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Hazelnut (*Corylus avellana* L.) genetic resources and nursery industry improvement by biotechnological approaches

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Riassunto

L’uso diffuso di polloni autoradicati, prelevati direttamente dagli impianti esistenti, condiziona i nuovi corileti, per l’assenza di garanzie sanitarie e per l’incertezza sui caratteri agronomici e qualitativi, in quanto spesso le varietà sono espressione di “cv-popolazioni” geneticamente difformi. Inoltre, le varietà coltivate sono il risultato di selezioni spontanee avvenute in passato. Il miglioramento del settore vivaistico attraverso la valutazione di genotipi provenienti da diverse aree corilicole e la messa a punto di protocolli razionali di propagazione e di miglioramento genetico non convenzionale attraverso le tecnologie “*in vitro*” sono gli obiettivi attuali per il miglioramento della corilicoltura.
Nel corso del dottorato sono stati condotti rilievi vegeto-produttivi a carico di varietà provenienti da tutto il mondo, conservati nel campo sperimentale “Le Cese”, situato presso la zona dei Monti Cimini, area estremamente vocata per la corilicoltura, che hanno portato alla caratterizzazione di alcune accessioni con caratteri di spiccato interesse per il miglioramento genetico, quali i calendari di fioritura e l’attitudine pollonifera.
Per quanto riguarda il miglioramento dei protocolli di micropropagazione si è giunti, secondo un approccio scientifico ed attraverso l’analisi minerale dei semi, alla formulazione di un mezzo di coltura “*ad hoc*” per il nocciolo, in particolare per la cv Tonda Gentile Romana.
La seconda parte del lavoro è stata svolta tenendo in considerazione l’utilizzo dell’azoto nelle sue diverse forme (quantità di azoto totale e dei rapporti tra fonti di azoto nitrico e ammoniacale), sia nei processi di crescita, sia nella fase di radicazione degli espianti.
La rigenerazione avventizia (radici, germogli, embrioni somatici) risulta essere ancora una tecnica particolarmente difficile da ottenere, tuttavia l’analisi istologica condotta sui calli ottenuti ha messo in evidenza importanti segni di rigenerazioni avventizie come testimoniato dall’osservazione di elementi vascolari quali tracheidi spiralate ed anulo-spiralate. L’ottimizzazione dei protocolli di rigenerazione attraverso l’impiego degli antibiotici quali molecole con funzione auxino-simile, ha permesso, per la prima volta in nocciolo, di ottenere organogenesi di germogli da tessuti maturi di cultivar.
Sono state condotte inoltre osservazioni preliminari sulla coltura di antere, tecnica utilizzata per l’ottenimento di individui aploidi e di-aploidi utili ai fini del sequenziamento del genoma; in particolare sono stati analizzati i principali fattori in grado di influenzare la callogenesi dei tessuti interni delle antere.

Parole chiave: nocciolo (*Corylus avellana* L.), risorse genetiche, micropropagazione, organogenesi di germogli, coltura di antere.

Abstract

The widespread use of rooted suckers, collected directly from the donor plants, affect novel hazelnut orchards, mainly due to the absence of health guarantees and genetic fidelity of the plants that, often, are the expression of cultivar-population. In addition, the cultivated varieties are the result of spontaneous selection from the past.

The improvement of a nursery industry through the agronomical evaluation of genotypes coming from different hazelnut-growing areas in the world, and the development of genetic improvement through non-conventional *in vitro* techniques are the main objectives of hazelnut cultivation.

During the PhD course, studies were conducted on vegetative traits of varieties from all over the world, preserved in the experimental field “Le Cese”, in a vocated area of central Italy, which led to the characterization of interesting traits such as flowering and suckers emission.

Regarding micropropagation protocols the formulation of a novel medium, suitable for the cv Tonda Gentile Romana, has been determined through mineral analysis of the seeds. Furthermore, the investigations taken into consideration the nitrogen source (amount of total nitrogen and the relationships between sources of nitrate and ammonium), both in the growth and rooting of explants.

Adventitious regeneration (roots, shoots, somatic embryos) is a technique particularly difficult to obtain from mature tissues of hazelnut. Histological analysis conducted on the calluses obtained showed significant evidences of shoot regeneration, as proved by the observation of vascular elements such as spiral and annulo-spiral tracheids. The optimization of the regeneration protocol through the use of antibiotics as molecules with auxin-like effect allowed to obtain, for the first time in hazelnut, shoot organogenesis from mature tissues of cultivar.

Preliminary observations on the anther culture, a technique used for obtaining haploid and di-haploid plants have been carried out, with the aim to use for MAS and genome sequencing; in particular, the main factors affecting the callus formation in hazelnut anther culture has been studied.

Keywords: hazelnut **(***Corylus avellana* L.), genetic resources, micropropagation, shoot organogenesis, anther culture.

General Introduction

1. Introduction

The hazelnut has been always cultivated for its beneficial properties and it has been considered a precious plant in many cultures throughout the course of history. Hazelnut is a native species of Asia, where it has been considered a "sacred nourishment", but it has also been used as wood for building for its good quality. The hazelnut has been frequently associated with legends about the "occult". Even the Greek mythology tells of the goddess Artemis, which had a sacred orchard collection of walnut and hazelnuts.
Later, under the Romans, the hazelnut was a symbol of fertility and happiness, and it was one of the most significant in the Celtic culture.

Today all these beliefs are part of the cultural and traditional heritage and, moreover, it is considered one of the most important tree crops in temperate zones. Its steady increase in production is due to its use primarily in the food, although in recent years the uses have been extended also in the field of cosmetics and medicine.

2. Description

The European hazelnut (*Corylus avellana* L.) (Figure 1) belongs to the genus *Corylus*, tribe Coryleae, family *Betulaceae*.

Figure 1: Chromolithography of hazelnut (*Corylus avellana* L.)

It is classified in the order *Fagales*, class *Magnoliopsida* (Cronquist, 1981). The genus *Corylus* currently count about 15 species, including *C. avellana*, *C. maxima* and *C. colurna*, that are considered the most interesting from a genetic point of view (Romismondo, 1976). The European hazelnut (*Corylus avellana* L.) is a diploid species *2n=2x*=22.

The hazelnut is a monoic plant, generally 5-8 m tall. Its buds are ovoid, obtuse and its leaves usually have 6-8 pairs of veins. The male flowers (Figure 2a) have two bracteoles while the female inflorescences (Figure 2b) are short and with red, pink or yellow stigmas (Ives et al., 2014).

Figure 2: Male (a) and female flowers (b) of hazelnut cultivar Tonda Gentile Romana

The fruit is a large nut surrounded by a tubular dentate involucres (Tutin, 1964). The hazelnut is characterized by a sporophytic self-incompatibility system (SSI) as all the species belong to the *Corylus* genera (Erdogan et al., 2004).

3. Distribution and production.

Hazelnut represent one of the most important fruit crops in Mediterranean basin. The hazelnut-growing in the World results 600,530 ha in 2013 and 773,828 tons in terms of total yield. The most important country for hazelnut production is Turkey, which supplies 64.6% of World production, equal to 500,000 tons of hazelnuts in shell and a cultivated area of 421,108 ha (FAO 2013). Italy is the second largest producer with 13.6% of the total, followed by the USA (5.5%) and Spain (1.3%). Other countries that produce hazelnut are Azerbaijan, Iran, Georgia and China. Over the last 40 years, the hazelnut cultivation increase has been particularly intense in Turkey (+728%) and in

Italy (+ 126%) (Cristofori, 2006). The highest yield per hectare was recorded in the United States (3.78 t ha^{-1}). In France and Georgia was about 2 t ha^{-1}, in Turkey was 1.88 t ha^{-1}, and in Italy 1.74 t ha^{-1} (Cristofori, 2006). In Europe, the average yield per hectare is about half respect to the United States. At the market level, Turkey is the country that exports the largest amount of its production (90%).

4. Italian hazelnut production

In Italy the annual consumption of hazelnuts is about 0.52 kg per capita (FAO, 2000). Hazelnut are the second most popular nuts worldwide just after almonds with a global production average at nearly one million tonnes (MT) annually (Contini et al., 2008). The cultivated area is around 70,000 hectares, mainly concentrated in four regions: Piedmont, Lazio, Campania and Sicily (Table 1).

Area of production	Area (x 1000 ha)	Production (x 1000 t)
Viterbo (Latium)	17.6	45.1
Avellino (Campania)	10.3	24.5
Napoli (Campania)	6.7	10.3
Salerno (Campania)	2.5	4.9
Messina (Sicily)	12.5	14.4
Cuneo (Piedmont)	8.6	11.5
Others	12.2	20.6
Italy	**70.4**	**131.3**

Table 1: Average area and production of the main Italian province

In the Viterbo province (Latium region), there are 1,200 farms (Proietti Zolla, 2006), and the hazelnut-growing area is extended for more than 17,000 hectares (ISTAT, 2007). The weather conditions, and the presence of volcanic lakes (Vico and Bracciano Lake), are some of the reasons that make this area highly suited for the hazelnut cultivation.

5. Hazelnut propagation

Plant propagation is divided into two major groups: gamic and clonal propagation. The gamic propagation (via seeds) does not ensure the fidelity respect to the donor plants because of the variability associated to cross-fertilization. The vegetative propagation (agamic), is highly adopted in woody plants since it allows to obtain true-to-type plants.

Hazelnut is vegetatively propagated and the method most commonly adopted in Italy is rooted suckers (Figure 3a). Layering (Figure 3b) and grafting (Figure 3c) on seedlings are also common practices, but none of these methods are satisfactory, because the first method is expensive and needs large areas in the field and the second method is still characterized by low plant production (Ercisli and Read, 2001, Cristofori et al., 2010).

Figure 3: Rooted suckers (a), layering (b) and grafting on seedlings (c) of hazelnut.

Actually, hazelnut propagation by cuttings (Figure 4a) is an interesting method to obtain a rapid propagation at a lower cost while improving the sanitary aspects in comparison to rooted suckers in commercial applications such as *in vitro* recalcitrant cultivars (Cristofori et al., 2010).

Notwithstanding, the *in vitro* propagation technique (Figure 4b) of hazelnut shows various problems for a wider application, due to the scarce adaptability in the establishment of sterilized explants and the scarce suitability of the explants to the *in vitro* conditions (Diaz-Sala et al., 1990; Bassil et al., 1991; Yu and Reed, 1995; Bacchetta et al., 2008). Micropropagation of mature tissues should be optimized for commercial applications in some Italian cultivars such as 'Tonda Gentile delle Langhe', ‘Tonda Gentile Romana’ and ‘Tonda di Giffoni’ that are mainly cultivated in Italy (Nas and Read, 2003; Damiano et al., 2005).

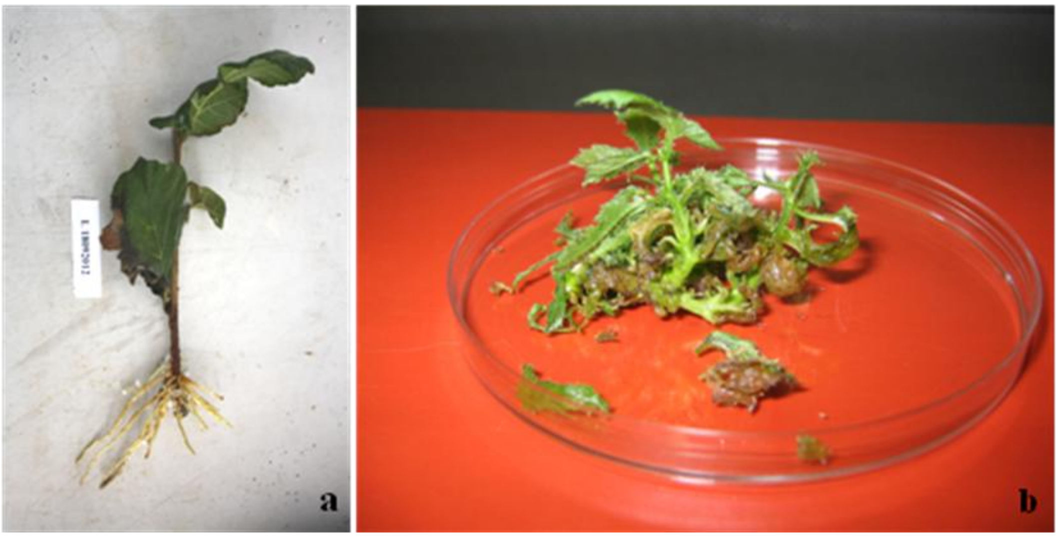

Figure 4: Hazelnut cv Tonda Gentile Romana leafy cuttings with newly formed adventitious roots (a). Twenty-five days-old hazelnut cv Tonda Gentile Romana micro-shoots (b).

6. Hazelnut genetic resources

The available hazelnut varieties have been selected over the time from natural wild populations in Europe, later spread to other parts of the World (Galderisi et al., 1999, Mehlenbacher, 1991). Nowadays there are about 400 local varieties, but World production in based on about 20 cultivars only (Cristofori, 2006).

The self-incompatibility and allogamy causes a high level of intra and inter-specific variability for the fruit traits. In addition, the variable weather conditions in several growing areas, do not correspond to greater variability of local varieties of hazelnut (Cristofori, 2006). One or few cultivars are used for most of the cultivated areas: "Tonda Gentile delle Langhe" ("Cosford" as pollinator) in Piedmont; "Tonda Gentile Romana" and "Nocchione" (as pollinator) in Latium; "Tonda di Giffoni", "San Giovanni" and "Mortarella" in Campania. Currently, the main problems of hazelnut cultivation are related to the industry requests for the seed traits (Tombesi and Limongelli, 2002), and resistance to biotic stress as *Pseudomonas avellanae* (Scortichini, 2006 Proietti Zolla, 2006). The main cultivars of hazelnuts are "Tombul", "Sivri", "Palaz", "Fosa", "Cakildak", in Turkey; "Tonda Gentile delle Langhe", "Tonda di Giffoni", "Tonda Gentile Romana", "San Giovanni", "Mortarella", "Riccia di

Talanico". "Camponica", "Comune di Sicilia" and "Minnulara" Italy; "Negret"and "Pauet" in Spain (Cristofori, 2006 Proietti Zolla, 2006). American cultivars like "Barcelona" were mainly used for fresh consumption, and recently are largely used for industry (Tombesi and Limongelli,2002).

7. Hazelnut germplasm conservation.

The hazelnut breeding programs focus to improve important traits that may represent an economical value, and for this reasons, the availability of gene pools as preserved collections of plants *ex situ* (*in vitro*-propagated) and *in situ* (germplasm collections in vocated areas) are necessary (Mehlenbacher, 1991). The collections of cultivars and local ecotypes existing in the World are present in about 20 countries (Cristofori, 2006). The largest collection in the world is located in the Giresun province (counts about 739 accessions with more than 700 genotypes). Other significant collections are located in Oregon (more than 480 varieties) and Villaviciosa in Spain (124 accessions). In Italy there are collections of cultivars and promising genotypes in Turin and Perugia and an interesting collection of varieties of about 50 accessions is located in the Vico Lake area, in Viterbo province (Cristofori, 2006).

