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Note

Micrografting of mature stone pine (*Pinus pinea* L.) trees

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Abstract – This paper describes an in vitro micrografting method for selected mature *Pinus pinea* L. trees. Needle fascicles of five selected clones were micrografted onto hypocotyls of two weeks-old germinated isolated embryos. Fascicle meristems outgrowth was recorded after one month of culture and the performance of the clones assayed was evaluated. Clones behave statistically different in establishment and development rates. Overall success of our protocol reached 43% of the graftings made.

micrografting / micropropagation / conifer / in vitro grafting / *Pinus pinea*

Résumé – Micro-greffage de clones sélectionnés de *Pinus pinea* L. Ce rapport décrit une méthode pour le greffage in vitro de *Pinus pinea* L. Des brachyblastes de cinq clones ont été greffés sur embryons isolés germés, âgés de deux semaines. Le développement des méristèmes fasciculaires a été quantifié pour chaque clone après un mois. Les pourcentages d'établissement et de développement sont statistiquement différents pour les cinq clones. Le taux de succès du greffage réalisé par notre méthodologie s'élève à 43 %.

micro-greffage / micro-propagation / conifer / in vitro greffage / *Pinus pinea*

Stone pine (*Pinus pinea* L.) is one of the most important tree species of the Mediterranean area. It has gained new attention in the last years due to the rural development programs granted by the European Union. Highly regarded owing to its ecological and landscape value, it lives in zones with bad soils and a strong summer drought. Stone pine has also a great economical importance, since it produces edible and valuable pine nuts or "piñon". This makes many aspects of its management similar to an agronomic tree. The existence of a wide potential for improvement and the great economic value of the pine nuts give good reasons for genetic breeding programs. These programs are based on the identification of excellent genotypes by establishing clonal banks with different provenances. The stone pine pruning possibilities are limited, as this species does not root as cutting, nor does it allow woody grafting. Thus these banks have to be established by grafting scions obtained from long shoot terminal buds at the precise time of spring flush initiation, or from other soft tissues like needled dwarf shoots [8]. Although it is easy to perform by skilled workers, grafting has a high percent of failure, leading to loss in time and land use, raising costs and making difficult to set up plantations.

In this paper we report the development of a micrografting protocol which, combined with the *P. pinea* improvement program, could allow a rapid build-up of plantations for genotype selection trials, improving the current situation of *P. pinea* breeding programs and reducing its costs.

Two weeks-old in vitro germinated embryos from stone pine (*P. pinea* L.) seeds were used as rootstocks. Seeds obtained from open pollinated trees in Spanish natural stands (ES-23/01 provenance) were provided by the Servicio de Material Genético del Ministerio de Medio Ambiente (Spain).

Seeds were surface sterilised by immersion in 7.5% (v/v) hydrogen peroxide solution for 45 min followed by three rinses in sterile double distilled water. After sterilisation seeds were kept in Petri dishes over a moistened sterile filter paper for 48 h at 4 ºC in darkness according to Humara et al. [6]. Each seed was dissected under aseptic conditions and the embryo transferred to glass test tubes (24 mm Ø × 95 mm) onto a cellulosic "Sorbarod^{TM}" plug imbibed with 5 mL of culture medium consisting of half strength macronutrients, micronutrients and vitamins of Woody Plant Medium [7] and supplemented with 10 g L^{-1} sucrose. The pH was adjusted to 6.5 prior to autoclaving. Embryos were maintained in a Sanyo Growth Cabinet MLR-350 (Japan) under a 16 h photoperiod under a photosynthetic photon flux of 100 µmol m^{-2} s^{-1} provided simultaneously by cool white light (Standard F36W/133 Sylvania, Germany) and Grow-Lux (F36W/GROT8 Sylvania, Germany) fluorescent lamps at 25/19 °C light/dark. Under these conditions most

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of the embryos developed within 2 weeks into 1–2 cm tall young seedlings suitable for grafting. Whole seeds were not used since their germination is not synchronous and have proven very difficult in vitro, furthermore by using only the embryo we miniaturize the seedling allowing its culture in glass tubes. Seedlings originated from whole seeds grow too fast and are unnecessarily big for micrografting purposes.

