soil moisture. Arct. Alp. Res. **20**, 461–465 (1988). — SWOFFORD, D. L. and SELANDER, R. B.: BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. Journal of Heredity **72**, 281–283 (1989). — TIGERSTEDT, P. M. A.: Studies on isozyme variation in marginal and central populations of *Picea abies*. Hereditas **75**, 47–60 (1973). — TRANQUILLINI, W.: Physiological Ecology of the Alpine Timberline. Ecological Studies, Volume 31. Springer-Verlag, New York (1979). — VILLALBA, R., VEBLEN, T. T. and OGDEN, J.: Climatic influences on the growth of subalpine trees in the Colorado Front Range. Ecology **75**, 1450–1462 (1994). — WENDEL, J. F. and WEEDEN, N. F.: Visualization and interpretation of plant isozymes. In: SOLTIS, D. E. and SOLTIS, P. S. (eds): Isozymes in Plant Biology. DUDLEY, T. R. (General Editor): Advances in Plant Sciences Series, Vol. 4, pp. 5–45. Dioscorides Press, Portland (1989). — WHITLOCK, C.: Vegetational and climatic history of the Pacific Northwest during the last 20,000 years: implications for understanding present-day biodiversity. Northwest Environmental Journal **8**, 5–28 (1992). — WOLF, P. G. and SOLTIS, P. S.: Estimates of gene flow among populations, geographic races, and species in the *Ipomopsis aggregata* complex. Genetics **130**, 639–647 (1992). — WOODWARD, A., SCHREINER, E. G. and SILSBEE, D. G.: Climate, geography, and tree establishment in subalpine meadows of the Olympic Mountains, Washington, U.S.A. Arctic and Alpine Research **27**: 217–225 (1995). — WORKMAN, P. L. and NISWANDER, J. D.: Population studies on southwestern Indian tribes. II. Local differentiation in the Papago. Amer. J. Human Genet. **22**, 24–49 (1970). — WRIGHT, S.: The genetical structure of populations. Ann. Eugen. **15**, 323–354 (1951). — WRIGHT, S.: The interpretation of population structure by F-Statistics with special regard to systems of mating. Evolution **9**, 395–420 (1965).

Genotypic and Environmental Variation of *Castanea crenata x C. sativa* and *Castanea sativa* Clones in Aptitude to Micropropagation

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Summary

Thirty five selected chestnut clones were micropropagated in six culture media. Data about the *in vitro* multiplication stage are presented: clonal variation, effect of culture media, interaction between both factors, variance components, clonal repetitivities and correlations between pairs of traits. Highly significant differences were found for all traits assessed. The genotype influence was the more important factor in all traits related with multiplication rates in clonal propagation. The high values of the clonal repetitivities indicate that these characteristics are genotype dependents and allow the prediction of the behaviour of the clones when one wants to start with *in vitro* propagation. High variability in growth rates and behaviour was found depending on the clone, so that multiplication coefficient ranged between 8.45 and 1.96 for hybrid clones. *Castanea sativa* clones have middle or low multiplication rates in comparison with hybrid clones. For shoot apex necroses results showed an important weight for the factor culture medium, the most favorable medium to avoid necrosis is GD; high levels of NH_4^+ and low levels of NO_3^-, HPO_4^{2-}, K^+, Mg^{2+} and NO_3^-/NH_4^+ ratio have a favourable influence, and there is very limited influence of the calcium concentration on the expression of shoot apex necrosis. Two types of necrosis have been observed, the first one "limited to apex" that allows to continue the growth of the explant by induction of branching and a second type "descending" for other clones that gradually extend down to the shoot and deteriorate it completely. For chlorosis results showed an important weight for the factor culture medium and also for interaction genotype-culture medium. The most favourable medium to avoid chlorosis is MS ($\frac{1}{2}NO_3$) and the worse medium is SH, low concentrations of HPO_4^{2-}, K^+, NO_3^-, and NO_3^-/NH_4^+ ratio and high levels of NH_4^+ promote absence of chlorosis. Correlations between micropropagation variables show that it is possible to select pairs of production traits and at the same time to improve against the appearance of apical necrosis.

Key words: micropropagation, *Castanea crenata x C. sativa*, *Castanea sativa, Castanea mollissima x C. sativa* clones, multiplication rates, clonal repetitivity, variance components, apical necrosis, chlorosis, nitrate:ammonium ratio.

Introduction

Chestnut is a species planted for quality wood production in acid, medium or good quality soils. Hybrid clones of *Castanea sativa* MILL. with *C. crenata* SIEB. et ZUCC. or *C. mollissima* BLUME resistant to ink disease, caused by several *Phytophthora* species, are recommended for plantations in areas affected by

this fungus. Clonal propagation of chestnut is used for the multiplication of plants resistant to *Phytophthora* sp. and selected by growing and crown form. Chestnut can be propagated by layering and cutting but micropropagation can provide an adequate method for mass propagation of selected genotypes.

To micropropagate a large number of clones, it is necessary to define both the culture media for multiplication and the clonal behaviour of this material. In consequence it is necessary to know *in vitro* traits as multiplication rates and appearance of shoots in different nutritional conditions with the aim to choose culture media. Another important question is that the best culture media for one clone are not necessarily the best for other clones. Repetitivity of clonal means is the parameter used to measure the degree of genetic determination of clonal behaviour. Numerous authors point out that genotype determines the aptitude to micropropagation in different species (AHUJA and MUHS, 1982; AHUJA, 1983; BERGMANN and STOMP, 1994; COLEMAN and ERNST, 1989; CHAUVIN and SALESSES, 1988; SÁNCHEZ and VIÉITEZ, 1991; SCALTSOYIANNES et al., 1994, 1997).

The objectives of this work are to study: 1) genetic variability in aptitude to micropropagation, assessed as the variability among clones and clonal repetitivity; 2) influence of the culture media on multiplication rates and appearance of shoots; 3) characteristics of the variables used to describe the multiplication stage. Finally we want to select clones with good proliferation behaviour and suitable culture media for multiplication.

Materials and Methods

1. – Experimental design

– *Plant material:* Thirty five chestnut clones were *in vitro* multiplicated. Thirty two are hybrid clones resistant to ink disease (*Phytophthora* spp.) (URQUIJO, 1956; VIÉITEZ, 1960): thirty-one of them are *Castanea crenata x C. sativa*, one is *Castanea mollissima x C. sativa* (clone CHR-162); twenty nine hybrid clones came from the collection of Centro de Investigacions Forestais de Lourizán (FERNÁNDEZ-LÓPEZ, 1996) and three hybrid clones came from INRA (clones CA-07, CA-118, CA-15) (SALESSES et al., 1993; SCHAD et al., 1952). Besides there are three clones of the species *Castanea sativa* (clones CHR-56, CHR-155 and MARA). The species or hybrid origin was determined by isozymes (FERNÁNDEZ-LÓPEZ, 1996).

In vitro cultures were initiated in spring from field-grown young stump shoots from trees more than thirty years old. Shoots were excised from plants, all leaves were removed and then were washed thoroughly in running tap water during 5 minutes. Node segments 1 cm were sterilized in a 0.15% solution of $HgCl_2$ for 2 minutes, rinsed three times in sterile deionized water and inoculated in tubes with 15 ml of HELLER's medium modified by VIÉITEZ (VIÉITEZ et al., 1983, 1986; BALLESTER et al., 1990) and consisting of HELLER's macronutrient formula with concentration of all its components multiplied by 1.25 and the addition of 1 mM $(NH_4)_2SO_4$ (Hm).

Multiplication was developed by axillary shoot production. Stem segments with at least one axillary or apical bud and one centimetre long were excised and inserted vertically into multiplication medium. The shoot cultures were subcultured in a four-weeks-cycle. Cultures were incubated in a growth chamber, at 25 °C and 16 hours of photoperiod, under white fluorescent lamps (Sylvania gro-lux 40W) with a Photon-flux density of 50 $\mu mol.m^{-2}.s^{-1}$.

