REPRODUCTIVE BEHAVIOUR AND CLONAL STUMP/ROOT PROPAGATION AND CONSEQUENCES FOR SUSTAINABLE GENETIC VARIABILITY IN CORK OAK AND HOLM OAK IN PORTUGAL

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Reproductive studies at cork oak and holm oak in Portugal aim to evaluate the effective size (Ne), flowering synchronisation, chance of inter-specific hybridization and fluctuation of seed years for support of management of seed sources, genetic improvement programmes and conservation of genetic resources.
The consequences for genetic variability from clonal stump/root propagation are highlighted. Holm oak is by rule more precocious, more stable and predictable than cork oak. The reproductive capacity is site-quality and tree-vigour dependent. For cork oak at Quinta da Serra plot (good site) the Ne ranges from 40%-80% while at Água Ferrenha (poor site) it varies from 10%-20%. Years of odd weather open possibilities for flowering phenological changes and inter specific cross. Evaluation of the rate of extensive clonage coming from stump/root sprouting in two stands with decline and one stand that suffered a fire hazard in 2003. On strong fire situation more than 2/3 of the trees are from clonal origin. No acorn collection should take place on poor seed years since the Ne is likely to be low.
The number of clones for clonal seed orchards should be at least the double of the aimed Ne. Clonal propagation at cork oak and holm oak shall be used sporadically and scattered either in time and space. On stands where extensive clonal propagation is in place the levels of kinship should be assessed by means of molecular markers. If high, sexually originated plants shall be introduced. Molecular tools are needed to support flowering/fruiting prediction.

Keywords: *Quercus suber*, *Quercus ilex* L. subsp. *Rotundifolia*, flowering, fruiting, effective size Ne.
Parole chiave: *Quercus suber*, *Quercus ilex* L. subsp. *Rotundifolia*, fioritura, fruttificazione, dimensione effettiva Ne.

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1. Introduction

Cork oak (*Quercus suber* L.) and holm oak (*Quercus ilex* L. subsp. *Rotundifolia* Lam.) are the two most important evergreen oaks in Portugal. Sexual reproduction, i.e. seed production, is the main source of sustainable genetic variability which is essential to face climatic changes, new economic traits and resistance to pests and diseases. Patches of decline ongoing at both species require recovery interventions, in which the genetic variability of the FRM shall be considered as a key component. Both species have unisexual flowers although cork oak may produce rare hermaphrodite flowers from which there is no report of fruit production. Cork oak fruiting is mainly annual but biennial fruiting happens in some trees and in some years in a process still poorly understood (Corti, 1955). Holm oak only produces annual acorns. Flowering and fruiting observations aim to evaluate the population effective size (Ne), the flowering synchronisation, the possibilities of inter-specific hybridization and the occurrence of mast seed years for assessment of genetic variability in seed sources and conservation of genetic resources.

2. Material and methods

Flowering and fruiting studies at cork-oak and holm-oak have been performed at various plots in Portugal where trees were randomly choose. Number of trees at each plot and years of observation are summarised at Table 1. At the various plots flowering and fruit production was evaluated by degrees of 1, 2 and 3 (Varela *et al.*, 2008) upon the maximum possible production: degree 1- null or negligible production of male flowers or fruits; degree 2- about half of the productive part of the crown exhibits male flowers or fruits; degree 3- the tree exhibits male flowers or fruits on large quantity.
The effective size Ne empirically is estimated to male flowering and annual fruits production considering the percentage of trees that bear reproductive structures in degree ≥ 2.
In some plots trees were also characterised by the perimeter at breast height (PBH) and competition ranked into 4 categories from 0 –isolated tree, to a maximum of 4 when the tree is totally surrounded by other trees. Biennial fruiting was studied at Quinta da Serra in 2011-2014.

2.1 Quinta da Serra, Azeitão

The plot includes 25 trees that are under long run population genetics studies since 1993 (except 2005 and 2006, 2007 it was only possible to collect data for acorn production due to lack of funding) for reproductive behaviour at the level of male flowering/ acorn production ability and flowering phenology. 1993-2014. The stand is uneven age and the spacing among trees is quite irregular. Quinta da Serra site is extremely suitable for cork oak in soil quality and deepness as well as in climate.