Five selected genotypes were used in this study, needle fascicles were obtained from twigs from the lower part of the crown of eleven-year old, grafted stone pines in the clone bank B23PH1 of the "Centro Nacional de Mejora Genética Puerta de Hierro", Madrid (Spain). This clone bank was established in 1992 with scions from plus trees selected for their superior cone yield in natural stone pine stands of the Catalan provenance (ES-23/06). Branches of these five superior clones, numbers 15, 21, 24, 36 and 54, were collected in February, March, April, May and July 2003 and stored no more than 20 d in plastic bags in darkness at 4 ºC until use. The day before grafting, branches were kept under running tap water overnight, and disinfected according to Ordás et al. [10]. Micrografting material was kept in antioxidant solution made of 100 mg L^{-1} ascorbic acid and 150 mg L^{-1} citric acid to prevent phenolic oxidation [10].

Micrografting procedure was carried out as follows. Whole brachyblasts in arrested growth (developed during the 2002 growth season) were dissected under aseptic conditions and cut leaving a 1 cm long basal part. After the removal of the sheath of the needle a cut was made to excise the brachyblast, and then a V-shaped cut of 2 mm orthogonal to the plane of the needle was performed (Fig. 1a). We used needles instead of isolated fascicle meristems because they are easier to manipulate and to avoid bud dehydration while having a sufficient level of miniaturization [1]. All manipulations were handled over sterile filter paper moistened with the antioxidating solution referred before with the aid of a physician magnifying glass. Seedlings were decapitated 1–2 mm below the insertion of the cotyledons and a 2–3 mm longitudinal cut was made. Scions were inserted into the split and the edges of the hypocotyl were held together with a ring made of 1 mm wall chromatography tubing (Fig. 1a). The cellulosic plug used as physical support allowed an easier manipulation without damaging rootstock, which has been proven to be detrimental according to Monteuuis [9]. The use of agar blocks to prevent rapid and intense dehydration of the scion [5] was also tested but no significant improvements were obtained (data not presented).

Micrografted needles were cultured in the same conditions as for germination. A completely randomised design with subsampling was applied using a minimum of 15 explants per clone and experiment. Data account for the total of five experiments carried out in February, March, April, May and July 2003. Data were collected 30 days after grafting, and the percentages of contamination, established and developed grafts were calculated. We define establishment as micrografts that survive after 30 days of culture and developed micrografts as those that exhibit a visible needle fascicle bud outgrowth. Contaminated explants were discarded and excluded from further analysis. Fungal contamination appeared in 40% of the tubes and was located mainly in the scions. Quantitative data (number of established/developed grafts) were analysed using Kruskall-Wallis H test for n independent group analysis and Mann-Whitney U test for 2 independent pairs. All statistical analyses were performed at the 5% level using the STATISTICA® software.