– *Culture media:* Due to the impossibility to make a broad spectrum approach (FOSSARD, 1976) or a Natural Model Approach (DURZAN, 1982) to this high number of clones, for initial selection of the culture media, the experience provided by other researchers that work with hybrid or European chestnut clones was considered (VIÉITEZ et al., 1982, 1983, 1986; BIONDI et al., 1981).

Six basal salts were tested: HELLER (1953) modified by VIÉITEZ (VIÉITEZ et al., 1983, 1986; BALLESTER et al., 1990) (Hm), MURASHIGE and SKOOG (1962) with half strength of nitrates (VIÉITEZ et al., 1983, 1986) (MS $^1/_2$ NO_3), GRESSHOFF and DOY (1972) (GD), LEPOIVRE (QUORIN and LEPOIVRE, 1977) (Lp), BLAYDES (1966) (B1), SCHENK and HILDEBRANDT (1972) (SH). *Table 1* shows ion compounds of the different culture media ($mmol.l^{-1}$). All media were supplemented with 30 $g.l^{-1}$ sucrose, agar 6 $g.l^{-1}$, vitamins, micronutrients and Fe-EDTA of MURASHIGE and SKOOG (1962) and 0.2 $mg.l^{-1}$ of 6-Benzylaminopurine, pH 5.6.

– *Number of blocks (or subcultures)* per combination clone/medium: three. In fact, four consecutive subcultures were carried out, but the analysed data come from the last three subcultures. Due to a different behaviour during the first subcultures, we began to measure the multiplication traits after the fifth subculture. For colour variable data only came from the last two subcultures of each culture medium.

– *Number of replications* in each subculture: twenty.

– *Size of the sample:* The data groups have the same size for all variables except for the shoot colour. So the models of ANOVA are absolutely balanced.

– *Size and characteristics of the explants:* To homogenize the type of explants and to reduce the experimental error, the first apical centimetre was used to perform tests; because of the fact that there is a high difference in the number of axillary buds, size and appearance of leaves among the explants coming from apical, middle or basal portion in the same shoot.

– *Variables:* variables for evaluation, recorded in each subculture were:

A. – Variables that indicate growth rates and the proliferation potential of a clone:

• *Number of shoots per explant* (NSH). This variable shows capacity of multiplication and the possible use of the bases of the explant for shoot cluster formation.

• *Length of the tallest shoot in centimetres* (LS). Indicates the proliferation capacity and the possibility to obtain microcuttings directly for rooting, without elongation stage.

• *Number of segments* (NS) of one centimetre with at least one apical or axillary bud per explant. Indicates the capacity of proliferation.

• *Percentage of Responsive Explants* (%RE). Percentage of explants that respond to the applied treatment in each subculture.

• *Multiplication coefficient* (MC). These data are the result of multiplying the mean number of segments of one centimetre per explant per subculture by the percentage of responsive explants (%RE) of each subculture (SANCHEZ and VIÉITEZ, 1991), expressed in frequencies.

B. – Variables indicating the quality of shoots:

• *Apical Necrosis* (AN). Presence or absence of shoot apex necrosis and the grade of deterioration of the shoots.

• *Colour.* Colour or more exactly presence of more or less chlorosis in the newly developed shoots. It was recorded as a cathegorical variable. Nine categories were established to quantify this variable; from dark green, the most favourable one, to pale yellow, the less advantageous one, related to presence of chlorosis. The Munsell color charts for plant tissues

(1977) was used in order to describe and analyse colour in terms of three attributes:chromatic colour, degree of lightness of a colour and strength or degree of departure of a particular colour. The higher values correspond to qualitative data which reflect a more favourable condition of the plant material and vice versa. A lot of *in vitro* publications mention problems of chlorosis but the study of this characteristic is unusual.

2. – Data Analysis

This is a factorial analysis of 35 clones and six culture media, with three consecutive subcultures in the combination clone-culture medium and 20 repetitions per subculture. In this wide group of data (12.600), variability in aptitude to micropropagation, sources of the variation, clonal repetitivities of traits and correlations both between multiplication variables and also correlations between each micropropagation variable and the ions concentration for culture media are studied.

Statistical Analysis: For analize the results these steps were followed:

Previously to make the analysis of variance, the fulfilment of *the assumptions of normality* (SHAPIRO-WILK W-test (1965) and *homogeneity of error* variance (BARTLETT's Test (1947) were tested (SAS Discrim procedures (proc univariate)). When the assumptions were not satisfied we studied whether it was useful to work with original data, transformed or mean values, and if F-values, significance levels and clonal repetitivity values would be altered depending on the type of data used.

Original data may be used for analysis of variance with variables as number of shoots per explant, length of the tallest shoot, number of segments of one centimetre per explant and multiplication coefficient whereas frequency data are used for apical necrosis and percentage of responsive explants variables; because in some cases transformed data don't satisfy the tests and the nonfulfilment of the assumptions by original data has a little effect on F-values, significance levels and clonal repetitivity with regard to transformed variables. Besides the sample sizes are large and equal and so there is little effect when the test of homogeneity is not satisfied. Original data make the interpretation of the results more easy. For colour variable there are very skewed distributions and very different variances in original data and Box-Cox (1964) transformations and means data do not improve adequately (MIRANDA-FONTAÍÑA and FERNÁNDEZ-LÓPEZ, submitted for publication).

Analyses of variance and means were generated using SAS (SAS Institute, Cary NC).

Models used in the analysis of variance: The models used for the analysis of variance were:

• For NSH, LS and NS variables in the study of behaviour of the population constituted of 35 clones and subject to six different nutritional conditions.

$$X_{ijkl} = \mu + C_i + M_j + CM_{ij} + B_{K(ij)} + \epsilon_{l(ijk)} \qquad \text{Model 1}$$

• Data Analysis of colour, %RE and MC variables in the study of behaviour of 35 clones subject to six nutritional conditions and for frequency data per subculture of AN.

$$X_{ijk} = \mu + C_i + M_j + CM_{ij} + \epsilon_{k(ij)} \qquad \text{Model 2}$$

M: Culture media (i=6), C: Clone (j=35), CM: Interaction between Clones and Culture media, B: Block/Subculture (k(ij)=3), ϵ: Residual effect.

Variance Components, Clonal Repetitivity and Means comparison:

To study the distribution of the variability among different factors, variance components were calculated using the mean square values and corresponding expected means square coefficients generated from PROC GLM, RANDOM/test option (SAS Inc., 1988). For all analysis sources of variation were considered to be significant at $\alpha = 5\%$.

If null hypothesis of equality of treatments is rejected, STUDENT-NEWMAN-KEULS test was performed to obtain groups of homogeneous treatments, both for culture media and for clones.

PEARSON correlation coefficients were determined to study the strength of relation both among *in vitro* multiplication traits. They also were determined between *in vitro* multiplication variables and ion and compounds concentration for each culture medium (PROC CORR). For the second type of correlation analysis mean data for clone-culture media combinations were used. The aim is to study the strength of relation between *in vitro* multiplication variables and milimol ion concentrations (detailed listing in *Table 1*).

Repetitivity of clonal means is the parameter used to measure the degree of genetic determination of clonal behaviour and it was calculated for each trait. Clonal repetitivity is defined as the quotient between clonal variance and phenotypic variance of clonal means. The variance among clones due to differences

Table 1. – Ion compounds of the different culture media (mmol.l^{-1}).

Components	GD	Lp	Hm	Bl	SH	MS (½NO_3^-)
N-total	12.91	37.94	10.82	39.11	27.33	30.00
NO_3^-	9.89	32.95	8.82	26.61	24.72	19.70
SO_4^{2-}	2.52	1.46	2.26	0.29	1.62	1.50
$H_2PO_4^-$	0.21	1.98	1.13	2.20	2.61	1.25
Cl^-	6.07	—	13.83	0.87	2.72	5.98
NH_4^+	3.02	4.99	2.00	12.50	2.61	10.30
K^+	13.92	19.78	12.57	12.96	24.72	10.65
Ca^{2+}	1.02	5.08	0.63	2.11	1.36	2.99
Mg^{2+}	1.01	1.46	1.26	0.29	1.62	1.50
Na^+	0.42	—	9.95	—	—	—
NO_3^-/NH_4^+ ratio	3.27	6.6	4.41	2.12	9.47	1.91

in their genotype can be considered as an estimate of the total genetic variance. However, there may be environmental effects on it, that is why a part of the environmental variance is transmitted to the descendants. This is the reason why clonal genetic variance can be an overestimate of the genetic variance and strictly speaking could be called clonal repetitivity (FALCONER, 1989). Clonal heritability is a heritability in the broad sense or degree of genetic determination (FALCONER, 1989). Clonal repetitivity allows the prediction of the behaviour of the clones in activities of micropropagation.