2.2 Other plots

Profiting from occasional funding reproductive studies were also performed at other plots in Portugal including the plot Quinta da Mitra, Évora that is in a mixed stand of cork oak and holm oak, Table 1.

3. Results

3.1 Quinta da Serra, Azeitão

The level of male flowering/fruiting ability varies considerably among trees. In the group of trees that has high reproductive ability trees T19, T20 and T24 are rather irregular as patent on the standard deviation. T12 is till 2014 the tree of the lowest capacity for acorn production.

The quantity of male flowering and acorn production varies considerably among years confirming the irregular reproductive behaviour of cork oak. The high acorn production that took happened consecutively in the years 2008, 2009 and 2010 shows that so far it is not possible to establish a reproductive behaviour pattern for masting. Trees are also characterized using the mean and standard deviation of acorn production 1993-2014 (Fig. 2). Until 2011 tree 20 showed a good and predictable acorn production which led to to be selected as mother-tree to the 2014 program of controlled crosses in cork oak (Varela and GenoSuber Consortium, 2014). Unexpectedly in the flowering season of 2014 tree T20 showed null production of female flowering and consequently null acorn production. These results rate tree T20 as the most irregular (higher standard deviation) which confirms the irregular reproductive behaviour of cork oak also at the individual level. The reproductive ability was also correlated with crown competition and trunk perimeter at breast height and data showed that individual tree acorn production ability has no correlation with PBH, $R^2 = 0.1677$ or with competition, $R^2 = 0.1437$.

The knowledge of the reproductive behaviour of cork oak trees at Quinta da Serra was an important aspect on the selection of that forest for controlled crosses for genetic mapping for support of the *Quercus suber* genome sequencing project GENOSuber (Varela and GenoSuber Consortium, 2014).

3.2 Other plots

Male flowering production and acorn production in cork oak and holm oak is compared in the same year in several plots in Portugal using the plot mean and the effective size Ne empirically estimated by the number of trees producing degree ≥ 2 and also for the number of trees with degree 3, Table 2. The year of 1994 was a non-mast year at Quinta da Serra (Fig. 1) with values of Ne (2+3)% for male flowering and acorn production respectively 70.83% and 16.67%. which shows the low reproductive profile of the year was not predictable by male flowering. Both at Quinta da Serra and MN Vimeiro I could verify that any tree reached the degree 3 at acorn production.

The studies of 1998 and 1999 in cork-oak and holm-oak plots showed that holm-oak as an higher reproductive profile than cork-oak. The comparison between the good plot of Quinta da Serra and the declining plot of Água Ferrenha in 2011 showed that declining trees have a low reproductive profile. Cork-oak and holm-oak are inter-fertile species but the chances of natural hybridization are lowered by the asynchronous flowering pattern that normally occurs. However some holm-oak trees may have an extended flowering that allows hybridization as it was verified in the mixed plot of Quinta da Mitra (Varela *et al*., 2008). Male flowering of cork oak and holm oak was also compared in 100 aments in 10 trees by the length and number of flowers. Cork oak aments mean length is 4.6 cm with a mean number of flowers of 21.2. No correlation was found between the length and the number of flowers: $R^2 = 0.297$. Holm oak aments mean length is 5.2cm and the mean number of flowers is 25.1 and no correlation was found between the length and the number of flowers: $R^2 = 0.132$. Holm oak aments are longer and bear more flowers which may increase the chances of gene flow by pollen.

3.3 Biennial fruiting

Biennial fruiting (Fig. 3a, 3b) is rare at Quinta da Serra. On the observations carried from 2000 to 2014 biennial fruiting was relevant only in 2013 (Tab. 3). In 2014 the four trees - T1, T4, T13 and T20 - selected as female parents for controlled crosses to support the species genome sequencing project GENOSuber (Varela and GenoSuber Consortium, 2014) the trees T1 and T20 had relevant production of biennial fruits (Tab. 4) which may explain the null female flowering and acorn production in 2014. The Sharp AB (alternate bearing) 2013-2014 in these trees may be explained by high use of photoassimilates and/or flowering inhibitory hormone response.