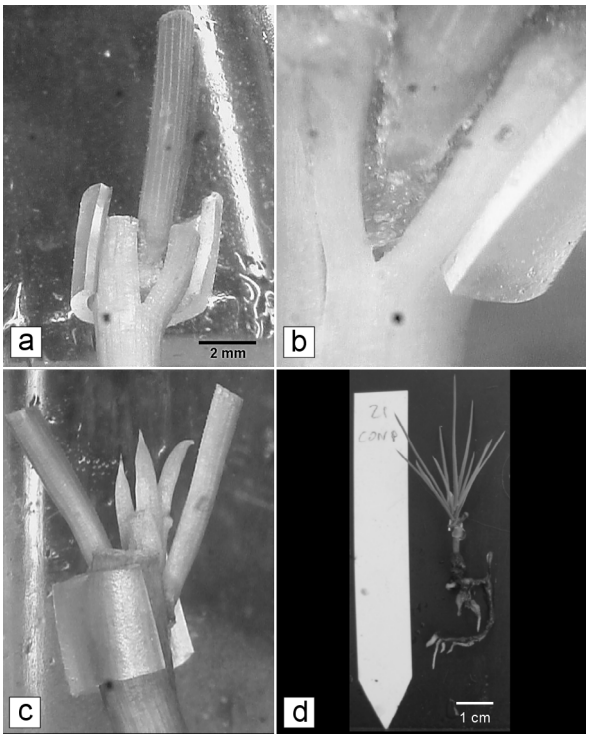

Figure 1. Different stages in *P. pinea* micrograft development: (a) one week old micrograft, (b) intermediate callus of a two weeks old micrograft, (c) three weeks old developed bud, (d) five months old grafted plantlet.

Established micrografts showed a visible intermediate callus within the first week of culture (Fig. 1a). Cells start to proliferate in the rootstock and then in the scion. Callus bridge stops to proliferate in 10–15 days, as soon as the graft cleft is filled (Fig. 1b). Fascicular bud development can be seen under the microscope in the second week and becomes clearly visible after three weeks (Fig. 1c). After four weeks no new developed grafts appear, although established scions remained alive until they were discarded (six months). Six weeks after the graft was made the culture medium was replenished by adding 2 mL of medium under aseptic conditions. For acclimatisation experiments at least 15 micrografts per tested month of collection were transferred to a sterile peat-perlite (1:4 v/v) mixture eight to ten weeks after micrografting and grown under decreasing initially high humidity allowing its transfer to normal humidity conditions one week later. Survival of micrografts after two months in nursery reached 98%. No plagiotropic growth was observed in micrografted plants at this point and rootstock has a well developed root system capable of sustaining further shoot outgrowth (Fig. 1d).

Average developmental success (out of contaminations) of this protocol is 43%, accounting for the 5 clones assayed, achieving a 61% with clone 15 (Tab. I). Clones behave statistically different both in establishment and development (Tab. I). It is noticeable that development is highly clone dependent, while establishment is more homogeneous. We have found a seasonal effect on

Table I. Rates of *P. pinea* micrograft establishment and development in the five clones assayed after four weeks of culture.

Clone	15		21		24		36		54		Total		
	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	*p*-value[a]
Established/total	51/59	86	49/67	73	50/73	68	44/64	69	30/55	55	224/318	70	0.0372
Developed/total	36/59	61	33/67	49	19/73	26	32/64	50	19/55	35	139/318	43	0.0005
Developed/established	36/51	71	33/49	67	19/50	38	32/44	73	19/30	63	139/224	62	0.0015

[a] Kruskall-Wallis H test *p*-value.

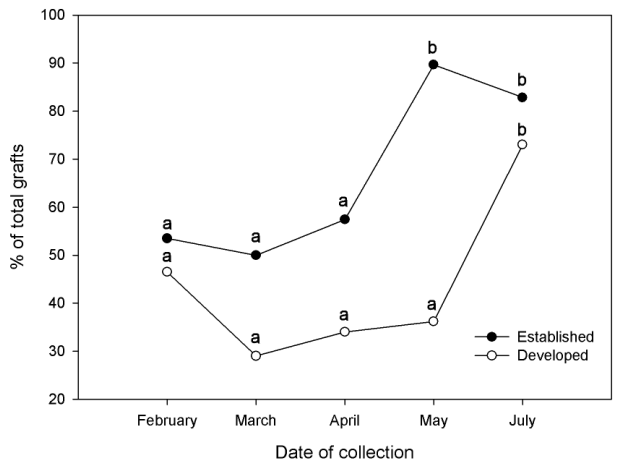

Figure 2. Effect of date of collection in *P. pinea* micrografting. Different letters indicate significant differences with Mann-Withney U test at $\alpha = 0.05$.