8. Hazelnut breeding programs in the World: main results

In Pennsylvania (USA) the breeder J.F. Jones used *Corylus americana* cv Rush (a cultivar resistant to cold stress and *Cryptosporella anomala*) in cross experiments with *Corylus avellana* cv Italian Red, obtaining an intra-specific new varieties cv. Bixby, and cross experiments with *C. avellana* cv. Barcelona obtaining the cv Buchanan.

Corylus americana produces small nuts but new selections were performed to produce large-fruits varieties such as cv Little page and Winkler. These cultivars and Rush were crossed by C.A. Reed and from the progeny were obtained the cv Reed and Potomac (Lunde et al., 2000).

Weschcke crossed the cv Winkler with a pollen mixture of *Corylus avellana* and from its progeny (which he called "hazilberts") the cv Carlola, Delores and Magdalene have been selected. Several intra-specific crosses between cultivars of *C. americana* (cv Rush x Winkler and Rush x Little page) or between cultivars of *C. avellana* (Barcelona x Medium-Long or Red Lombert or Daviana or Purple Aveline) have been realized in the first years of 20th century, but these produced progeny of little value. Inter-specific crosses between *C. americana* cv Rush and different cultivars of *C. avellana* provided interesting offspring of good value since they combined plant resistance to biotic and abiotic stress of cv Rush with some valuable fruit traits of *C. avellana* (fruit size, flavour, roundness). Crosses between cv Rush and cv Cosford provided offspring characterized by high yield; cv Rush x cv Red Lambert crosses produce offspring with inflorescences resistant to very low temperature. Also interesting were the offspring of Rush x Barcelona and Rush x Ballwiller crosses. Furthermore, the cv Barcelona in the 1930s had attracted the interest of American hazelnut-growers until the years 1960s, when it started to lose interest, when the activity of germplasm collection, evaluation and clonal selection began. Some new clones such as Butler and Lausing seem to be very promising as inter-compatible with cv Barcelona since they produce large fruits too. (Lagerstedt, 1980).

Gellatly, (1956) in Canada crossed *C. colurna* (hazel tree) with *C. avellana* (European hazelnut) to combine some interesting traits of the two species: vigorous tree and absence of suckers, typical of *C. colurna*. The progeny that Gellatly obtained, called "trazels", resulted highly variable and showed some new combinations of characters, but the fruits were small and with thick shell, similar to *C. colurna*. He also worked on crosses between some selections of the endemic species *C. cornuta*, which survive to temperatures of -50 ° C, with *C. avellana*, with the aim to combine the characteristics of the parents. The progeny obtained, indicated "Filazels", allowed to enrol them in the register of selection.

In Turkey, in 1962, was founded in Giresun the "Giresun Filbert Research Institute" to strengthen the hazelnut research programs. In Italy, the business of breeding was initiated by Prof. Romismondo in the 1960s at the University of Turin. The goal was to improve the cultivar Tonda Gentile delle Langhe (TGDL). Crossings were performed with cv Cosford, characterized by thin shell and fruits well distributed on the branches and inter-compatible with the TGDL. The progeny showed the fruit traits of Cosford indicating that the elongated shape and thin shell are controlled by dominant genes.

Tombesi (1966) investigate the compatibility in hazelnut varieties and observed for the first time the low percentage of viable pollen grains produced by italian cultivars compared to the american varieties. He reports a germination rate that varies from 0.5 to 6.2% in 20 cultivars studied. The high pollen sterility is due to aberrations in the anaphase during the first meiotic division. Treatments to pollen with low temperatures (till -18 °C) reduce the germination rate from 53 to 12%. In some cases swedish ecotypes of *C. avellana* maintained the pollen viability even after germination treatments to -31 ° C.

In Spain, the most important novel cultivar is "Negret" which has the advantage of being partially self-compatible. Other varieties compatible with "Negret" and characterized by high yield are the cv Grossal, Ribeta and Artelleta.

In France in 1960 were established a collection of cultivars of potential interest and the most promising have proved the cv Fertile de Contard or Barcelona, Negret and Tonda Gentile delle Langhe. They were performed crosses between these cultivars and the cv “Merville de Bolwiller, Duchilly and Cosford with the aim to select clones characterized by early ripening, cold stress resistance and fruit shape for confectionery industry.

Recently, the selection of new varieties for hazelnut industry, and also resistant to Estearn Filbert Blight (EFB) caused by *Anisogramma anomala*, has been developed at Oregon State University (OSU) with the constitution of six new varieties: Willamette, Lewis, Clark, Sacajava, Santiam and Yamhill. Two new cultivars, Corabel and Feriale, has been released by INRA Bordeaux, and the University of Turin recently released the new variety “Daria”.

In the IRTA de Mas Bover (Spain) are actually under investigation some promising low sucker emission rootstocks (Dundee and Newberg, two open-pollinated *C. colurna* seeds, Tonda Bianca and the Negret clone MB-69) to solve the problems related to high sucker emission (Figure 5) of the common hazelnut varieties used (Rovira et al., 2014).

A new “ever-growing” genotype (EVG-d) has been recently obtained at Tuscia University, characterized by a fail of growth cessation and enter dormancy. It does not require chilling units to sprout and might contribute to the expansion of the hazelnut crop in warm climatic region devoid of air temperature events below 0°C (Catarcione et al., 2009).

Figure 5: In the grafting region it is possible to observe the higher vigour of the rootstock *C. colurna* than the grafted hazelnut (a). Twelve years-old hazelnut cv Tonda Gentile Romana grafted onto *Corylus colurna* rootstock "Dundee" (b).

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Aims of the work

The hazelnut cultivation, today, is undergoing an increase in terms of cultivation areas, especially in the country where the availability of large agricultural areas and low labour costs allow hazelnut to be one of the most profitable tree crops such as Chile, South Africa and China.

The main problems that characterize the traditional hazelnut-growing areas are:

1. The lack of a wide range of varieties;
2. The lack of a rational nursery industry in Italy, but generally, in all other hazelnut-growing countries, where there are not optimized large-scale propagation techniques;
3. The widespread use of self-rooted suckers, directly collected from the donor plants in the field, resulting in genetic instability, long juvenile phase and lack of sanitary guarantees;

Despite all the problems described above, research are still very poor. Apart from the Oregon State University which since 40 years worked in breeding and selection of new pathogen-resistant varieties (mainly Eastern Filbert Blight-*Anisogramma anomala*) and released a few tens of varieties, there are few other institutions working on hazelnut genetic improvement and on nursery industry.

Therefore, the research need to be improved, starting from the evaluation and selection of varieties well adapted in the own growing environments and, at the same time, working in the genetic improvement, through conventional and unconventional techniques, to release novel varieties with interesting traits. In fact, as for the most tree species, classic breeding is going to be quickly replaced by MAS and others novel techniques.

The activity carried out in this work are listed as follow:

1. First evaluations on vegetative and reproductive performance of hazelnut varieties in a core collection of the Latium Region;
2. The improvement of micropropagation medium of hazelnut (Corylus avellana L.) cv Tonda Gentile Romana by using analytical data from seeds;

3. The effects of nitrogen source, concentration and NH_4^+:NO_3^- ratio influence micropropagation of hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana;
4. In vitro adventitious shoot organogenesis in hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana;
5. In vitro anther culture of hazelnut (*Corylus avellana* L.) in attempt to obtain haploids or di-haploids .

Evaluations of vegetative and reproductive performance of hazelnut varieties in a core collection of Latium Region.

1.Introduction

The hazelnut cultivation needs to be improved in order to obtain satisfactory yields and quality and to reduce the costs of production. The producers tend to achieve these goals and Italy will also proceed in this direction, and varieties represent one of the key factors for success.

The World production is based on approximately 20 main varieties selected from wild plants. They are well adapted to the own areas of cultivation, usually characterized by mild climate, high rainfall and cool summers.

In Italy, about 72,000 ha are interested by hazelnut cultivation, mainly located in four regions. In recent years, Italian production has reached about 128,000 ha (Franco et al., 2014).

In Campania region the cultivation is situated particularly in Avellino province, followed by Naples, Caserta and Salerno, were the main varieties used are Mortarella, San Giovanni, Tonda di Giffoni, Camponica, Riccia di Talanico, Tonda Bianca and Tonda Rossa. In Latium region are located more than 25% of the national production areas, concentrated in the Viterbo province, a typical hazelnut-growing area. The main variety is the "Tonda Gentile Romana", followed by "Nocchione" and "Tonda di Giffoni". In Piedmont the cultivation is based almost exclusively on cv "Tonda Gentile delle Langhe", and in Sicily the hazelnut cultivation is concentrated in Messina province, where several varieties are used, as Comune di Sicilia, Minnulara, Nostrale and Montebello.

No variety is completely satisfactory for agronomical traits and for the market request; for this reason the research have to improve the genetic characteristics of *Corylus avellana*, or the breeders have to employ different species of the same gene pool characterized by important traits such as the low sucker emission of *C. colurna* or cold hardiness of *C. eterophylla*.

The main varieties that have been recognized worldwide for their interesting features are: Tombul, Tonda Gentile delle Langhe, Tonda Romana, Tonda di Giffoni, Negret and Barcelona. However, they are limited by the growing areas with regard to productivity, environmental conditions, qualitative traits or disease resistance.

Hazelnuts are grown for two markets, kernels and in-shell; the cultivars suitable to each request are different (Solar and Stampar, 2009).

Phenology is the study of periodic biological events in the plant world that are influenced by the environment, especially temperature changes driven by weather and climate (Lieth, 1974). The time of vegetative stages are closely related to the climate of the observed site and the weather during the month before a phenological event, since in deciduous trees, including hazelnuts, the dates of flowering and leafing at the end of winter or early spring are a function of the chilling requirement during the post-rest phase (Crepinsek et al., 2012). Hazelnut flowering time varies greatly according to the climate and is very temperature-dependent. During flowering, both male and female inflorescences can be observed on the same tree (Germain, 1994). The peak flowering periods of male and female flowers may not overlap on the same cultivar (Balwin, 2004), and dichogamy is present in almost all the cultivars (Mehlenbacher, 1991), and usually male catkins precede female flowers (protandry) or the pistilate flowers mature firstly (protogyny).

The natural growth habit of hazelnut is a multi-stem bush, and suckering represents a problem in orchard management (Tous et al., 1994; Cristofori et al., 2014; Rovira et al., 2014). Studies related to rootstocks have been limited in hazelnut; the researchers of Corvallis University (Oregon State University - USA) have been produced in the past years the first hybrids USOR 7-71 (Newberg) and USOR 15-71 (Dundee), originated from a large group of open-pollinated *C. colurna* seeds and selected on the basis of bark characteristics which differ from those of typical *C. colurna* (Lagersted, 1993a). They have been evaluated as rootstocks for ‘Ennis’ (Lagersted, 1993b) and the selections imparted vigour to the scion cultivar, were non suckering, and were consistently among the top yielders in comparison to the 45 rootstocks selections evaluated for that trait (Thompson et al., 1992). Furthermore, seedlings of *C. colurna* were evaluated as a rootstocks compared with four own rooted cultivars (‘Rimski’, ‘Istarki Dugi’, ‘Tonda Gentile Romana’ and ‘Cosford’). Results showed larger nuts and higher fruit weight in trees grafted on *C. colurna* seedlings and these trees were more vigorous and more productive (Miletic et al., 2009).

Recently, results obtained in hazelnut rootstock trial in Spain show that rootstocks have a clear positive influence on tree growth, suckers emission and yield. Non suckering rootstocks (‘Dundee’, ‘Newberg’ and ‘MB-69’) improve the agronomic performances of cv Negret N·9 (Rovira et al., 2014).

In this study has been described the flowering and leafing of 48 cultivars, cultivated in a core collection established in a typical growing area located in Viterbo province. In addition, the cultivars were classified in order to evaluate the sucker emission attitude, suggesting their use as a low suckers emission rootstocks (Cristofori et al., 2014).

2. Materials and methods

2.1. Plant material

The collection field was established by ARSIAL and CRA (Rome) in a typical hazelnut-growing area of Viterbo province (Italy) during the year 2000, at “Le Cese”, surroundings the basin of Vico Lake, on a sandy-clay-loam soil characterized by sub-acid pH (6.1), a good organic matter content (2.26%) and low total calcium content (7.4 $g \cdot kg^{-1}$). The collection includes 48 italian and foreign cultivars, as listed in Table 1 where, according to the recent findings, the varieties Montebello, Nocchione, Santa Maria del Gesù and probably Barrettona, could be synonyms (Boccacci et al., 2006). Each cultivar is represented by three multi-stemmed bush trained as open vase and spaced 4m x 5m, irrigated through a sub-irrigation system and managed with standard orchard management techniques.

2.2. Vegetative traits and floral phenology

Suckers emission has been expressed as five classes of attitude (very low, low, medium, high and very high).

The leafing dates at the phenological stage C, for the period 2013-2015 (Germain and Sarraquigne, 2004). Male flowering were recorded at stage Fm1 (start), Fm2 (peak) and Fm3 (end), and female flowering at stage Ff1 (start), Ff2 (peak) and Ff3 (end) as described in the Figure 1 (Germain and Sarraquigne, 2004).

3.Results and discussions

As reported in Table 1, the cultivars showed an high suckers emission variability. Almost all Italian cultivars showed an high emission of suckers. A very high suckers emission was observed for the cv Barrettona, Camponica, Napoletanedda, Tonda di Giffoni, Riccia di Talanico and in the foreign cv Barcelona and Grifoll, whereas the cv Palla Grossa, Closca Molla and Hynich showed a very low and a low suckers emission respectively. These cultivars could be suitable as rootstocks with low suckers emission, which are useful to graft the cv Tonda Gentile Romana, since this cultivar is the most important in Latium and shows a medium-high suckers emission attitude (Figure 2).
Flowering time and leaf bud-break (Figure 3 a,b) of each cultivar were observed over the period of 2013-2015. The cultivars Tonda Gentile delle Langhe, Tonda Rossa, Karidaty and Sivri showed a very early shed pollen, concentrated in the mid of December, contraywise to Pallagrossa, Hynich, Vermellet, Merveille de Bollwiller and Bearn that showed a very late male flowering, starting in the second part of February.
For female anthesis, the earliest cultivars were Nostrale, Tonda di Giffoni, San Giovanni, Nociara, Nocchione and Piazza Armerina that showed a styles emergence from female inflorescences at the end of December, whereas the latest ones were Bearn, Pallagrossa, Daviana, Cosford and Hynich.
Some cultivars as Vermellet, Tonda Bianca, Segorbe, Tonda Rossa, Daviana, Negret and Cosford showed a very marked proterandry.
Grifoll, Tombul, Camponica, Tonda di Giffoni, San Giovanni, Gunslebert, Grossal and Napoletanedda, showed an early leaf bud break, occurred in the last decade of March, meanwhile Daviana and Cosford were very late for this trait, occurring in mid April (Figure 3 a,b).

4.Conclusions

The 48 cultivars showed an high diversity in their phonological behaviour in this vocated area (Viterbo, Italy). The cultivars Hynich, Palla Grossa and Closca Molla have shown a very low attitude to emit suckers, suggesting that can be taken in account their use as a non-suckers rootstocks for other hazelnut cultivars.

Floral phenology considerably varied among the cultivars studied, giving important information about the overlap of male and female flowers, to take into account since hazelnut is characterized by a self-incompatibility system.

Spring phenological phase occurs in a relative short period, starting from the 4th week of February to the 3rd week of April.

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6. Tables and Figures

Table 1: Origin and suckers-emitter attitude of the cultivars growth up in the collection field at "Le Cese" (Viterbo, Italy).

