



MICROPROPAGATION OF ELITE GENOTYPES OF *CASTANEA SATIVA* (MILL.)

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ABSTRACT

Sprouted buds of eight mature plus-trees of *Castanea sativa* were collected from two natural populations (Mt. Paiko and Mt. Hortiatis, Greece). Explants were cultured on Murashige and Skoog (MS) medium (three concentrations of macro and micro-elements) combined with three concentrations of BAP (6-Benzylaminopurine). Results were evaluated based on three multiplication traits: (a) number of shoots produced, (b) length of the longest shoot segments and (c) percentage of responsive explants. A high genotype-dependant variation for the number of shoots produced per explant was detected. MS medium at full salt strength and 0.2 mg l⁻¹ BAP provided the best results based on the multiplication traits measured. Elite mature tree P-2 proved to be highly favourable for multiplication. Rooting was attempted on 1/2 MS medium at five different NAA concentrations. Successful rooting (56%) was obtained by using 0.50 mg l⁻¹ NAA in vermiculite medium after eight weeks. These results indicate that the P-2 genotype can be micropropagated from the protocol developed for ornamental purposes, clonal testing and tree improvement applications.

Keywords: European chestnut, plus-trees, MS media, rooting media, multiplication traits, clonal testing.

Academic Discipline And Sub-Disciplines

Plant Genetics

SUBJECT CLASSIFICATION

Tissue Culture, Micropropagation

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INTRODUCTION

European chestnut (*Castanea sativa* Mill.) is one of the more widespread broad-leaved tree species in southern Europe and Asia Minor where it is used for high quality timber and starch containing nut production in managed forests and fruit orchards. It appears as natural (in autochthonous pure and mixed with other broadleaves stands) and naturalized forest, coppice forest and orchards, the latter dedicated to nut production, representing an important and vital integral part of the rural economy (Aravanopoulos et al. 2001). Moreover, *C. sativa* presents considerable ornamental qualities and in places not favourable for nut production (central Europe, the United Kingdom, parts of North America), is used as an ornamental tree (Plants for a Future 2014). Its qualities include a majestic dome shaped canopy, yellow-white and strongly fragrant catkins produced in the summer, green spiked burs that contain the nut which ripe in the fall, deeply serrated, large and dark green foliage turning golden bronze before falling and a twisted, ribbed, retiform bark that takes a conspicuous form with age (FAO (2014).

However, *C. sativa* is under pressure from a number of environmental and anthropogenic threats, that include diseases such as chestnut blight caused by *Cryphonectria parasitica* (Murr) and ink disease caused by *Phytophthora cinnamomi* (Rand), as well as environmental degradation of natural ecosystems. Specially designed European-wide breeding programs aim at developing genetically improved chestnuts for a multitude of applications including timber production, nut production, ecosystem stabilization and ornamental use, calling for the selection and use of novel cultivars and varieties (Fernandez-Lopez et al. 2005, Tchatchoua and Aravanopoulos 2010a, 2010b). Quantitative genetics results from a provenance-progeny test located at Taxiarchis, Greece which is part of a European-wide provenance-progeny trials network (Fernandez-Lopez et al. 2005), indicated that plus-trees from Greek provenances could be considered as elite trees and can be used in further multipurpose chestnut breeding programs for the genetic improvement of wood and nut production, as well as aesthetic form (Tchatchoua and Aravanopoulos 2010b). Henceforth, tissue culture propagation methods were initiated on elite genotypes for clonal selection procedures. Tissue culture allows elite individuals to be directly tested as clones, (instead of indirect testing, typical of progeny tests). Besides saving time, since it does not require trees to produce seed, this strategy also allows a higher number of individuals to be tested per unit of time and space. In addition, the potential of vegetative propagation techniques to deliver high genetic gains is evident, as they offer the possibility of capturing the total genetic variation (i.e. both additive and non-additive components).