Expected mean square tables with the corresponding coefficients were calculated as follows:

Expected mean square table for variables analyzed with *model* of analysis 1:

Source of Variation	MS	Expected Mean Squares
Clone	MS_1	$\sigma^2_e + K_1\ \sigma^2_{B(C*CM)} + K_2\ \sigma^2_{C*CM} + K_4\ \sigma^2_C$
Culture Medium	MS_2	$\sigma^2_e + K_1\ \sigma^2_{B(C*CM)} + K_2\ \sigma^2_{C*CM} + K_3\ \sigma^2_{CM}$
Clone *Culture Medium	MS_3	$\sigma^2_e + K_1\ \sigma^2_{B(C*CM)} + K_2\ \sigma^2_{C*CM}$
Block (Clone*Culture Medium)	MS_4	$\sigma^2_e + K_1\ \sigma^2_{B(C*CM)}$
Error	MS_5	σ^2_e

Variance among clones: $s^2_C = (MS_1 - MS_3) / K_4$;
Variance among culture media: $s^2_{CM} = (MS_2 - MS_3) / K_3$;
Variance due to interaction of clones and culture media:
$s^2_{C*CM} = (MS_3 - MS_4) / K_2$;
Variance among blocks in the interaction of clone and culture medium:
$s^2_{B(C*CM)} = (MS_4 - MS_5) / K_1$
Error Variance : $s^2_e = MS_5$
Total Variance: $s^2_t = s^2_C + s^2_{CM} + s^2_{C*CM} + s^2_{B(C*CM)} + s^2_e$

Expected mean square table for variables analyzed with *model* of analysis 2:

Source of Variation	MS	Expected Mean Squares
Clone	MS_1	$\sigma^2_e + K_1\ \sigma^2_{C*CM} + K_3\ \sigma^2_C$
Culture Medium	MS_2	$\sigma^2_e + K_1\ \sigma^2_{C*CM} + K_2\ \sigma^2_{CM}$
Clone *Culture Medium	MS_3	$\sigma^2_e + K_1\ \sigma^2_{C*CM}$
Error	MS_4	σ^2_e

$s^2_C = (MS_1 - MS_3) / K_3$; $s^2_{MC} = (MS_2 - MS_3) / K_2$;
$s^2_{C*MC} = (MS_3 - MS_4) / K_1$; $s^2_e = MS_4$;
$s^2_t = s^2_C + s^2_{CM} + s^2_{C*CM} + s^2_e$

Clonal Repetitivities (Rc) were calculated:

- For variables analysed with *model* 1:

$R_C = s^2_c\ K_4 / (s^2_e + K_1\ s^2_{B(C*MC)} + K_2\ s^2_{C*CM} + K_4\ s^2_C)$

- For variables analysed with *model* 2:

$R_C = s^2_c\ K_3 / (s^2_e + K_1\ s^2_{C*CM} + K_3\ s^2_C)$

3. – Rooting of shoots and acclimatization

For rooting, the lower leaves were removed from the microcuttings and the bases of elongated, excised shoots were dipped into a solution of IBA (1 $g.l^{-1}$) and placed into a moist and sterilized subtratum of perlite and composted pine bark (mix 2:1) in polystyrene trays (MIRANDA and FERNÁNDEZ, 1990), immediately watered and covered with a policarbonate sheet (3 mm) to maintain moisture and kept in a growth chamber under the same conditions as for the foregoing phases.

After eight weeks in growth chamber the plants were acclimatized in a microtunnel with a translucent polythene, placed in a greenhouse with controlled temperature of 24 ± 3 °C. The tunnels are provided with a fog system with air at 8 atmospheres and high relative humidity (MIRANDA and FERNÁNDEZ, 1992). The plants were successfully transplanted into containers of 500cc., filled with mixture of peat (Terraplant-2): perlite: composted pine bark (Dermont) or remained in the same polystyrene trays fertilized with a MURASHIGE and SKOOG salt solution, for further growth and development in greenhouse.

Results and Discussion

When plant material is genetically so different there is not an unique medium advisable to carry out the multiplication stage. But a series of common characteristics became evident in a part of clones when a concrete medium was used, so *table 2* shows mean data for the total population of clones. (1) *Medium GD* gave rise to develop a high number and good appearing shoots and is the best medium to avoid shoot-tip necrosis and good to avoid chlorosis. (2) *MS* (*½ nitrates*) *medium* promoted a middling number of shoots per explant, but these shoots are longer than the shoots developed with other culture media. This culture medium has the higher multiplication coefficients. A low number of clones have few, but very long shoots with internodes longer than one centimetre, and comparatively lower values in number of shoots of one centimeter per explant (clones CHR-155). Almost for this medium the presence of chlorosis and apical necrosis is very low. An unfavourable initial characteristic is the production of vitrified shoots in two clones, CHR-149 and CA-07, an aspect mentioned by VIEITEZ et al. (1986) with other chestnut clones. *Figure 1* (a) shows the effect of MS (½ nitrates) medium on shoot proliferation of six chestnut clones after four weeks, *figure 1* (b) clone CHR 114 cultivated on six different basal media. (3) *Lp medium* promotes a high number and long shoots, but with chlorosis.(4) *Hm medium* shows a medium behaviour in multiplication rates and high values of apical necrosis and chlorosis; in spite of that there is a group of clones that perfectly grow in this medium. (5) *Bl medium* has mediocre results. (6) For all variables the worse and inadvisable medium is *SH* both for proliferation growth and in general aspect of the cultures.

An overview of the analysis of variance and variance components results shows similar guidelines among variables that indicate growth rates (*Tables 3* and *4*) but different tendencies for quality variables (*Table 5*).

For *number of shoots per explant, length of the tallest shoot, number of segments of one centimetre per explant, multiplication coefficient* and *percentage of responsive explants* (*Tables 3* and *4*) analyse shows that the model is highly significant for all evaluated parameters at 1‰, both for main factors (clone and culture medium) and for the interaction clone-culture medium. F-values of the interaction are comparatively much less important than F-values for the main factors. Variance components indicate that the factor clone supports a very important part of the variability (with 57% of variability for the multiplication coefficient). The high values of the clonal repetitivities indicate that these proliferation potential characteristics are genotype dependents and allow the prediction of the behaviour of the clones when one wants to plan an *in vitro* study or propagation. In clonal studies in plantations values of clonal variance rarely have so high values. When these variables are correlated with ions and compounds concentrations in different culture media, values of correlations (*Table 6*) are low, but they have significant and negative values for levels of phosphate, potassium and nitrate/ammonium ratio, so low concentrations promote best growth, like in MS (½ NO_3) or GD media; highest proliferation rates on media containing low total N levels and low nitrate/ammonium ratios were found by PIAGNANI and ECCHER (1988) with other chestnut clones. The unfavourable effect of a high NO_3^-/NH_4^+ ratio on *in vitro* growth were mentioned by

Table 2. – Effect of the different culture media on total population of clones, for the in vitro multiplication variables: *number of shoots per explant (NSH), length of the tallest shoot (LS), Number of segment of one centimetre per explant* (NS), *multiplication coefficient (MC), percentage of responsive explants* (%RE), Apical necrosis *(AN)* and *colour* of shoots. STUDENT-NEWMAN-KEULS test. Mean values of 12.600 data NHS, LS and NS; mean values of 630 data for %RE, MC, AN.

