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The effect of $CuSO_4$ for establishing *in vitro* culture, and the role nitrogen and iron sources in *in vitro* multiplication of *Corylus avellana* L. cv. Tonda Gentile Romana

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ABSTRACT

In the present work, we have used copper sulphate ($CuSO_4 \cdot 5H_2O$) enriched medium for effective control of visible and latent contamination. Among the different concentrations used, 1.25–2.5 mg/L resulted the most appropriate. In addition, the role of different nitrogen source and concentrations (NH_4NO_3 and KNO_3), as different iron source (FeEDTA and FeEDDHA) has been investigated in the proliferation and rooting phases of European hazelnut (cv. Tonda Gentile Romana). The normal concentration of nitrogen present in Murashige and Skoog medium is too high for hazelnut micropropagation cv. Tonda Gentile Romana. A reduction of total nitrogen, accompanied by a reduction of ammonium forms, resulted in a better quality of the shoots. Similar results have been obtained when the common iron source FeEDTA has been replaced by the same concentration of FeEDDHA. 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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Hazelnut; micropropagation; copper sulphate; iron; nitrogen supply

1. Introduction

Hazelnut (*Corulys avellana* L.) is a woody species of increasing interest because of its industrial production in both fresh market and processing industries. Furthermore, it is known that hazel fruit contains taxanes, molecules used in the production of chemotherapy drugs (Bestoso et al. 2006). In the last years, the economic potential of this species has been increased since the hazelnut oil has been proposed even as biodiesel (Xu et al. 2007; Demirbas 2008).

Usually, hazelnuts are propagated by suckers' layering from vigorous donor plants. However, the increasing interests in hazelnut cultivation, which led to the need of large number of plant availability, and the presence of some diseases recently encountered in some hazelnut district (i.e. Dieback of hazelnut in the Viterbo province), are underlined the risk connected with suckers' layering propagation, unable to satisfy the massive demand of high quality plant material.

Propagation by cuttings, a propagation techniques widely used for fruit trees, have not produced protocols able to ensure a large-scale propagation (Kantarci and Ayer 1994; Solar et al. 1994; Cristofori et al. 2010; Contessa et al. 2011a; Contessa et al. 2011b; Contessa et al. 2014; Tombesi et al. 2015), because hazelnut are very genotype dependent and due to the annual weather condition and the health and vigor of donor plants, that are fundamental for the success of cuttings (Contessa et al. 2011a; Contessa et al. 2011b, Contessa et al. 2014).

In vitro technologies, such as micropropagation, can be used to produce plants of existing and novel varieties, to be multiplied exponentially in a short time period, as well as a tool for implementing plant breeding biotechnological approaches in woody species, particularly if mature tissues from valuable cultivars are used (Silvestri et al. 2016; Karasawa et al. 2016).

The hazelnut has yet been considered a species characterized by a recalcitrant behaviour (Nas and Read 2004; Damiano et al. 2005; Bacchetta et al. 2008; Caboni et al. 2009) since the *in vitro* response is most exclusively genotype-dependent (Hand et al. 2014; Hand and Reed 2014; Akin et al. 2017). Hazelnut varieties are generally difficult to initiate into culture due to internal microbial contaminants and lack of juvenility material (Hand et al. 2016). Recently Hand et al. (2016) reported that the surface disinfestation technique is important, and they recommend the use of first three nodes below the apex of fast-growing greenhouse plants. Often, the surface sterilization is not sufficient, since the endogenous contaminant can arise after many days in culture, avoiding the maintenance of aseptic conditions that represent the single factors responsible for maximum losses in plant tissue culture (Javed et al. 2017). Copper is an essential elements for plants and possess a wide range antimicrobial efficacy but the plant response varies among species. The *in vitro* establishment is not the sole problem encountered in hazelnut propagation, due to the recalcitrance of hazelnut to the *in vitro* conditions.

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Many basal salts have been used for hazelnut micropropagation. Yu and Reed (1993) compared the most commonly used: DKW (Driver and Kunijuki 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), Hand and Reed (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 $Ca(NO_3)_2$ (1.5× 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 Murashige and Skoog (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.

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 (Sotiropoulos et al. 2005), the nitrogen concentration, and the ratio 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. 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). In addition, nitrogen forms and concentration, as reported by many authors in numerous species (Kerbauy 1993; Hinnen et al. 1989; Evans 1993; Woodward et al. 2006) affected the rooting.

