Gametic embryogenesis through isolated microspore culture in Corylus avellana L

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Abstract

Haploid technology is a valuable tool to obtain homozygosity particularly in woody plants, in order to support plant breeding. Hazelnut, the fourth worldwide nut tree, is a monoecious, anemophilous species and it is characterized by a sporophytic incompatibility system (Germain and Dimoulas, 1978), not allowing to obtain homozygous plants through conventional methods, involving several self-pollination. In this study, gametic embryogenesis was applied to hazelnut and, particularly, isolated microspore culture was performed on four different cultivars. Two culture media were tested and four temperature stress treatments were applied to the isolated microspores, cultivated at the vacuolated stage of development. For the first time in our knowledge, early embryos were recovered in hazelnut *via* isolated microspore culture in all cultivars. The assessment of the zygosity condition of embryos carried out using SSR markers showed that analysed embryos were homozygous indicating that they developed from haploid microspores. The response to the culture treatments, was, however, genotype-dependent, as previously reported in experiments regarding male gamete embryogenesis in other fruit crops.

Keywords: Haploid, hazelnut, gametic embryogenesis, temperature stress.

Introduction

Hazelnut (*Corylus avellana* L.), belonging to the Betulaceae family of the order Fagales and native to European regions, Turkey and the Caucasus mountains (Kafkas and Dogan, 2009), is the sixth worldwide tree nut, with a production of more about 858,000 tons (FAOSTAT, 2013), behind cashew, Persian walnut, chestnut, almond and pistachio. All cultivated forms of hazelnut are diploid with a monoploid number of chromosomes n=x=11. It is a monoecious, dichogamous, self-incompatible and wind pollinated plant (Rovira et al. 1993). The incompatibility system is sporophytically determined and controlled by a single locus with multiple alleles (Martins 2010). Incompatibility is a main factor limiting genetic improvement, and it prevents to reach homozygosity by conventional methods, involving several self-pollination cycles.

Gametic embryogenesis is recognized as an important tool for plant breeding, making possible to develop, in only one step, completely homozygous lines, from heterozygous parents (Bueno et al. 2006; Seguì-Simaro and Nuez 2008; Islam and Tuteja 2012; Datta 2005; Solis et al. 2008; Germanà 2011a). This opportunity is particularly useful for breeding in woody species, in which it is not possible to obtain homozygosity through conventional methods (Germanà 2011b), because of the long juvenile phase, the high degree of heterozygosity, the large size and, often, of the self-incompatibility (Germana` 2006; 2009).

Haploid (Hs) and doubled haploid (DHs) plants, are very useful to fix parental lines to produce F1 hybrids, for rapid introgression of new characteristics through backcrossing, to develop population molecular maps, to fix characteristics through transformation and mutagenesis (Datta 2005; Szareijo and Foster 2006; Soriano et al. 2013) and to do reverse breeding (Dirks et al. 2009; Ferrie and Möllers 2011). In addition, haploids and double-haploids are important for genomic studies and genome sequencing (Aleza et al. 2009; Dirks et al. 2009; Ferrie and Möllers 2011; Foster et al. 2007; Germanà et al. 2013; Talón and Gmitter 2008), for physical mapping (Leeuwen et al. 2003; Kunzel et al. 2000; Wang et al. 2001), genetic mapping (Houssain et al. 2007; Chu et al. 2008; Zhang et al. 2008), and for the integration between physical and genetic mapping (Kunzel et al. 2000; Wang et al. 2001).

Gametic embryogenesis represents a unique system where, thanks to the plant totipotency, a single immature gamete switches from the gametophytic pathway toward the sporophyte formation (Prem et al 2012). Gametic embryogenesis, that can be achieved from female (gynogenesis) or male (microspore embryogenesis) gametes, rarely occurs in nature (Silva 2012), and it is usually induced subjecting immature gametes to some kinds of stress. Particularly, temperature stresses, hot and cold, are the most effective and commonly used ways to promote microspore embryogenesis in several species (Ferrie and Caswell 2010; Germanà 2011 a; Shariatpanahi et al. 2006; Silva 2012). Specifically, the involvement of heat shock proteins (HSPs), when temperature treatments are applied in the microspore embryogenesis, has been described, but the molecular mechanism of the induction and the relationship between different stresses remain unclear. However, it is supposed that the increased levels of HSPs may be associated with the embryogenic potential acquisition (Karami and Saidi 2010; Silva 2012).

