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Selection and identification of Spanish elite clones for Mediterranean pine nut as orchard crop

S. Mutke[1,2,*], C. Guadaño[3], S. Iglesias[4], D. Leon[4], S. Arribas[4], J. Gordo[5], L. Gil[6] and G. Montero[1,2]

[1]INIA-CIFOR, Forest Research Centre, Ctra. La Coruña, km 7.5, 28040 Madrid (Spain)
[2]iuFOR, Sustainable Forest Management Research Institute UVa-INIA (Spain)
[3]TRAGSA. CNRGF Puerta de Hierro, Ctra. La Coruña, km 7.5, 28040 Madrid (Spain)
[4]MAPAMA, DG Rural Developm. and Forest Policy, Gran Vía de San Francisco, 4, 28005 Madrid (Spain)
[5]Junta de Castilla y León, Duque de la Victoria, 8, 47001 Valladolid (Spain)
[6]UPM, Technical University of Madrid, Forestry College, Ciudad Universitaria s/n, 28040 Madrid (Spain)
*e-mail: mutke@inia.es

Abstract. Since 19th century, Mediterranean stone pine area has been expanding to nearly one million hectares. Half of this increase has been on private lands in the last decades, especially in Portugal and western Turkey, where its current extension is 176,000 and 195,000 ha, respectively fourfold the original areas. For establishing profitable stone pine plantations as Mediterranean pine nut crop, grafting improved genetic materials is of major interest. Since 1990, several hundred Spanish clones have been evaluated in grafted common garden trials. They had been selected for superior cone production by plus tree prospection in forests. Analyses of cone yield series in the common trials have allowed ranking these pre-selected clones. However, the legal admission of clones in the National Register of basic materials for the production of reproductive material certified as ‘qualified’ or ‘tested’ requires individual identifiability by distinctive characters. In 2015, the Spanish Ministry for Agriculture, Food and Environment has released the first 15 elite clones individually characterised by molecular markers. Field trial data have allowed estimating genetic gain in 10-30% over average cone yield. This catalogue of basic materials has been a first step in building a supply chain, followed by the establishment of mother plant orchards for certified ‘tested’ or ‘qualified’ scion supply to commercial nurseries and plantations. As next step, external quality standards must be defined for grafted plants obtained in nursery.

Keywords. *Pinus pinea* – Forest genetic materials – Grafting – Elite clones.

I – Introduction

Plantations of Mediterranean stone pine, *Pinus pinea* L., for soil protection and forest restoration, as well as for producing Mediterranean pine nuts, have expanded threefold the area occupied by the species in the Mediterranean area, exceeding currently 960,000 hectares. Half of this increase has been done by private landowners in the last 40 years, especially in Portugal and western Turkey, where maritime influence with annual rainfalls exceeding 500 mm favours cone productivity (Loewe-Muñoz *et al*., 2016). The current and ongoing expansion of stone pine in both countries is already more than fourfold the original area to 176,000 and 195,000 hectares, respectively (ICNF, 2013; Kilci *et al*., 2014; Santos, 2015; Can, 2016). Due to the excellent world market expectation for pine nut kernel, one of the world’s most expensive nuts, for instance Portuguese forest owners have invested in the last fifteen years more than 40 million euros in stone pine plantations and forests (Calado, 2014).

In Spain, expansion of stone pine area has been nearly threefold during 20th century to 490,000 ha, but afforestation with this species aimed mainly at soil protection and forest restoration on wasteland, barren slopes and dunes. Until the European Common Agricultural Policies reform in 1992 favouring farmland set-aside, in Spain stone pine plantation on arable land had been minor, due to less favourable climate that limits revenue expectations from cone yields. Therefore in Spain, virtually all cones are harvested still from pine forests, not in orchards. Anyway, little efforts have been made for its proper domestication as a nut crop. No defined cultivars are known, an absence maybe related to the extremely low genetic diversity found in the species (Prada *et al*., 1997; Vendramin *et al*., 2008; Mutke *et al*., 2012).

Cultivation of stone pine as agroforestry system or orchard crop, including grafted plantations, allows the forest owners getting revenues from annual cone yields even on lands not adequate for most agricultural crops. Moreover, resistance of stone pine to the pine wilt nematode *Bursaphelenchus xylophilus*, one of the most dangerous pest that has been provoking decay of maritime pine *P. pinaster*, facilitates the restauration of affected pinewoods in Portugal by changing the main species to stone pine (Nunes-da-Silva *et al*., 2015; Zas *et al*., 2015).

For establishing profitable plantations as pine nut crop, reproduction of grafted plants is of major interest for allowing massive propagation of genetic improved material, aiming toward domestication of this forest tree (Mutke *et al*., 2013). The standardised use of grafted trees requires the implementation of a legal, technical and commercial framework for supplying legally admitted forest reproductive materials under strict quality standards. The present contribution exposes the state of the art in Spain for establishing grafted *Pinus pinea* orchards.

II – Clone selection in grafted comparative field trials

Since the early nineties, several Spanish regional and national programmes for selecting outstanding stone pines have been developed by forest administrations for enhancing cone production (Abellanas *et al*., 1997; Iglesias 1997; Mutke *et al*., 2000). Within this framework, several grafted comparative clonal trials have been established for Mediterranean stone pine in Spain, evaluating the performance of candidate clones (Table 1). The ortets of these candidate clones had been selected as plus trees in pine forests, by phenotypic traits, namely their outstanding cone yield and good environmental adaption (Gordo, 2004). Comparative trials targeted for characterization of the cone yield of each clone, in quantity and quality, in common garden conditions. The relevance of genetics *vs* environment factors for seed-yield, quantity and quality was evaluated, for instance the clonal degree of genetic determination H^2 and the expected genetic gain for individual clones (Mutke *et al*., 2003b, 2005a, 2007a).

