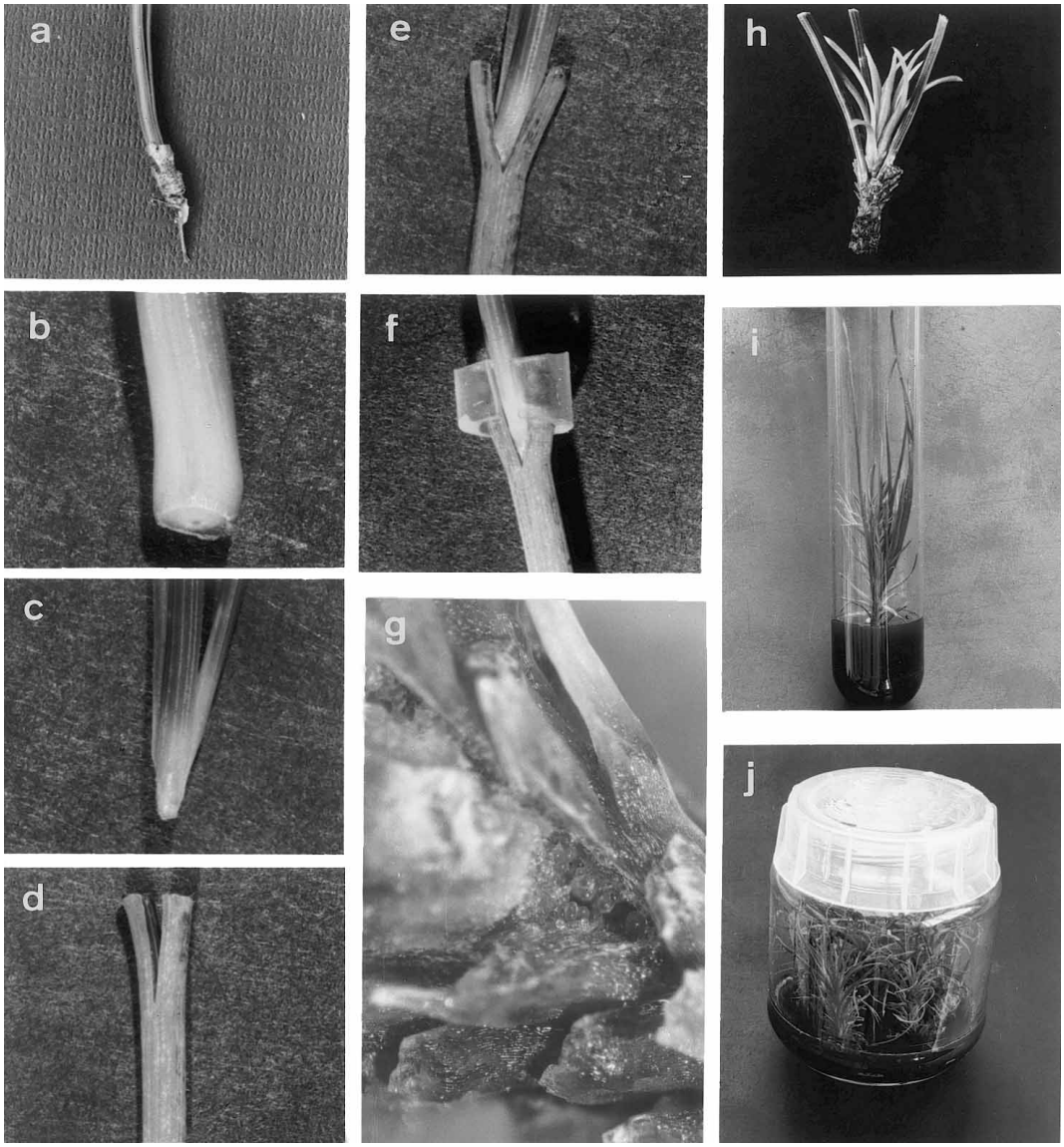


**Figure 1**. Micrografting technique steps. (a) needle fascicle excised from the macroblast (see needle sheath in the basal portion). (b) needle without brachyblast (5 ×). (c) needle with two longitudinal cuts (3 ×). (d) cleft of the rootstock (4 ×). (e) scion-rootstock assembly (4 ×). (f) maintenance of the structure with an elastic silicone ring (4 ×). (g) formation of the scion-rootstock callus (30 ×). (h) development and elongation of needles from the axillary bud of the scion. (i) mature radiata pine in vitro established after reinvigoration. (j) Mature radiata pine microshoots.

indicates months of development starting from active growth (1 month; b1) to mature developed needles (11 months; b11) and completely mature needles (13 months; b13).