In many crops, anther culture is often the method of choice for H and DH production because of its simplicity (Sopory and Munshi 1996). Nevertheless, the technique of isolated microspore culture, performed by removing somatic anther tissue and requiring better equipment and more skills compared to the first technique, provides a better system for investigating cellular, physiological, biochemical and molecular processes involved in pollen embryogenesis (Nitsch 1977; Reinert and Bajaj 1977; Germanà, 2011b).

In this study, the technique of isolated microspore culture was applied to five hazelnut genotypes, testing two basal media and four temperature stresses, with the aim to obtain micropore-derived embryo regeneration.

Materials and Methods

Plant material and pollen developmental stage

Immature catkins (Fig. 1a) were harvested in February from trees of the following cultivars of *Corylus avellana* L.: 'Tonda Gentile Romana' (2 clones: **TR and GR**), 'Imperatrice Eugenia' (**IE**), 'Minnulara' (**M**) and 'Carrello' (**C**).. Catkins with anthers (Fig. 1a) mostly containing microspores at the vacuolated/uninucleated stage (Fig. 1b) were selected, assuming that, like in many other fruit crops (Germanà 2011 a; 2011b), this stage of development is the most responsive for gametic embryogenesis also for hazelnut. For this reason, a preliminary investigation was carried out to select the appropriate catkin size staining anthers with few drops of 4',6-diamidino-2-phenylindole (DAPI) solution (1 mg/mL) and observing them under fluorescence microscope (Zeiss, Axiophot, Germany). Catkins with anthers containing microspores at vacuolated/uninucleate stage were selected and, as common pre-treatment, were placed in darkness at 4 °C for two weeks.

Catkins sterilization and microspore isolation and culture

After two weeks of 4 °C pretreatment, 5 catkins for each genotype were surface sterilized by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion for 20 min in 25% (v/v) sodium hypochlorite solution (about 1.5% active chlorine in water) with few drops of Tween® 20, and then rinsed three times with sterile distilled water. After that, the material was maintained in immersion in gentamicin antibiotic solution (0.2%) for 30 minutes. The antibiotic solution was removed and the microspore isolation was performed following the procedure reported by Kumlehn et al., (2006) with limited modifications. Isolated microspores were cultured at the concentration of 100,000 microspores per mL and a final volume of 1.0 mL per each Petri dish (3001-type Petri dishes, BD Biosciences) was placed.

Petri dishes, sealed with Parafilm®, were placed at 26±1 °C, in the dark for the first 30 days and later under cool white fluorescent lamp (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 µmol m^{-1} s^{-1} and a photoperiod of 16 light hours.

Experimental design

In order to evaluate the influence of medium composition on the embryogenic induction of the five genotypes, isolated microspores were cultured on two different media: **P** (Germanà et al. 1996) and **N6** (Germanà and Chiancone 2003). Moreover, because it is well known that usually stress enhances microspore response, isolated microspores cultured in both media, were subjected to the following thermal stress, just after isolation:

1) 35 °C for 30 minutes (**H),**
2) 40 °C for 60 minutes **(H+)**,
3) -20 °C for 30 minutes **(F)**,
4) -20 °C for 60 minutes **(F+)**.

Height Petri dishes (repetitions) were prepared per each combination (genotype, medium, thermal stress).