Cultivar	Origin	Suckers emission	Cultivar	Origin	Suckers emission
Annusa Racinante	Sicily (IT)	high	**Minnolara**	Sicily (IT)	high
Apolda	Unknown	low-medium	**Montebello**	Sicily (IT)	low-medium
Avellana Speciale	Campania (IT)	high	**Morell**	Spain	high
Barcelona	France	very high	**Napoletanedda**	Sicily (IT)	very high
Barrettona	Unknown	very high	**Negret**	Spain	high
Bearn	Unknown	low-medium	**Nocchione**	Latium (IT)	high
Camponica	Campania (IT)	very high	**Nociara**	Sicily (IT)	low-medium
Carrello	Sicily (IT)	medium	**Nostrale**	Sicily (IT)	medium-high
Closca Molla	Spain	low	**Palla Grossa**	Unknown	low
Comen	Greece	high	**Piazza Armerina**	Sicily (IT)	high
Comune di Sicilia	Sicily (IT)	low-medium	**Racinante**	Sicily (IT)	high
Cosford	England	low	**Riccia di Talanico**	Campania (IT)	very high
Daviana	England	high	**San Giovanni**	Campania (IT)	high
Ennis	USA	medium	**Santa Maria del Gesù**	Sicily (IT)	high
Fructo Rubro	Unknown	medium	**Segorbe**	France	medium-high
Gironell	Spain	medium	**Sivri A**	Turkey	high
Grifoll	Spain	very high	**Tombul**	Turkey	high
Grossal	Unknown	high	**Tonda Bianca**	Campania (IT)	high
Gunslebert	Germany	medium	**Tonda di Giffoni**	Campania (IT)	very high
Hynich	Unknown	very low	**Tonda Gentile delle Langhe**	Piedmont (IT)	medium-high
Jeans	England	medium-high	**Tonda Gentile Romana**	Latium (IT)	medium-high
karidati	Turkey	high	**Tonda Rossa**	Campania (IT)	low-medium
Lounge d'Espagne	England	low-medium	**Vermellet**	Unknown	medium-high
Merveille	France	medium	**Vermellet SP**	Unknown	medium-high

Figure 1: Female flowering at stage Ff1-start (a), Ff2-peak- (b) and Ff3-end-(c) and male flowering at stage Fm1-start-(d), Fm2-peak-(e) and Fm3-end-(f), as described by Germain and Sarraquigne, 2004.

Figure 2: Comparison among a medium-high suckers-emitter cultivar (Tonda Gentile Romana) and other very low suckers-emitter cultivars (Hynich, Palla Grossa, Closca Molla).

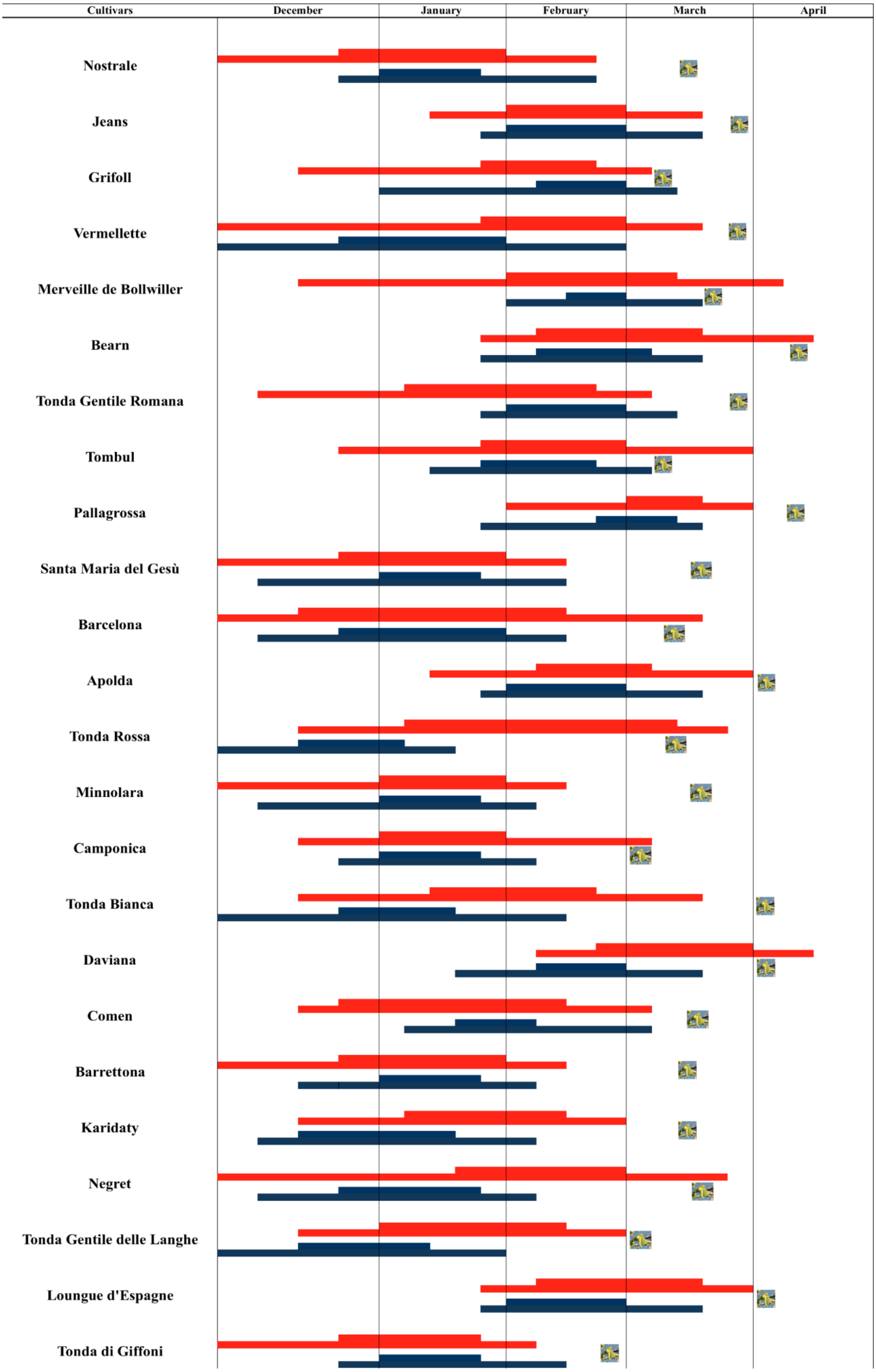

Figure 3a: Average dates of flowering and leaf bud-break observed in 48 hazelnut cultivars at Le Cese- Vico Lake- in Viterbo Province. (female flowering, male flowering, leaf bud-break data).

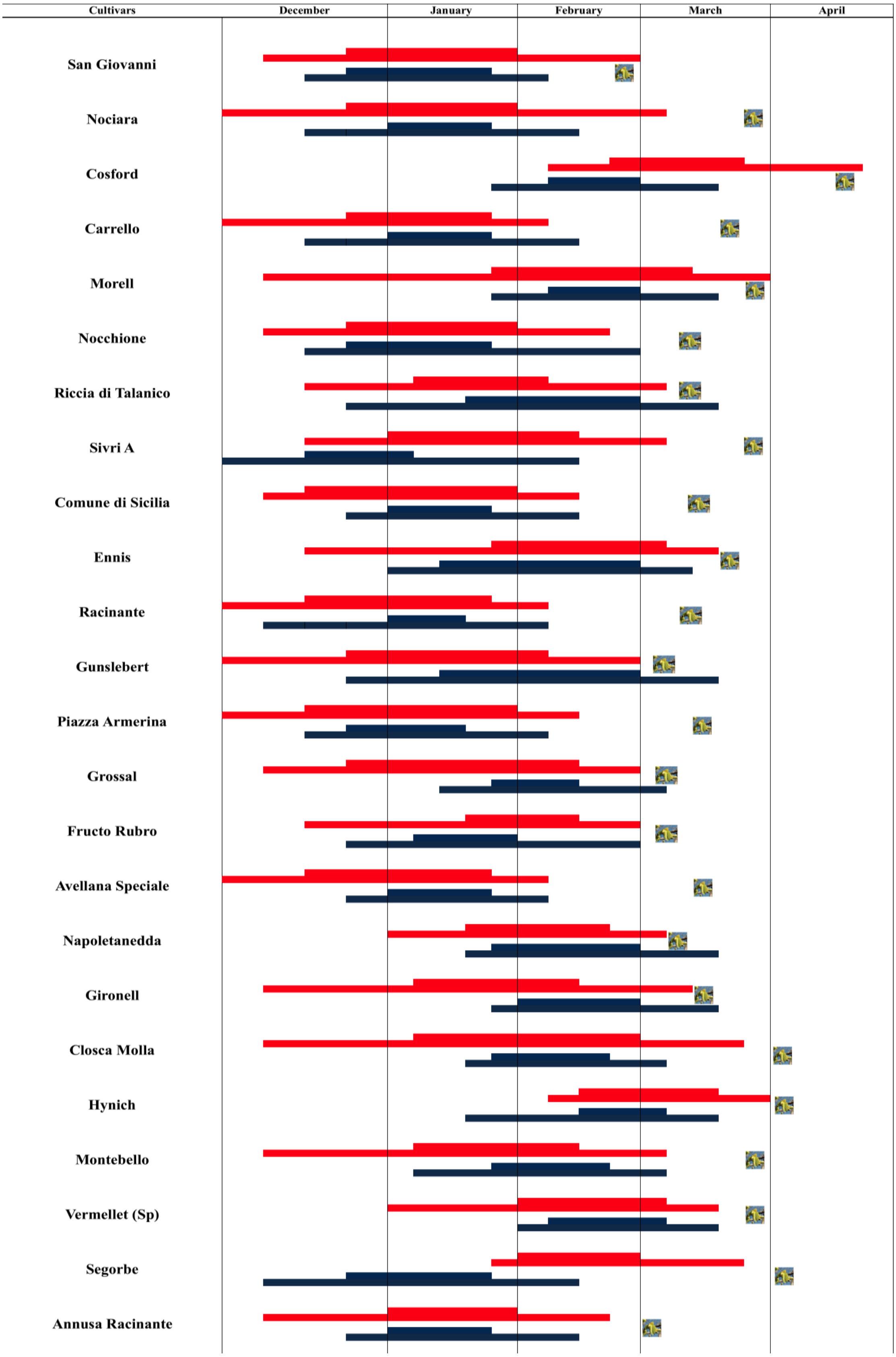

Figure 3b: Average dates of flowering and leaf bud-break observed in 48 hazelnut cultivars at Le Cese- Vico Lake- in Viterbo Province. (▬ female flowering, ▬ male flowering, leaf bud-break data).

The improvement of micropropagation medium of hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana by using analytical data from seeds

1. Introduction

Micropropagation has been proposed as an alternative large-scale multiplication method to traditional propagation, by rooted suckers, with the aims to improve the rapid diffusion of certified material.

Until now, successful protocols for micropropagation of hazelnut has been published for both embryonic and juvenile material (Anderson, 1984; Perez et al., 1987). Numerous protocols for hazelnut variety micropropagation have been proposed (Garrison et al., 2013; Bacchetta et al., 2008; Damiano et al., 2005; Nas and Read, 2004; Yu and Reed, 1995), but once suitable for a wide range of cultivars has not been identified yet and, moreover, still missing propagation methods that allow a large-scale propagation as required for commercial purpose, due to the recalcitrant behaviour of this species, and the difficulties of hazelnut to adapt in *in vitro* conditions (Contessa et al., 2011).

Various problems affect the hazelnut *in vitro* culture micropropagation, including the lack of multiplication, short shoots production, chlorosis, and milky white exudation (Hand et al., 2014; Nas and Read, 2001; Al Kai, 1984) and, furthermore, the number of sterile explants derived from mature tissues is limited due to contamination and low proliferation index (Bassil et al., 1992; Diaz Sala et al., 1990; Messeguer and Mele, 1987). Bacteria and fungi, combined with the strong damage produced by the sterilizing agents, are normally the main problem associated to the *in vitro* establishment of aseptic culture. Since these factors depend on the age, phytosanitary treatments and seasons, it is very difficult to find a unique satisfactory method.

Recently, Hand et al. (2014), Hand and Reed (2014) have employed a computer assisted technique and a surface design statistical software with the aim to model multiple factors and their influence on the overall outcome based on plant response. They conclude that overall quality of the varieties used in the experiment were influenced by ammonium and nitrate nitrogen, mesos and minors. Less amount of $Ca(NO_3)_2$ improved multiplication, while higher amount increased shoot length of plantlets. Additionally, NH_4NO_3, $Ca(NO_3)_2$, mesos and minors have showed significant effects on hazelnut growth and multiplication. Hand and Reed (2014) also found that there were many significant interactions among the minor nutrients, in particular for B, Mo and Zn, that increase overall shoot quality, length and multiplication. Anyway the authors concluded that the results were genotype-dependent and all cultivars require minor nutrient concentration compared to the media commonly used (Bacchetta et al., 2008; Hand et al., 2014; Hand and Reed, 2014).

The phyto-hormones are the main components in the culture medium to reach the success of proliferation. The length of the shoots and the multiplication rate are influenced by concentration of cytokinins (Bassil et al., 1992). The most employed cytokinin is BAP (6-Benzylaminopurine) at various concentrations and with different results for each cultivars and/or type of explants. Andres et al. (2002) reported the presence of high levels of endogenous cytokinins in the leaves of hazelnut during the spring, when the morphogenic potential is high. The leaves collected from the branches developed after forced outgrowth of field-grown material in autumn showed an high ratio of two cytokinins, 2-iP (isopentenyl adenine) and Z (zeatin), similar to the ratio found in the young leaves of hazelnut, suggesting that the ratio 2iP/Z can be considered a good indicator of the potential for *in vitro* establishment.
The chemical composition of the medium plays an important role in the success of micropropagation, since a sub-optimal culture medium is the base of the physiological disorders of the tissues (Nas and Read, 2004). In order to formulate an optimized culture medium in recalcitrant species, a novel approach based on the mineral composition of the seeds has been successfully applied (Rugini, 1984, Bacchetta et al., 2008; Nas and Read, 2004), since the seeds store mineral and food reserve essential for starting the vegetative growth if factors preventing germination are eliminated (Bewley and Black, 1978).

2. Materials and methods

2.1. Experiment I: seed analysis and formulation of novel medium

Twenty-four fruits randomly harvested from a field-grown plant were shelled and dried at 105±1°C for 48 hours, until constant dry weight has been reached. Then, the ashes have been obtained by incineration into porcelain crucibles in a muffle furnace at 520°C. Minerals were determined by ICP methods. Data have been summarize as minimum, maximum, mean value, and standard deviation (±SD). Furthermore, twenty-four fruits were treated as described above after removal of the pellicle (blanched hazelnut) in order to verify its influence on the mineral content.
Mineral contents of *Olea europaea* L. and *Prunus dulcis* L. seeds and the respective media of olive (Rugini, 1984) and almond medium (Murashige and Skoog, 1962) have been choose as reference species for calculating the concentrations of macro and

microelements for formulation of hazelnut medium. The procedure have been applied as follow: 1) determination of anions and cations in OM and MS media; 2) evaluation of the ratio between the concentration of mineral elements found in seeds of hazelnut cv Tonda Gentile Romana compared to those of almond and olive seeds; 3) calculation of the cation and anion amounts to set up their concentration in new developing medium.