NSH		LS (cm)		NS		%RE		MC		AN		Colour	
GD	3,01 a	MS	3,23 a	MS	4,38 a	Hm	96,14 a	MS	4,25 a	SH	0,37 a	MS	7,94 a
Lp	2,88 b	Lp	2,88 b	Lp	4,23 b	MS	95,71 a	Lp	4,03 ab	Hm	0,19 b	GD	7,20 b
Hm	2,77 c	GD	2,80 c	Hm	4,11 b	GD	94,38 b	Hm	4,00 ab	Bl	0,18 b	Lp	5,97 c
MS	2,53 d	Hm	2,79 c	GD	4,11 b	Bl	93,00 c	GD	3,94 b	Lp	0,16 bc	Hm	5,67 d
BL	2,48 d	Bl	2,45 d	Bl	3,56 c	Lp	92,52 c	BL	3,38 c	MS	0,12 c	Bl	4,87 e
SH	2,47 d	SH	2,45 d	SH	3,35 d	SH	86,76 d	SH	3,08 d	GD	0,06 d	SH	2,92 f

Within each column, values followed by the same letter are not significantly different at the 5% significance level.

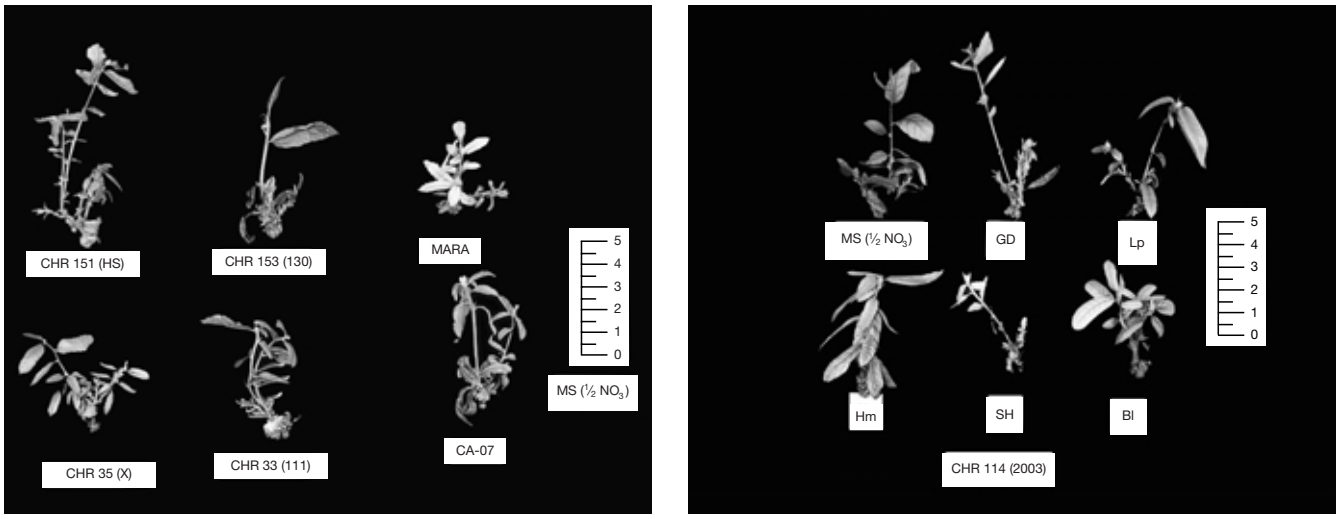

Figure 1. – In vitro multiplication of chestnut: (a) Six clones at the end of a subculture period on MS (½ nitrates); (b) Clones CHR 114 cultivated on six different basal media.

Table 3. – F-values and significance levels resulting from analysis of variance, Variance components and Clonal Repetitivities for variables *number of shoots per explant* (NSH), *length of the tallest shoot* (LS) and *number of segment of one centimetre per explant* (NS). Model of analysis number 1. Number of observations in dataset 12.600.

	d.f.	Variables								
		NSH			LS			NS		
		F	Var	%	F	Var	%	F	Var	%
Clone	34	13,36***	0,275	14,53	10,88***	0,44	17,86	15,30***	1,709	24,72
Culture Medium	5	12,52***	0,05	2,48	11,39***	0,08	3,21	8,05***	0,144	2,09
Clone*Culture Medium	170	2,33***	0,08	4,38	2,86***	0,173	7,06	2,73***	0,454	6,58
Block(Clone*CultMedium)	420	2,72***	0,118	6,23	3,56***	0,201	8,17	3,90***	0,584	8,45
Error	11970		1,373	72,38		1,569	63,70		4,023	58,16
σ^2_t			2,463	100		2,463	100		6,917	100
R_c			0,92			0,91			0,93	

***) Pr>F = 0.0001

VIÉITEZ et al. (1983, 1986), and it also was indicated that the presence of the ammonium ion seems to be essential for optimum *in vitro* growth of chestnut, so long as its concentration is lower than in MS medium.

The percentage of variance attributable to the block factor is the variance due to variations inside each clon-culture medium combination. For *number of shoots per explant, length of the tallest shoot* and *number of segments of one centimetre per*

Table 4. – F-values and significance levels resulting from analysis of variance, Variance components and clonal Repetitivities for variables *multiplication coefficient (MC)* and *percentage of responsive explants (%RE)*. Model of analysis number 2. Number of observations in dataset: 630.

	d.f.	Variables					
		%RE			MC		
		F	Var	%	F	Var	%
Clone	34	7.74***	33.695	38.6	15.70***	2	56.8
Culture Medium	5	13.61***	10.793	12.4	8.86***	0.2	5
Clone*Culture Medium	170	4.65***	23.536	27	3.10***	0.5	15.7
Error	420		19.285	22.1		0.8	22.4
σ^2_t			87.309	100		3.5	100
R_c			0.87			0.94	

***) Pr>F = 0.0001

Table 5. – F-values and significance levels resulting from analysis of variance, Variance components and clonal Repetitivity for variables *Apical necrosis (AN)* and *colour of shoots.* Model of analysis number 2.

	d.f.	AN			d.f.	Colour		
		F	Var	%		F	Var	%
Clone	34	4.339***	0.007	17.01	34	1.74*	0.267	4.04
Culture Medium	5	29.637***	0.010	25.19	5	49.52***	3.002	45.4
Clone*Culture Medium	170	2.253***	0.007	16.99	170	51.26***	2.128	32.18
Error			0.020	40.81	7177		1.215	18.37
σ^2_t			0.041	100			6.612	100
R_c			0.77				0.42	

explant (*Table 3*) the block factor shows significant differences among the three consecutive subcultures, because of the fact that when a culture medium promotes a good development this effect tends to be accentuated in the successive subcultures and vice versa. For this reason we consider that it is not adequately to work with data coming from results of only one subculture when the plants don't remain a minimum time under these tested nutritional conditions.

For *apical necrosis* and *colour* results are significant for all parameters (*Table 5*). For these variables, that indicate quality of shoots, F-values and variance components show an important weight for the factor culture medium and also for the clone-culture medium interaction in colour variable. Clonal repetitivity values are high but comparatively lower than for variables that indicate proliferation potential. The interaction clone-culture medium is a genotype-environment interaction. This interaction shows that the different culture media have several effects in each genotype or that some clones have more sensitivity to differences in the culture medium.