development (Fig. 2). This should be considered carefully, it could be due to physiological differences or, more likely, to the little optimisations of the procedure and acquired dexterity of manipulator. Even though success may vary from laboratory to laboratory notwithstanding genotype influence, this technique has proven to be efficient with all the material tested. Although there is a great variability in micrografting methods our technique showed a higher efficiency than the reported values in *P. radiata* [4], possibly due to the different rootstock used, since juvenile seedlings are more suitable for grafting than older or unrooted material as reported by Monteuuis [9]. It is noticeable that using fascicular buds, the present protocol gives similar results to those obtained with apical meristems in other conifers such as *Larix* [3] and *Picea* [9]. The use of needles instead of apical buds is more practical as they are more abundant and easier to manipulate.

While micrografting is not practical on a large scale for a majority of species [2], it can be very useful in stone pine breeding programs, especially for establishing field trails and seed orchards. In this context our approach can be used to evaluate scion-rootstock interaction easily by using half sibling seeds. Traditional grafting of stone pine is done by skilled workers (about 120 grafts/day) and can be performed only during few weeks in spring, limited by growth phenology of rootstocks and scions. Although micrografting is made at lower rate (50 grafts/day), it has proven effective before, during and after spring flush (Fig. 2), overcoming these seasonal limitations and thus having a potentially greater output. Actually, the main problem of traditional tip grafting consists in the low success rate combined with this temporal limitation. The process implies also a delay of several years: rootstocks are normally several years old, nursery-grown seedlings, and grafting in open-air condition may imply less than 40% of successful grafts (adverse meteorological post-grafting conditions can produce even 100% failures (Sven Mutke, personal communication)). In vitro micrografting of a selected tree can be made in a faster way reducing traditional costs by saving nursery space and reducing scion cost, since each twig can provide 20 to 40 micrografting suitable needles. Ex vitro acclimated plants are to be tested to determine its real agronomical value. To our knowledge this is the first report on in vitro grafting method for *Pinus pinea*.

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REFERENCES

[1] Bonga J.M., von Aderkas P., Rejuvenation of tissues from mature conifers and its implications for propagation in vitro, in: Ahuja M.R., Libby W.J. (Eds.), Clonal Forestry, Vol. 1, Springer-Verlag, Berlin Heidelberg New York, 1993, pp. 182–200.

[2] Bonga J.M., von Aderkas P., Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment, Tree Physiol. 20 (2000) 921–928.

[3] Ewald D., Kretzschmar U., The influence of micrografting in vitro on tissue culture behavior and vegetative propagation of old European larch trees, Plant Cell Tissue Organ Cult. 44 (1996) 249–252.

[4] Fraga M., Cañal M.J., Aragonés A., Rodríguez R., Factors involved in *Pinus radiata* D. Don. micrografting, Ann. For. Sci. 59 (2002) 155–161.

[5] Jonard R., Micrografting and its applications to tree improvement, in: Bajaj Y.P.S. (Ed.), Biotechnology in agriculture and forestry, Vol. 1, Springer-Verlag, Berlin Heidelberg New York, 1986, pp. 31–48.

[6] Humara J.M., Marín M.S., Parra F., Ordás R.J., Improved efficiency of *uidA* gene transfer in stone pine (*Pinus pinea*) cotyledons using a modified binary vector, Can. J. For. Res. 29 (1999) 1627–1632.

[7] Lloyd G.B., McCown B.H., Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture, Proc. Inter. Plant Prop. Soc. 30 (1980) 421–427.

[8] Mutke S., Gordo J., Gil L., The stone pine breeding program in Castile-Leon (central Spain), FAO-CIHEAM Nucis Newsletter, 9 (2000) 50–55.

[9] Monteuuis O., Effect of technique and darkness on the success of meristem micrografting of *Picea abies*, Silvae Genet. 43 (1994) 91–95.

[10] Ordás R.J., Tavazza R., Ancora G., In Vitro morphogenesis in the globe artichoke (*Cynara scolymus* L.), Plant Sci. 71 (1990) 233–237.