The effect of the season when tissues are collected was assayed using as scions needles taken from basal portions of different aged trees (14–40 years of age, AA) in summer, autumn, winter and spring.

Tree architecture and branch scion position were evaluated by using b11 scions taken in January from mature trees. Scions used were selected from basal and apical levels in the tree. Scions taken from three different

positions (basal, middle and apical) along the annual growth of macroblast were also analysed. Needles used as scions were collected in different branches in the basal portion of the tree.

In order to study the effect of the apical dominance in the micrografting response of the b11 scions, the terminal bud of basal branches of mature trees (AA) was removed in October 1998, and the closed b11 needles to the end of the branch were collected and micrografted in February 1999.

### 2.5. Sterilisation

Scions composed of basal parts of needles containing an axillary bud (≈ 40 mm) were sterilised by dipping into 70% ethanol (in sterile conditions) for 30 s. These were washed with sterile water, dipped into a solution of Tween 20, 2.5% (v/v) and sodium hypochlorite for 15 min and then washed four times with sterile water. The b1 explants were sterilised whole, without removing their bracts. Due to the high sensitivity of the scion to the sterilisation process, several ranges of sodium hypochlorite (1, 5, 12.5 and 25 g $L^{-1}$) were tested.

### 2.6. Culture conditions

In all the cases, the different steps of micrografting were carried out in sterile tubes (20 × 150 mm), containing 10 ml of culture media, at 25 ± 2 ºC, 70–80 µmol $m^{-2}$ $s^{-1}$ light intensity and a 16:8 (day/light) photoperiod. The micrografts were cultured far 10 d in a stimulation culture medium called QLS1 composed of 1/3 diluted macroelements of QL medium [15]; microelements; $Fe^{2+}$ and vitamins of MS medium [13]; 30 g $L^{-1}$ sucrose, 0.8% agar and pH 5.8. In addition, the medium was supplemented with 2.69 mm naphtalenacetic acid (NAA) and 22.19 mM benzyladenine (BA). Later, micrografting systems were transferred to development medium (QLP) for 30 days. QLP composition was QLS1 but without phytohormone supplementation.

Proliferation of microshoots was achieved in a QLY, QL1, QLP sequence culture medium. QL1 was composed of QLS1 salts supplemented with 0.1 mg $L^{-1}$ indolebutyric acid (IBA), 0.2 mg $L^{-1}$ BA and 3 g $L^{-1}$ of activated charcoal. QLY medium was composed of QLS1 salts supplemented with 0.1 mg $L^{-1}$ IBA and 1 mg $L^{-1}$ BA.

### 2.7. Quantification of results

Micrografting response was quantified according to the following four criteria: (1) establishment (callus formation after 10 days culture) (*figure 1g*), (2) consolidation, or vascular formation between scion and rootstock (non-necrotic scions after 30 days culture), (3) development (outgrowth after 45 days) (*figure 1h*) and (4) the ability to initiate serial culture (*figure 1i, j*).

### 2.8. Statistical

Results correspond to 15 micrografts for each treatment. Results were processed with a SPSS® package using the contingency analysis utility for each qualitative variable. $\chi^2$ tests ($P < 0.05$) were performed for each variable. At a later stage and once the significant differences between variables were proved, a comparison of these variables in pairs with the $\chi^2$ test ($P < 0.05$) was carried out.

## 3. RESULTS AND DISCUSSION

Success of micrografting selected *P. radiata* elite trees is strongly influenced by the handling procedure both before, during and after surface sterilisation has taken place.

To ensure micrografting success the needle sheath was removed (*figure 1b*) just prior to surface sterilisation, and a small piece of brachyblast near the base of the scion was retained. In addition, after surface sterilisation, basal tissues must be removed. As it was previously reported for *Pinus nigra* [14], these actions increase scion viability by eliminating phenol exudation and necrosis of tissues normally associated with sterilising agents. It was shown that 5 g $L^{-1}$ was the optimal sodium hypochlorite concentration (*table I*). Other concentrations decreased scion viability.

**Table I.** Effect of the sodium hypochlorite concentration on the explant viability ($n$ = 15).

| [sodium hypochlorite] (g $L^{-1}$) | Contamination (%) | Necrosis (%) |
|---|---|---|
| 1 | 68 ± 15 | 29 ± 2 |
| 5 | 18 ± 5 | 28 ± 6 |
| 12.5 | 20 ± 10 | 62 ± 12 |
| 25 | 13 ± 7 | 85 ± 2 |