3.4 Clonal propagation by stump and root sprouting

Stump and root sprouting happen spontaneously in all *Quercus* but in Nature it happens randomly mixed with sexual reproduction which has positive effects for adaptation and evolution as older genotypes coexist and cross with new genotypes coming from recombination. Stump and root sprouting may easily lead to the formation of several clones of the pre-existing tree that after some years are phenotypically indistinguishable. However when Human induced stump and root sprouting is overused to propagate interesting phenotypes and to make stand recovery after forest fires it may biasing the balance clonal versus seed recruitment with negative effects for genetic variability, adaptation and evolution. If over practice of cloning is suspected it is necessary to evaluate the genetic relationship of the trees using molecular markers.

4. Discussion

Cork oak has a very irregular reproductive behaviour with sharp differences on Effective Size (Ne) among mast and poor-seed years. The low Ne of poor-seed years leads to indicate that no seed harvesting should take place in those years. Whenever decline induces poor seed production and consequently risky levels of low Ne, reforestation recovering actions should consider seed recruitment on ecologic similar stands with higher Ne.

5. Perspectives

Management of seed supply: Seed harvesting for afforestation or recovery of populations in decline shall not take place in poor seeding years as the low Ne increases the probabilities of inbreeding. Clonal propagation by stump and root sprouting shall be blended with seed recruitment to assure sustainable genetic diversity along generations.
Population for conservation of genetic resources shall have an effecttive size ≥ 50. Small and marginal populations of high conservation value with low effective size shall be managed to increase Ne (Varela and Eriksson, 1995).
To deep the knowledge on the reproductive behaviour of cork oak there is the need to understand the molecular basis of flowering, annual and biennial fruiting. Models for holm-oak seed production are likely more easy to establish.

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Table 1. Observation plots and main characteristics in soil, climate, trees density, vigour and average production of male flowering and acorn.

plot	*species*	*years_observation*	*N. of trees*	*edapho climatic conditions*	*stand density*	*vigour*
Quinta da Serra, Azeitão	cork-oak	1993-2014	25	deep fertile soil; climatic conditions very suitable	medium to low	very good
MN Vimeiro, Alcobaça	cork-oak	1994; 1998	20	deep fertile soil; suitable climatic conditions	medium to low	very good
Quinta da Mitra, Évora	cork-oak / holm-oak	1997-2000	C-oak-39 H-oak-25	poor soil; long, hot and dry summers	medium	medium
Ermida Gerês	cork-oak	1993	20	deep fertile soil; suitable climatic conditions	high	good
Monte Morais, Macedo de Cavaleiros	cork-oak	1993	20	Serpentine soil, hot and dry summers, cold winters	medium	medium
Água Ferrenha, Grandola	cork-oak	2011-2013	30	poor and shallow soil; long, hot and dry summers	low	in decline

Table 2. Male flowering production and acorn production in several plots in Portugal are compared using the mean and the effective size Ne empirically estimated by the number of trees producing degree ≥ 2 (Ne (2+3)%) and also for the number of trees with degree 3 (Ne (3)%).

		Cork oak												*Holm oak*	
		Q. Serra		*MN Vimeiro*		*Q Mitra*		*Monte Morais*		*Ermida Gerês*		*Água Ferrenha*		*Q Mitra*	
		Fl ♂	*Fr ♀*	*Fl ♂*	*Fr ♀*	*Fl ♂*	*Fr ♀*	*Fl ♂*	*Fr ♀*	*Fl ♂*	*Fr ♀*	*Fl ♂*	*Fr ♀*	*Fl ♂*	*Fr ♀*
1993	mean	2,50	2,29					2,1	1,75	2,1	1,75				
	Ne (3)%	13,0	9,0					30,0	20,0	25	20,0				
	Ne (2+3)%	95,83	91,67					80,0	55,0	75,0	55,0				
1994	mean	2,21	1,17	2,20	0,95										
	Ne (3)%	50,0	0	50,0	0										
	Ne (2+3)%	70,83	16,67	75	25										
1998	mean	2,54	2,67	1,25	1,20	2,44	1,29							2,24	2,17
	Ne (3)%	75,00	79,17	20,0	5,0	22,0	2,0							8,0	11,0
	Ne (2+3)%	79,17	87,50	50	35	76,92	28,21							84,0	60,0

(Table 2. Continued)

1999	mean	2,04	1,38			1,74	1,00							2,28	2,28
	Ne (3)%	75,00	79,17			10,0	0							13,0	13,0
	Ne (2+3)%	66,67	25,0			41,03	0							76,0	76,0
2011	mean	1,96	1,25									1,53	0,45		
	Ne (3)%	33,3	0									30	0		
	Ne (2+3)%	62,5	25,0									40,0	10,0		

Table 3. Annual and biennial fruiting at Quinta da Serra plot in the years 2012, 2013 and 1014.