To take advantage of different forms of nitrogen, both NO_3^- and NH_4^+ were used as inorganic nitrogen sources, at the concentrations used by Murashige and Skoog (1962), since the nitrogen usually found in hazelnut kernels (2200-2500 mg N/ 100 g kernel) is too high and toxic if replaced at all in the culture media (Nas and Read, 2004).

The novel formulation has been supplemented with vitamins suggested in Nas and Read medium (NRM) (Nas and Read, 2002), and 200 mgL^{-1} myo-inositol, gibberellic acid (GA_3) 0.5 mgL^{-1}, indole-3-butyric acid (IBA), 6-benzyladenine (BAP) 1 mgL^{-1}, zeatin (Z) 0.5 mgL^{-1} and sucrose (3%).

2.2. Experiment II: Alluminium (Al^{3+}) effects on callus production

Since the high variability found in Aluminium concentration of each single fruits analysed, fast-growing calli culture of hazelnut leaf tissues were established in order to detect the effect on growth and vitality of different concentration of aluminium (Al^{3+}). The leaf discs were grown on B5 medium (Gamborg et al., 1968), supplemented with 2,4-D (1 $mg \cdot L^{-1}$), pH 5.8 and gelled with Difco Bacto agar 0.55%. The plates were maintained in darkness at 24°C±1°C. Aluminium was added as $AlCl_3$ at concentration of 0 (control), 10, 100, 500 and 1000 mM. After 10 days the new developed calli were measured by determination of fresh weight. All observations and measurements were carried out in Petri dishes containing ten explants. All the experiments have been conducted with three replications. Data were subjected to analysis of variance (ANOVA). The mean were separated with Duncan's test ($P \leq 0.05$), using R software package (http://cran.rpoject.org).

2.3. Experiment III: Effect of the ethylenediamine di-2-hydroxy-phenylacetic acid (Fe-EDDHA) on hazelnut micro-shoot quality.

The experiments have been carried out to investigate the different iron sources on *in vitro* shoot multiplication and quality of the cv Tonda Gentile Romana.

Two chelated forms of iron, ethylenediaminetetraacetic acid (Fe-EDTA), the most common iron source in the majority of media used, and ethylenediamine di-2-hydroxy-phenylacetic acid (Fe-EDDHA), have been tested to determine the effect on shoot development.

Shoots were excised into single node segments and placed into jars each containing 100 mL of medium. Fifteen explants were placed in each one, and three jars for each treatment have been used. Explants were maintained in a growth chamber at 24±1°C with a 16-h photoperiod of 40 µmol m^{-2} s^{-1} provided by fluorescent lamps. Data were recorded after 40 days. Data for analysis included shoot height, number of nodes per shoots, number of shoots per explants, chlorophyll (Chl) content, and Chl a/Chl b ratio. The explants were grown on Tonda Romana medium (Table 3) supplemented with vitamins suggested in Nas and Read medium (NRM) (Nas and Read, 2002), and 200 mgL^{-1} myo-inositol, giberellic acid (GA_3) 0.5 mgL^{-1}, indole-3-butyrric acid (IBA), 6-benzyladenine (BAP) 1 mgL^{-1}, zeatin (Z) 0.5 mgL^{-1} and sucrose (3%). Iron sources has been supplied as FeEDTA at 36.70 $mg{\cdot}L^{-1}$(100 mM), the same amount present in MS medium (Murashige and Skoog, 1962), and Fe-EDDHA at 43.52 $mg{\cdot}L^{-1}$(100 mM) and 87.04 $mg{\cdot}L^{-1}$(200 mM).

Data were subjected to analysis of variance (ANOVA). The mean were separated with Duncan's test (P≤0.05), using R software package (http://cran.rpoject.org).

3. Results and discussion

3.1. Experiment I: seeds analysis and formulation of novel medium

Determination of mineral contents in seeds of hazelnut cv Tonda Gentile Romana have been demonstrated that there are not differences between entire and blanched hazelnut (Table 1). The determination of mineral seed composition is important since it depend on varieties and growing conditions such as soil. Among the fruits harvested, singly analysed, the minimum, maximum and mean value of macroelements (Ca, Fe, K, Mg,

P) and microelements (Al, Cd, Cr, Cu, Mn, Ni, S, Zn) are summarize in Tab. 1, An high variability have been observed particularly for Aluminium (Al^{3+}), ranging between 1.779 to 7.799 $\mu g \cdot g^{-1}$, with an average of 3.942 $\mu g \cdot g^{-1}$.

In Table 2 have been showed the concentration of macro- and microelements in MS (Murashige and Skoog, 1962) and OM (Rugini, 1984) media, which are suitable with almond and olive respectively, compared with the concentrations calculated for the implementation of the novel medium for hazelnut cv Tonda Gentile Romana. Many differences have been observed among the species. Differences were found for Ca, P, Mg and Zn, which showed lower values respect to both olive and almond. The Mn element showed higher values (21.320 $mg \cdot L^{-1}$) than the almond (5.493 $mg \cdot L^{-1}$) and olive (5.500 $mg \cdot L^{-1}$).

The novel medium, named "Tonda Romana Medium", developed on the basis of these differences, is reported in Table 3. In comparison with MS (Murashige and Skoog, 1962) and OM (Rugini, 1984). This medium showed a significative reduction of $Ca(NO_3)_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4, accompanied by the addition of K_2SO_4, not present in the other media. Among the microelements, the B has been slightly increased in the form H_3BO_3 and the Mn has been significantly increased by adding $MnSO_4 \cdot 7H_2O$ at 65.590 $mg \cdot L^{-1}$, 5-fold greater than that reported in MS and OM (16.90 $mg \cdot L^{-1}$). The Zn was drastically has been reduced by reducing the content of $ZnSO_4 \cdot 7H_2O$. Sucrose was chosen as carbon source, at 30 $g \cdot L^{-1}$.

3.2. Experiment II: Alluminium effects on callus production

Calli growth of Tonda Gentile Romana explants were stimulated in the presence of low concentration of Al^{3+}compared to those grown in absence of Al (Fig. 1). The weight of the callus tissues were significantly higher in the presence of Aluminium at 10 mM (50±1.9 mg fw). When the concentration of Al^{3+}has been increased (100, 500 and 1000 mM), the weight of calli resulted drastically reduced, with numerous necrotic areas among the brownish callus. At 1000 mM of Al^{3+}, the leaf discs used for the experiments started to growth, but rapidly stop the cell division and died after few days.

The variability in the adaptation to Al^{3+}can be observed between plants and between cultivar of the same bean species (Massott et al., 1999). The mechanisms proposed to explain alterations caused by Al^{3+} include 1) decreased cell division, 2)interaction of Al^{3+} with essential elements (Ca, Mg, P), resulting in decreased absorption and translocation leading to deficiency symptoms, and c) translocation of toxic amounts of

Al to the aerial part of the plant (Foy, 1988). No much works have been reported about aluminium effects in *in vitro* propagation, but our results seems to be in line with those reported for many fruit species in hydroponics conditions where usually a low concentration of aluminium provoke an initial increase of the growth (Pereira et al., 2003). Further investigation are necessary to clarify the mechanism involved in the growth of hazelnut under Al-stress.

3.3. Experiment III: Effect of the ethylenediamine di-2-hydroxy-phenylacetic acid (Fe-EDDHA) on hazelnut micro-shoot quality.

The explants grew in the presence of Fe-EDDHA resulted longer and with more nodes per explants (Tab.4) compared to those grew in medium supplemented with Fe-EDTA. In all Fe-EDDHA concentrations, the number of shoots and chlorophyll contents were similar and higher than the control medium enriched with Fe-EDTA, with larger leaves. In particular, the shoot dry weight resulted greater than the control in all the Fe-EDDHA treatments. Furthermore, the explants grew on the control medium appears light green.

Fe-EDTA represents the major iron source (chelator) largely used in *in vitro* culture, including hazelnut micropropagation (Messeguer and Mele, 1987; Diaz-Sala et al., 1990,1994; Bassil et al., 1991; Yu and Reed, 1995; Damiano et al., 2005; Bacchetta et al., 2008), while in various other woody species including interspecific hybrids of hazelnut (Van der Salm et al., 1994; Molassiotis et al., 2003; Ciccotti et al., 2008; Garrison et al., 2013) a better response have been obtained through the use of Fe-EDDHA. The stability of chelated iron is fundamental to maintain the availability of Fe in the medium having usually a pH ranging between 5.4 to 6. However, Fe-EDTA photo-oxidizes at pH 5.7 (Hangarter and Stasinopoulos, 1991) and forms insoluble ferric oxide, unavailable for the shoots (Lindsay and Schwab, 1982; Garrison, 2013).

Hangarter and Stasinopoulos (1991) correlated the photo-oxidation of Fe-EDTA and the consequent formation of formaldehyde to the poor quality and growth of the explants. In addition, the better growth observed may arise from the less energy and the time requested to uptake Fe-EDDHA by hazelnut shoots, as reported by Alcañiz et al. (2005), so the chelated remain available for a longer period in culture.

The increasing in chlorophyll content in hazelnut, according to the observation made on the interspecific hybrid hazelnut by Garrison et al. (2013) and Van der Salm (1994), reflecting a greater photosynthetic activity which help the acclimatization phase. The high Chl a/ Chl b ratio in the medium supplemented with Fe-EDTA (control) highlight the Fe-stress due to a reduction of chlorophyll b associated to a reduction of light use efficiency, as reported for *Pyrus communis*, were the Fe-stress cause a decrease of photosynthesis (Morales et al., 2000).

4. Conclusions

The novel formulation of mineral elements resulted beneficial in shoot growth of this variety, however the amount and formulation of nitrogen (N) in preliminary test was not completely satisfactory and was the object of the following chapter The main goal of hazelnut nursery industry and breeding programmes is the rapid propagation of clonal material for a wide range of genotypes. Increasing the quality of shoots allows to reduce the costs of Micropropagation, which seems a very important technique to overcome the traditional hazelnut propagation, which up to now is carried out by the not rational technique based on rooted suckers and only in few case by semi-hardwood cuttings .

Local genotypes show contradictory responses to *in vitro* culture and, often, demonstrate a contradictory behaviour. The formulation of a specific medium for each cultivar should allow to design an optimum medium for one or few elite varieties.

This study provides the advantage to study a novel formulation medium, starting from seed analysis of a single genotype (Tonda Gentile Romana).

In addition, starting from the results obtained the growth-promoting role of Aluminium at low concentration on callus culture of cv Tonda Gentile Romana has been demonstrated. In the novel formulation, aluminium has not been yet tested since in the common agar used in *in vitro* propagation, aluminium is present in trace as contaminant. Further investigations are necessary to better understand the effects of Aluminium in hazelnut micropropagation.

Also it has been demonstrated that iron source plays a key role in hazelnut *in vitro* culture, in particular the Fe-EDDHA, that produces a better growth of hazelnut cv Tonda Gentile Romana.

The novel formulation of mineral elements resulted beneficial in shoot growth of this variety, however the amount and formulation of nitrogen (N) in preliminary test was not completely satisfactory and was the object of the following chapter.

5. References

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6. Tables and Figures

Table 1: Mineral contents of hazelnut seeds cv Tonda Gentile Romana. Data are represented as minimum, maximum, mean value and standard deviation. Statistical analysis refers to the mean values of each elements in the entire and blanched hazelnut.

	Mineral content (µg g^{-1} dw)												
	P	**K**	**Mg**	**Ca**	**S**	**Fe**	**Zn**	**Mn**	**Cu**	**Al**	**Ni**	**Cr**	**Cd**
Min	3248.34	2292.07	984.06	808.92	412.03	23.458	15.909	12.174	7.532	1.779	0.896	0.316	0.002
Max	5404.13	3795.24	1395.77	1378.84	992.17	52.241	27.752	52.320	18.005	7.799	1.851	0.690	0.011
Mean	4104.11	2818.79	1116.47	1073.08	736.88	29.516	19.868	19.830	10.459	3.942	1.102	0.434	0.004
SD	493.44	420.64	111.31	159.88	129.95	6.311	2.878	8.781	2.314	1.620	0.204	0.098	0.002
Entire hazelnut	4240.31	2963.39	1140.88	1047.55	715.28	31.250	20.280	21.790	10.780	4.020	1.160	0.460	0.005
Blanched hazelnut	3967.90	2674.19	1092.07	1098.60	158.48	27.790	19.460	17.870	10.140	3.871	1.040	0.410	0.005
P<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 2: Comparison between the chemical composition of anions and cations (mg L^{-1}) of MS medium (Murashige and Skoog, 1962), OM medium (Rugini, 1984) and novel derived “Tonda Romana Medium” .

Elements	Murashige and Skoog medium	Olive medium	Tonda Romana Medium
NH_4^+	371.846	371.846	**371.846**
NO_3^-	2443.393	2443.393	**2443.393**
Ca	119.897	222.160	**50.536**
K	783.799	785.700	**751.137**
P	38.693	77.180	**18.220**
Mg	36.455	147.700	**22.246**
S	52.260	203.690	**80.294**
Cu	0.006	0.080	**0.006**
Fe	5.584	5.584	**5.584**
Mn	5.493	5.500	**21.320**
Zn	1.956	3.240	**0.585**

Table 3: The composition of Tonda Romana medium in comparison with MS (Murashige and Skoog, 1962) and Olive Medium (Rugini, 1984).

Elements	MS	OM	Tonda Romana Medium
Macroelements (mg·L^{-1})			
NH_4NO_3	1650.000	412.000	**1650.000**
$Ca(NO_3)_2·4H_2O$	-	1968.000	-
$CaCl_2·4H_2O$	440.000	332.160	**190.460**
$MgSO_4$	-	732.600	-
$MgSO_4·7H_2O$	370.000	-	**225.600**
KNO_3	1900.000	1100.000	**1840.000**
KH_2PO_4	170.000	340.000	**80.050**
KCl	-	500.000	-
K_2SO_4	-	-	**73.000**
Microelements (mg·L^{-1})			
H_3BO_3	6.200	12.400	**6.800**
$CuSO_4·5H_2O$	0.025	0.250	**0.022**
$MnSO_4·H_2O$	16.900	16.900	**65.590**
$Na_2MoO_4·2H_2O$	0.250	0.250	-
$ZnSO_4·7H_2O$	8.600	14.300	**2.570**
$Zn(NO_3)_2·6H_2O$	-	-	-
$FeSO_4·7H_2O$	27.800	-	-
Na_2EDTA	37.300	-	-
FeNaEDTA	-	36.700	**36.700**
KI	0.830	0.830	**0.850**
$CoCl_2·6H_2O$	0.025	0.025	**0.030**
Vitamins (mg·L^{-1})			
Thiamine (B1)	0.100	0.500	**0.600**
Riboflavin (B2)	-	-	**0.210**
Nicotinic acid (B3)	0.500	5.000	**1.150**
Pyridoxine (B6)	0.500	0.500	**0.600**
α-Tocopherol (E)	-	-	**20.000**
Vitamin C	-	-	**1.000**
Glycine	2.000	2.000	**0.850**
Folic acid	-	0.500	-
Biotin	-	0.050	-
L-Glutammine (mg·L^{-1})	-	2190.000	-

Table 4: Effect of iron type and concentrations on shoot development of single-node explants of *in vitro*-grown hazelnut cv Tonda Gentile Romana. Different letters in the same column indicate significative differences among treatments using Duncan's test (P≤0.05).