Vegetative propagation of chestnut has proved to be challenging. Rooting of greenwood and hardwood cuttings has proved largely unsuccessful (Cummins 1970). Vegetative propagation by layering was developed for some clonal rootstocks (Ridley and Beaumont 1999) however this method is laborious and time-consuming. Various grafting and budding methods have been applied in chestnut, being heavily dependant on factors such as time of grafting, age, growth vigour of rootstock, quality and quantity of scion, graft incompatibility and susceptibility to chestnut blight (Ferrini and Pisani 1994). Grafting results have been generally inconsistent (Osterc et al. 2005). Tissue culture can provide an adequate method for mass propagation of elite genotypes in *C. sativa*. In the past decades tissue culture application in *C. sativa* has recorded some successes (Rodriguez 1982; Sanjose et al. 1982; Cheure et al. 1883; Qu-guang et al. 1986) The success of this method is variable and generally depends on: (a) genotype (Giovannelli and Giannini 1999), (b) rejuvenation and pre-treatments applied (Martins and Pais 2005) and (c) nutritional solutions and growth regulators used (Vieitez and Vieitez 1980; Vieitez et al., 1983; Osterc et al. 2005). The aim of the present study is to investigate the feasibility of micro-propagation of elite mature Greek genotypes of *C. sativa* suitable for ornamental purposes and wood production.

1. MATERIALS AND METHODS

2.1 Origin of explants

Scions were collected from stump sprouts of eight mature plus-trees from two old-growth natural *C. sativa* populations located in Mt. Paiko and Mt. Hortiatis, Greece. Tree diameter was measured by a Nestle Best Nr 300060 calliper, tree height with a Vertex IV and Transponder T3 instrument and a large increment borer was employed to record tree age (Table 1). The scions were dipped in small plastic jugs for sprouting in a glass house of the Laboratory of Forest Genetics and Tree Breeding, Aristotle University of Thessaloniki, Greece. After sprouting, young shoots about 2-3 cm long were excised from the axillary meristem put in labelled plastic bags and transferred to the laboratory.

Table 1. Description of the mature *Castanea sativa* plus-trees used in micropropagation experiments.

Tree code	Location	Altitude (m)	Height (m)	Diameter (cm)	Age
H-2	Mt. Hortiatis	520	30.0	35	60
H-B	Mt. Hortiatis	550	11.0	62	80
P-2	Mt. Paiko	700	15.3	103	500
P-4	Mt. Paiko	720	13.5	61	350
P-5	Mt. Paiko	730	12.0	60	250
P-8	Mt. Paiko	750	16.3	107	200
P-A	Mt. Paiko	630	15.2	35	47
P-B	Mt. Paiko	600	14.5	30	60
Average		650	16	61.6	193.4

2.2 Phase 1: Sterilization and inoculation of explants

Leaves were removed from the young shoots and washed thoroughly in running tap water. They were sterilized in a 0.15% solution of $HgCl_2$ for 2 min, rinsed three times in sterile deionised water and single-node segments were placed in tubes containing 50ml of MS (Murashige and Skoog 1962) medium for shoot initiation. Each culture tube contains five single - nodes with four repetitions per genotype. All media were supplemented with 30 g l^{-1} of sucrose, 7.1 g l^{-1} agar, vitamins, micronutrients and Fe-EDTA of Murashige and Skoog (1962), 0.1 mg l^{-1} of BAP (6-Benzylaminopurine). The pH was adjusted to 5.6 prior to autoclaving. The propagation chamber was maintained at 25°C and 16 hours of photoperiod, under white fluorescent lamps throughout the experiment..

2.3 Phase 2: Multiplication phase

Multiplication was developed by axillary shoot production. Stem segments at a length of at least 2 cm, bearing at least one axillary or apical bud, were excised and inserted vertically into the multiplication medium.