Data collection and statistical analysis

Isolated microspores in culture were weekly observed by a stereo-microscope (Leica MZ 125) and by an inverted microscope (Zeiss West Germany). In addition, examinations were performed by a fluorescence microscope (Zeiss, Axiophot, Germany), after staining with 4',6-diamidino-2-phenylindole (DAPI). Because changes in the microspore development distribution were observed for a long time, it was decided to report the final monitoring of the different features, registered after 20 months of culture. At this time, per each genotype and per each combination medium/thermal stress, 450 microspores/structures (three repetitions with 150 elements each) were counted, in order to individuate the different structural features: uninucleated microspores, rarely binucleated with two different nucleus sizes beginning their normal gametophytic pathway, binucleated with two equal-size vegetative-type nuclei just starting their sporophytic pathway, trinucleated, and also multinucleated microspores. Moreover, the number of embryos and calli produced per each Petri dish were registered. These values were used to calculate means and to perform the statistical analysis. Differences among genotypes were tested by one-way analysis of variance (ANOVA) with Conover-Inman's test ($p \leq 0.05$). To individuate the influence of the factors "Medium" and "Thermal treatment", means were analysed by two-way ANOVA, and mean separation was performed using Tukey's multiple comparison tests at ($p \leq 0.05$).

Detection of homozygosity

Simple Sequence Repeat (SSR) markers were used to assess the genetic condition of embryos obtained from pollen microspore culture and to determine their origin (gametic or somatic).

DNA extraction

Embryos were collected from the culture medium by an insulin syringe, frozen in liquid nitrogen and ground using steel beads in a Tissuelyser (QIAGEN®, Germany). DNA extraction for genotyping was performed as described in Doyle and Doyle (1987) with minor modifications.

PCR amplification and SSR allele sizing

Five fully characterized SSR loci were used for assessing the origin of the embryos: CaT-B107, CaT-B503, CaT-B504, CaT-B505, CaT-B507 (Boccacci et al., 2005). PCR was performed, as described by Boccacci et al. (2008), in two steps in a total volume of 10 µl containing 20 ng of DNA, 0.25 U of KAPA Taq DNA polymerase (KAPABIOSYSTEMS, Wilmington Massachusetts, USA) 1 µl of 10X PCR buffer, 200 µM nucleotide mix and 0.5 mM of each primer. PCR conditions were as follows: an initial denaturation step at 95° C for 3 min followed by 32 cycles of denaturation (30 s at 95°C), annealing (45 s at 52° C), and extension (90 s at 72° C). The final elongation step was at 72°C for 30 min. Two µl of the amplification product were used as template for a second PCR, carried out for 28 cycles with the same conditions of the first one.

PCR products were analyzed on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Data were processed using GeneMapper Software (ver. 4.0; Applied Biosystems) and alleles were defined by their size in base pairs, by comparison with the size standard (GeneScan-500 LIZ, Applied Biosystems).

Data obtained were compared with the hazelnut database of DISAFA.

Results and Discussion

The microspore culture monitoring carried out by fluorescence microscope after 20 months of culture and after DAPI staining, showed uninucleated (Fig. 1b), binucleated presenting nuclei with asymmetrical division (Fig. 2a), binucleated revealing nuclei with symmetrical division (Fig. 2b), trinucleated (Fig. 2c), and multinucleated stage (Fig. 2d); the performances of the different genotypes are reported in table 1-6. Actually, the presence of binucleated microspores with asymmetrical division of the nucleus, indicating of the gametophytic pathway, was very sporadic: never more that 3% and in three genotypes, (C, IE and M), less than 1% of the total (Table 1).

The statistical analysis carried out to study the *in vitro* isolated microspore development in the five hazelnut genotypes tested, showed different responses among the genotype (Table 1). For this reason, the influence of the two parameters "Medium" and "Thermal treatment" on hazelnut microspore development was carried out separately per each genotype. Particularly, regarding the "Medium" influence on the multinucleated microspores production, the P medium was the most valuable in the genotypes C, M and TR (Tables 2, 5 and 6). In the other genotypes, differences between the two media were not statistically significant. Two way ANOVA evidenced also that in the cultivars C and M, the formation of multinucleated microspores was mainly influenced by the factor "Medium", while different results were recorded for the genotypes GR, IE and TR, where a significant interaction was observed between the two factors. IE, GR and C, were the genotypes with the highest percentages of multinucleated microspores.

For GR, the factor "Treatment" was the main source of variability in the multinucleated microspore induction (Table 3). Particularly, statistically significant differences were observed between the treatment F+ and the treatment H, while the other treatments induced intermediate responses. The analysis of interaction evidenced a statistically significant difference in the thermal treatment H, where N6 induced better response than P (32.0% *vs* 19.8%) (data not shown).