	2012		*2013*		*2014*	
	annual fruit	*biennial ♀*	*annual fruit*	*biennial ♀*	*annual fruit*	*biennial ♀*
T12	1	0	1	3	0	0
T20	1	0	3	3	0	0
T1	1	0	3	2	0	0
T7	3	0	3	2	0	0
T14	0	0	2	2	0	1
T16	0	0	2	2	0	0
T25	2	0	1	2	1	0
T19	0	0	2	1	1	0
T22	1	0	2	1	1	0
T4	3	0	3	1	2	0
T21	1	0	2	1	2	0
T23	1	0	2	1	2	0
T24	2	0	3	1	2	0
T18	2	0	2	1	3	0
T3	1	1	3	0	0	0
T6	1	0	2	0	0	0
T9	3	0	1	0	0	0
T2	3	0	3	0	1	0
T5	3	0	1	0	1	0
T8	1	1	1	0	1	0
T11	3	1	1	0	1	0
T15	2	0	3	0	1	0
T10	3	0	2	0	3	0
T13	3	0	2	0	3	0
T17	1	0	1	0	3	0

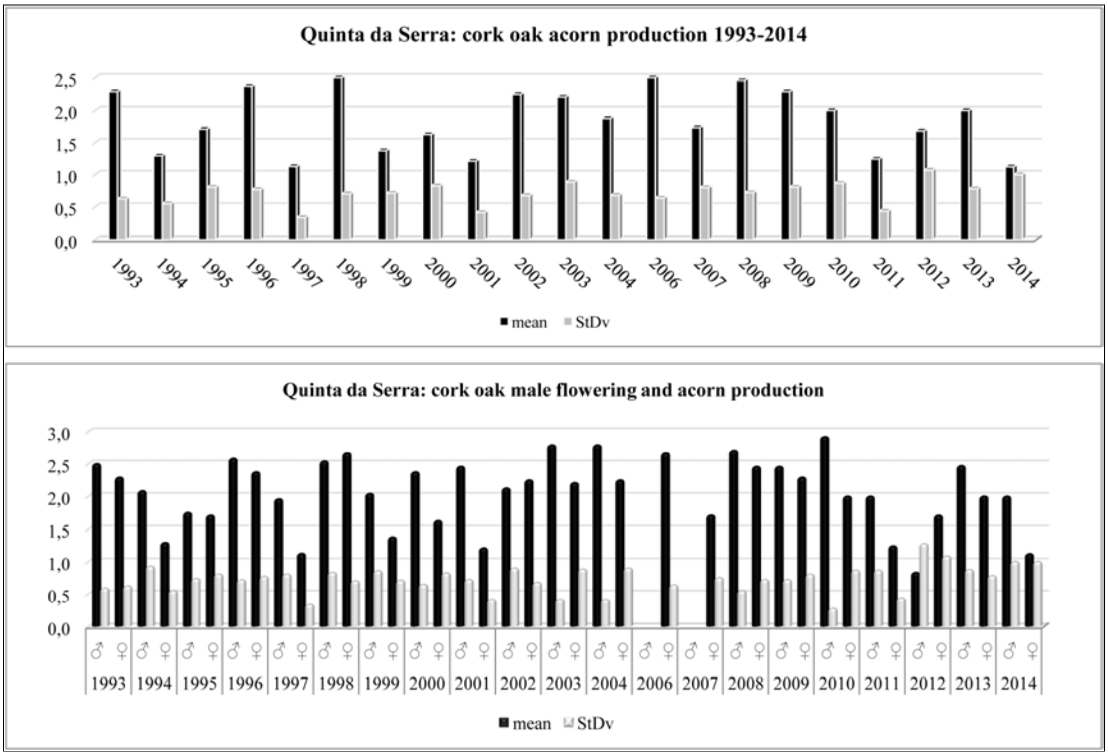

Figure 1 a, b. Male flowering and acorn production at the plot of Quinta da Serra from 1993-2014. Mast year: mean acorn production above 2; 1.5 to 2the year is rated medium, below 1.5 the year is rated non-mast. In 22 years of observation there were seven mast-years (1993, 1996, 1998, 2003, 2004, 2006, 2008, 2009); seven years were of medium acorn production 1995, 2000, 2002, 2007, 2010, 2012 and 2013. Eight years were non-mast. Masting happened in one third of the years but the data do not yet allow to establish a production cycle model of acorn production since masting could be observed in two consecutive years, 2008 and 2009, followed by a medium year 2010. Male flowering is by rule higher that acorn production except in 1998.