The culture media used were: MS (Murashige and Skoog, 1962) with half strength of nitrates (MS- $\frac{1}{2}NO_3$) (Vieitez et al. 1983), $\frac{1}{2}MS$ and MS. BAP (6-benzylaminopurine) concentrations used as growth regulators were: BAP 0.1 mg l^{-1} , BAP 0.2 mg l^{-1} and BAP 0.5 mg l^{-1} . The shoots were sub-cultured every four weeks for multiplication and elongation under same condition of light as described above. The number of explants used per medium per BAP concentration was 20. In total, 180 combinations ($20 \times 3 \times 3$) were used for the three media and three hormone concentrations per subculture. Four subcultures were carried out and during each sub-culturing the recorded variables were: (a) number of shoots produced, (b) length of the longest shoot segments, which indicates the possibility of obtaining shoots that can be used directly for rooting and (c) percentage of responsive explants, which indicates how explants respond to the different treatments applied (Fig. 1).

Please, check the best culture media in Results



(a)

(b)

(c)

Figure 1. Micropropagation sequence in *Castanea sativa* mature plus-trees: (a) explants in MS initiation culture medium, (b) shoot from culture medium, (c) separation and transfer of culture shoots.

2.4 Phase 3: Rooting

Shoots greater than 2 cm long were excised and placed in sterilised vermiculite where a solution of $\frac{1}{2}MS$ medium supplemented with five (0.0 mg l^{-1} , 0.10 mg l^{-1} , 0.25 mg l^{-1} , 0.50 mg l^{-1} , and 1.0 mg l^{-1}) different concentrations of NAA was added. The number of replications employed for the rooting experiment was 16 for each treatment and a total sample size of 80 was produced during this stage with only one factor considered, i.e. the different concentrations of NAA in the vermiculite medium used. The variables measured after eight weeks were: (a) number of roots, (b) length of each root and (c) percentage of rooted shoots. Rooted shoots were removed from the agar medium, washed in running tap water and transferred in plastic pots for acclimatisation in a mixture of perlite and vermiculite.

2.5 Statistical analysis

One-way analyses of variance (ANOVA) were carried out. The data set was balanced with approximately normal distribution, hence no transformation was required. The type III model at the 5% significant level was employed and the means among factors were compared using the Duncan Multiple Range Test (DMRT) where appropriate. The SPSS (version 15) statistical software package was used (SPSS 2006).

2. RESULTS AND DISCUSSION

3.1 Effect of genotype on *Castanea sativa* tissue culture

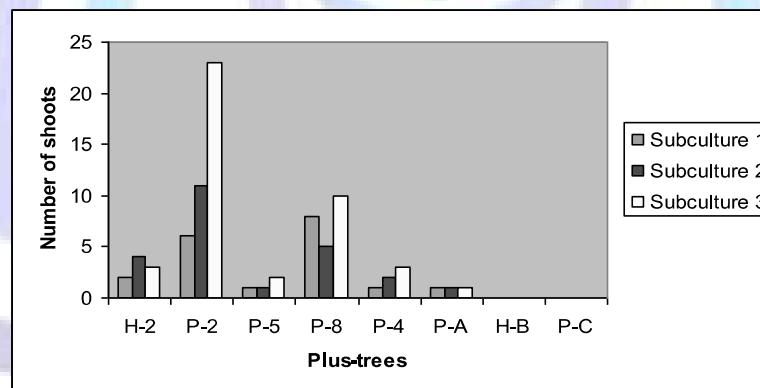
The selected individuals were mature or over-mature plus-trees of high growth and aesthetic value. The age of the trees ranged between 47 and 500 years old, with an average of 193.4 years; tree height averaged 16 m and diameter 61.6 cm (Table 1). All eight mature elite plus-trees were successfully introduced in tissue culture. For shoot regeneration, the number of shoots for the P-2 and P-8 elite-trees, was observed to be very high compared to the other plus-trees that were used (Table 2, Fig. 2). A genotype effect was also observed regarding growth of the elite trees in culture. The effect