Apical necrosis has been attributed to several causes, like a deficiency of cytokinins or calcium (ABOUSALIM and MANTELL, 1994; PIAGNANI et al., 1996; SHA et al., 1985; VIÉITEZ et al., 1989). But in this study with a wide group of clones and equal concentrations of cytokinin in all cases, there is an important weight for the factor culture medium over the expression of this characteristic with 25.19% of the total variability, while 17% both due to the factor clone and the interaction clone-culture medium. So with regard to the culture medium for GD medium 25% of the clones don't show shoot apex necrosis and 47% of clones have less than 5% of necrotic shoots (*Figure 2*). Contrary for SH medium nearly the fourth part of clones have more than sixty percent of shoots with apical necrosis. When each clonal mean value is correlated with ions and compounds concentration of each culture medium (*Table 6*), a favourable influence for NH_4^+ levels is observed and very limited influence of the calcium concentration on the expression of this trait is observed. On the contrary higher PEARSON correlations and significant probability values were obtained for ions and compounds like NO_3^-, HPO_4^{2-}, K^+, Mg^- and NO_3^-/NH_4^+ ratio, indicating that high concentrations of these compounds promote elevated percentages of shoot apex necrosis. With regard to the scarce influence of calcium concentration, two media with low levels of this ion have one the most favourable results (GD) and the other one the worst results (SH), and Lp with higher calcium values (Lp) have high apical necrosis frequencies (*Figure 2*). As conclusion for the clones studied in this work apparently calcium level is not an important factor in the expression of the apical necrosis.

Table 6. – PEARSON correlation coefficients between *in vitro* multiplication variables and ion and compounds concentration for each culture medium. Number of observations in dataset 210.

	NSH	LS	NS	%RE	MC	AN	Colour
N-total	−0.1671[a] 0.0153[b]	−0.0401 0.5624	0.0563 0.4168	−0.1157 0.0946	−0.0636 0.3587	0.1227 0.0760	−0.1457 0.0348
NO_3^-	−0.1308 0.0583	−0.0698 0.3139	−0.0737 0.2876	−0.1954 0.0045	−0.0857 0.2160	0.2308 0.0007	−0.2770 0.0001
SO_4^{2-}	0.1954 0.0045	0.0933 0.1777	0.0969 0.1614	0.1137 0.1004	0.1023 0.1393	−0.0974 0.1595	0.1709 0.0125
$H_2PO_4^-$	−0.1907 0.0055	−0.2123 0.0020	−0.1809 0.0086	−0.2870 0.0001	−0.1903 0.0057	0.4915 0.0001	−0.6428 0.0001
Cl^-	0.1162 0.8671	0.0659 0.3413	0.0625 0.3673	0.1788 0.0094	0.0782 0.2587	0.0192 0.7813	0.0499 0.4713
NH_4^+	−0.1682 0.0147	0.0361 0.6026	0.0023 0.9731	0.0926 0.1810	0.0075 0.9139	−0.1435 0.0356	0.1775 0.0100
K^+	−0.1008 0.1452	−0.1633 0.0178	−0.1447 0.0361	−0.3133 0.0001	−0.1571 0.0227	0.5255 0.0001	−0.6177 0.0001
Ca^{2+}	0.0143 0.8361	0.1233 0.0744	0.0908 0.1899	0.0085 0.9018	0.0843 0.2235	−0.0618 0.3727	0.1618 0.0189
Mg^{2+}	0.0347 0.6161	0.1331 0.0541	0.0733 0.2899	−0.0462 0.5053	0.0698 0.3138	0.1883 0.0062	−0.0068 0.9210
Na^+	0.2112 0.0021	0.0211 0.7610	0.0690 0.3193	0.1819 0.0082	0.0793 0.2522	−0.2377 0.0005	0.2024 0.0032
NO_3^-/NH_4^+ ratio	−0.0471 0.4968	−0.1523 0.0273	−0.1355 0.0499	−0.3165 0.0001	−0.1492 0.0306	0.4808 0.0001	−0.5626 0.0001

[a]) PEARSON correlation coefficient: r

[b]) p-value: significance probability for testing the null hypothesis that the true correlation in the population is zero

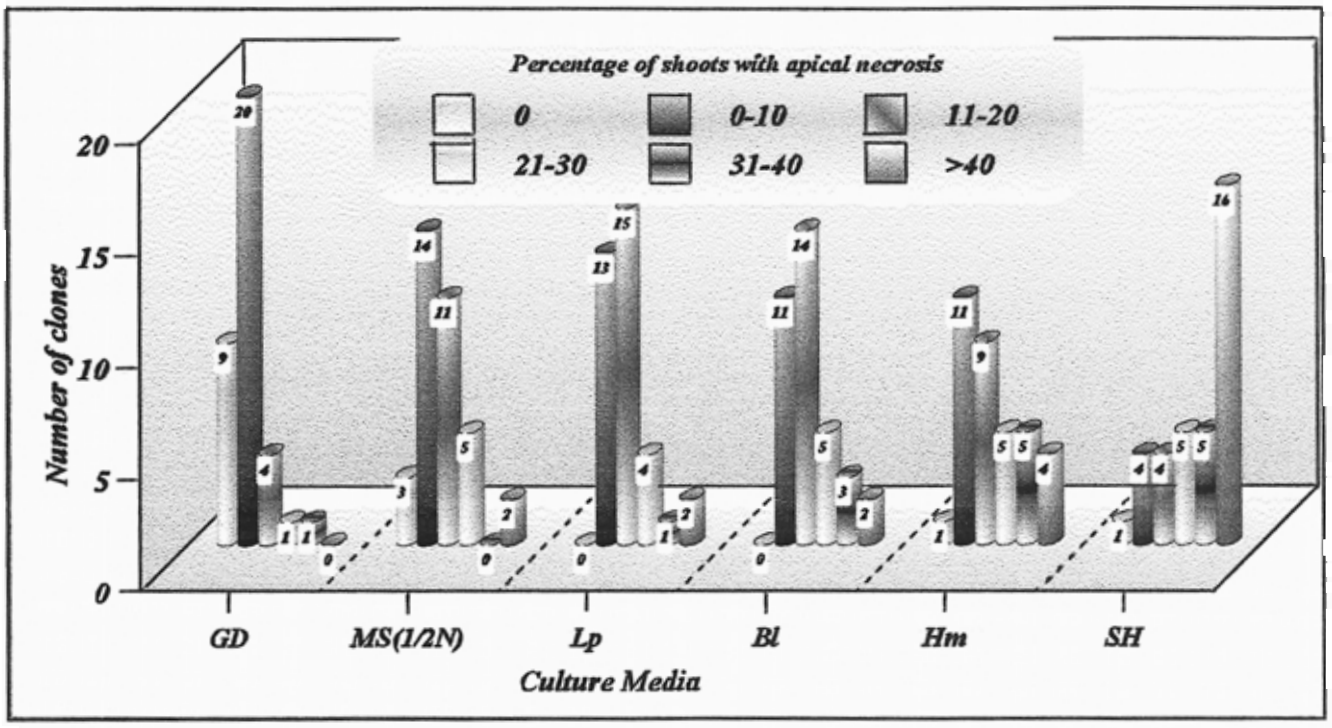

Figure 2. – Influence of the culture media on the number of clones that showed a concrete percentage of shoots with apical necrosis.

When a detailed analysis of apical necrosis was made, we have observed that this trait was obvious when the cultures were between the third and fourth week. Moreover quantitative and qualitative differences between types of apical necrosis have been detected. So in some clones, a first type that can be called "limited to apex" was found and that allowed to continue the growth of the explant by induced branching (clone CHR-151) and in other clones a second type "descending" that gradually extended down to the shoot and completely deteriorated it (clone CHR-149).

With regard to colour or chlorosis of shoots, various types of chlorosis have been observed both between different clones growing in the same nutritional conditions and for the same clone growing in different nutritional conditions. The most important factor on the expression of this characteristic is the interaction clone-culture medium. Four kinds of chlorosis can be described as follows: the first type is a chlorosis that appears in all shoots, in a second type the yellow hue increases from the base to the apex of the shoots, another one affects to a part of leaves, between veins, and finally another type affects to complete leaves but not to the stem. The most favourable medium to avoid chlorosis is MS (½ NO_3) and the worst medium is SH, in which sixty percent of clones showed intensive chlorosis. Highly significant PEARSON correlations were obtained for ions and compounds like HPO_4^{2-}, K^+, NO_3^-, and NO_3^-/NH_4^+ ratio, so that high concentrations of these compounds promote chlorosis. On the other side its seems that high levels of NH_4^+ have a positive effect on the prevention of this unfavourable trait (*Table 6*).