Iron source (μM)	Shoot height (mm)	No. nodes per explants	Chlorophyll content (mg chl g^{-1} fw)	Chl a/Chl b	Dry weight (%)
EDTA (100)	8.4 b	2.98 b	1.82 b	2.98 b	18.61 a
EDDHA (100)	24.2 a	6.69 a	2.52 a	2.62 a	20.08 a
EDDHA (200)	20.1 a	3.01 b	2.31 a	2.63 a	19.25 a

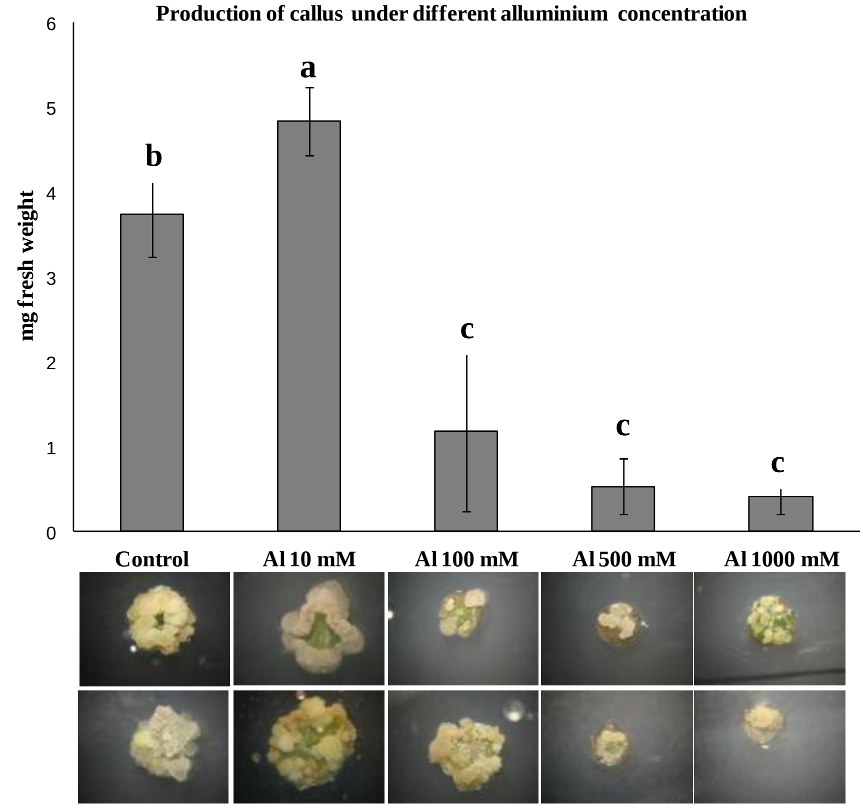

Figure.1: Effect of aluminium concentration on callus development from leaf disc of *in vitro*-grown hazelnut cv Tonda Gentile Romana. Different letters indicate significative differences among treatments using Duncan's test (P≤0.05).

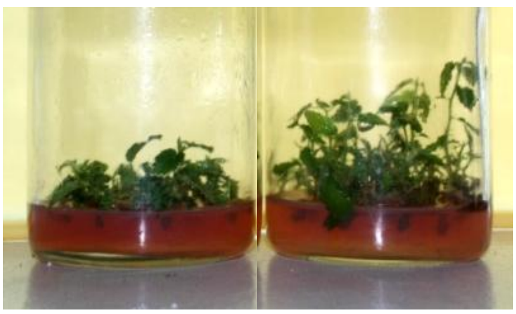

Figure .2: Shoot of hazelnut cv Tonda Gentile Romana growing in novel formulation medium.

The effects of nitrogen source: concentration and NH_4^+/NO_3^- ratio on micropropagation of hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana

1. Introduction

Nitrate and ammonium ions are the most common nitrogen source used for *in vitro* propagation (Murashige and Skoog, 1962; Niedz, 1994). The different forms of nitrogen affect the endogenous levels of cell metabolites as well as proteins, organic acids and plant hormones (Preece, 1995; Sotiropoulos et al., 2005) and the N concentration and the proportions of its forms may influence cell division, differentiation, growth and development of tissue cultures. In addition Nitrogen supply affect also chlorophyll content, Rubisco activity, electron transport rate, photosynthetic rate, anthocyanin production, fresh mass, soluble protein concentration and osmotic pressure of the cell sap (Guidi et al., 1998; Jain et al., 1999; Mashayekhi-Nezamabadi, 2000). Furthermore, species susceptible to ammonium nutrition grew without toxicity symptoms if the concentration of ammonium is moderate (Maynard and Barker, 1969). An unbalance of ammonium concentration in the culture medium lead to NH_4^+-toxicity which is the result of NH_4^+-induced mineral nutrient deficiency caused by the impaired uptake of metal ions, alterations in the osmotic balance and modified phytohormone metabolism (Gerendas et al., 1997).

Also the rooting resulted affect by nitrogen forms and concentration, as reported by many authors in numerous species ((Kerbauy, 1993; Hinnen et al., 1989; Evans, 1993; Woodward et al., 2006).

Many basal salts have been used for hazelnut micropropagation. Yu and Reed (1993) compared the most commonly used: DKW (Driver and Kuniyuki, 1984), WPM (Lloyd and McCown, 1980) and Anderson medium (Anderson, 1984) and found that DKW was superior respect to the others.

Recently, Hand et al. (2014) studied the required mineral nutrient concentrations for micropropagation of five cultivars of *C. avellana* and found that nitrogen requirement is strongly cultivar-dependent. In particular, increased $Ca(NO_3)_2$ (1.5x DKW medium) significantly promoted the shoot multiplication and length of all the five varieties employed and, furthermore, it improved the overall quality of two varieties.

The nitrogen type and concentration even affect the quality response of each cultivar in different ways: in particular one variety responded best to low NH_4NO_3 and high $Ca(NO_3)_2$ level; two of them required high NH_4NO_3 and high $Ca(NO_3)_2$, while one required high amount $Ca(NO_3)_2$ and low NH_4NO_3 concentrations (Hand et al., 2014). Nas and Read (2004) used concentrations of $Ca(NO_3)_2$ and NH_4NO_3 lower than the MS

medium but higher than DKW medium, while Bacchetta et al. (2008) formulated a novel medium (HM) with the same concentration of NH_4NO_3 used in MS medium.
Starting from the contradictory results published, the screening of nitrogen requirements for each cultivars is important specially for the recalcitrant varieties such as Tonda Gentile Romana. The objective of this research was to study the effects of different nitrogen sources on growth and chlorophyll content of this important hazelnut cultivar.

2. Materials and methods

2.1. Plant material

Shoot material used for the following experiments, both proliferation and rooting, derived from in vitro culture of hazelnut on media "Tonda Romana Medium" reported in the previous chapter, supplemented with 6-benzyladenine (BAP) 1 mgL^{-1}, zeatin (Z) 0.5 mgL^{-1}, gibberellic acid (GA_3) 0.2 mgL^{-1}, and maintained in a growth chamber at 24±1°C with a 16-h photoperiod of 40 µmol m^{-2} s^{-1} provided by fluorescent lamps.

2.2. Shoot growth in proliferation medium

Experiments were performed to examine the effect of different NH_4^+ and NO_3^- combination on shoot growth and quality of the propagated hazelnut.
Ten shoots of about 20 mm in length were transferred into each jars containing, replicated three times 100 mL of medium. The content of NH_4^+ and NO_3^- were modified as reported in Table 1. The pH of all the media tested was adjusted to 5.8 before autoclaving at 121°C for 20 minutes.
The photosynthetic pigments were extracted in acetone 80% and centrifuged twice at 5000 g per 15 minutes. The concentrations of chlorophylls and carotenoids were determined spectrophotometrically
Data were subjected to analysis of variance (ANOVA). The mean were separated with Duncan's test ($P \leq 0.05$), using R software package (http://cran.rpoject.org).

2.3. Rooting medium with different Nitrogen source and ratio

The effect of different nitrogen sources and concentrations was examined by transferring the shoots on half-strength MS media modified in terms of ammonium and nitrate as described in table 2.

All measurements were carried out in jars containing 100 mL of medium and ten explants. All the experiments have been conducted with three replications. Rooting data were recorded as percentage, transformed by the arcsin square root, and subjected to analysis of variance (ANOVA).

The mean were separated with Duncan's test ($P \leq 0.05$), using R software package (http://cran.rpoject.org).

3. Results and discussion

3.1. Shoot growth in proliferation medium

Explants growth resulted significantly affected by different ammonium and nitrate combination (Figure 1). In particular, the best results have been obtained with combination B (Table 1), in which both N-NH_4 and N-NO_3 were reduced respect to the control (A). In particular, node number per explants was significantly higher than the others media used (Figure 2c). Chlorophyll a, chlorophyll b and total chlorophyll content are similar to the control (A).

The growth medium D, where N-NH_4 and N-NO_3 were present in equal concentration, negatively influenced the node number per explants, resulting significantly lower respect to the medium A (control) and medium B.

The growth medium C, where N-NH4 and N-NO3 are present at 50 and 10 mM respectively, gave the worst results both in terms of shoot growth and shoot quality; shoot per explants and node number per explants were significantly lower than all the other media. Total chlorophyll and chlorophyll b showed a significative reduction and, some shoots, displayed toxicity symptoms.

In this work, internode length and chlorophyll a content, seems to be not affected by the N-NH_4 and N-NO_3 concentrations.

Many woody species show toxicity symptoms due to NH_4^+ ions (Yan et al., 1992). Previous experiments in tobacco cell cultures showed that cells proliferated better in a medium containing low amounts of ammonium and, in strawberry and carrot cultures, the low ammonium concentrations increase the dry mass accumulation (Hidder et al. 1994).

The effects on the chlorophyll content are in line with those reported by Hsu et al.(2003) in rice, where NH_4^+ are responsible of ethylene sensitivity and, consequently, chlorophyll losses.

3.2. Rooting

Rooting was significantly affected by nitrogen concentration and source. Highest rooting was found at lower nitrogen levels (Table 2), in particular in the rooting media A and B, lacking NH_4NO_3, containing 475 and 950 mg L^{-1} KNO_3 respectively (Figure 3a, b).

In the medium D, E and F (control), the rooting resulted very low (Table 2), and the roots did not elongate (Figures 3d, e, f). These results are in line to those reported in other species (Woodward, 2006; Grimes and Hodges, 1990; Sriskandarajah et al., 1990; Chattopadhyay et al., 1992), The variations in the nitrogen sources were able to improve the rooting, especially when nitrate was the sole nitrogen source.

4. Conclusions

Nitrogen forms and concentrations resulted important in the growth and rooting of hazelnut. The normal concentration of nitrogen present in MS medium (60mM) is too high for hazelnut micropropagation cv Tonda Gentile Romana.

A reduction of total nitrogen (40 mM), accompanied by a reduction of ammonium forms, resulted in a better quality of the shoots.

Furthermore, an increase in rooting occurs when the amount of nitrogen was reduced in the rooting medium, particularly when the NH_4NO_3 was not present.

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6. Figures and Tables

Table1: Ammonium and nitrate concentrations (mM) replaced in “Tonda Romana Medium”

Ammonium and Nitrate concentrations (mM)				
Media	**A**	**B**	**C**	**D**
$N\text{-}NH_4$	20	10	10	25
$N\text{-}NO_3$	40	30	50	25

Table2: Ammonium nitrate and potassium nitrate concentrations (mgL^{-1}) replaced in rooting medium used. The medium F correspond to the amounts present in MS (Murashige and Skoog, 1962). Percentage followed by different letters are significantly different (Duncan’s test, P=0.05)

Ammonium nitrate and potassium nitrate concentrations (mgL^{-1})						
Media	**(A)**	**(B)**	**(C)**	**(D)**	**(E)**	**(F)**
NH_4NO_3	-	-	412.00	412.00	825.00	825.00
KNO_3	475.00	950.00	475.00	950.00	475.00	950.00
Rooting (%)	**77.0 a**	**62.5 a**	**0**	**0**	**0**	**11.1 b**

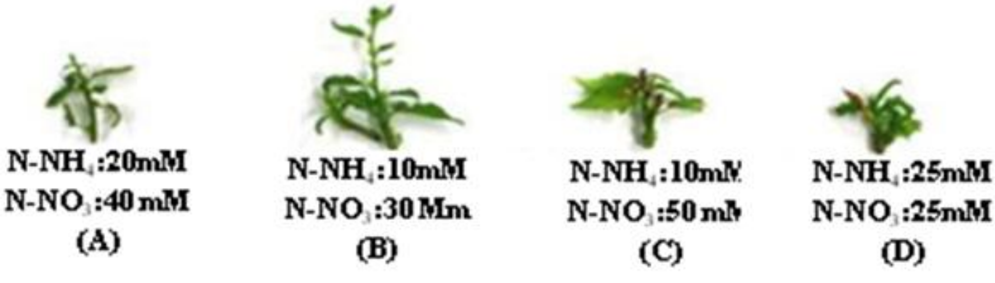

Figure1: Shoots of hazelnut cv Tonda Gentile Romana grown on different ammonium and nitrate concentration.

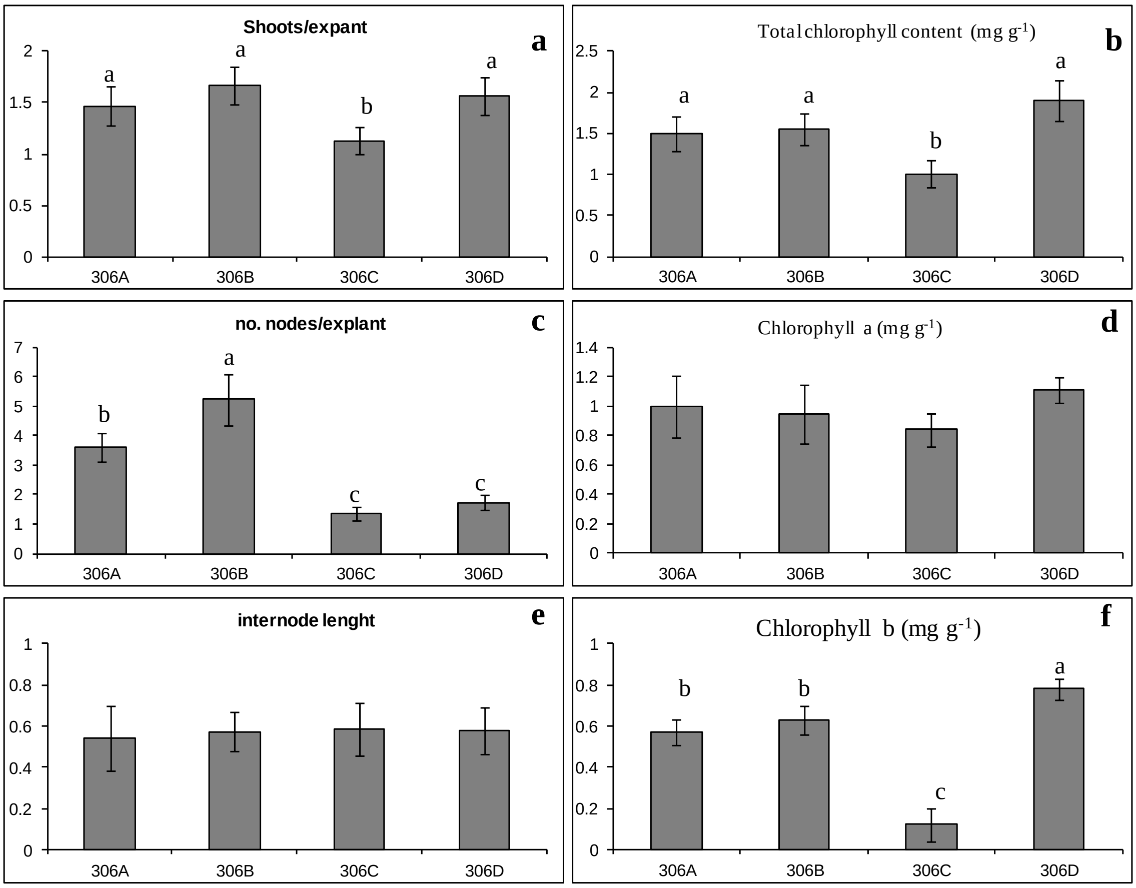

Figure 2: Growth and quality of hazelnut cv Tonda Gentile Romana grown on different ammonium and nitrate concentration. Shoot per explants (a), node number per explants (c), mean internode length (e), total chlorophyll content (b), chlorophyll a (d) and chlorophyll b (f). Bars indicate with different letters are significantly different (Duncan's test, P=0.05).