was manifested in the number of shoots per explant. The highest regenerated shoots per explant were obtained in genotype P-2. Miranda and Fernandez-Lopez (2001) also showed that micropropagation of different *C. sativa* clones, as well as hybrid clones of *C. sativa* x *crenata* and *C. sativa* x *mollissima* was genotype-dependent. Even when Ballester et al. (2001) used several different mineral media he observed that it was impossible to identify a general purpose medium for all the clones. Similar results are common in other angiosperms as well. For instance, Scaltsoyiannes et al. (1997) found a significant genotype effect in micropropagation experiments of *Juglans regia*. Ahuja (1983) used 48 aspen clones, 22 of which exhibited growth and differentiation in culture, while the remaining clones showed little or no growth. Coleman and Ernst (1990) examined 15 genotypes of *Populus deltoides* and found that six were recalcitrant to shoot regeneration. Srivastava et al. (1991) found that regeneration success is mainly genotype dependant in *Populus tomentosa*. Furthermore, Scaltsoyiannes et al. (2009) also found differences among genotypes in the successful micropropagation of *Prunus avium*.

Table 2. Plus-tree *Castanea sativa* genotypes and mean number of regenerated shoots produced

Plus-tree	Mean	S.E
H-2	3.0000	± 0.57735
P-2	13.3333	± 5.04425
P-5	1.3333	± 0.33333
P-8	7.6667	± 1.45297
P-4	2.0000	± 0.57735
P-A	1.0000	± 0.00000
H-B	.0000	± 0.00000
P-C	.0000	± 0.00000
F-Value	6.131	
p-Value	001	

Figure 2. Effects of *Castanea sativa* mature plus-tree genotypes on the number of shoots produced in tissue culture.



Usually, juvenile explants are most readily established in culture, while they grow and proliferate at a more rapid rate than adult explants tissue. Nevertheless, the genotypic effect in this experiment resulted in the proliferation of only the P-2 elite mature tree, regarding *in vitro* multiplication. In the genus *Castanea*, Chevre and Salesses, (1987) found that truly juvenile shoot tips from seedlings could be explanted and propagated by shoot culture, but adult material (for example of the hybrid *C. crenata* x *C. sativa*) require to be rejuvenated in advance. *C. sativa* shoot tips isolated from seedlings obtained by embryo culture, resulted in shoots which proliferated more rapidly and rooted more easily than those derived from axillary bud explants (Piagnani and Eccher 1988). Moreover, Chevre et al. (1983) noted a difference in the axillary shoots obtained from juvenile and mature buds of this species.

3.2 Effect of media and concentrations on shoot length and shoot number of *Castanea sativa*

The analysis of the effects of media and concentrations on the size of the longest shoots and the number of shoots produced, showed that both media and their concentrations had highly significant effects. Among the different media employed, MS was the best followed by MS-1/2NO₃ (Fig. 3, 4, Table 3). The best results regarding BAP concentrations were obtained at 0.1 mg l⁻¹ BAP, followed by 0.2 mg l⁻¹ BAP, while the least effective was 0.5 mg l⁻¹ BAP

(Fig. 5, Table 4). The medium and concentration that produced the longest shoots also produced the largest number of shoots. While MS was the best medium identified, it also indicated the present of vitrified shoots. This result indicates that a high concentration of nitrate ($1650 \text{ mg l}^{-1} \text{ NH}_4\text{NO}_3$) in the medium favourably influenced the number of shoots produced. However, negative effects of a high $\text{NO}_3^- / \text{NH}_4^+$ ratio on *in vitro* growth were mentioned by Vieitez et al., (1983) in chestnut, Gitonga et al. (2008) in *Macadamia integrifolia*, and more generally by Griffis et al. (1983) in fruit trees, and McCown and Sellmer (1987) in woody species. On the other hand, high proliferation rates on media containing low total nitrogen levels and low nitrate/ammonium ratios were observed (Miranda-Fontaina and Fernandez-Lopez 2001, Piagnani and Eccher 1988, Riffaud and Cornu 1981), in contrast to the present findings. The best medium for clones and hybrids of *C. sativa* studied by Miranda and Fernandez-Lopez (2001) was the GD medium (Gresshoff and Doy 1972), while the MS- $\frac{1}{2}\text{NO}_3$ medium was the second best on the number of shoots per explant as in the present study. Mullins (1987) recorded the highest multiplication rate for *C. sativa* clones using a combination of MS- $\frac{1}{2}\text{NO}_3$ and BA (0.1 mg l^{-1}).