Diverse response was observed in the cultivar IE concerning the same parameter, in which none of the factors had a predominant influence (Table 4), but differences were observed in the response to thermal treatments between the two media: P better than N6 in H+ (+34.7% *vs* 24.7%) and in F (40.0 % *vs* 23.3%), whereas N6 better than P in F+ (30.0% *vs* 22.7%) (data not shown).

On the contrary, for TR, both factors ("Medium" and "Treatment") had a significant influence on the multinucleated microspore induction. Analyzing separately the factors, P medium induced a statistically significant higher percentage of multinucleated microspores than N6; while, regarding the thermal treatments, two groups were individuated: Co and H induced statistically higher response than H+, F and F+ (Table 6). Furthermore, regarding the interaction, main differences were observed in the Control where the P medium induced a statistically higher response than N6 (37.3% *vs* 22.0%)

After three-four months of culture, stereo- microscope observations revealed new structures: light brown calli (Fig. 3) that increased in quantity and volume and, also, the formation of globular embryos was detected (Fig. 4 a). Moreover, different shapes of embryos were observed: elongated (Fig. 4 b) and also often embryos with a suspensor-like structure (Fig. 4c). This kind of structure was observed also in Citrus in the embryos obtained through isolated microspore culture, but not by anther culture.

Previously, in fruit tree crops, Hofer et al (1999) reported embryo regeneration, through isolated microspore culture, in apple and, recently (Chiancone et al., 2015), the production of gamete-derived embryos obtained by in the same way in Citrus was reported.

Because of the data distribution, it was not possible to apply the two-way ANOVA to the average number of calli and embryos registered per Petri dish, but for the novelty and relevance of these results, it is interesting to describe the performance of the different cultivars. The statistical analysis of the average calli number per Petri dish confirmed the morphogenic potential of TR, that, together with GR, produced the highest number of calli (Fig. 5a). Actually, the highest average callus number per Petri dish was recorded in the genotype 'Tonda Gentile Romana' TR, in the treatment H+ in combination with the N6 medium (13.7) (Fig. 5f). As reported for the microspore development, also for calli production, the response to the different media and to the thermal treatments was genotype-dependent. The cold thermal treatments positively influenced callus production: specifically, F+ treatment induced the highest average number of calli per Petri dish in C, IE (in combination with P medium), GR (in combination with both P and N6 media); while, the hot thermal treatments, and particularly, the H+, seemed to produce a higher callus number in M (in combination with N6) and in TR (in combination with P and N6) (data not shown).

The embryo achieving does not appear to be influenced by the treatments in the same way than calli. Really, with the exception of M, in which the combination of H+ thermal treatment with N6 medium, was proven to be suitable both for callus and embryo achieving. For the other cultivars, the results were different: the highest average embryo number were recorded in the control, in combination with P medium for C and TR, with N6 medium for GR, and in the H+ treatment in combination with N6 medium for IE (Fig. 5b-f).

Generally, regarding the effect of the medium composition, P medium induced a little higher percentage of multinucleated microspores and also a higher number of embryos. Not always at the genotype with the very high number of multinucleated microspores corresponded the higher microspore-derived embryo number (for example, IE).

Regarding the stress, many research highlighted its importance in inducing microspore embryogenesis. However, in this study the higher number of embryos was obtained in both P and N6 control (without additional stresses other that 4 °C before isolation), except for IE.

Detection of homozygosity

Genetic analysis showed that the hazelnut embryos had a single allele at each of the five SSR loci analysed (Figs. 6 and 7) The allele found at each locus matched either one of the 2 alleles of the parent genotype (in the case of the figures 'Minnulara' was the cultivar of origin of the microspores). Being the embryos very small, ploidy level could not be determined; for this reason we can postulate that the embryos are very likely haploids but there is no evidence they may not be double haploids. For sure the embryos analysed originated from a haploid microspore.