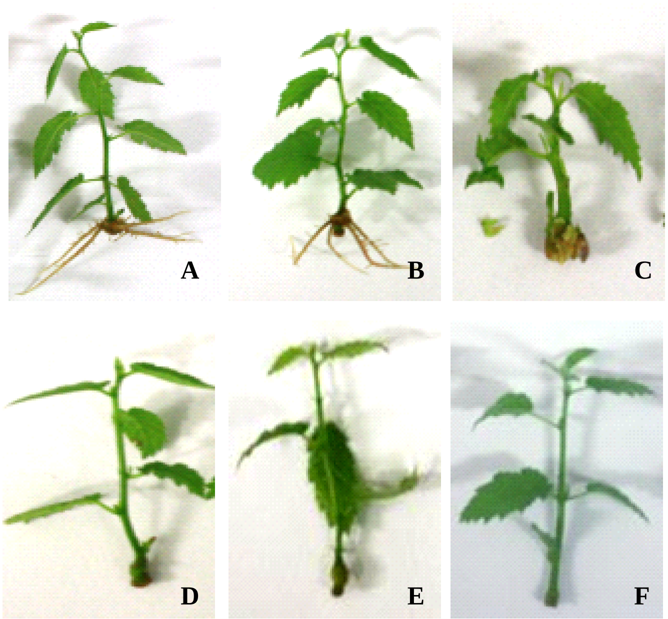

Figure3: Plantlet of hazelnut cv Tonda Gentile Romana rooted on different ammonium nitrate and potassium nitrate concentration.

In vitro adventitious shoot organogenesis in hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana

1.Introduction

Adventitious shoot organogenesis or somatic embryogenesis are the key process for rapid genetic improvement, but neither somatic embryogenesis nor adventitious shoot regeneration have been achieved using mature tissues.

The first report on hazelnut *in vitro* culture has been published by Radojevic et al. (1975) for embryoid induction., through a callus culture isolated from mature embryos cultured for over two years and capable to reach secondary somatic embryogenesis. Small round nodules appears on the callus surface cultivated in a modified MS medium supplemented with kinetin (KIN) and 2,4-Dichlorophenoxyacetic acid (2,4-D) after several months of culture, which resulted as pro-embryonic structures. For the first time the authors understood that in hazelnut the growth regulator 2,4-D did not inhibit the embryoids induction, whilst it arrest their further development into plantlets.

Anatomical and histochemical studies on callus revealed that embryos originated in the epidermal layer of the nodules (Radojevic et al., 1975; 1979). Rodriguez et al. (1984) report that cotyledonary node segments cultured in the presence of (IBA) (1 mgL^{-1}) plus BAP (0.1 mgL^{-1}) or BAP (1 mgL^{-1}) plus IBA (0.1 mgL^{-1}) resulted in somatic embryogenesis induction. Subsequent proliferation has been successfully maintained for five subcultures in the presence of BAP (0.1 mgL^{-1}); following this procedure an efficient regeneration (55%) has been reached and maintained in 60% of the explants used. In the last years many others papers have been published concerning adventitious regeneration and somatic embryogenesis in hazelnut (Perez et al., 1983; Perez et al., 1986;Berros and Rodriguez, 1994; Berros et al., 1995; Centeno et al., 1997; Rodriguez et al., 2000; Ayun et al., 2009) but never starting from mature tissues of cultivars.

In *Agrobacterium*-mediated transformation experiments the antibiotics, normally β-lactams, are added in the regeneration media with the aim to “disinfest” the explants from the bacteria used for transformation, since the β-lactams inhibit peptidoglycan cross-linking during cell wall synthesis in bacteria. Peptidoglycans are not produced by the plants and it would not be expected to affect plant growth, but the reports published demonstrate otherwise (Nauerby et al., 1997; Bosela, 2009). It has been demonstrated that, often, carbenicillin cause an auxin-like effects on callus growth (Robert et al., 1989; Santos and Salema, 1989; Chang and Schmidt, 1991; Holford and Newbury, 1992; Nakano and Mii, 1993; Yepes and Aldwinkle, 1994; Lin et al., 1995; Nauerby et al., 1997; Suzuki et al., 1998; Yu et al., 2001) and cefotaxime stimulates adventitious organogenesis or somatic embryogenesis (Rugini and Caricato, 1995; Mathias and Boyd, 1986; Zaghmout and Torello, 1992; Pius et al., 1993; Nakano and Mii, 1993; Rao et al., 1995; Danilova and Dolgikh, 2004; Mathias and Mukasa, 1987; Okkels and Pedersen, 1988; Predieri et al., 1989; Valobra and James, 1990; Borelli et al., 1992; Yepes and Aldwinckle, 1994; Suzuki et al., 1998; Estropa et al., 2001; Bhau and Wakhlu., 2001; Tang et al., 2004).

The goal of this work was to develop an *in vitro* shoot organogenesis protocol for hazelnut cv Tonda Gentile Romana, using the antibiotics as trigger during proliferation stage.

2. Material and methods

2.1.Experiment I: Adventitious shoot induction

The explant source came from established axenic culture of hazelnut cv Tonda Gentile Romana, grown on "Tonda Romana Medium" reported in Table 1, supplemented with 6-benzyladenine (BAP) 1 mgL^{-1}, zeatin (Z) 0.5 mgL^{-1}, giberellic acid (GA_3) 0.2 mgL^{-1}, and maintained in a growth chamber at 24±1°C with a 16-h photoperiod of 40 µmol m^{-2} s^{-1} provided by fluorescent lamps.

For shoot regeneration, leaves, petioles, internodes and stipules were cultured on MS (Murashige and Skoog, 1962), supplemented with sucrose 30 g $L^{-1}$and plant agar 0.55%;the pH of medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. After sterilization, the media were supplemented with three different hormone combination as reported in Table 2. Ten explants for each tissues (leaf, petioles, internodes and stipules) were cultured in Petri dishes with 25 mL of medium. Explants were maintained in dark condition for a week and then where transferred to a growth at the same environmental conditions as described above. After four weeks, the calli were transferred to a new medium consisting of half-strength MS (Murashige and Skoog, 1962), supplemented with sucrose 30 g L^{-1}, plant agar 0.55% and BAP at 0.5 mgL^{-1}.

Some calli were longitudinally sectioned using a microtome, and the 10-12 µm thick slides where stained with aniline blue (1%) and observed with a microscope (Leitz Dialux 22/22 EB-Ernst Leitz Wetzlar GMBH).

2.2.Experiment II: Adventitious shoot induction through a double-phase proliferation treatment.

On the basis of the results obtained in the previous experiment, to assess the effectiveness of antibiotics to enhance the adventitious shoot organogenesis in hazelnut cv Tonda Gentile Romana, a double-phase propagation system (solid/liquid) by adding an aqueous solution of antibiotics has been established. Fifteen days-old shoots of hazelnut cv Tonda Gentile Romana, growing on medium as mentioned above, at the end of proliferation phase 15 mL of liquid solution containing vancomicine, cefotaxime or carbenicilline at 1000 mgL^{-1} concentration where added on the solid medium.

After fifteen days the shoots were recorded, and the explants were cultured in adventitious shoot induction medium C, as described above. After twenty-days, calli were transferred to a new medium consisting of half-strength MS (Murashige and Skoog, 1962), supplemented with sucrose 30 g $L^{-1}$and 0.55% of plant agar and 0.5 mgL^{-1} of BAP.

Data were subjected to analysis of variance (ANOVA). The mean were separated with Duncan's test ($P \leq 0.05$), using R software package (http://cran.rpoject.org). Regeneration frequency were expressed as percentage.

3. Results and discussion

3.1.Experiment I: Adventitious shoot induction

The cultured explants displaying callus formation started with a yellowish swelling on the excised portion of the explants (Figure 1b), midribs (Figures 1 c, d) and petioles (Figures 1a).

The callus formation rate was different according to the induction media used. Callus formation began after seven days of culture (when the explants were transferred to light conditions). In the induction medium A has been observed an early callus formation, but only in the induction medium C nodule formation have been formed (Figures 7a, b).

Stipules became brown in few days and only in a few cases they produced small calli. When the explants were transferred to half-strength MS (Murashige and Skoog, 1962) medium, supplemented with sucrose 30 g L^{-1}, 0.55% plant agar and 0.5 mgL^{-1} of BAP, on the callus surface appeared spots of red pigments (Figures 2 a, b), probably due to an anthocyanins biosynthesis and accumulation (Figure 2c) stimulated by the exogenous and endogenous cytokinins, as reported in other species (Paulraj et al., 2014; Raaman et al., 2013; Mulinacci et al., 2008; Crouch et al., 1993).

Histological observations at 10 magnifications carried out on the thin slides sectioned from callus of stipules (Figure 3) did not revealed the presence of vascular elements, and calli appeared necrotic (Figures 3 a,b,c). Observations at 40 magnifications (Figures 3 c,d,e) confirmed the presence of necrotic cells and in only one explant (Figure 3e) a vascular element seems to be visible. The calli derived from leaves (Figures 4a, b), internodes (Figure 4c) and petioles (Figure 4d), manifested the presence of vascular elements since from 10 magnifications. At 40 magnifications the presence of tracheary elements (Figures 4 e, f, g, h) were visible particularly annular and helical tracheids, the typical elements defining the transport systems and structure of vascular plants, confirming the attempts of the explants to regenerate adventitious shoots.

3.2.Experiment II: Adventitious shoot induction after a double-phase proliferation treatment.

A double-phase proliferation treatment has been examined using the same concentration (1000 mgL^{-1}) of three different antibiotics (Carbenicilline, Vancomycin and Cefotaxime). In all cases, the presence of antibiotics affected the growth of hazelnut cv Tonda Gentile Romana (Figure 5).

The growth of hazelnut of shoots under different antibiotics was reported in Table 3. Cefotaxime seems to inhibit the growth of the young plantlets causing, in few days, the browning of the explants and abnormal shoot morphology. Both carbenicilline and vancomycin significantly enhanced the growth of the shoots, in particular the shoot resulted 2-fold higher than the control, while no significative differences were found in the node number. The internode length were also affected, resulted significantly longer than the control counterpart. Furthermore, the plantlets treated with vancomycin and carbenicilline showed bigger leaves. Our observation are in line according to many authors that described the effects of different antibiotics on growth enhancement in various species (Yepes et al., 1994; Tamprasert and Reed, 1997; Kaur et al., 2008; Mancharda et al., 2011).

The explants cultured in liquid double-phase with antibiotics were used to repeat the regeneration experiment. The pre-treatments of the tissues with antibiotics strongly affect the regeneration. As showed in Table 4, cefotaxime pre-treated explants were able to regenerate new shoots from petioles (25%) (Figure 7c) and leaves (43%) (Figure 7d), carbenicilline from leaves (33%) (Figure 7f), and vancomycin from stipules (16%). No regeneration events were obtained in the controls.

The young regenerated explants were excised and transferred to the proliferation medium, were they continued to proliferate (Figure 7e) and the derived shoots rooted (Figure 6) similarly to those of control.

4.Conclusions

This work indicates that the protocol described is able to induce adventitious shoot regeneration from mature tissues of hazelnut cv Tonda Gentile Romana, an important commercial cultivars which, which similarly to the other varieties, resulted recalcitrant to regeneration. Previous reports showed that organogenesis and somatic embryogenesis in hazelnut were possible only from zygotic tissues, because the juvenility of the tissues plays a key role in facilitating the induction of these processes. The role of cefotaxime, carbenicilline and vancomicine in stimulating the adventitious shoot regeneration is not clear yet, however they seem to be important in promoting regeneration in recalcitrant woody species as olive cultivar (Rugini et al., 1995) and hybrid aspens (Bosela et al., 2015).

The availability of an efficient regeneration protocols in hazelnut varieties, particularly for Tonda Gentile Romana, the most important cultivars in the Latium region, open the ways to the application of a numerous techniques suitable for a rapid genetic improvement in hazelnut breeding.

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6. Tables and Figures

Table 1: The composition of Tonda Romana medium.

Elements	Tonda Romana Medium
Macroelements ($mg \cdot L^{-1}$)	
NH_4NO_3	1650.000
$Ca(NO_3)_2 \cdot 4H_2O$	-
$CaCl_2 \cdot 4H_2O$	190.460
$MgSO_4$	-
$MgSO_4 \cdot 7H_2O$	225.600
KNO_3	1840.000
KH_2PO_4	80.050
KCl	-
K_2SO_4	73.000
Microelements ($mg \cdot L^{-1}$)	
H_3BO_3	6.800
$CuSO_4 \cdot 5H_2O$	0.022
$MnSO_4 \cdot H_2O$	65.590
$Na_2MoO_4 \cdot 2H_2O$	-
$ZnSO_4 \cdot 7H_2O$	2.570
$Zn(NO_3)_2 \cdot 6H_2O$	-
$FeSO_4 \cdot 7H_2O$	-
Na_2EDTA	-
FeNaEDTA	36.700
KI	0.850
$CoCl_2 \cdot 6H_2O$	0.030
Vitamins ($mg \cdot L^{-1}$)	
Thiamine (B1)	0.600
Riboflavin (B2)	0.210
Nicotinic acid (B3)	1.150
Pyridoxine (B6)	0.600
α-Tocopherol (E)	20.000
Vitamin C	1.000
Glycine	0.850
Folic acid	-
Biotin	-
L-Glutammine ($mg \cdot L^{-1}$)	-

Table 2: Hormonal combination added after sterilization to the medium consisting of MS (Murashige and Skoog, 1962), supplemented with sucrose 30 g L^{-1} and plant agar 0.55%

Hormone	Medium A (mgL^{-1})	Medium B (mgL^{-1})	Medium C (mgL^{-1})
BAP	2	1	1
NAA	0.5	-	-
IBA	-	0.01	2.00
KIN	-	2	2

Table 3: Effects of cefotaxime, carbenicilline and vancomicine on double-phase proliferation of hazelnut cv Tonda Gentile Romana on some vegetative parameters

Antibiotics	Shoot height (mm)	No. nodes per explants	Internode length (mm)
Control (water)	26.2 **b**	4.2 **a**	6.2 **a**
Cefotaxime	11.0 **c**	2.0 **b**	5.5 **a**
Carbenicilline	57.8 **a**	5.1 **a**	11.3 **b**
Vancomicine	59.2 **a**	5.7 **a**	10.4 **b**

Table 4: Shoot regeneration from petioles, leaves, internodes and stipules of hazelnut cv Tonda Gentile Romana according to the pre-treatment used.