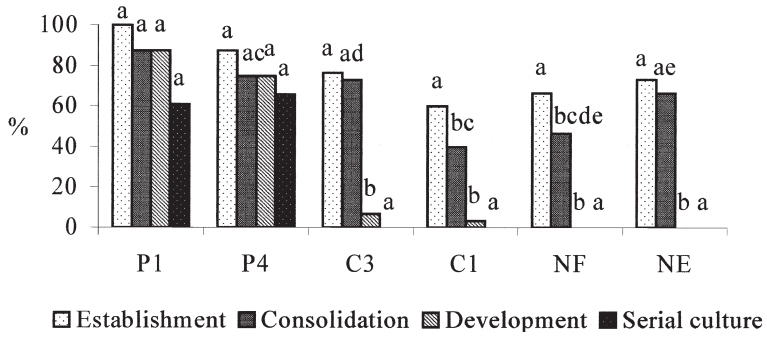


**Figure 2.** Micrografting response of different aged and reinvigorated state trees (see text for definition of plant code). Different letters for the same variable indicate significant differences ($\chi^2$ test with $P < 0.05$).

In *Pinus radiata* high concentrations of auxins and cytokinins were required for early development of the micrograft in vitro. This differed from *Sequoia*, in which exogenous gibberelin and cytokinins do not influence the reinvigoration effect of the rootstock on the scion [8].

We followed the performance of differently aged trees (P1 and P4; C3, C1, NF, NR and AA) to ascertain the effect of maturation on micrograft production (*figure 2*). Scions taken from juvenile trees (P1 and P4) easily and quickly underwent all the micrografting steps. Close to 90% of the scions grew and could then be used for serial propagation.

At first, few micrografts from scions from adult trees (C3, C1, NF, NR and AA) reached the goal of elongation but their progress depended on the morphogenic competence of the tree (*figure 2*).

Once the tree age effect was demonstrated, we proceeded to analyse several factors involved on the successful micrograft production. The first one was needle developmental stage (*figure 3*). It was observed that b11 needles showed the highest outgrowth and shoot development. Needles older than 11 months, collected just before the spring growth, showed high establishment and consolidation responses (60–70%) however, no development was observed. This shows that inductiveness does not guarantee further development.

The second factor studied was the seasonal period of collection. This was of paramount importance for success in micrografting of mature scions (*figure 4*). We verified that the winter period represents the time at which the scions are most receptive to being micrografted. This may be the result from the physiological status of the donor plant and hormone levels at the time of excision.

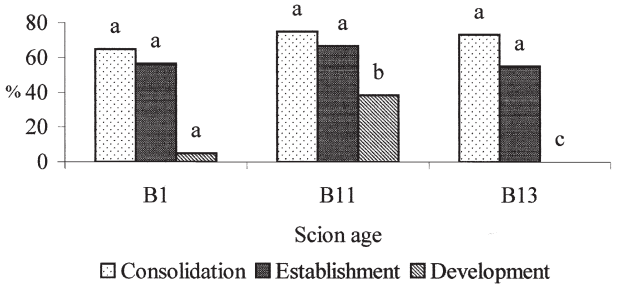


**Figure 3.** Micrografting response of 1-month-old (b1), 11-month-old (b11) and 13-month-old (b13) scions taken from mature trees (AA). Different letters for the same variable indicate significant differences ($\chi^2$ test with $P < 0.05$).

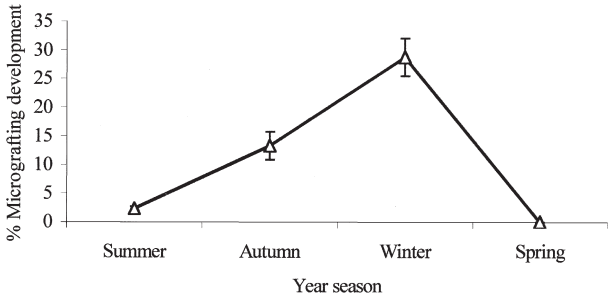


**Figure 4.** Incidence of time collection on micrografting development of scions taken from mature trees. Results correspond to the mean value of 15 experiments and its standard deviation.

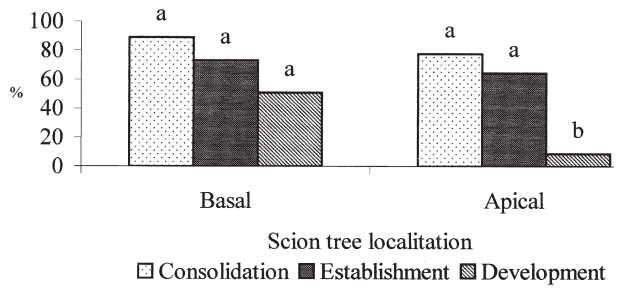


**Figure 5.** Micrografting response of scions taken from apical and basal parts of mature trees. Different letters for the same variable indicate significant differences ($\chi^2$ test with $P < 0.05$).