Table 1. Test sites of comparative clonal trials for *Pinus pinea* in Spain

Code	Municipality (province)	Mean annual temperature [°C]	Mean annual rainfall [mm]	Nº of clones tested (since)
B23PH	Madrid (Madrid)	14.2	440	331 (1991)
B23MN1	Quintanilla de O. (Valladolid)	10.1	508	98 (1991)
B23MN2	Tordesillas (Valladolid)	12.3	450	66 (1998)
EC-1	Almonte (Huelva)	16.2	642	120 (1990)
EC-2	Villaviciosa de C. (Córdoba)	15.7	745	120 (1990)
EC-3	Aroche (Huelva)	15.0	792	120 (1993)
EC-4	Arcos de la Frontera (CA)	17.8	740	120 (1993)

Comparison of estimated clonal values among test sites allows also estimating genotype by environment interactions, that is, the relative performance of evaluated genotypes (clones) in different agro-climatic zones.

Recorded data from grafted replicates of each clone for growth and cone yields in the trials, as well as cone and seed characterisation, have allowed ranking productively the pre-selected genotypes. Results of evaluated trials (Table 1) allowed estimating the degree of genetic determination for the annual average cone yield (kg/tree) between 15-38%, resulting in an expected genetic gain of 12-39% if selecting the top 10% of the tested genotypes from each provenance. The genetic correlations between cone yield and cone or seed size were always positive (r = 0.17-0.47), hence no trade-off between crop quantity and quality was observed (Mutke *et al*., 2005a; Mutke *et al*., 2007b; Guadaño and Mutke, 2016).

Comparing the performance of each clone in different comparative trials, the genotype by environment interactions were found to be significant, with some clones performing well at one test site, but only average at others, and *vice versa*. This result implies that the network of trials must be extended into different agro-climatic zones for a neater characterisation of each genetic material (Guadaño and Mutke, 2016).

III – Distinctness and identification of clones by molecular markers

One of the mandatory requirements for the inclusion of clones in National Register of Basic Materials for producing Forest Reproductive Materials is the identifiability of each clone by distinctive characters approved and registered with the official body, following the *Council Directive 1999/105/EC on the marketing of forest reproductive material, Annex IV*. Those distinctive characters can be morphological, phenological or molecular markers, and are required for allowing future post-hoc identification of marketed planting stocks as traceability control of providers and traders.

Nevertheless, in case of stone pine, morphological, phenological and molecular diversity has been found extremely low (Mutke *et al*., 2003a, 2005b, 2010, 2012; Vendramin *et al*., 2008). Only recently, a set of molecular markers, six polymorphic nuclear microsatellites (nSSRs) (Pinzauti *et al*., 2012), has allowed profiling the elite clones selected in the Spanish programmes.

Table 2. Estimated genetic gain for registered stone pine clones over the average cone yield in the respective comparative trial (Guadaño and Mutke, 2016)

Clone	Name of Spanish unit of approval	Genetic gain
	'Qualified' Basic Materials (evaluated at only one test site)	
2004	CL-Q-23/Hoyo de Pinares	19%
2048	CL-Q-23/Almorox	19%
2068	CL-Q-23/San Martín de Valdeiglesias	24%
3029	CL-Q-23/El Provencio	31%
3048	CL-Q-23/Pozoamargo	21%
3057	CL-Q-23/Casas de Haro	23%
3063	CL-Q-23/El Picazo	22%
6010	CL-Q-23/Santa Coloma de Farners	9%
6015	CL-Q-23/Llagostera	11%
6053	CL-Q-23/Dosrius	9%
	'Tested' Basic Materials(evaluated at 2-3 test sites)	
1011	CL-C-23/Portillo-11	+25-27%
1012	CL-C-23/Portillo-12	+12-29%
1073	CL-C-23/La Vega	+12-17%
1123	CL-C-23/Íscar	+11-20%
1201	CL-C-23/Valdegalindo	+15-18%

50 Spanish stone pine clones had been singled out for outstanding cone production in the grafted comparative trials, and passed hence to be the next phase to be genotyped by molecular markers, following the protocol of Pinzauti *et al*. (2012) that combines six nSSRs.

Nevertheless, the six markers used allowed only a distinctive characterisation for some of these clones, while other, even better performing clones didn´t show an own distinct genetic profile in these six markersa and in consequence could not be admitted legally as basic materials.

In 2015, the first fifteen Spanish elite clones have been released and registered officially in the National Catalogue of Basic Materials for *Pinus pinea* by the *Resolución de 21 de abril de 2015 de la Dirección General de Desarrollo Rural y Política Forestal* (B.O.E. 13/05/2015) and *Resolución de 3 de diciembre de 2015 de la Dirección General de Desarrollo Rural y Política Forestal* (B.O.E. 19/12/ 2015) (Table 2).

IV – Conclusions

The approval of the first stone pine clones in the Spanish National Register of Basic Materials will allow marketing their scions and grafted plants as *qualified* and *tested* forest reproductive materials under European regulation (*Council Directive 1999/105/EC on the marketing of forest reproductive material*, whose Annex I includes *Pinus pinea)*.

However, the availability of elite clones as registered basic materials is only the first step in the supply chain for planting stock. The next step is the establishment of officially admitted mother plant hedges to supply scions to commercial nurseries and plantations, followed by the definition of commercial standards for external quality of planting stocks, such as scions, rootstocks and grafted plants. Another pending research line is the phenotypic characterisation of all clones in different agroclimatic zones.

In a recently published monograph, the authors of the present contribution have drawn the balance of their professional experiences gained by their implication in genetic improvement programmes for stone pine in Spain over last 25 years (Guadaño and Mutke, 2016).

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