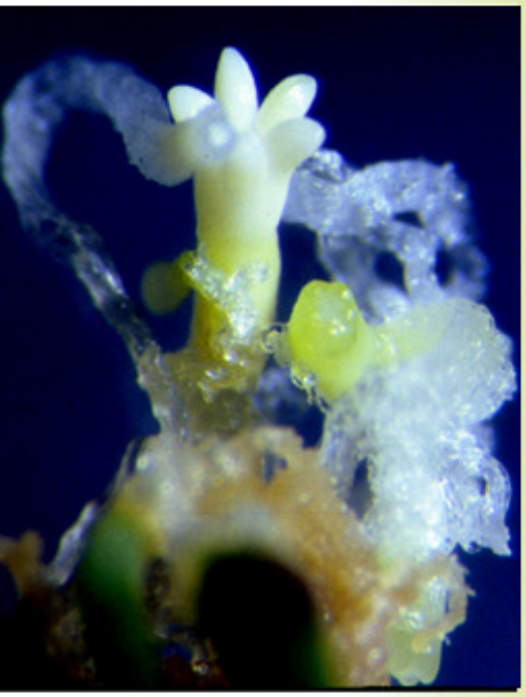
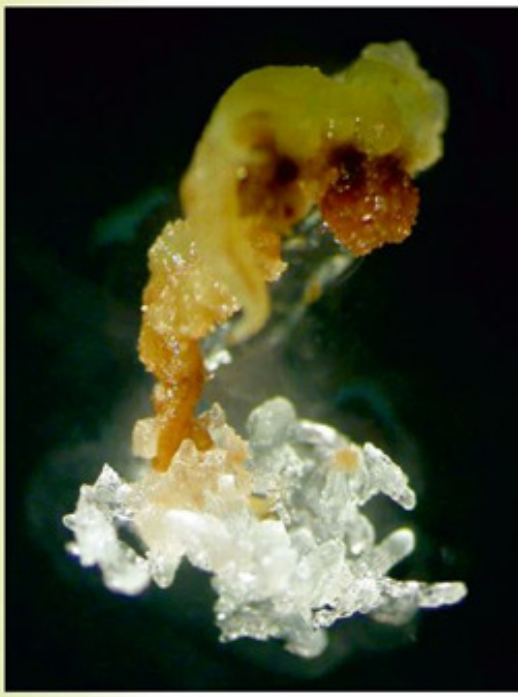
IUFRO

Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management





Proceedings

Second International Conference of the IUFRO Working Party 2.09.02

June 25 - 28, 2012 • Brno, Czech Republic

IUFRO WORKING PARTY 2.09.02
SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

Proceedings of the IUFRO Working Party 2.09.02 conference
"Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management"
June 25-28, 2012. Brno, Czech Republic

Micropropagation of a recalcitrant pine (*Pinus pinea* L.): An overview of the effects of ectomycorrhizal inoculation

C. Ragonezi[1,4], A. T. Caldeira[2, 3,7], M. do Rosário Martins[2, 4,7], D. Teixeira[2, 4,7], L. S. Dias[5], O. Miralto[4, 5], E. Ganhão[5], K. Klimaszewska[6], A. Zavattieri[4, 5]

[1]*Plant Breeding and Biotechnology Laboratory, ICAAM, University of Évora, Largo dos Colegiais nº 2, 7002-554 Évora, Portugal;*
[2]*Department of Chemistry, University of Évora, Largo dos Colegiais nº 2, 7002-554 Évora, Portugal ;*
[3]*CQE, University of Évora, St. Romão Ramalho nº59, 7000-671 Évora, Portugal;*
[4]*Institute of Mediterranean Agricultural and Environmental Sciences (ICAAM), University of Évora, Portugal;*
[5]*Department of Biology, University of Évora, Largo dos Colegiais nº 2, 7002-554 Évora, Portugal;*
[6]*Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, 1055 du P.E.P.S., P. O. Box 10380, Stn. Sainte-Foy, Québec, QC G1V 4C7, Canada;*
[7]*Hercules Laboratory, University of Évora, Palácio do Vimioso, Largo Marquês do Marialva, 8, 7000-809 Évora, Portugal*
Corresponding author: Carla Ragonezi email: cazi04@yahoo.com.br

Keywords: acclimatization, co-culture, ectomycorrhiza, *in vitro* adventitious rooting, micropropagation, stone pine.