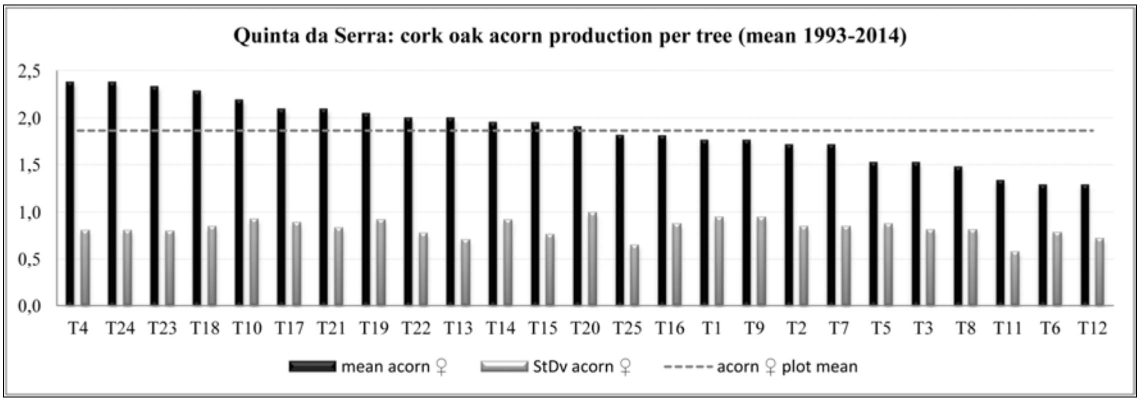

Figure 2. Acorn production per tree assessed by degree of production at Quinta da Serra, Azeitão from 1993-2014. These results rate tree T20 as the most irregular (higher standard deviation) though it is the tree with the greater PBH and isolated. T10 shows a very good fruiting capacity despite of competition degree of 2 and PBH under mean.

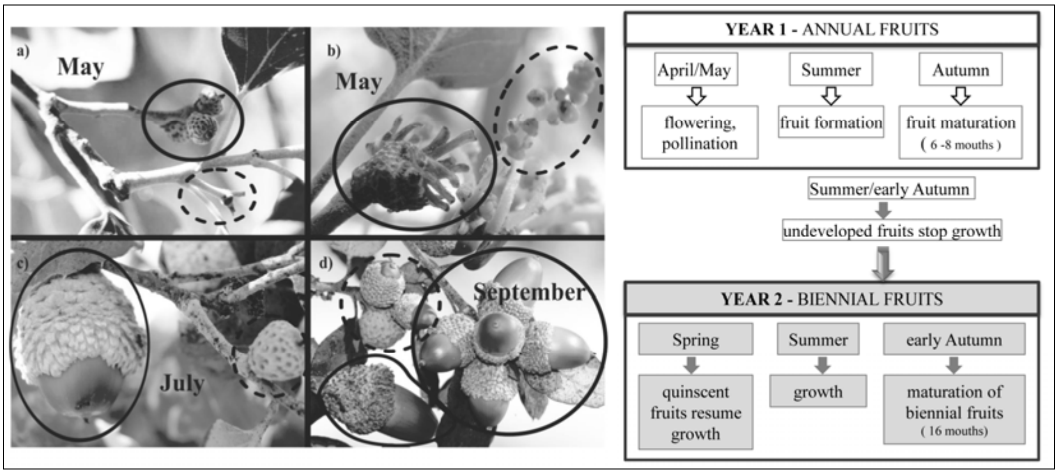

Figure 3-a, b, c, d. Annual and biennial fruiting in cork oak. Biennial fruiting happens when some of the fruits from the current year stop growth by the summer (year 1). These fruits remain quinscent till coming spring (year 2) when another flowering season happens (a, b). In May of year 2 the tree displays simultaneously the quinscent fruits from last flowering season and the annual flowering of the current year (a) and the quinscent biennial fruits resume growth and the vegetative activity is visible by the exuberant growth of the scales of the capsule (b) that in some cases get reddish colour. During the Summer of year 2 the two type of fruits co-exist and grow (c, d). By early autumn of year 2 biennial fruits complete maturation. Annual fruits take 2-3 more months and complete maturation by mid/late Autumn. The exuberant growth of the scales of the biennial fruits is a distinctive feature upon annual acorns.