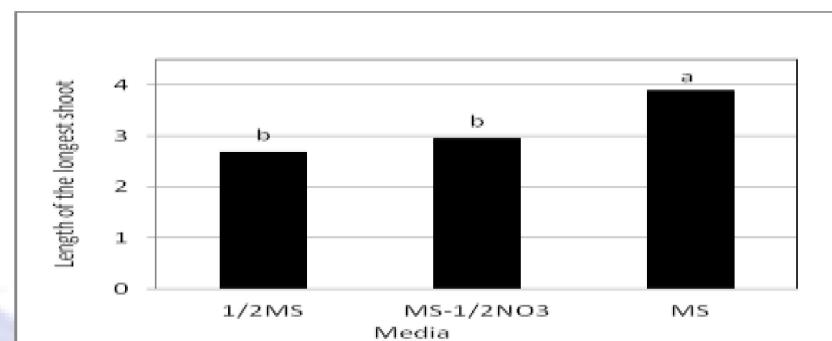


Figure 3. Effect of media on length of shoots on the *Castanea sativa* mature plus-tree genotypes.

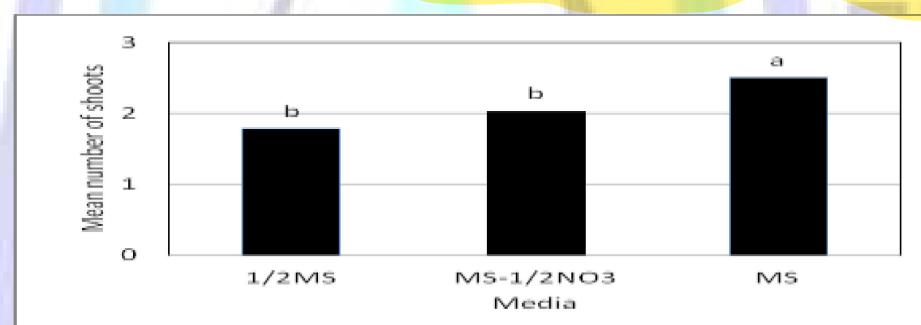


Figure 4. Effect of media on number of shoots on the *Castanea sativa* mature plus-tree genotypes.

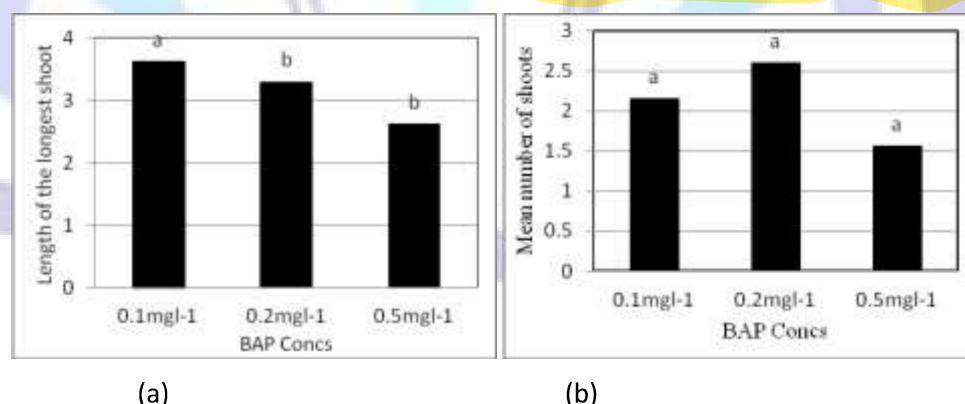


Figure 5. Effect of BAP concentration on shoot length (a) and shoot number (b) of *Castanea sativa* mature plus-tree genotypes.

