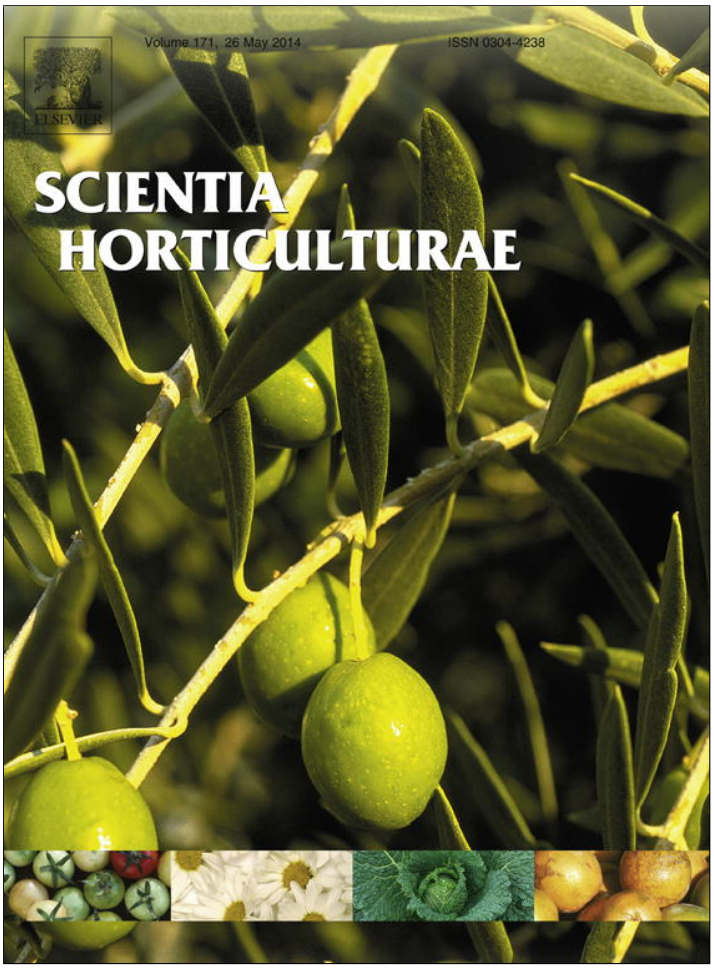

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Short communication

Effect of coconut water and growth regulator supplements on *in vitro* propagation of *Corylus avellana* L

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ABSTRACT

Corylus avellana L. represents an economically important crop in the European Union. Several protocols of hazelnut micropropagation have been tested but have not yet provided effective methods to allow large-scale propagation for commercial purposes, due to poor proliferation and yield. The aim of this work was to study the *in vitro* effects of coconut water (CW) in combinations with gibberellins and cytokinins on proliferation and growth of hazelnut *in vitro* and the effect of explants orientation on shoot growth. Single axillary buds from mature trees of 'Tonda Gentile delle Langhe' cultivar collected from suckers and crown were cultured on a modified DKW semi-solid basal medium enriched with coconut water, BAP, IAA and GA_3 in several combinations. The addition of 20% coconut water increased the number of adventitious shoots per explant and the combination of 2 mg/L BAP, 0.01 mg/L IAA and 0.5 mg/L GA_3 promoted multiplication and shoots elongation. Shoot multiplication and growth were also affected by explant orientation. The highest rates occurred with sucker explants in the horizontal position.

1. Introduction

Micropropagation could be a valuable method for large scale hazelnut multiplication to accelerate the distribution of certified material and new cultivars from breeding programs. Many micropropagation protocols have been tested (Bacchetta et al., 2008; Damiano et al., 2005; Garrison et al., 2013; Nas and Read, 2004; Yu and Reed, 1995), but a protocol adequate for most cultivars has not yet been identified. Furthermore, the methods have not yet been developed to allow a large-scale propagation, as required by commercial purposes due to the recalcitrant behaviour of the species and the low propensity of hazelnut to adapt to *in vitro* conditions (Contessa et al., 2011).

Growth regulators are one of the most important components of *in vitro* culture media for the success of hazelnut micropropagation. Shoot length and proliferation rate are influenced by concentration of cytokinin (Bassil et al., 1992), which is involved in the regulation of vegetative growth. The cytokinin most used is BAP (6-Benzylaminopurine) at various concentrations with different results for each cultivar and plant starting material.