Groups of clones with similar behaviour were obtained with STUDENT-NEWMAN-KEULS test (*Table 8*) after applied models of analyses 1 and 2, but only taking into account the best four culture media (GD, Hm, MS, Lp). There is a high variability of behaviour depending on the clone and this is a very important factor when one wants to carry out a multiplication of an interesting group of clones and plans a profitable chestnut micropropagation, since multiplication rates determine the costs of micropropagation (MIRANDA and FERNÁNDEZ, 1993). The multiplication coefficient ranged between 8.45 and 1.96 for the clones CHR-151 and CHR-167 respectively. *Castanea sativa* clones (CHR-155, CHR-56, MARA) didn't show a common guideline of behaviour: so MARA has a very low multiplication rate and on the contrary CHR-155 has a mean multiplication coefficient of 4.53. Generalizations can not be made about *Castanea sativa* clones which always have multiplication rates lower than any hybrid clone.

Correlations between micropopagation variables (*Table 7*) showed that variables as NS, LS, NSH and %RE have very high values of correlations coefficients. The r-values for MC against NSH and %RE variables are near of 1 since MC came from the combination between them. Apical necrosis shows negative and low r-values, but p-values are significant at 1‰. Correlations show that it is possible to select simultaneously pairs of production traits and at the same time to improve against the appearance of apical necrosis.

Due to the similar pattern of behaviour among variables and, on the other side, the significant interaction among them, one may think that it will be enough for a study of selection of clones to make the assessment of a single variable like multiplication coefficient since the advantageous results of this variable will include the others and will save overworks. However this argument is not valid for an *in vitro* propagator. To micopropagate a clone it is necessary to evaluate any trait that may indicate the possible modification of a basal salt or a concentration of hormone and if there is the necessity to pass the elongation stage as individual or cluster of shoots when the size of the tallest shoot is not suitable to obtain directly microcuttings.

Shoots of all clones were rooted and acclimatized. In spring the plants were established in nursery for bare root cultivation. These plants obtained by micropropagation are in clonal plantations with the objective of clonal selection.

Conclusions

In spite of the fact that there is not an unique medium advisable to carry out the multiplication stage for this concrete genetic material, when one wants to produce a high number of shoots per explant the best media are GD, Lp and Hm with values of 3.01, 2.88 and 2.77 respectively. The best media to avoid both, apical necrosis and chlorosis and to improve the general appearance are GD and MS. To increase the length of shoots MS (½ nitrates) medium gives the best results and reveals a high percentage of responsive explants, in spite of this

Table 7. – Phenotypic PEARSON correlation coefficients between pairs of tissue culture traits. Number of observations in dataset 630.

	LS	NS	%RE	MC	AN	Colour
NSH	0.5780 [a] 0.0001 [b]	0.7867 0.0001	0.6745 0.0001	0.7928 0.0001	-0.2236 0.0001	0.2038 0.0030
LS		0.9151 0.0001	0.6634 0.0001	0.9165 0.0001	-0.3008 0.0001	0.2941 0.0001
NS			0.6944 0.0001	0.9972 0.0001	- 0.2774 0.0001	0.2621 0.0001
%RE				0.7326 0.0001	-0.3888 0.0001	0.3292 0.0001
MC					-0.2902 0.0001	0.2723 0.0001
AN						-0.3697 0.0001

[a]) PEARSON correlation coefficient: r

[b]) p-value: significance probability for testing the null hypothesis that the true correlation in the population is zero

Table 8. – STUDENT-NEWMAN-KEULS test for variables *number of shoots per explant* (NSH), *length of the tallest shoot* (LS), *number of segment of one centimetre per explant (LS), number of segments of one centimetre* (NS), *multiplication coefficient* (MC) and *percentage of responsive explants* (%RE), Apical Necrosis (AN) and colour, but only taking into account the best for culture media (GD, Hm, MS, LP). Within each column, values followed by the same letter are not significantly different at the 5% significance level. Models of analysis: 1 for NSH, LS and NS; 2 for MC, %RE, AN and color.