The production of homozygous plants in fruit trees of high commercial value like hazelnut is highly desired to help increase the breeding efficiency and reduce the time to obtain new varieties. But, this is not possible in woody plants, like hazelnut, characterized by long juvenile cycle, high degree of heterozygosity, large size and with incompatible systems. The only way to obtain isogenic lines is through haploid technology. Actually, microspore embryogenesis is a promising tool in plant breeding since it permits to obtain full homozygous plants in just one step using heterozygous parents (Germanà 2011 a, b). This technology can be of great interest

to increase the research and plant breeding of *Corylus avellana*, a species not very often subject of improvement (Dunstan and Thorpe 1986).

Conclusions

In our knowledge, this is the first time that haploid technology was applied to hazelnut and that homozygous early embryos were regenerated through isolated microspore culture. The response to the culture media and treatments, was, however, highly genotype-dependent, as previously reported in experiments regarding tissue culture in hazelnut as well as microspore embryogenesis in other fruit crops. Actually, each genotype presented different behaviour, even if, all of them produced calli and, more important, microspores-derived early embryos. Really, even if it is not possible to individuate the absolute best medium or thermal treatment stress, this result represents a major step forward in the knowledge on gametic embryogenesis in fruit crops. Further studies are, however, necessary to better understand the process of gametic embryogenesis in this species and to optimize the rate of microspore-derived embryos and, in addition, to obtain their germination into plantlets.

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Tables

Table 1 Influence of genotype on isolated microspore development, after twenty months of culture.

Genotype	Uninucleated microspores	Binucleated microspores (SND)	Binucleated microspores (ASD)	Trinucleated microspores	Multinucleated microspores
	%	%	%	%	%
'Carrello'	27.7 a	28.4 ab	1.9 a	12.9 b	29.1 ab
Tonda G. Romana GR	30.0 a	25.3 bc	2.9 a	11.8 b	30.0 ab
'Imperatrice Eugenia'	23.0 b	23.5 c	0.1 b	22.0 a	31.4 a
'Minnulara'	23.4 b	31.1 a	0.6 b	21.6 a	23.3 b
Tonda G. Romana TR	27.6 a	27.1 ab	0.6 b	19.7 a	25.0 b

Per each column, values followed by different letters are statistically different.
One-way ANOVA, Conover-Inman's test, $p \leq 0.05$.
SND: Symmetrical nucleus division
AND: Asymmetrical nucleus division

Table 2 Influence of medium composition and thermal treatment on 'Carrello' isolated microspore development, after twenty months of culture.

Factors		Uninucleated microspores	Binucleated microspores (SND)	Binucleated microspores (ASD)	Trinucleated microspores	Multinucleated microspores
		%	%	%	%	%
Medium (M)	P	27.6 a	25.5 b	2.6 a	12.6 a	31.7 a
	N6	27.7 a	31.2 a	1.2 a	13.3 a	26.5 b
M p value		0.962	0.003	0.073	0.593	0.014
Treatment (T)	Co	23.7 b	29.7 a	1.7 a	13.7 a	31.3 a
	H	34.0 a	26.2 a	2.1 a	11.0 a	26.7 a
	H+	29.4 ab	26.4 a	1.4 a	11.4 a	31.4 a
	F	26.7 ab	29.3 a	2.0 a	14.0 a	28.0 a
	F+	24.7 ab	30.3 a	2.3 a	14.7 a	28.0 a
T p value		0.025	0.398	0.928	0.400	0.412
M X T p value		0.425	0.330	0.658	0.175	0.145

Per each factor and per each column, values followed by different letters are statistically different.
Two-way ANOVA, Tukey's test, $p \leq 0.05$.
SND: Symmetrical nucleus division
AND: Asymmetrical nucleus division
P medium: Germanà at al 1996; N6 medium: Germanà and Chiancone, 2003.
Co: no thermal treatment; H**:** 35 °C for 30 minutes**;** H+: 40 °C for 60 minutes; F: -20 °C for 30 minutes; F+: -20 °C for 60 minutes.

Table 3 Influence of medium composition and thermal treatment on 'Tonda Gentile Romana' GR isolated microspore development, after twenty months of culture.