Iron is an important element for development and quality of plant material. It is involved in many pathways as photosynthesis and respiration, but the plants can take up iron only in specific forms (Hell and Stephan 2003). At pH 5.6–6.0, usually adjusted in plant tissue culture medium, the unchelated ferric iron forms insoluble ferric oxides, unavailable to plant tissues (Garrison et al. 2013). To enhance its solubility Fe^{3+} are usually added as chelated forms (Fe-EDTA and Fe-EDDHA), but Fe-EDTA is the iron form contained in all the main salt media usually marketed.

Fe-EDTA represents the most used iron source in micropropagation protocols, also in hazelnut (Yu and Reed 1993; Damiano et al. 2005; Bacchetta et al. 2008); however, Fe-EDDHA was found more suitable for some interspecific hybrids of hazelnut (Garrison et al. 2013).

Starting from the contradictory results published the screening of macro and microelement requirements as nitrogen and iron for each cultivar is important, especially for the recalcitrant varieties such as Tonda Gentile Romana, which shows some problems for a large-scale propagation.

This work aimed to optimize a protocol for *in vitro* establishment able to reduce the bacterial contaminants and increase the hazelnut *in vitro* establishment.

Although the present work did not consider the importance of ion confounding (Akin et al. 2017), the general approach for optimization of tissue culture medium by using salts as factors, proved to be a useful tool to study the media composition.

2. Materials and methods

2.1. Plant material

Plant materials "Tonda Gentile Romana" were used for *in vitro* establishment. The variety has been propagated by cuttings, potted in 1 L pots and maintained in a growth chamber until controlled environmental conditions (24 ± 1 °C; 16 h light/8 h dark, RH 35%), with artificial fluorescent lamps (40 μmol m^{-2} s^{-1}).

2.2. Explant collection, surface sterilization, and $CuSO_4$-enriched media

The first three nodal segments were used as explants, discarding the shoot apex. All leaves have been removed and nodal segments were immerged for 1 h in aqueous solution containing 250 mg/L ascorbic acid, 250 mg/L citric acid, 5 mg/L GA_3, and 0.1% PPM. Then, the explants have been surface sterilized in a 20% commercial bleach plus few drops of Tween 20 for 30 min and rapidly rinsed twice with sterile distilled water.

The culture establishments have been performed in 100 × 20 mm glass tube containing 5 mL of medium composed of half-strength MS medium, 20 g/L sucrose, 6 mg/L 6-benzylaminopurine, 0.1 mg/L naphthalene acetic acid, 0.1 mg/L thidiazuron and 0.55% plant agar (Duchefa, Haarlem, Netherlands). Medium pH has been adjusted to 5.8 with KOH 1 M and autoclaved at 121 °C for 20 min. The culture media were supplemented with different concentrations of $CuSO4{\cdot}5H_2O$ (0.0, 1.25, 2.5, and 5 mg/L). The contamination rates and number of bud sprouted have been collected.

The cultures have been 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. Fifty explants for each media have been considered.

2.3. Propagation of explants under different concentration of NH_4^+/NO_3^- and iron source

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 jar containing 100 mL of medium, replicated three times. For all experiments, the nitrogen content differs in inorganic nitrogen levels. Treatments have been prepared as follow: A-(1650 mg/L NH_4NO_3 and 1900 mg/L KNO_3), B-(825 mg/L NH_4NO_3 and 1900 mg/L KNO_3), C-(825 mg/L NH_4NO_3 and 4000 mg/L KNO_3), D-(2000 mg/L NH_4NO_3). The pH of all the media tested was adjusted to 5.8 before autoclaving at 121 °C for 20 min.

Furthermore, iron was supplied either as ethylenediamine tetra acetic acid-ferric-sodium salt (FeEDTA) or ethylenediamine bis(2)-hydroxyphenylacetic acid (Fe-EDDHA) in the form of Sequestrene 138 (PhytoTechnology Laboratories, Lenexa, KS, USA), at 100 and 200 mg/L.

The photosynthetic pigments were extracted from 100 mg of fresh leaves. The extraction has been performed in 15 mL test tubes with 4 mL of methanol 100%, heated 10 min in a water bath to 65 °C and then stored at 4 °C for 24 h. After centrifugation at 5000*g* per 5 min, the concentrations of chlorophyll contents were determined spectrophotometrically with a spectrophotometer EVO 60 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and chlorophyll a, chlorophyll b, total chlorophyll, and *Chla/Chlb* ratio calculated according to Lichtenthaler (1987). Five samples for each treatment were analysed and repeated three times. The dry matter (DW) was determined using 10 leaves for each treatments by heating at 105 ± 2 °C until constant weight. The evaluation of growth parameters has been performed by the number of shoots per explant, the number of nodes and the mean internode length after four weeks of culture.