Clon	NSH	Clon	LS	Clon	NS	Clon	AN	Clon	%RE	Clon	MC	Clon	Color
CHR-151	4,48 a	CHR-151	5,12 a	CHR-151	8,45 a	CA-118	0,35 a	CHR-62	100,00 a	CHR-151	8,45 a	CHR-155	7,19 a
CA-07	3,77 b	CHR-161	4,38 b	CHR-161	7,12 b	CHR-12	0,31 ab	CHR-33	100,00 a	CHR-161	7,04 b	CHR-149	7,17 a
CHR-39	3,62 bc	CHR-62	3,89 c	CHR-62	6,05 c	CHR-2	0,27 bc	CHR-151	100,00 a	CHR-62	6,05 c	CA-118	6,83 b
CHR-161	3,47 cd	CHR-155	3,88 c	CA-07	5,95 c	CHR-167	0,24 cd	CA-118	99,58 ab	CA-07	5,87 cd	CHR-44	6,81 b
CHR-162	3,40 cd	CHR-33	3,68 cd	CHR-162	5,70 cd	CHR-149	0,20 cde	CHR-121	99,16 abc	CHR-162	5,56 cde	CA-15	6,63 bc
CA-118	3,39 cde	CHR-31	3,48 de	CHR-33	5,35 de	CHR-120	0,20 cde	CHR-161	98,75 abcd	CHR-33	5,37 cde	CHR-55	6,61 bcd
CHR-33	3,21 cde	CHR-153	3,44 def	CHR-31	5,26 def	CA-07	0,19 cde	CHR-77	98,75 abcd	CHR-31	5,18 cdef	CA-07	6,60 bcd
CHR-48	3,14 efg	CHR-162	3,39 defg	CA-118	5,14 efg	CHR-47	0,19 cde	CHR-44	98,33 abcde	CA-118	5,12 cdefg	CHR-136	6,57 bcd
CHR-12	3,10 fgh	CHR-77	3,34 defgh	CHR-12	5,01 efg	CHR-169	0,19 cde	CHR-155	98,33 abcde	CHR-12	4,87 defgh	CHR-162	6,55 bcd
CA-15	3,09 fgh	CA-07	3,33 defgh	CHR-48	4,79 fgh	CHR-32	0,18 cde	CA-07	98,33 abcde	CHR-48	4,69 efghi	CHR-33	6,40 cde
CHR-53	3,07 fgh	CHR-12	3,32 defgh	CHR-121	4,63 gh	CHR-31	0,18 cdef	CHR-53	97,91 abcde	CHR-77	4,59 efghi	CHR-12	6,40 cde
CHR-44	2,99 fghi	CA-118	3,26 efgh	CHR-77	4,63 gh	CHR-56	0,17 defg	CHR-39	97,91 abcde	CHR-121	4,59 efghi	CHR-153	6,38 cde
CHR-62	2,95 fghij	CHR-44	3,20 efgh	CHR-155	4,61 gh	CHR-55	0,17 defgh	CHR-136	97,91 abcde	CHR-53	4,55 efghi	CHR-117	6,37 cde
CHR-32	2,93 fghij	CHR-53	3,19 efgh	CHR-53	4,60 gh	CHR-151	0,16 defghi	CHR-31	97,91 abcde	CHR-155	4,53 efghi	CHR-151	6,35 cde
CHR-121	2,83 ghijk	CHR-117	3,13 efgh	CHR-44	4,42 hi	CHR-13	0,15 defghij	CHR-162	97,50 abcdef	CHR-44	4,35 efghij	CHR-120	6,28 def
CHR-149	2,78 hijkl	CHR-48	3,09 fgh	CHR-35	4,25 hij	CHR-117	0,15 defghijk	CHR-114	97,50 abcdef	CHR-35	3,96 fghijk	CHR-39	6,26 def
CHR-155	2,71 ijkl	CHR-121	3,06 fgh	CHR-117	4,02 ijk	CHR-114	0,13 efghijkl	CHR-48	97,50 abcdef	CHR-136	3,93 ghijk	CHR-121	6,17 efg
CHR-114	2,71 ijkl	CHR-13	3,01 gh	CHR-136	3,99 ijk	CHR-3	0,09 fghijklm	CHR-12	97,50 abcdef	CHR-117	3,89 ghijk	CHR-31	6,14 efg
CHR-35	2,65 jklm	CHR-35	2,99 h	CHR-153	3,95 ijkl	CHR-77	0,09 fghijklm	CHR-149	97,08 abcdef	CHR-149	3,86 ghijkl	CHR-62	6,00 fgh
CHR-31	2,62 jklm	CHR-149	2,69 i	CHR-149	3,86 jkl	CHR-35	0,09 fghijklm	CHR-117	96,66 abcdef	CHR-153	3,83 hijkl	CHR-169	5,99 fgh
CHR-136	2,53 klmn	CHR-114	2,62 ij	CHR-39	3,84 jkl	CHR-28	0,09 fghijklm	CHR-153	96,25 abcdef	CHR-39	3,76 hijkl	CHR-167	5,88 gh
CHR-153	2,51 klmn	CHR-136	2,62 ij	CHR-114	3,65 klm	CHR-48	0,08 ghijklm	CA-15	93,75 abcdefg	CHR-114	3,58 hijklm	CHR-13	5,78 h
CHR-55	2,51 klmn	CHR-28	2,50 ijk	CHR-13	3,61 klm	CHR-33	0,08 hijklm	CHR-32	93,33 bcdefgh	CA-15	3,39 ijklmn	MARA	5,54 i
CHR-77	2,49 klmn	CHR-32	2,41 ijkl	CA-15	3,60 klm	CHR-136	0,07 ijklm	CHR-169	92,91 cdefghi	CHR-13	3,25 jklmno	CHR-47	5,50 i
CHR-28	2,47 lmn	CA-15	2,39 ijkl	CHR-32	3,47 klm	CHR-39	0,07 ijklm	CHR-28	92,50 defghij	CHR-32	3,24 jklmno	CHR-161	5,43 ij
CHR-117	2,46 lmn	CHR-169	2,37 ijklm	CHR-28	3,39 ml	CHR-62	0,07 ijklm	CHR-35	92,08 efghij	CHR-28	3,13 jklmnop	CHR-114	5,31 ijk
CHR-56	2,45 lmn	CHR-39	2,34 ijklm	CHR-169	3,15 mn	CHR-53	0,07 ijklm	CHR-120	91,66 fghij	CHR-169	2,95 kl mnop	CHR-56	5,19 jkl
CHR-169	2,42 lmn	CHR-120	2,28 jklm	CHR-56	2,85 no	CHR-153	0,07 ijklm	CHR-56	90,41 ghijk	CHR-56	2,63 lm nop	CHR-53	5,07 klm
CHR-47	2,32 mno	CHR-56	2,16 klmn	CHR-120	2,82 no	CHR-162	0,07 ijklm	CHR-3	87,91 hijkl	CHR-120	2,61 lmnop	CHR-35	5,01 lm
CHR-3	2,30 mno	MARA	2,11 lmno	CHR-3	2,72 no	CHR-155	0,07 ijklm	CHR-13	87,91 hijkl	CHR-3	2,40 m nop	CHR-28	4,88 m
CHR-120	2,25 no	CHR-3	2,08 lmno	CHR-47	2,64 no	CA-15	0,06 ijklm	CHR-2	87,50 ijkl	CHR-47	2,31 nop	CHR-2	4,81 m
CHR-167	2,20 no	CHR-47	2,06 lmno	CHR-55	2,48 o	CHR-121	0,05 jklm	CHR-47	87,08 jkl	CHR-55	2,19 nop	CHR-48	4,80 m
CHR-2	2,06 op	CHR-112	2,00 mno	CHR-2	2,46 o	CHR-161	0,05 klm	CHR-55	85,83 kl	CHR-2	2,15 nop	CHR-77	4,79 m
MARA	2,03 op	CHR-167	1,83 no	MARA	2,44 o	CHR-44	0,04 lm	MARA	85,00 l	MARA	2,11 op	CHR-32	4,45 n
CHR-13	1,90 p	CHR-55	1,78 o	CHR-167	2,33 o	MARA	0,03 m	CHR-167	83,33 l	CHR-167	1,96 p	CHR-3	4,19 o

fact there is a group of clones that need an elongation stage to obtain microcuttings with good quality. Always the worse and inadvisable medium was SH for both variables that indicate proliferation growth and general aspects of the cultures.

Highly significant differences were found for all traits assessed. The genotype influence was the more important factor for traits related with multiplication rates in clonal propagation. For variables that indicate the quality of shoots results showed an important weight for the factor culture medium and also for the interaction clone-culture medium. The high values of the clonal repetitivities indicate that these traits [COMMENT1]are genotype dependent and allow the prediction of the behaviour of the clones when one wants to carry out *in vitro* propagation. High variability in growth rates and behaviour was found depending on the clone, so the multiplication coefficient ranged between 8.45 and 1.96 for hybrid clones.

Shoot apex necrosis for this group of clones and with equal concentrations of cytokinin, shows an important weight for the factor culture medium. So a favourable influence of NH_4^+ levels and very limited influence of the calcium concentration are observed. For chlorosis variable four levels have been observed. Both for shoot apex necrosis and chlorosis high concentrations of ions and compounds like HPO_4^{2-}, K^+, NO_3^-, and NO_3^-/NH_4^+ ratio promote elevated percentages and on the other side its seems that high levels of NH_4^+ have a positive effect on the prevention of this unfavourable traits. Two types of necrosis have been observed. The first one "limited to apex" allows to continue the growth of the explant and a second type "descending" that gradually extend down along the shoot.

When one tries to multiply a concrete clone it is necessary to make the individual analysis of this clone and to leave aside the results of the total population, because the best culture media for one clone are not necessarily the best for other clones. *Castanea sativa* clones have middle or low multiplication rates in comparison with hybrid clones.

Correlations shows that it is possible to select simultaneously pairs of production traits and at the same time to improve against the production of apical necrosis.

References

ABOUSALIM, A. and MANTELL, S. H.: A Practical Method for Alleviating Shoot-Tip Necrosis Symptoms in In vitro Shoot Cultures of Pistacia vera cv. Mateur: J. Hort. Sci. **69**, 357–365 (1994). — AHUJA, M. R.: Somatic cell differentiation and rapid clonal propagation of aspen. Silvae Genetica **32** (3–4): 131–135 (1983). — AHUJA, M. R. and MUHS, H. J.: Control of growth and differentiation in tissue and protoplast derived callus in different genotypes of aspen. In: Proc. 5th Int. Cong. Plant Tissue and Cell Culture. Pp. 177–178 (1982). — BALLESTER, A., SAN-JOSÉ, M. C. and VIÉITEZ, A. M.: Vitrification and Ba-induced anomalies in *in vitro* chestnut cultures. 1990. Suelo y Planta. **1**(1): 69–82 (1990). — BARTLETT, M. S.: The use of transformations. Biometrics **3**: 39–45 (1947). — BERGMANN, B. A. and STOMP, A. M.: Effect of the genotype on *in vitro* adventitious shoot formation in *Pinus radiata* and correlations between pairs of phenotypic traits during *in vitro* shoot development. Plant Cell Tissue and Organ Culture **39**: 185–194 (1994). — BIONDI, S., CANCIANI, L., DE PAOLI, G. and BAGNI, N.: Shoot formation from bud cultures of mature chestnut. Proc. Coll. Int. Cult.`In Vitro Essences For. IUFRO, Fontainebleau, France, AFOCEL, pp. 180–185 (1981). — BLAYDE, D. F.: Interaction of kinetin and various inhibitors in the growth of soybean tissues. Physiol. Plant **8**: 748–753 (1966). — BOX, G. E. P., COX, D. R.: An analysis of transformation. Journal of the Royal Statistical Society, Series B **26**: 211–243 (1964). — CHAUVIN, J. E. and SALESSES, G.: Advances in chestnut micropropagation (*Castanea* sp.). Acta Horticulturae, No 227, 340–345. International Symposium on vegetative propagation of woody species, Pisa, Italy, 3 to 5 Sep. 1987 (1988). — COLEMAN, G. D. and ERNST, S. G.: *In vitro* shoot regeneration of *Populus deltoides:* effect of cytokinin and genotype. Plant Cell Reports **8**: 459–462 (1989). — DURZAN, D.: Somatic embryogenesis and sphaeroblasts in conifer cell suspensions. Proc. 5th Int. Con. Plant Tissue and cell culture. Tokyo. Ed. A. FUJIWARA. Publ. The Japanese Association for Plant Tissue Culture, pp. 113–114 (1982). — FALCONER, D. S.: Introduction to quantitative genetics. Third edition. Longman