With regards to the different concentrations of BAP used, it is noted that BAP has been found to promote axillary bud proliferation in *Castanea* in (Vieitez and Vieitez 1980). Riffaud and Cornu (1981) also used successfully a $4 \times 10^{-6} \text{ M}$ BAP concentration in *Prunus avium* micropropagation. The effect of cytokinins on tissue or organ cultures can vary according to the particular compound used, the type of culture, and the variety of plant from which it was derived and whether the explant is derived from juvenile or mature tissue (Sanchez et al. 1997). In *Corylus avellana*, 5 mg l^{-1} BAP gave the best rate of shoot multiplication from juvenile explants, but 10 mg l^{-1} zeatin was required for nodal sections of plants in the adult phase (Messeguer and Mele 1987). In many species shoots formation can be stimulated by a balance between auxin and cytokinin as demonstrated by Grinblat, (1972) in *Citrus*.

Table 3. Length of the longest shoot and number of shoots per media in *Castanea sativa*.

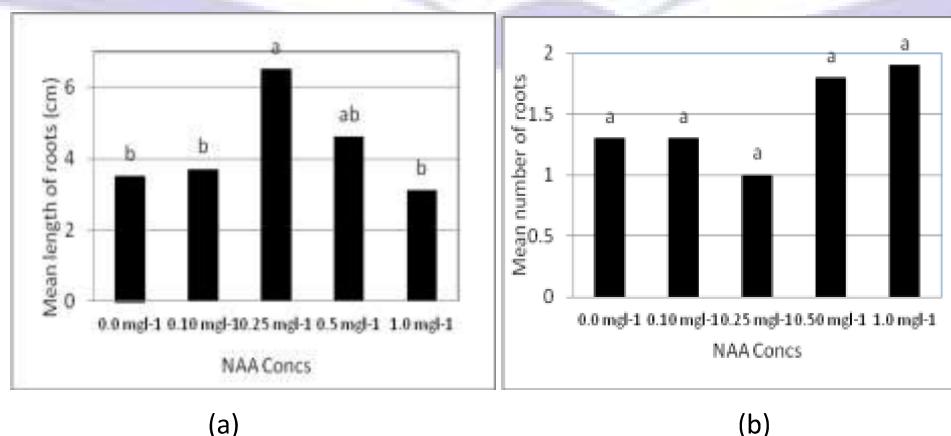
Media	Length of the longest shoot (cm)	Mean number of shoots
½ MS	2.68 ± 0.176	1.78 ± 0.134
MS-1/2 NO ₃	2.95 ± 0.188	2.03 ± 0.143
MS	3.89 ± 0.231	2.50 ± 0.165
F-Value	6.446	12.721
p-Value	.002	000

Table 4. Length of the longest shoot and number of shoot per BAP concentration in *Castanea sativa*.

BAP Concentration	Length of the longest shoot (cm)	Mean number of shoot
0.1 mg l ⁻¹	3.61 ±0.219	2.15 ± 0.145
0.2 mg l ⁻¹	3.29 ± 0.186	2.60 ± 0.121
0.5 mg l ⁻¹	2.61 ± 0.197	1.56 ± 0.169
F-Value	10.095	6.073
p-Value	0.000	.002