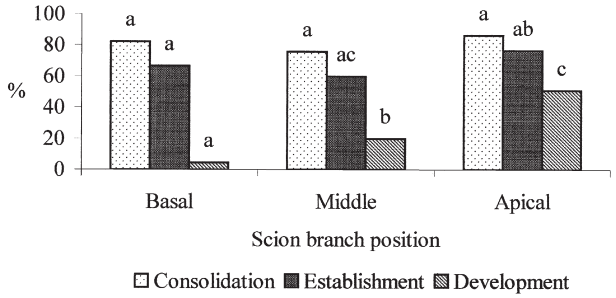


**Figure 6.** Incidence of the lack of close links between the apical bud and the scion on the micrografting response. Different letters for the same variable indicate significant differences ($\chi^2$ test with $P < 0.05$).

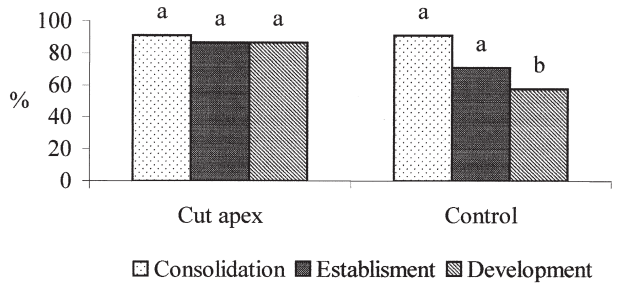


**Figure 8.** Effect of the apical dominance elimination on the micrografting response of b11 scions taken from mature trees. Different letters for same variable indicate significant differences ($\chi^2$ test with $P < 0.05$).

The scions location within the tree can also influence micrografting. An average of 50% of scion outgrowth was achieved when needles (b11) were taken from the basal branches (*figure 5*) whereas, only 10% was observed when scions were isolated from the apical parts.

Finally, scion location along the annual growth of the macroblast (*figure 6*) also affected the micrografting response. It was shown that the most reactive scions were those located at the apical terminal end. A gradual decrease on micrografting development was observed as scion position became more distant from the lateral apex.

Among other factors, the apical dominance [3, 10] could be the reason of the location-related scion response. It was described that the auxin synthesised in the apical bud inhibits the growth of the axillary buds [2], and so the location of the scion into the tree becomes decisive for the micrografting success.

Using optimal micrografting conditions, we studied effect of true age on grafting success (*figure 7*). In vitro establishment ability using micrografting depends on the tree age since outgrowth decreases during ageing. But the development of the micrografts also depends on a cumulative amount of parameters; among them, ex vitro graft (C3) further increases the levels reached by the in vitro technique. Results show a higher ability of NF over NR to initiate serial cultures, which seems to indicate that more than the chronological age, the morphogenic state of the donor tree is critical for the micrografting-induced response.

Despite the higher micrografting responses of ex vitro reinvigorated materials, consecutive grafting is a tedious and long-time technique, being usually necessary more than 5 years in order to obtain enough reinvigoration to allow vegetative propagation. However, there are other possibilities, which allow the improvement of the mature micrografting response: when the apical bud was

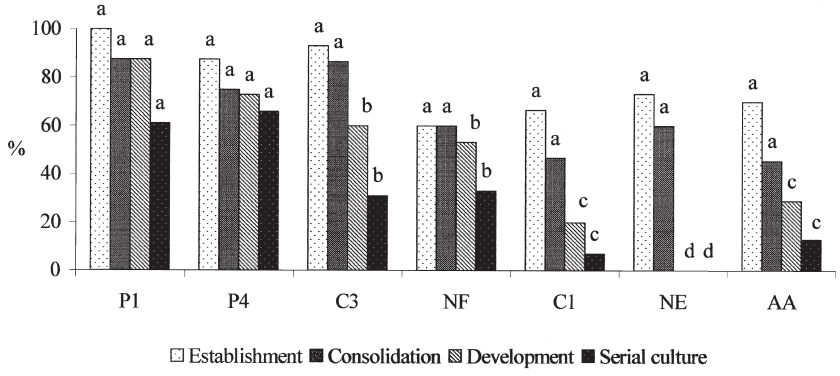


**Figure 7.** Micrografting response and ability to initiate serial cultures of terminal b11 scions taken in January from basal portions of different aged trees. Different letters for same variable indicate significant differences ($\chi^2$ test with $P < 0.05$).

excised, the needles located just below it showed the highest development response (*figure 8*) (80%), as opposed to 50% development of controls.

Finally it is important to remark that, as the micrografting technique allows the in vitro establishment of adult trees, the mature in vitro established material (*figure 1j*) showed similar growth rates to the juvenile ones at the end of 6 months (data not presented).

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