Andrés et al. (2002) reported the presence of high endogenous cytokinin levels in leaves of hazelnut during spring when the morphogenetic potential is elevated. Leaves collected from branches that were produced after the forcing of dormant autumn buds, showed high iP-type/Z-type cytokinin ratio, similar to that detected in juvenile hazel leaves, suggesting that the ratio of iP-type/Z-type cytokinins may be a good index of *in vitro* potential of hazelnut materials. Van Overbeek et al. (1941) introduced coconut water (CW) as new component of the nutrient medium for callus culture. Coconut water is widely used in the plant tissue culture industry due to its growth regulatory properties and cytokinin-type activity that support cell division and promote rapid growth (Arditti, 2008; Yong et al., 2009). In particular, coconut water contains high levels of Z-type cytokinin (*trans*-zeatin riboside, *trans*-zeatin *O*-glucoside, dihydrozeatin *O*-glucoside, *trans*-zeatin, dihydrozeatin, *trans*-zeatin riboside-5′-monophosphate), iP-type cytokinin (*N*6-isopentenyladenine), kinetin, kinetin riboside and *ortho*-topolin in addition to sugars, vitamins, minerals and amino acids (Aguilar et al., 2009; Yong et al., 2009).

The aim of this study was to promote the proliferation and growth rate of 'Tonda Gentile delle Langhe' (TGL) (syn. 'Tonda Gentile Trilobata') shoots *in vitro* using different supplements to the basal medium. In particular, this work focuses on the evaluation of the effects of coconut water in different combinations with gibberellins, cytokinins, and auxins using a modified DKW (Driver and Kuniyuki, 1984) medium.

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2. Material and methods

2.1. Plant material

Plant material was sampled from 15 years old plants of 'Tonda Gentile delle Langhe' cultivar grown in Cravanzana (Piemonte, NW Italy) in the Langhe district (latitude 44°34′, longitude 8°07′, altitude 550 m a.s.l.). One year old suckers and branches (about 40 cm long) of the lower crown were collected in February and disinfected with 70% ethanol for 1 min, 2.5% bleach solution with a few drops of Tween 20 (polyoxy-ethylene sorbitan monolaurate, Sigma Aldrich Co., St. Louis USA) for 15 min and rinsed with tap water. Subsequently branches were dipped in 1% (w/v) benomyl (Benlate, DuPont) solution for 50 min and allowed to dry under laminar flow. The plant material was wrapped in black plastic bag and stored in a cold chamber at 4 °C for 62 days in darkness. In May, 5–6 bud cuttings were immersed in deionized water and placed in a growth room (16/8 h photoperiod, light intensity 37.5 $\mu E\,m^{-2}\,s^{-1}$ at 23 ± 2° C) for 4 weeks to activate shoot development.

2.2. Establishment of in vitro culture

Shoots of 10 cm length were collected and cut into single or double node segments, after the leaves were trimmed off.

The explants were washed with antibacterial soap for 30 min and rinsed with tap water. Sterilization was performed by immersion in 70% ethanol for 10 s. Afterwards, the explants were surface-sterilized for 30 min with 1% sodium hypochlorite with three drops of Tween 20; the material was rinsed in sterile distilled water three times for 5, 10 and 20 min. To reduce tissue browning the explants were immersed for 2 h in a filter sterilized solution of 200 mg/L ascorbic acid.

After sterilization, all explants were recut at the basal end to enhance the absorption of nutrients and placed individually into 20 × 150 mm culture tubes containing 10 mL of semi-solid culture medium. Plant material was cultured for 5 days on a DKW medium, modified by substituting 200 mg/L FeEDDHA (Sequestrene 138 Fe, *Phyto*Technology Laboratories™, Shawnee Mission, KS) for FeEDTA, reducing macronutrients to 25%, and adding 0.5% activated charcoal and 1% glucose to the medium.

Explants were then transferred to establishment culture medium composed of DKW medium modified by substituting 200 mg/L Fe EDDHA for FeEDTA, reducing macronutrients to 80%, and adding 2% glucose and 2.5 mg/L BAP. All culture media were adjusted to pH 5.8 before adding 0.8% (w/v) granulated agar (BD Difco™, NY, USA) and autoclaving at 121 °C for 20 min. The same medium without growth regulators was used as control. The explants were placed in a growth chamber at 23 ± 2° C with a 16 h photoperiod (light intensity 37.5 $\mu E\,m^{-2}\,s^{-1}$). After 40 days, shoots were subcultured for further proliferation.