RIASSUNTO

Comportamento riproduttivo e clonale ceppo / propagazione radice e le conseguenze per la variabilità genetica sostenibile in sughero e leccio in Portogallo

Gli studi su antesi e fruttificazione, per quercia da sughero e leccio, sviluppati in Portogallo in varie parcelle durante diversi anni mirano a valutare la dimensione effettiva delle popolazioni (Ne), la sincronizzazione delle fioriture, le possibilità di ibridazione interspecifica e la cadenza delle annate di pasciona. La capacità di sughera e leccio di moltiplicarsi per polloni da ceppaia e radice è di frequente utilizzata dai tecnici forestali per il recupero post-incendio e per propagare fenotipi interessanti ormai vetusti. Sono discusse le conseguenze per la variabilità genetica e la possibilità di senescenza genetica.

I risultati contribuiscono alla valutazione della variabilità genetica nell'ambito di ulteriori utilizzazioni quali le linee guida per la collezione di semi in programmi di afforestazione, il pascolamento nei sistemi silvopastorali, la gestione vivaistica e la conservazione delle risorse genetiche. I risultati sulle *performances* di fioritura e fruttificazione evidenziano che il leccio è più precoce, ha un comportamento riproduttivo più stabile e prevedibile e registra con minore frequenza annate con scarsa produzione di seme rispetto alla sughera. La capacità riproduttiva dipende dalla fertilità stazionale e dalla vigoria delle piante. La quercia da sughero nella parcella di Quinta da Serra (buona fertilità stazionale) ha fatto registrare valori di Ne compresi tra il 40 e l'80%, mentre nella stazione a bassa fertilità di Água Ferrenha essa varia tra il 10 e il 20%. Per lo stesso anno, nella parcella di Quinta da Mitra, dove sughera e leccio sono consociati, quest'ultima specie ha sempre evidenziato valori di Ne più elevati. In annate particolari sotto il profilo climatico, alcuni individui delle due specie possono evidenziare sincronizzazione nelle fioriture e la possibilità di ibridazione. In diversi popolamenti a sviluppo clonale, la moltiplicazione di individui geneticamente identici è largamente usata per propagare fenotipi interessanti che hanno raggiunto un'età avanzata e per i quali non è possibile ricorrere alla moltiplicazione per seme a causa di incendi o fenomeni di degrado.

La capacità del genere *Quercus* di ricacciare da gemme dormienti localizzate sulla ceppaia o sulle radici spesso da origine a differenti individui che formano un gruppo clonale, che si origina da un albero morto o ceduato, causando la decrescita del numero di individui geneticamente non imparentati, nei popolamenti naturali. Nel caso di realizzare importanti progetti di afforestazione o tutte le volte che si ricerca l'incremento della variabilità genetica, non si devono raccogliere le ghiande in annate a bassa produzione di seme perché si riscontrano bassi valori di Ne. Gli studi rivelano che la selezione di piante madri per realizzare collezioni clonali, si dovrebbero basare sulla conoscenza dei processi riproduttivi per saggiare la loro *performances* di fioritura e fruttificazione. La propagazione clonale di sughera e leccio dovrebbe essere usata sporadicamente nell'ambito di un modello casuale sia nel tempo che nello spazio, poiché livelli molto bassi di reclutamento dei semenzali, alla fine daranno luogo a bassi livelli nella variabilità genotipica portando alla formazione di popolamenti con ridotta capacità di riproduzione gamica.

Nei popolamenti dove l'estensiva propagazione clonale è stata favorita, i livelli di similarità genetica dovrebbero essere saggiati per mezzo di marcatori molecolari. Se il

livello relazionale impone decise azioni, bisogna intervenire con l'inserimento di piante originatesi per via sessuale, al fine di aumentare la diversità genetica.

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