Factors		Uninucleated microspores	Binucleated microspores (SND)	Binucleated microspores (ASD)	Trinucleated microspores	Multinucleated microspores
		%	%	%	%	%
Medium (M)	P	32.7 a	22.8 b	3.4 a	11.4 a	29.6 a
	N6	27.2 b	27.7 a	2.4 a	12.2 a	30.5 a
M p value		0.010	0.005	0.320	0.598	0.501
Treatment (T)	Co	29.0 a	26.3 a	2.0 a	14.3 a	28.3 ab
	H	34.8 a	23.8 a	4.6 a	10.9 a	25.9 b
	H+	29.0 a	25.0 a	2.0 a	11.7 a	32.3 ab
	F	28.2 a	25.9 a	3.0 a	11.9 a	30.9 ab
	F+	28.7 a	25.3 a	3.0 a	10.3 a	32.7 a
T p value		0.050	0.852	0.500	0.523	0.023
M X T p value		0.010	0.272	0.157	0.694	0.000

Per each factor and per each column, values followed by different letters are statistically different.
Two-way ANOVA, Tukey's test, $p \leq 0.05$.
SND: Symmetrical nucleus division
AND: Asymmetrical nucleus division
P medium: Germanà at al 1996; N6 medium: Germanà and Chiancone, 2003.
Co: no thermal treatment; H**:** 35 °C for 30 minutes**;** H+: 40 °C for 60 minutes; F: -20 °C for 30 minutes; F+: -20 °C for 60 minutes.

Table 4 Influence of medium composition and thermal treatment on 'Imperatrice Eugenia' isolated microspore development, after twenty months of culture.

Factors		Uninucleated microspores	Binucleated microspores (SND)	Binucleated microspores (ASD)	Trinucleated microspores	Multinucleated microspores
		%	%	%	%	%
Medium (M)	P	23.2 a	22.9 a	0.0 a	20.3 a	34.5 a
	N6	22.4 a	22.5 a	0.0 a	22.1 a	33.9 a
M p value			0.514	0.173	0.486	0.294
Treatment (T)	Co	19.3 a	23.7 a	0.0 a	20.7 a	36.3 a
	H	23.0 a	21.3 a	0.3 a	20.3 a	37.3 a
	H+	26.7 a	27.3 a	0.0 a	16.3 a	29.7 a
	F	20.0 a	21.7 a	0.0 a	26.7 a	31.7 a
	F+	26.0 a	23.7 a	0.3 a	23.7 a	26.3 a
T p value			0.337	0.570	0.096	0.209
M X T p value			0.823	0.570	0.544	0.024

Per each factor and per each column, values followed by different letters are statistically different.
Two-way ANOVA, Tukey's test, $p \leq 0.05$.
SND: Symmetrical nucleus division
AND: Asymmetrical nucleus division
P medium: Germanà at al 1996; N6 medium: Germanà and Chiancone, 2003.
Co: no thermal treatment; H**:** 35 °C for 30 minutes**;** H+: 40 °C for 60 minutes; F: -20 °C for 30 minutes; F+: -20 °C for 60 minutes.

Table 5 Influence of medium composition and thermal treatment on 'Minnulara' isolated microspore development, after twenty months of culture.

Factors		Uninucleated microspores	Binucleated microspores (SND)	Binucleated microspores (ASD)	Trinucleated microspores	Multinucleated microspores
		%	%	%	%	%
Medium (M)	P	25.6 a	24.5 b	0.0 a	19.7 a	30.1 a
	N6	29.6 a	28.2 a	1.0 a	19.4 a	21.8 b
M p value		0.067	0.000	0.415	0.245	0.000
Treatment (T)	Co	24.3 a	23.0 a	0.3 a	22.7 a	29.7 a
	H	28.3 a	25.7 a	0.7 a	16.0 b	29.3 a
	H+	31.0 a	28.0 a	0.3 a	18.3 ab	22.3 a
	F	26.3 a	28.1 a	0.4 a	21.2 a	23.9 a
	F+	28.0 a	27.0 a	0.7 a	19.7 ab	24.7 a
T p value		0.059	0.282	0.289	0.010	0.944
M X T p value		0.008	0.234	0.500	0.008	0.714

Per each factor and per each column, values followed by different letters are statistically different.
Two-way ANOVA, Tukey's test, $p \leq 0.05$.
SND: Symmetrical nucleus division
AND: Asymmetrical nucleus division
P medium: Germanà at al 1996; N6 medium: Germanà and Chiancone, 2003.
Co: no thermal treatment; H**:** 35 °C for 30 minutes**;** H+: 40 °C for 60 minutes; F: -20 °C for 30 minutes; F+: -20 °C for 60 minutes.