Stone pine (*Pinus pinea* L.) is an economically important forest species in some regions of the Iberian Peninsula. Portugal and Spain have nearly 500,000 ha of stone pine stands, representing 85% of the worldwide distribution. The trees are exploited mainly for their wood, resin and pinion. The first two products have diminished in importance over the years but there is an increased demand for the pinion in the food industry. In addition to its enormous profitability as a seed producer, stone pine plays a positive role in soil protection, dune fixation and also as a pioneer species particularly in cork and holm oak degraded ecosystems. At present, the stone pine plantations are a major source of income for forestry holdings. The investments and research programs have targeted breeding, reforestation, forest management and harvesting. In 1988, the Portuguese National Forestry Station initiated a breeding program whose main objective was to genetically improve the quality and quantity of pinion (Barreira and Alpuim, 1988). Since then, various actions were initiated within integrated R&D projects such as PAMAF 2090 "Improvement of *Pinus pinea* L. for the production of edible seeds in Southern Portugal" and PIDDAC 212 "Improvement of *Pinus pinea* L. for pinion production". At an early stage of implementation of these breeding programs Provenance Regions were first delimited (Carneiro et al., 1998). These regions served for the basic identification and selection of reproductive material in accordance with seed certification guidelines. Simultaneously, selected stands of good producers of pinion were subsequently registered in the National Catalogue of base Materials (CNMB: http://www.dgrf.min-agricultura. pt).

The maternal inheritance of desirable characteristics such as cone weight, number of seeds per cone and seed length is considerably high in stone pine, thus encouraging the selection of seeds from selected trees (Alpuim, 1994; Carneiro, 2002; Evaristo et al., 2008). Two different approaches were studied under the PAMAF 2090 project for multiplication of the selected material: grafting and micropropagation. Grafting is arduous and generates high variability due to scion-rootstock interaction that varies production levels and, therefore, is not suitable for large-scale multiplication of elite cultivars. Micropropagation has been shown to be feasible in other conifer species and therefore in 1997 micropropagation studies funded by PAMAF 2090 began in The Breeding and Biotechnology Laboratory of ICAAM (LMBT), University of Évora (partner in the project). For a decade, the research group at ICAAM tried to enhance all phases of the stone pine micropropagation especially adventitious rooting of *in vitro* produced shoots and acclimatization. During this time, continuous increments in the multiplication rate and rooting frequency were achieved by varying the culture medium composition and physical conditions. Significant advance

was finally obtained by testing different combinations of carbon source, light and temperature during the induction and expression phases of the adventitious root formation (Zavattieri et al., 2009). With the new combination of chemical and physical factors it was possible to increase the rooting percentage from 30 to over 75 in several tested clones. Despite these promising results, the growth of adventitious roots could not be sustained rendering the acclimatization of plants either difficult or impossible. In a parallel research project, the group at the LMBT demonstrated that some fungi were beneficial in overcoming the root growth cessation when co-cultured *in vitro*. From a random sample of 12 fungi derived from soil samples of a pine stand from Mata de Valverde (Alcácer do Sal, Southern Portugal) at least nine were repeatedly capable of enhancing root growth (Oliveira et al., 2003). These results prompted a new interesting study on the effect of beneficial microorganisms in overcoming the problems with adventitious rooting and acclimatization in stone pine.

In 2007 the Portuguese Science and Technology Foundation approved and financed the project PTDC/AGR-CFL/71437/2006 "***Analysis and mastering*** of root growth signalling by ectomycorrhizal fungi in *Pinus pinea* L." coordinated by the LMBV and with the scientific advice of Dr. Krystyna Klimaszewska (Canadian Forest Service) to advance the biotization research between stone pine and ectomycorrhizal fungi. First results of *in vitro* biotization showed improvement in various root parameters during *in vitro* co-culture of *Pinus pinea* with *Pisolithus arhizus* before physical contact occurred (Fig. 1). Significant differences were found in the number of branches, in the number of roots plus branches, in total length of roots, in total length of roots plus branches, in average root length and in the length of the longest root in inoculated plants compared with non-inoculated plants (Table 1). The roots of inoculated plants also grew better in vermiculite and during acclimatization in mixed substrates compared with control plants resulting in the development of a vigorous root system. Overall, mycorrhizal inoculation increased the survival rate of the regenerated plantlets during acclimatization (Ragonezi et al., 2012). To confirm that the plants inoculated *in vitro* were actually mycorrhized; histological and microscopic observations of the roots were carried out after one month of acclimatization in sterile vermiculite (Fig. 2).