2.4. Rooting of explants under different concentration of NH_4^+/NO_3^-

In order to study the response to different nitrogen sources and concentrations on root induction, 2–3 cm individual shoots were excised at the end of a 4 weeks proliferation period on the medium HM (as described above). The rooting media consisted of half-strength MS medium gelled with 0.6% plant agar and supplemented with 3% sucrose, 1 mg/L indole-3-butyric acid. The media varied within them in inorganic nitrogen levels as follow described: A-(475 mg/L KNO_3), B-(950 mg/L KNO_3), C-(412 mg/L NH_4NO_3 and 475 mg/L KNO_3), D-(412 mg/L NH_4NO_3 and 950 mg/L KNO_3), E-(825 mg/L NH_4NO_3 and 475 mg/L KNO_3), F-(825 mg/L NH_4NO_3 and 950 mg/L KNO_3). All the experiments have been conducted with three replications by using 100 mL of medium and ten explants. The cultures were initially maintained for a week in dark conditions at 24 ± 1 °C, and then were exposed to the same light and temperature conditions as described above. After 21 days in culture on the rooting medium, the percentage of rooted shoots were recorded.

Rooted plantlets have been transferred in Jiffy pots and covered with polyethylene film to maintain high relative humidity. After three weeks the number of survived plants were recorded.

2.5. Statistical analysis

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). Data recorded as percentage have been transformed by arcsine square root prior to be subject to ANOVA.

3. Results and discussion

3.1. Explant collection, surface sterilization, and $CuSO_4$-enriched media

The nodal segments discarding the shoot apex were used as explants, as suggested by Hand et al. 2016 (Figure 1(a)). After 15 days in culture, the nodal segment start to growth (Figure 2(b)).

As showed in Figure 1, the addition of copper sulphate strongly reduced the bacterial contaminations in the culture medium. 2.5 mg/L $CuSO_4 \cdot 5H_2O$ (Figure 2(c)) significantly decreased the contamination frequency in the establishment phase, but maintaining a high bud sprouting (Figure 1). The highest concentrations (5.0 mg/L) reduce significantly the contamination rate, but at the same time negatively affected the bud sprouting and, for this reason, this concentration has been considered not suitable (Figure 1).

3.2. Propagation of explants under different concentration of NH_4^+/NO_3^-

Shoot growth resulted significantly affected by different ammonium and nitrate combination (Table 1). In particular, the best results have been obtained with the nitrogen concentration combination B (825 mg/L NH_4NO_3: 1900 mg/L KNO_3), in which both N-NH_4 and N-NO_3 were reduced respect to the control (1650 mg/L NH_4NO_3: 1900 mg/L KNO_3);

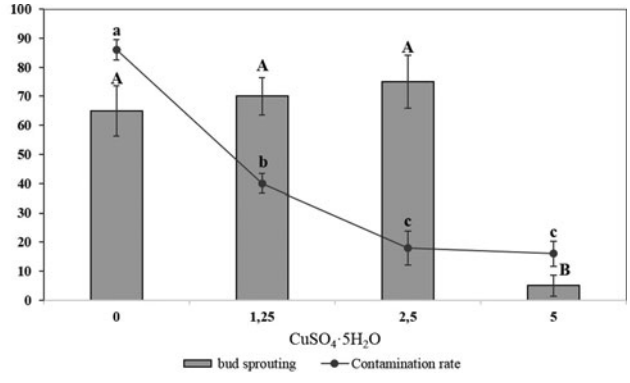

Figure 1. Effect of different concentration of copper sulphate on bud development and contamination rate of hazelnut cv Tonda Gentile Romana after 21 days in culture. Bars and line represent the mean ± standard deviation. Mean denoted by different letters within bars or line are significantly different at $P \leq 0.05$ using Duncan's test.