Table 6 Influence of medium composition and thermal treatment on 'Tonda Gentile Romana' TR isolated microspore development, after twenty months of culture.

Factors		Uninucleated microspores	Binucleated microspores (SND)	Binucleated microspores (ASD)	Trinucleated microspores	Multinucleated microspores
		%	%	%	%	%
Medium (M)	P	25.6 b	24.5 a	0.0 a	19.7 a	30.1 a
	N6	29.6 a	28.2 a	1.0 a	19.4 a	21.8 b
M p value		0.035	0.303	0.204	0.794	0.002
Treatment (T)	Co	24.3 a	23.0 a	0.3 a	22.7 a	29.7 a
	H	28.3 a	25.7 a	0.7 a	16.0 a	29.3 a
	H+	31.0 a	28.0 a	0.3 a	18.3 a	22.3 b
	F	26.3 a	28.1 a	0.4 a	21.2 a	23.9 b
	F+	28.0 a	27.0 a	0.7 a	19.7 a	24.7 b
T p value		0.222	0.221	0.709	0.364	0.007
M X T p value		0.010	0.010	0.954	0.330	0.043

Per each factor and per each column, values followed by different letters are statistically different.
Two-way ANOVA, Tukey's test, $p \leq 0.05$.
SND: Symmetrical nucleus division
AND: Asymmetrical nucleus division
P medium: Germanà at al 1996; N6 medium: Germanà and Chiancone, 2003.
Co: no thermal treatment; H**:** 35 °C for 30 minutes**;** H+: 40 °C for 60 minutes; F: -20 °C for 30 minutes; F+: -20 °C for 60 minutes.

492 **Figure legends**

493 Fig. 1: a) Catkins and one anther of *Corylus avellana* L., cv. 'Imperatrice Eugenia', at the developmental stage
494 used for microspore isolation; b) Uninucleated microspore of cv. 'Imperatrice Eugenia' (DAPI staining) (Bar
495 represents10μm).

496 Fig. 2: a) Binucleated pollen, symmetrical division (DAPI staining), cv. 'Imperatrice Eugenia'; b) Trinucleated
497 microspore (DAPI staining), cv. 'Imperatrice Eugenia'; c-d) Multinucleated microspore (DAPI staining), cv.
498 'Carrello' (c) and 'Tonda Gentile Romana' TR (d) (Bars represent10μm).

499 Fig. 3: Microspore-derived calli of cv. 'Tonda Gentile Romana' GR (Bars represent 100μm).

500 Fig. 4 Microspore-derived embryos of cv. 'Tonda Gentile Romana' GR: a-c) different stages of development
501 (Bars represent 20μm).

502 Fig. 5: a) Influence of the genotype on the regeneration of calli and embryos of five hazelnut genotypes (One
503 way ANOVA, Conover-Inman's test $p \leq 0.05$) Within each parameter (callus or embryo), values followed by
504 different letters are statistically different at $p \leq 0.05$ according to Conover-Inman's test; b-f): Influence of the
505 medium composition (P and N6) and of thermal treatments (35 °C for 30 minutes (H), 40 °C for 60 minutes
506 (H+), -20 °C for 30 minutes (F), -20 °C for 60 minutes (F+)) on the production of calli and embryos of
507 'Carrello' (b), 'Tonda Gentile Romana' GR (c); 'Imperatrice Eugenia' (d), 'Minnulara' (e) and 'Tonda Gentile
508 Romana' TR (f).

509 Fig. 6 Genetic profile at SSR locus CaT-B505 of the cultivar ''Minnulara'' (above) and of the microspore-
510 derived embryo (below). The embryo shows a single allele (128 bp) shared with the parental genotype.

511 Fig. 7 Genetic profile at SSR locus CaT-B107 of the cultivar ''Minnulara'' (above) and of the microspore-
512 derived embryo (below). The embryo shows a single allele (114 bp) shared with the parental genotype.

513