Scientific and Technical (1989). — FERNÁNDEZ-LÓPEZ, J.: Variabilidad isoenzimática, morfológica y selección clonal en *Castanea sativa* MILLER, *Castanea crenata* SIEB. et ZUCC., *Castanea mollissima* e híbridos interespecíficos. Tesis doctoral, Univ. Polit. de Madrid (1996). — FOSSARD, R. A. DE.: Tissue culture for plant propagators. University of New England, Armidale, Australia (1976). — GRESSHOFF, P. M., DOY, C. H.: Development and differentiation of haploid *Lycopersicon esculentum.* Planta **107**: 161–170 (1972). — HELLER, R.: Recherches sur la nutrition minérale des tissus végétaux cultivés *in vitro*. Ann. Sci. Nat. Bot. Biol. **14**: 473–497 (1953). — MIRANDA, M. E. and FERNÁNDEZ, J.: Estudio de las condiciones para el enraizamiento en brotes de castaños híbridos obtenidos por cultivo *in vitro*. II. Congresso Forestal Nacional. Novembro 1990. Porto, Portugal. Vol. I., pp. 433–436 (1990). — MIRANDA, M. E. and FERNÁNDEZ, J.: The micropropagation of chestnut tree: in vivo establishment and post-propagation growth. Mass production technology for genetically improved fast growing forest tree species. Symposium Bordeaux, France 1992. Tome I. pp. 421–426 (1992). — MIRANDA, M. E. and FERNÁNDEZ, J.: Cost components analysis of micropropagating chestnut plants. In: PARDOS, J. A., AHUJA, M. R. and ELENA ROSSELLO, R.: Biotechnology of trees. Proceedings of the IUFRO Working Party S2.04–07 Somatic Cell Genetics, Valsain, Spain 18 to 22 October 1993. pp. 39–44 (1993). — Munsell® Color Charts for Plant Tissues: Munsell Color. Macbeth division of Kollmorgen Instruments Corporation, 4'05 Little Britain Road, New Windsor, New York 12553–6148 (1977). — MURASHIGE, T. and SKOOG, F.: A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. **15**: 473–497 (1962). — PIAGNANI, C. and ECCHER, T.: Factors affecting the proliferation and rooting of chestnut *in vitro*. Acta Horticulturae **227**, 384–386. International Symposium on vegetative propagation of woody species, Pisa, Italy, 3 to 5 sep. 1987 (1988). — PIAGNANI, C., ZOCCHI, G. and MIGNANI, I.: Influence of Ca^{2+} and 6-benzyladenine on chestnut (*Castanea sativa* MILL.) *in vitro* shoot-tip necrosis. Plant Science **118** (1996). — QUORIN, M., LEPOIVRE, PH. and BOXUS, PH.: Un premier bilan de 10 années de recherches sur les cultures de méristèmes et la multiplication *in vitro* des fruitiers ligneux. C. R. Rech. 1976 à 1977. Stn. Cult. Fruit Maraîchères, Gembloux, pp. 93–117 (1977). — SALESSES, G., RONCO, L., CHAUVIN, J. E. et CHAPA, J.: Amélioration génétique du chataignier. Mise au point de tests d'evaluation du comportement vis-à-vis de la maladie de léncre. Lárboriculture Fruitiere **458**: 23–31 (1993). — SÁNCHEZ, M. C. and VIÉITEZ, A. M.: In vitro morphogenetic competence of basal sprouts and crown branches of mature chestnut. Tree Physiology **8**: 59–70 (1991). — SAS® Institute Inc.: SAS®/STAT User's Guide: Statistic Version 6.4th edn. SAS Institute Inc., Cary, N.C. (1990). — SCALTSOYIANNES, A., PANETSOS, K. P., ECONOMOU, A. and TSOULPHA, P.: Micropropagation of the pine hybrid *Pinus brutia* (TEN) x *P. halepensis* (MILL.) by culturing fascicle shoots. Ann. Sci. For. **51**, 175–182 (1994). — SCALTSOYIANNES, A., TSOULPHA, P., PANETSOS, K. P. and MOULALIS, D.: Effect of genotype on micropropagation of walnut trees (*Juglans regia*). Silvae Genetica **46**, 6, 326–332 (1997). — SCHAD, C., SOLIGNAT, G., GRENTE, J. et VENOT, P.: Recherches sur le chataignier à la Station de Brive. Annales de l'amelioration des plantes, III: 369–458 (1952.) — SCHENK, R. U. and HILDEBRANDT, A. C.: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. **50**: 199–204 (1972). — SHA, L., MCCOWN, B. H. and PETERSON, L.: Occurrence of shoot-tip necrosis in shoot cultures. J. Am. Soc. Hort. Sci. **110**: 631–634 (1985). — SHAPIRO, S. S. and WILK, M. B.: An analysis of variance test for normality. Biometrika **52**: 591–611 (1965). — URQUIJO, P.: La regeneración del castaño. Bol. Pat. Veg. Ent. Agr. XXII, 217–232 (1956). — VIÉITEZ, E.: Obtención de castaños resistentes a la enfermedad de la tinta. Centro Regional de Enseñanzas y experiencias Forestales de Lourizán, Pontevedra (1960). — VIÉITEZ, A. M., BALLESTER, A., VIÉITEZ, M. L. and VIÉITEZ, E.: In vitro plantlet regeneration of mature chestnut. J. Hortic. Sci. **58**: 457–463 (1983). — VIÉITEZ, A. M., SÁNCHEZ, C. and SAN-JOSÉ, C.: Prevention of shoot-tip necrosis in shoot cultures on chestnut and oak. Scientia Horticulturae **41**, 151–159 (1989). — VIÉITEZ, A. M. and VIÉITEZ, M. L.: *Castanea sativa* plantlets proliferated from axillary buds cultivated *in vitro*. Sci. Hortic. **18**: 343–351 (1982). — VIÉITEZ, A. M.,VIÉITEZ, M. L. and VIÉITEZ, E.: Chestnut (*Castanea* spp.) In: Y.P.S. BAJAJ (Editor): Biotechnology in Agriculture and Forestry. Vol. I: Trees I. Springer-Verlag, Berlin, pp. 393–414 (1986).

Testing the Conservation of *Quercus spp.* Microsatellites in the Cork Oak, *Q. suber* L.

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Summary

The transferability of microsatellite (SSR) loci from *Quercus* spp. to cork oak (*Quercus suber* L.) was investigated. Semi-automated analysis of fluorescently-labelled PCR fragments was used to test 24 primer sets developed for *Q. myrsinifolia* BLUME, *Q. petraea* (MATT.) LIEB. and *Q. robur* L. in 41 cork oak trees from four stands covering the main area of distribution of the species in Spain. Successful cross-species events occurred for 13 loci (54%). Two of them were monomorphic and another two appeared as multilocus. High levels of genetic variability were detected both for the number of alleles, 62 (7.5 per polymorphic locus, with a maximum number of 19 in locus ssrQpZAG110) and for the expected heterozygosity (mean H_E = 0.648). These results were much higher than those previously reported by other authors using allozyme loci. The usefulness of the SSR loci successfully amplified for studies on population genetics of cork oak is discussed.

Key words: Fagaceae, *Quercus suber,* cork oak, microsatellites.

Introduction

Cork oak (*Quercus suber* L., Fagaceae) is one of the most important evergreen oak species in the western part of the Mediterranean Basin. The geographic distribution of the

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