Table 1. *Means ± standard errors of variables with significant differences between control and inoculated plants (Ragonezi et al., 2012). Differences were investigated separately for each stage by exact or approximate Student's t tests after checking for homocedasticity and at the probability level P=0.05. Sample size in control was n=10 except in vitro where n=13; in inoculated plants always n=13.*

Growth stage	Variable	Control	Inoculated	P
In vitro	Change in number of branches	0 ± 0	1.4 ± 0.6	0.018
	Change in number of roots plus branches	0 ± 0	1.4 ± 0.6	0.018
Vermiculite	Change in total length of roots (mm)	3.8 ± 3.1	29.2 ± 7.2	0.003
	Change in total length of roots plus branches (mm)	2.8 ± 4.2	32.3 ± 7.1	0.002
	Change in average root length (mm)	0.9 ± 1.2	9.7 ± 3.4	0.014
	Change in length of the longest root (mm)	1.0 ± 2.0	14.7 ± 3.7	0.002
Mixed substrate, 2nd measurement	Change in number of roots	-1.0 ± 0.3	-0.1 ± 0.1	0.001
	Change in number of roots plus branches	-1.0 ± 0.3	-0.1 ± 0.1	0.001
	Change in total length of roots (mm)	-4.2 ± 8.8	17.4 ± 7.6	0.038

Fig. 1 *In vitro biotization in double phase medium composed of two solid phases (upper thin solid phase for fungi; lower solid phase modified WPM for root growth development) during in vitro co-culture of Pinus pinea with Pisolithus arhizus (Ragonezi et al., 2012).*

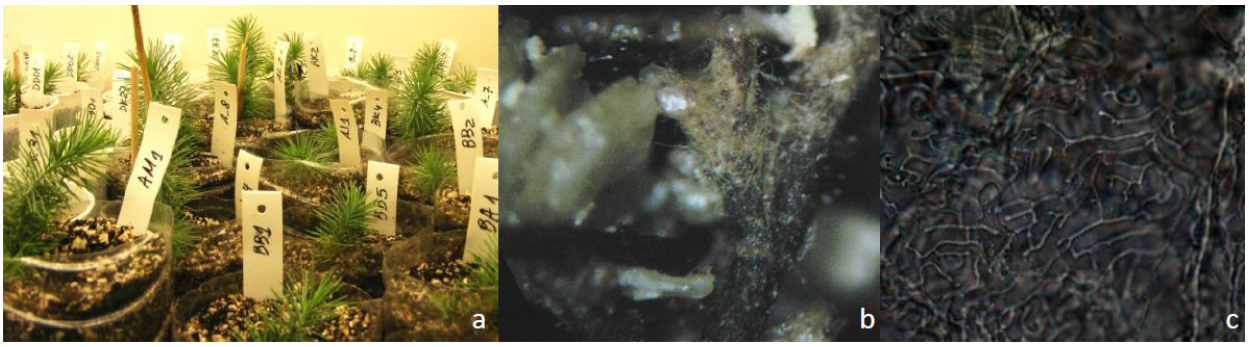


Fig. 2. *2a Inoculated Pinus pinea plants during acclimatization in mixed substrates; 2b Ectomycorrhiza derived from inoculated plants with Pisolithus arhizus, covered with brownish mycelia; 2c Transversal section of the root showing the Hartig net, optical microscopy observation 1250X magnification.*

To better understand the positive effects of fungal inoculation on root growth parameters before the establishment of physical contact between these partners, the biochemical compounds released by the roots were analysed. The analysis was facilitated by developing a co-culture system of stone pine plantlets and the fungus in a two-phase semi-solid/liquid medium (now under provisional patent No. 105239 of the National Institute of Industrial Property, INPI (Fig. 3).