	Adventitious shoot organogenesis (%)			
Type of explant	**Control**	**Carbenicilline**	**Cefotaxime**	**Vancomicine**
Petioles	0	0	25%	0
Leaves	0	33%	43%	0
Internodes	0	0	0	0
Stipules	0	0	-	16%

Figure 1: callus formation started with a yellowish swelling on petioles (a),on excised portion of the explants (b), and midribs (c, d).

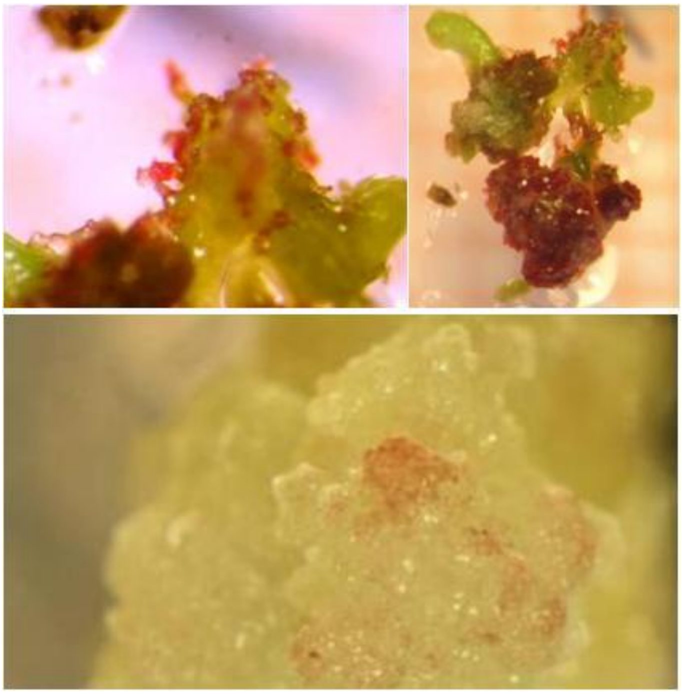

Figure 2: Explants showing callus formation red spots on the surface (a, b). Spots of red pigments appeared on callus surface probably due to anthocyanins biosynthesis and accumulation (c).

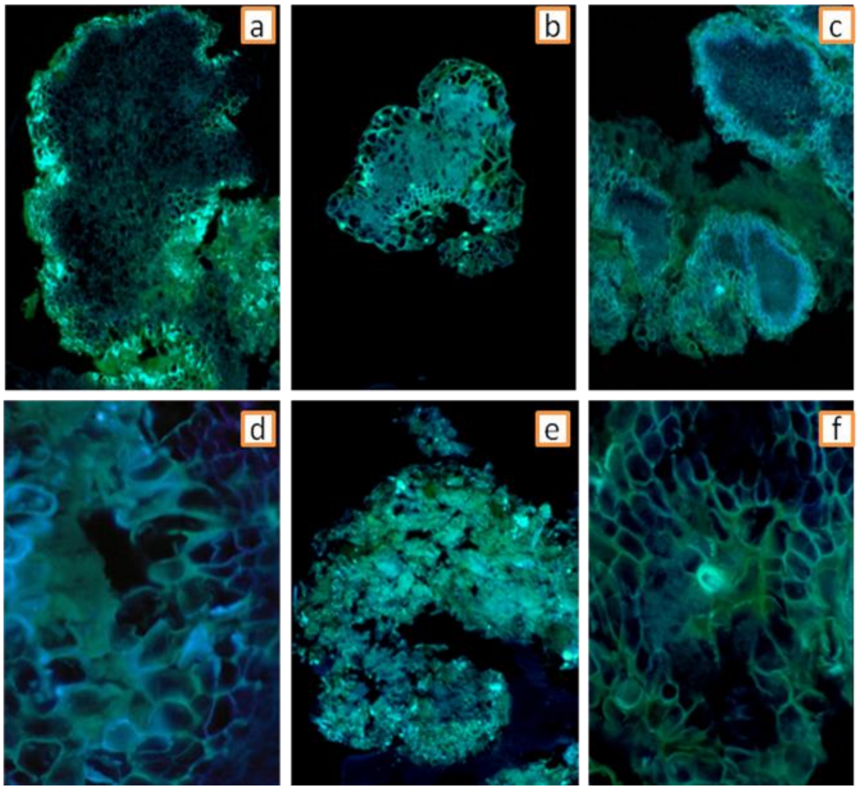

Figure 3. Histological observation of calli derived from stipules, treated with 1% aniline blue, observed with optical microscope at 10 (a,b,c) and 40 (d,e,f) magnifications.

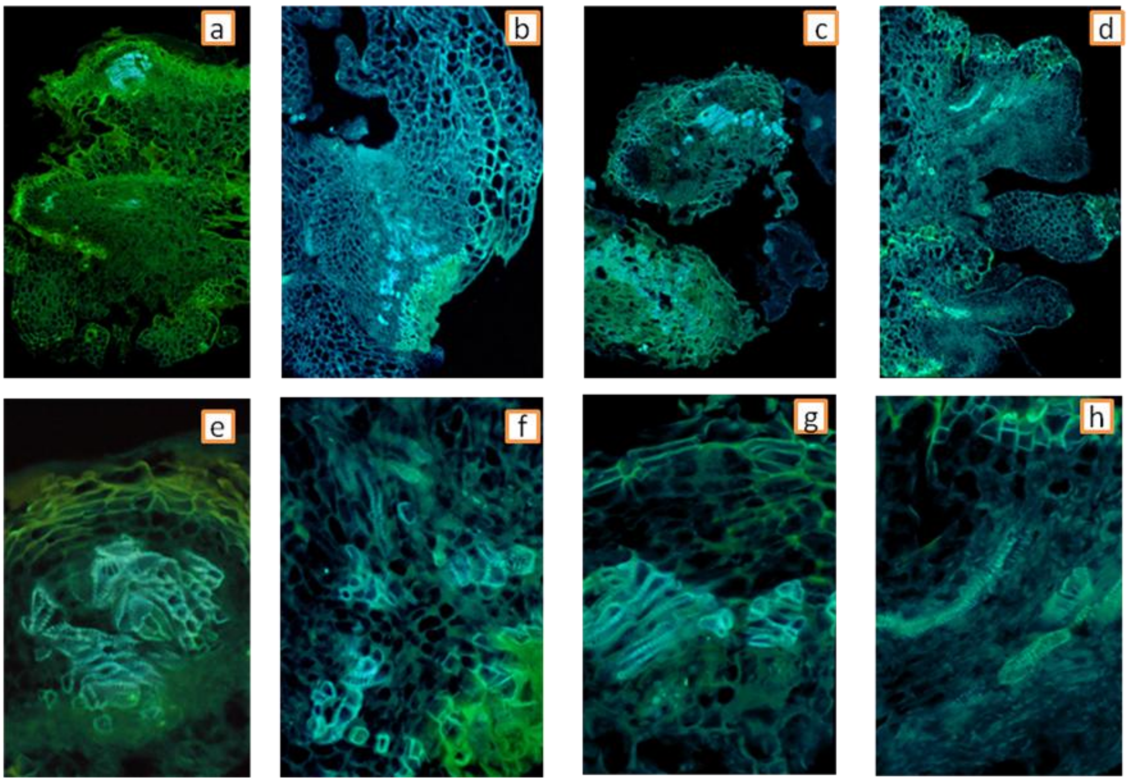

Figure 4. Histological observation of calli derived from internodes and leaves tissues, treated with 1% aniline blue, observed with optical microscope at 10 (a,b,c,d) and 40 (e,f,g,h) magnifications.

Figure 5: *In vitro* proliferation of hazelnut cv Tonda Gentile Romana on double-phase media. Significantly higher proliferation rates were obtained in the treatments with carbenicilline and vancomycin than control and cefotaxime.


Figure 6:A regenerated shoots rooted and transplanted in Jiffy pot for the acclimatization phase.

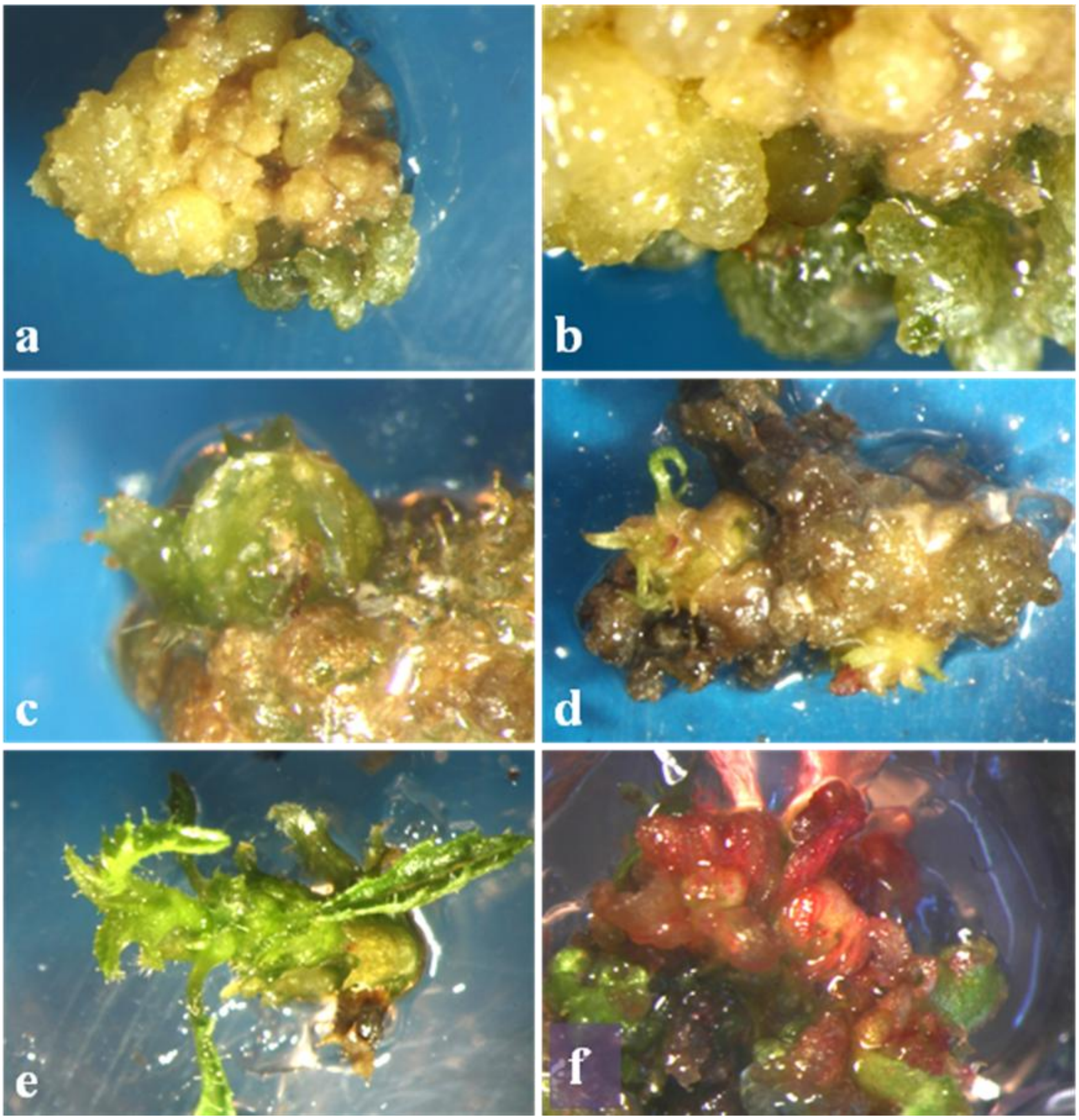

Figure 7: Nodule formation obtained in the induction medium (a and b). A small bud appeared on callus derived from petioles of shoot pre-treated with cefotaxime (c). Buds emerging from browned callus derived from leaves of shoot pre-treated with cefotaxime (d). Regenerated explant excised and transferred in the proliferation medium, were they continued to growth and convert into plantlets (e). cluster of bud arise from callus derived from leaves of shoot pre-treated with vancomicine (f).

In vitro anther culture of hazelnut (*Corylus avellana* L.) for haploids and di-haploids

1.Introduction

The breeding in the fruit species is based on conventional (clonal selection, controlled hybridization, mass selection, induced mutations) or on biotechnological methods employing embryo culture, in vitro fertilization, somaclonal variation, somatic hybridization, genetic transformation and haploid production (Germanà et al., 2006).

All this techniques are still not used in hazelnut and only a paper exists, dealing with protoplast culture.

Following duplication of chromosome number of haploidy genotypes is the fastest way to reach homozygosis. They are useful to fix traits in a desirable combination, to facilitate breeding and to apply marker development, speeding up population mapping and MAS (Marker Assisted Selection).

The importance of haploids in genetic improvement started with the discovery of the *in vitro* culture of immature anthers of *Datura innoxia*, which the normal gametophytic development were able to switch into sporophytic development, inducing somatic embryos with haploid chromosome number.

The haploid plant production through anther culture are usually obtained after surface-sterilization of flowers in sterile conditions followed by anther isolation and culture in liquid or solid media (Sunderland et al., 1984).

The most important factors influencing the efficiency of anther culture are represented by physiological stage and development conditions of the donor plants, since they affect the endogenous hormonal content and nutritional status of anthers. It was demonstrated that, i.e., nitrogen starvation conditions often yields embryogenic pollen grains having a large vacuole, few starch grains and a thin exine wall (Heberle-Bors, 1984). Photoperiod and temperature also influence significantly the embryogenic response of anthers (Heberle-Bors, 1989).

Variations in anther culture response is also genotype-dependent as described by numerous authors; i.e. Bajaj (1990) and Germanà (2007) studied the most responsive cultivar in wheat and citrus respectively.

The “embryogenic period” of anthers usually starts from the first mitosis to bi-cellular stage of pollen and rapidly lose the capacity at beginning of starch storage (Raghavan, 1990; Touarev et al., 2001).

Several pre-treatments represent a key factor in successful anther cultures. The commonly used pre-treatments are cold or heat shocks, starvation in nitrogen and sucrose, osmotic levels and water stresses (Shariatpanahi et al. 2006). The hot-shock treatment (41°C) resulted in aquiring embryogenic competence of anthers in *Brassica napus* (Binarova et al., 1997), pepper (Barany et al., 2001) and tobacco (Touraev et al., 1996). Also the colchicine has been used as stress inducer in various crops such as wheat, maize, rice, sugar beet and sorghum (Germanà, 2011 a,b).

The medium composition is also an important factor in anther culture. B-5 (Gamborg, 1968), MS (Murashige and Skoog, 1962) and N6 medium (Chu, 1978), with minor changes are the most used. The MS is mostly employed in solanaceous crops, while N6 has been mostly used in cereals (Chu, 1978).