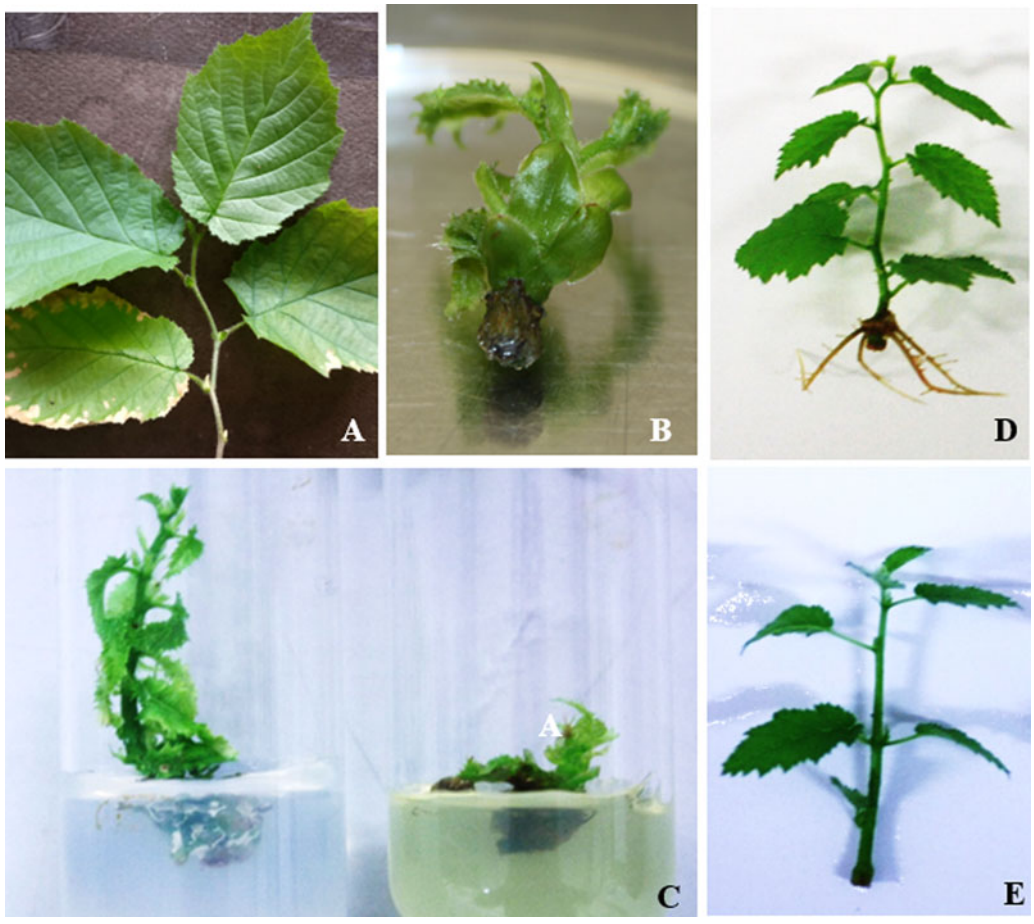

Figure 2. Explants of cv Tonda Gentile Romana used for in vitro establishment (a). Disinfected explant growing in the medium after 21 days of culture (b). Media containing $CuSO_4 \cdot 5H_2O$ at 2.5 mg/L (on the left) compared with control (on the right) (c). Plantlets derived from rooting experiment in rooting medium lacking NH_4NO_3 (d) and control rooting medium F (e).

Table 1. Effect of nitrogen source and concentrations on shoot development of single node explants of *in vitro* grown hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana.

N sources and concentrations (mg/L)	Shoots/explant	Nodes/explant	Internode length (mm)	Total chlorophyll (mg/g fw)	Chrophyll *a* (mg/g fw)	Chrophyll *b* (mg/g fw)	Chl *a*/Chl *b*	Dry matter (%)
A NH_4NO_3: 1650 KNO_3: 1900	1.46 ± 0.51^{a}	3.60 ± 0.9^{b}	5.4 ± 0.3	1.70 ± 0.21^{a}	$1.10 \pm 0.19^{a,b}$	0.37 ± 0.06^{b}	2.97 ± 0.12^{b}	19.8 ± 2.2
B NH_4NO_3: 825 KNO_3: 1900	1.66 ± 0.48^{a}	5.2 ± 0.8^{a}	5.7 ± 0.3	1.95 ± 0.20^{a}	1.45 ± 0.11^{a}	0.63 ± 0.07^{a}	2.30 ± 0.19^{c}	19.7 ± 1.1
C NH_4NO_3: 825 KNO_3: 4000	1.13 ± 0.35^{b}	1.4 ± 0.5^{c}	5.9 ± 0.4	1.21 ± 0.17^{b}	0.84 ± 0.09^{b}	0.22 ± 0.08^{b}	3.82 ± 0.54^{a}	18.9 ± 1.9
D NH_4NO_3:- 2000 KNO_3:-	1.56 ± 0.50^{a}	1.7 ± 0.5^{c}	5.7 ± 0.2	2.01 ± 0.25^{a}	$1.13 \pm 0.11^{a,b}$	0.61 ± 0.05^{a}	1.85 ± 0.15^{d}	20.1 ± 1.5