3.3 Effect of NAA concentration on *in-vitro* rooting of *Castanea sativa*

We investigated different concentration of NAA for rooting ability using the P-2 elite tree. Both the highest mean number of roots and the mean length of roots were recorded in a concentration of 1.0 mg l⁻¹ NAA and 0.25 mg l⁻¹ respectively (Fig. 6, Table 5). In addition, the highest percentage (56.25 %) of rooting was recorded in a concentration of 0.5 mg l⁻¹ NAA (Fig. 7, Table 5). Among the concentrations of NAA employed, the lowest number of roots was observed in 0.25 mg l⁻¹ NAA, the shortest root length was observed in 1.0 mg l⁻¹ NAA and the lowest percentage of roots was observed in 0.25 mg l⁻¹ NAA. The analysis of variance showed that the number of roots produced differed significantly among NAA concentrations ($p=0.002$), while for root length there was no significant difference among NAA concentrations. Similar rooting percentages were obtained in microcuttings of *Castanea dentata* by dipping in 5-10 mM indolebutyric acid for one minute and cultivated in half strength MS basal medium and adding 0.2 g l⁻¹ charcoal for two weeks. In cultures established from juvenile and mature-phase debladed petioles, auxin induces cell division in explants of both mature and juvenile origin, but only the latter further develops forming adventitious roots (Murray et al. 1994). Differences observed in rooting response of easy to root and difficult to-root apple cultivars concerned the capacity to rapidly organize new cells into primordial and not just the capacity to reactivate cell division after wounding and auxin treatment (Collet et al. 1994). Auxin has been known to be involved in the process of adventitious root formation (Haissig and Davis 1994), while the interdependent physiological phases comprising the rooting process are associated with changes in endogenous auxin concentration (Heloir et al. 1996). This work, to the best of the authors' knowledge, is the first reported on elite mature trees from Greek populations.


Figure 6. Effect of NAA concentration on length of roots (a) and number of roots (b) of *Castanea sativa* mature plus-tree genotypes.

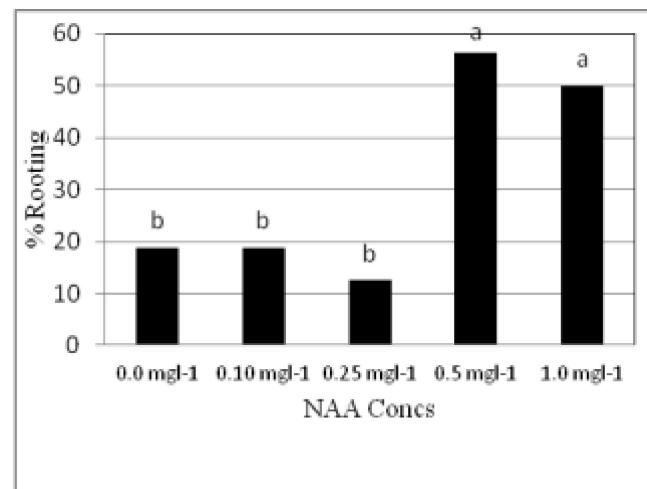


Figure 7. Effect of NAA concentrations on rooting percentage of *Castanea sativa* mature plus-tree genotypes.

Table 5. Percentage rooted, mean length of the roots and number of root per NAA concentration in *Castanea sativa*.

NAA Concentration	% rooted	Mean length of the root (cm)	Mean number of roots
0.0 mg l⁻¹	18.75	3,5 ± 0.25	1,3 ± 0.51
0.1 mg l⁻¹	18.75	3,7 ± 0.25	1,3 ± 0.66
0.25 mg l⁻¹	12.5	6,5 ± 0.13	1.0 ± 0.69
0.5 mg l⁻¹	56.25	4,6 ± 0.26	1,8 ± 0.59
1.0mg l⁻¹	50	3,1 ± 0.17	1,9 ± 0.55
F-Value	3.380	4.741	2.336
p-Value	0.013	0.002	0.063



(a)

(b)

Figure 8. Rooted shoots of *Castanea sativa* mature plus-tree genotypes by using a concentration of 0.50 mg l⁻¹ NAA (a) and 1 mg l⁻¹ NAA (b).



3. CONCLUSION

The main objective of this work was to develop a tissue culture protocol for different *Castanea sativa* mature and over-mature plus-tree genotypes of high growth and aesthetic value that originated from two Greek populations. Results indicated that micropropagation of selected mature genotypes, such as the 500 years old P-2 elite mature tree from Mt. Paiko, is achievable and can provide a useful tool for the mass propagation of selected mature plus trees for wood and nut production, ornamental use, as well as for tree improvement purposes. This study also indicates that further research on sources of explants, media, hormones and their concentrations will be highly promising for the successful multiplication of elite mature trees.

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