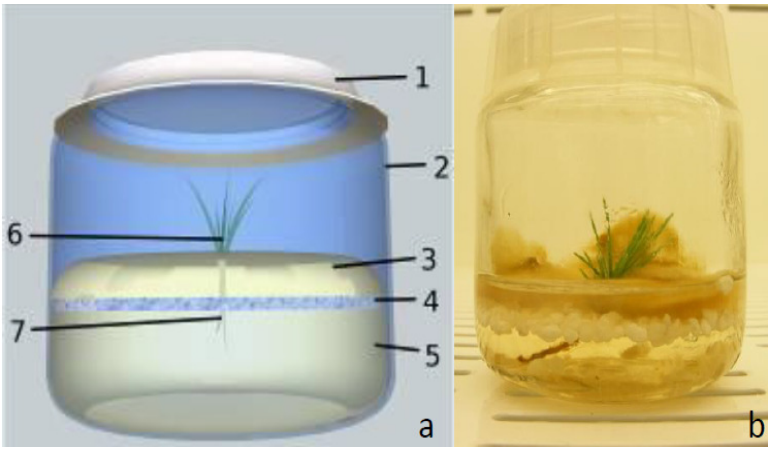


Fig. 3 *3a 1 - Cover, 2 - Flask, 3 and 4 - WPMS (Woody Plant Medium - Lloyd and McCown, 1981 - solid phase - with perlite facing down - 4), 5 - WPML (liquid phase), 6 - Microshoot, 7 - Root system (Provisional Patent INPI Nº 105239). 3b Double phase semi-solid/liquid medium co-culture system between Pinus pinea plantlets and the ectomycorrhizal fungus Pisolithus arhizus (Ragonezi et al. submitted).*

References

Alpuim M, Rocha ME (1994) Breve estudo biométrico sobre as características da pinha e semente de *Pinus pinea* L. In *Actas do III Congresso Florestal Nacional*, Figueira da Foz.

Barreira LL, Alpuim M (1988) Contribuição para um programa de melhoramento de pinheiro manso. *In Actas do "Encontro sobre Pinheiro manso"*. SPCF, Alcácer do Sal, 25-26 de Novembro.

Carneiro MM, Alpuim M, Rocha ME, Carvalho MAV (1998) Delimitação e caracterização de regiões de proveniência de *Pinus pinea* L. Silva Lusitana 6(2): 129-263.

Carneiro A (2002) Avaliação da variabilidade e controlo genético de características biométricas das pinhas e da semente da *Pinus pinea* L. Relatório de fim de curso de Engenharia Florestal. Universidade Técnica de Lisboa - Instituto Superior de Agronomia, p. 58.

Evaristo I, Tenreiro R, Costa R (2008) Characterisation of Biometric Parameters and Fatty Acids Content of *Pinus pinea* L. Pine Nuts of Portuguese Populations. Silva Lusitana [online]. 16(1):. 1-19.

Lloyd G, McCown, B (1981) Commercially feasible micropropagation of mountain laurel, Kalmia latifolia, by the use of shoot tip culture. Proc Plant Prop Soc 30:421-427.

Oliveira P, Barriga J, Cavaleiro C, Peixe A, Zavattieri Potes A (2003) Sustained *in vitro* root development obtained in *Pinus pinea* inoculated with ectomycorrhizal fungi. Forestry 76: 579-587.

Ragonezi C, Caldeira AT, Martins MR, Dias LS, Santos-silva C, Ganhão E, Miralto O, Pereira I, Louro R, Klimaszewska K, Zavattieri MA (2012) *Pisolithus arhizus* (Scop.) Rauschert improves growth of adventitious roots and acclimatization on *in vitro* regenerated plantlets of *Pinus pinea*. Prop. Orn. Plants 12:139-147.

Ragonezi C, Teixeira D, Caldeira AT, Martins MR, Santos-Silva C, Ganhão E, Klimaszewska K, Zavattieri A (2012) O-coumaric acid ester, a potential signaling molecule detected during early *in vitro* co-culture between *Pinus pinea* plantlets and the ectomycorrhizal fungus *Pisolithus arhizus*. Symbiosis (submitted).

Zavattieri A, Lima M, Sobral V, Oliveira P, Costa A (2009) Effects of carbon source, carbon concentration and culture conditions on in vitro rooting of *Pinus pinea* L. microshoots. Acta Hortic. 812: 173-180.