Data represent mean value ± SD. Different letters among the same column indicate significant differences among treatments with Duncan's test ($P \leq 0.05$).

in this medium, the node number resulted higher than the control (5.8 vs. 3.6). In the media C and D, the mean node number resulted drastically reduced, showing a very low number of neo-formed phytomers (1.4 and 1.7, respectively). The mean internode length and the dry matter did not shown significant differences among the treatments. The number of shoot per explants was not affected by treatments, except in the medium C (825 mg/L NH_4NO_3: 4000 mg/L KNO_3), where this value resulted significantly lower. The total chlorophyll content resulted drastically reduced in the medium C (1.21 mg/g fw), while in the others media the mean values range between 1.70 and 2.01 mg/g fw. Similar results have been observed for chlorophyll a, for which the highest content was recorded in the medium B, not significantly different to the media A and D while the lowest one was observed in medium C (0.84 mg/g fw). On the contrary, the *Chl b* contents have been affected by the treatments; in particular, *Chl b* resulted higher in media B and D (0.63 and 0.61 mg/g fw respectively) than the control A and medium C (0.37 and 0.22 mg/g fw, respectively). The *Chl a/Chl b* ratio, which can be provide a further information about the physiological status of the shoots, resulted significative affected by the medium; in particular the shoots grown in the medium C showed the highest ratio (3.82), while the lowest one has been observed in medium B and D (2.30 and 1.85, respectively).

Table 2. Effect of iron source (FeEDTA or FeEDDHA) on shoot development of single node explants of *in vitro* grown hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana.

Irone source (mg/L)	Shoot height (mm)	nodes/explant	Internode length (mm)	Total chlorophyll (mg/g fw)	Chrophyll *a* (mg/g fw)	Chrophyll *b* (mg/g fw)	Chl *a* / Chl *b*	Dry matter (%)
Fe-EDTA (36.70)	14.4 ± 2.4^{b}	3.10 ± 0.6^{b}	4.6 ± 0.6	1.82 ± 0.24^{b}	1.24 ± 0.12^{b}	0.42 ± 0.08^{b}	2.98 ± 0.23^{a}	21.2 ± 0.9
Fe-EDDHA (100)	24.2 ± 1.9^{a}	6.71 ± 0.7^{a}	5.5 ± 0.3	2.52 ± 0.21^{a}	1.62 ± 0.06^{a}	0.69 ± 0.08^{a}	2.34 ± 0.12^{b}	19.8 ± 1.6
Fe-EDDHA (200)	20.1 ± 1.1^{a}	$4.05 \pm 0.4^{a,b}$	5.9 ± 0.5	2.31 ± 0.18^{a}	1.67 ± 0.13^{a}	0.62 ± 0.05^{a}	$2.63 \pm 0.15^{a,b}$	20.5 ± 1.8

Data represent mean value ± SD. Different letters among the same column indicate significant differences among treatments with Duncan's test ($P \leq 0.05$).

Concerning the iron source used, the shoots grown with FeEDDHA showed better traits when compared with FeEDTA (Table 2). Shoots appeared longer and characterized by a higher number of internodes when the iron source used was FeEDDHA in both concentrations (100 and 200 mg/L). In the same manner, the total chlorophyll content, *Chl a*, *Chl b*, and *Chl a/Chl b* ratio have showed different results from the control (36.70 mg/L FeEDTA). In particular, the shoots cultivated with 100 mg/L EDDHA are characterized by the lowest value of *Chl a/Chl b* ratio (2.34) respect to Fe-EDTA (2.98), while no differences have been observed between Fe-EDDHA at 100 and 200 mg/L. No differences in terms of dry matter have been observed.

3.3. Rooting

Rooting was significantly affected by nitrogen concentration and source (Table 3). Highest rooting was found at lower nitrogen levels, in particular in the rooting media A (475 mg/L KNO_3) (Figure 1(d)) and B (950 mg/L KNO_3), the media lacking NH_4NO_3, 87% and 72% respectively.