The two main functions of growth regulators in anther culture are to induce embryogenesis (Bajaj, 1990; Bajaj et al., 1977) and to identify the fate of embryogenic pathways (Ball et al., 1993). Many investigations, among the most growth regulators employed, demonstrated that 2,4-D promotes and enhances the callus growth, while NAA and IAA are involved in direct embryogenesis (Liang et al., 1987). Also polyamines resulted involved in the improvement and frequency of microspore embryogenesis such as in clementine (Chiancone et al., 2006), cucumber (Kumar et al., 2004) and wheat (Rajyalakshmi et al. 1995). There are still many species recalcitrant to anther culture, mostly in woody species, as recently reviewed by Dunwell (2010), Germana (2011a,b), Touraev et al. (2009) and Wedzony et al. (2009).

2.Materials and methods.

Immature catkins were harvested from branches of selected hazelnut plants of the varieties Nocchione, Tonda di Giffoni and Tonda Gentile Romana when the pollen reached the uninucleate-early bi-nucleate stage. The material was surface-sterilized by vigorous shaking in ethanol 70% for 30 seconds, immersion for 10 min in 20% solution of commercial bleach with few drops of Tween 20, and three washes in sterile distilled water.

The catkins were stored in sealed plastic bags and maintained for 3 days at 4°C in darkness.

For the experiments, the catkins were dissected in sterile condition under laminar flow hood and the immature anthers were extracted by using a needle.

The anthers were placed in different media varying the basal salts and growth regulators used. Two basal media, N6 (Chu, 1978) and BN (Bourgin and Nitsch, 1967), supplemented with sucrose 3%, and plant agar 6%. The pH was adjusted to 5.8 before autoclaving (20 min, 121°C).

After sterilization, three different growth regulator combinations were added: 1) 2,4-D 1 mgL^{-1}, IAA 0,5 mgL^{-1}, ZEA 1 mgL^{-1}, 2) BAP 2,5 mgL^{-1}, NAA 0,1 mgL^{-1}, 3) 2,4-D 1 mgL^{-1}, KIN 1 mgL^{-1}.

Forty-eight anthers were placed in each dishes and three dishes for each cultivar and medium were prepared. Petri dishes were sealed with parafilm, incubated at 24±1°C for 21 days in dark condition and then were subcultured in Petri dishes containing a medium consisting in half-strength MS (Murashige and Skoog, 1962) supplemented with sucrose 3%, IAA 0,5 mgL^{-1} and TDZ 1 mgL^{-1} and placed in light condition.

The number of greenish, swollen and callusing anthers were recorded as percentage, transformed by arcsine square root, and analysis of variance were performed on all data to estimate the effects of cultivars, basal salts and growth regulator combinations.

Some anther calli were collected and treated with colchicine solution at 0.05% for 5 hours at room temperature; then the calli were fixed using absolute alcohol:glacial acetic acid (3:1) for 24 hours, hydrolysed in the 1N HCl at 60°C for 8 minutes, then stained in 1% acetocarmine solution. An Leitz Dialux 22/22 microscope was used for observations.

3. Results and discussion

Different feature has been described in anther culture as marker of the initiation of a morphogenetic response and the change of the developmental pathway; the swelling of anthers have been described in different systems as the first anatomical change accompanying the morphogenic response, however, the non-responsive anthers decreased in volume and turgidity (Germanà et al., 2006).

The number of greenish anthers in induction medium after 21 days of culture resulted significantly affected by the cultivar, basal salts and growth regulator combinations. The analysis of variance also demonstrated significant interactions among the parameters investigated (cultivar x basal salts; cultivars x hormone combinations; basal salts x hormone combinations) (Table 1).

In all the cultivars tested (Tonda Gentile Romana, Tonda di Giffoni and Nocchione) most of the anthers appeared swollen (Figures 1b,c) in comparison to those at the beginning of culture (Figure 1a). The percentage of swollen anthers seems to be not affected by the cultivar, but they were significantly affected by the basal salts (BN gave the best results in Nocchione, while Tonda di Giffoni and Tonda Gentile Romana showed a better response in N6-Chu basal salts). The growth regulators significantly affected the percentage of swollen anthers, in particular the combination 2,4-D 1 mgL^{-1}, IAA 0,5 mgL^{-1} and ZEA 1 mgL^{-1} gave the best results in terms of swollen anthers. A significant interaction between cultivars and growth regulators used were also observed (Table 1).

Callus formation started from different part of the anthers, from the external (Figures 1e,f) or from inner tissues (Fig. 1d), but inner callus formation occurred only in the swollen anthers.

In Table 2 were reported the callus formation of the three cultivars. The "Tonda Gentile Romana" showed the lowest percentage of callus formation. Regarding the basal salts, a significant difference has been found between the two basal salt combinations tested; in general the best response was observed in BN medium (Bourgin and Nitsch, 1967).

Regarding hormone combination consisting of, 2,4-D 1 mgL^{-1}, IAA 0,5 mgL^{-1}, ZEA 1 mgL^{-1}, gave the best results but not statistically different from the combination consisting of 2,4-D 1 mgL^{-1}, KIN 1 mgL^{-1}. The growth regulator combination BAP 2,5 mgL^{-1} and NAA 0,1 mgL^{-1} showed the lowest callus induction among all the cultivar used. Furthermore, the analysis of variance demonstrated a significant interaction among cultivar and growth regulator, and basal salts x growth regulator (Table 2).

Chromosome observation (at 100 magnifications) showed that in all the cultivars tested the haploid calli were not formed. However, all the cells analysed showed an high chromosome instability (Figures 2a,b).

The results presented in this work, using three hazelnut cultivars, confirmed the remarkable influence of the genotypes in callus formation, as reported for many others species. Regarding the basal salts and growth regulator used, no clearly information has been obtained, since the analysis of variance revealed a marked interaction between the factors studied. Finally, we could speculate that at least in hazelnut, the presence of 2,4-D plays a key role in the callus formation, while the growth regulator combination consisting of BAP 2,5 mgL^{-1} and NAA 0,1 mgL^{-1}, gave the lowest percentage of callus formation.

4. Conclusion

The regeneration of microspore-derived plants or embryos from anthers culture are an important aim in the breeding of fruit crops, particularly for hazelnut, which has not been much studied yet with biotechnological approaches.

No haploids tissues have been obtained, although many cells showed chromosome number instability. Regarding the callus formation from inner part of anthers, medium and growth regulator combination have been identified. This information could be useful for future investigation.

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6. Figures and Tables

Table 1: Greenish anthers and swollen anthers percentage in anther culture in Corylus avellana L. cultivars Nocchione, Tonda di Giffoni and Tonda Romana, in different induction medium. Analysis of variance were performed to estimate effect of cultivars, basal salts and hormones used, and interaction among them.

Effect of cultivar, induction medium (basal salt and hormones) on the browning and swallen of anthers

cultivar	basal salt	hormones	green anthers (%)	swallen anthers (%)
Nocchione	BN	2,4-D (1), IAA (0.5), ZEA(1)	55	82
	N6-CHU	2,4-D (1), IAA (0.5), ZEA(1)	80	82
	BN	BAP (2,5), NAA (0,1)	16	55
	N6-CHU	BAP (2,5), NAA (0,1)	5	33
	N6-CHU	KIN (1), 2,4-D (1)	36	50
	BN	KIN (1), 2,4-D (1)	86	80
Tonda di Giffoni	BN	2,4-D (1), IAA (0.5), ZEA(1)	72	62
	N6-CHU	2,4-D (1), IAA (0.5), ZEA(1)	95	76
	BN	BAP (2,5), NAA (0,1)	30	26
	N6-CHU	BAP (2,5), NAA (0,1)	17	57
	N6-CHU	KIN (1), 2,4-D (1)	68	56
	BN	KIN (1), 2,4-D (1)	52	77
Tonda Gentile Romana	BN	2,4-D (1), IAA (0.5), ZEA(1)	25	49
	N6-CHU	2,4-D (1), IAA (0.5), ZEA(1)	85	75
	BN	BAP (2,5), NAA (0,1)	24	49
	N6-CHU	BAP (2,5), NAA (0,1)	7	64
	N6-CHU	KIN (1), 2,4-D (1)	14	43
	BN	KIN (1), 2,4-D (1)	3	44

ANALYSIS OF VARIANCE

Variable: greenish anthers percentage

Factors	SS	*dF*	MS	F	ProbF	
cv	1,101	2	0,550	23,289	0,0000001	**
medium	0,188	1	0,188	7,963	0,0066673	**
hormone	3,285	2	1,643	69,511	0,0000000	**
cv x basal salts	0,160	2	0,080	3,387	0,0411471	*
cv x hormones	0,751	4	0,188	7,946	0,0000411	**
basal salts x hormones	0,749	2	0,375	15,852	0,0000038	**
Residual	1,276	54	0,024			
Total	8,061	71	0,114			

ANALYSIS OF VARIANCE

Variable: swallen anthers percentage

Factors	SS	*dF*	MS	F	ProbF
cv	0,112	2	0,0558	1,689	0,194282875
medium	0,293	1	0,2930	8,875	0,004325076 **
hormone	0,663	2	0,3317	10,047	0,000195211 **
cv x basal salts	0,111	2	0,0553	1,673	0,197175531
cv x hormones	0,417	4	0,1042	3,155	0,021076127 *
Residual	1,783	54	0,0330		
Total	3,743	71	0,0527		

Table 2: Callus formation in anther culture in Corylus avellana L. cultivars Nocchione, Tonda di Giffoni and Tonda Romana, in different induction medium. Analysis of variance were performed to estimate effect of cultivars, basal salts and hormones used, and interaction among them.

Effect of cultivar, induction medium (basal salt and hormones) on the anther callus			
cultivar	**basal salt**	**hormones**	**callus (%)**
Nocchione	BN	2,4-D (1), IAA (0.5), ZEA(1)	67
	N6-CHU	2,4-D (1), IAA (0.5), ZEA(1)	39
	BN	BAP (2,5), NAA (0,1)	11
	N6-CHU	BAP (2,5), NAA (0,1)	19
	BN	KIN (1), 2,4-D (1)	55
	N6-CHU	KIN (1), 2,4-D (1)	41
Tonda di Giffoni	BN	2,4-D (1), IAA (0.5), ZEA(1)	77
	N6-CHU	2,4-D (1), IAA (0.5), ZEA(1)	40
	BN	BAP (2,5), NAA (0,1)	9
	N6-CHU	BAP (2,5), NAA (0,1)	7
	BN	KIN (1), 2,4-D (1)	24
	N6-CHU	KIN (1), 2,4-D (1)	50
Tonda Gentile Romana	BN	2,4-D (1), IAA (0.5), ZEA(1)	42
	N6-CHU	2,4-D (1), IAA (0.5), ZEA(1)	13
	BN	BAP (2,5), NAA (0,1)	0
	N6-CHU	BAP (2,5), NAA (0,1)	0
	BN	KIN (1), 2,4-D (1)	4
	N6-CHU	KIN (1), 2,4-D (1)	5

ANALYSIS OF VARIANCE

variable: Callus formation percentage

Factors	**SS**	**DF**	**MS**	**F**	**ProbF**
cv	1,091	2	0,545	50,160	9,03140E-13 **
medium	0,106	1	0,106	9,724	2,98877E-03 **
hormone	1,750	2	0,875	80,471	1,67672E-16 **
cv x medium	0,015	2	0,008	0,706	4,98273E-01
cv x hormone	0,305	4	0,076	7,019	1,39785E-04 **
medium x hormone	0,511	2	0,256	23,506	5,82738E-08 **
Residual	0,555	51	0,011		
Total	4,520	71			

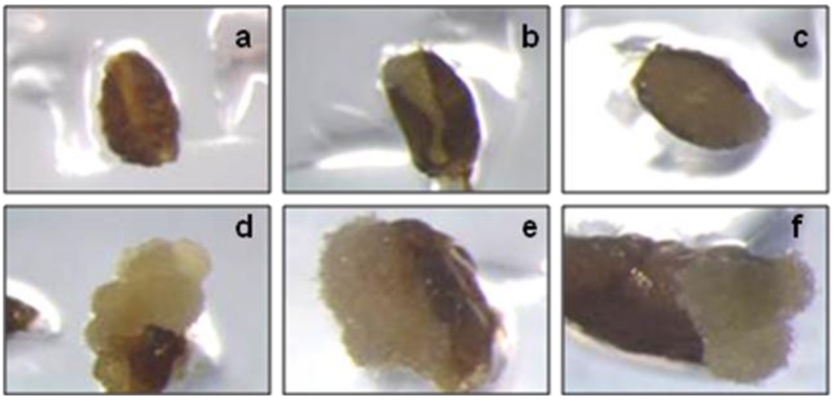

Figure 1: Anther culture of hazelnut cv Nicchione a) not developed anthers after 21 days of culture; b,c) swollen anthers after 21 days of culture; d) callus formation from external tissues of anther; e,f) callus formation from inner tissues of anthers

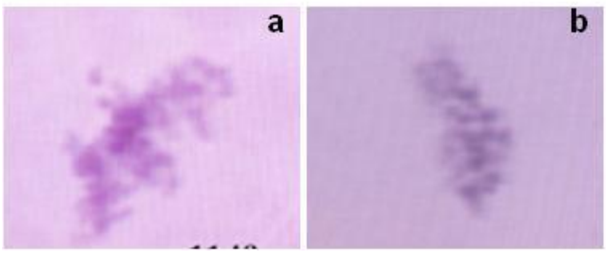

Figure 2: Chromosomes in cells of calli derived from cv Nocchione. No haploid cells were found, but all the cells showed an high chromosome instability (a,b).

General conclusions

Hazelnut is an important nut crop, and the largest producers are Turkey, Italy, Spain and USA, but recently it has been introduced in countries as China, Chile and Australia.

The world production is based on approximately 20 old varieties, and in Italy the hazelnut cultivation are located in four regions, where the cultivar availability consists of only few cultivars commonly propagated using rooted suckers.

The *in vitro* propagation technique is widely used in woody species, but in the case of hazelnut there are still problems for a much wider application of the *in vitro* propagation.

Furthermore, hazelnut is characterized by long juvenile period that makes the conventional breeding extremely time-consuming.

For the mentioned reasons more work should be done for genetic improvement and nursery industries. The evaluation of the available genetic resources and the development of *in vitro* biotechnology open new perspectives for hazelnut cultivation.

The study of 48 cultivars grown in a core collection has allowed the identification of some interesting genotypes for low suckers emission and for phenological traits with the aim to extend the number of varieties suitable for ongoing climate changes, since hazelnut is one of the most sensitive plants because the flowering occur during winter.

The studies carried out on micropropagation confirm that each cultivar requires its own specific medium. Our investigations ended with the formulation of novel suitable for the cultivar Tonda Gentile Romana, trough the analysis of several factors including mineral elements in the seeds, iron source, nitrogen amount and ammonium and nitrate ratio.

Regarding the contribution of biotechnology in genetic improvement, to our knowledge, this is the first report on the adventitious shoot organogenesis in hazelnut from mature tissues. The availability of an efficient regeneration protocol allows the application of numerous techniques for hazelnut genetic improvement.

Finally, the protocol developed to get callus initiation from the inner part of the anthers, will be very useful for future investigation on anther culture with the aim to produce haploid tissues.