In the media E and F (control; Figure 1e), the rooting resulted very low, and the roots did not elongate. The plantlets obtained in the media lacking NH_4NO_3 showing a better acclimatization, confirming the importance of the medium used in the acclimatization phase of young plantlets produced *in vitro*. The media C and D gave better results respect to media E and F, while their survival percentages did not differ from the media A and B.

4. Discussion and conclusions

The results of the present study showed that the applications of $CuSO_4 \cdot 5H_2O$ at 1.25 to 2.5 mg/L is useful in controlling the endogenous contaminants during the establishment of *in vitro* cultures.

The main objective of *in vitro* propagation of recalcitrant species such as hazelnut is the opportunity to have a protocol adapted to a large number of commercial varieties. The improvement of growing media is important not only to produce a higher quality material, but also for obtaining shoots easy to handling in *in vitro* operations. In this work, we analysed the effect of different nitrogen supplies (KNO_3 and NH_4NO_3) and iron source (FeEDTA vs. FeEDDHA) in the propagation chain of European hazelnut. The reduction to half concentration of ammonium nitrate compared to the control, produced shoots with a greater internode number, higher chlorophyll *a* and *b* contents and a good *Chl a/Chl b* ratio.

Table 3. Effect nitrogen sources and concentrations on rooting and acclimatization of micro-cuttings derived from *in vitro* grown hazelnut (*Corylus avellana* L.) cv Tonda Gentile Romana.

N sources and conc. (mg/L)		Rooting (%)	Survival (%)
A	NH_4NO_3: -KNO_3: 475	87 ± 9^{a}	84 ± 3^{a}
B	NH_4NO_3: - KNO_3: 950	72 ± 8^{a}	78 ± 6^{a}
C	NH_4NO_3: 412 KNO_3: 475	52 ± 3^{b}	81 ± 8^{a}
D	NH_4NO_3: 412 KNO_3: 950	58 ± 6^{b}	81 ± 5^{a}
E	NH_4NO_3: 825 KNO_3: 475	33 ± 8^{c}	62 ± 3^{b}
F	NH_4NO_3: 825 KNO_3: 950	22 ± 8^{c}	51 ± 6^{b}

Data represent mean value ± SD. Different letters among the same column indicate significant differences among treatments with Duncan's test ($P \leq 0.05$).

The data obtained confirm what has been observed in many others woody species that showed 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 increased the dry mass accumulation (Hidder et al. 1994). In addition, the effects on the chlorophyll contents are in line with the literature. As reported by Hsu et al. (2003) in rice, the amount of NH_4^+ are responsible of ethylene sensitivity and, consequently, chlorophyll losses.

Similar results have been obtained changing the iron source; FeEDDHA at both concentrations (100 and 200 mg/L) increase the shoot height and node number and produce better quality of the shoots, as demonstrated by the higher total chlorophyll, chlorophyll a and chlorophyll b contents. As reported by many authors (Hangarter and Stasinopoulos 1991; Garrison et al. 2013), FeEDTA could become unavailable in the medium due to its photo-oxidation at pH 5.7 and to its degradation after light exposure. Furthermore, the best performance of FeEDDHA in hazelnut propagation could be attributed to less energy requirements for its decomposition (Alcañiz et al. 2005). The micropropagated shoots characterized by high chlorophyll contents are capable of photosynthesis, which allows better rooting and acclimatization (Kanechi et al. 1998). Furthermore, the shoots cultivated on medium containing 100 mg/L FeEDDHA showed the lower *Chl a/Chl b* ratio, which could mean a better acclimatization ability. As reported in other species (Spiller and Terry 1980; Morales et al. 2000), Fe-stressed shoot possess lower chlorophyll content and higher *Chl a/Chl b* ratio, due to a loss of chlorophyll b with a consequent reduction of light harvesting complex.

Concerning the rooting phase, the results are in line to those reported in other species (Woodward et al. 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. Anyway, further investigations are necessary, since the results obtained concerning the quality of explants during multiplication phase are important but results on multiplication response (number of shoots/explant) are also very critical to the development of protocols aimed to mass propagation.

Thus, optimization of *in vitro* culture conditions, including nitrogen and iron source can have a significant impact for obtaining high quality propagated material, able to better adapt to *ex vitro* conditions.

Author contribution

Cristian Silvestri was responsible for conception and design of experiments, data analysis, and drafting of the manuscript and edited the paper. Eddo Rugini took care of study conception, design, and drafting the manuscript. Valerio Cristofori took care of study conception and design and edited the manuscript. All authors read and approved the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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