2.3. Proliferation of explants

After 6 weeks, the material was transferred into glass jars with 50 mL of modified semi-solid DKW medium (macronutrients reduced to 80%). In the proliferation phase, growth regulators were selected according to Bassil et al. (1992), Messeguer and Mele (1987), and Yu and Reed (1995) to determine the optimum combination for shoot multiplication. BAP and IAA (indole-3-acetic acid) were added in a single dose (2 mg/L and 0.01 mg/L, respectively) whereas GA_3 (gibberellic acid) was tested at different concentrations (0.1, 0.3 and 0.5 mg/L) to promote elongation, alone or in combination with coconut water (10, 20 and 30%). The coconut water was extracted from commercial fresh coconut fruit (*Cocos nucifera* L.) and added to the culture media with 3% glucose. The same medium without growth regulators was used as control. The media were adjusted to pH 5.8 before adding 0.7% (w/v) granulated agar (BD Difco™, NY, USA) and autoclaving at 121 °C for 20 min.

Explants subcultivated using the medium with coconut water at 20%, BAP (2 mg/L), IAA (0.01 mg/L) and GA_3 (0.5 mg/L) were grown in horizontal or vertical position to test the effect of orientation on invigoration of ontogenically adult material. Every 30 days, explants were transferred into fresh culture medium.

2.4. Statistical analysis

Data were collected 4 months after the beginning of culture establishment and each explant was scored for the number of shoots per explant and shoot length (mm). Three explants per pot, replicated 10 times, were used for each combination. The experiments were conducted in a complete factorial randomized scheme.

Analyses of variance was performed to estimate the effect of (BAP + IAA)/GA_3, CW and the interaction (BAP + IAA)/GA_3 × CW on the number of shoots per explants and shoot length. Significant differences ($p \leq 0.05$) among means were determined using Bonferroni's test at a fixed level of $\alpha = 0.05$. ANOVA was conducted to compare the use of terminal and axillary buds as source of explant on the number of shoots per explants and shoot length. ANOVA was done using IBM SPSS Statistics software (version 20 for Windows, SPSS Inc., Chicago, USA).

3. Results

3.1. Establishment of in vitro culture

In the preliminary step, aimed at obtaining shoot development from 5–6 buds cuttings forced to sprout, crown material showed a poor ability to sprout, short shoot elongation (<2 cm) and a high mortality of the shoots. This prevented the use of crown material for the experiment. On the contrary, suckers proved to be useful explant sources due to their juvenile characteristics.

Differences in growth of the shoots were observed along the branch. Shoots from buds in the terminal position showed greater growth ability and vigor compared with those in the basal position, and were more suitable for culture establishment.

The treatment of the branches before storage and the sterilization method used before culture establishment led to lower *in vitro* contamination rates (8%) compared with the results obtained in literature (Bacchetta et al., 2008; Díaz-Sala et al., 1990). The ascorbic acid treatment and the use of 5% activated charcoal were not effective in preventing tissue browning and exudate release; 56.5% of not contaminated explants were completely necrotic after 40 days.

3.2. Proliferation of explants

Different concentrations of (BAP + IAA)/GA_3 had a synergistic effect with coconut water on shoot development after 4 months from culture establishment (Table 1).

(BAP + IAA)/GA_3 and CW both affected shoot number and shoot length and an interaction between (BAP + IAA)/GA_3 and CW was revealed for all the considered parameters. An ascending trend of shoot number and shoot length was found with increasing (BAP + IAA)/GA_3 and CW concentration.

In general, the explants in media with CW at 20% reached the highest number of shoots per explant; the explants in media with 20% and 30% CW showed the greatest shoot growth. The best result was obtained using the medium with coconut water at 20%, BAP (2 mg/L), IAA (0.01 mg/L) and GA_3 (0.5 mg/L), yielding an average multiplication rate of 5.3 shoots per bud, and a shoot length of 36.8 mm at the fourth subculture.

Explants in the horizontal position responded better than explants in the vertical orientation both for the number of shoots

Table 1
Number of shoots per explant and length (mm) of shoots. Effect of (BAP + IAA)/GA3, CW and interaction (BAP + IAA)/GA3 × CW on number of shoots per bud and length (mm) of shoots.

% Coconut water	(BAP + IAA)/GA_3 (mg/L)			
	0 + 0/0	2 + 0.01/0.1	2 + 0.01/0.3	2 + 0.01/0.5
No. of shoots per explant				
0	1.0_b	1.5_c	1.4_c	1.8_d
10	1.2_b	3.6_a	3.5_b	3.9_c
20	2.8_a	3.8_a	4.1_a	5.3_a
30	2.7_a	3.3_b	3.8_b	4.4_b
(BAP + IAA)/GA3	<0.001			
CW	<0.001			
(BAP + IAA)/GA3 × CW	<0.001			
Length (mm) of shoots				
0	7.5_d	9.0_d	15.8_d	27.8_c
10	9.0_c	11.3_c	19.5_c	31.5_b
20	10.2_b	12.8_b	21.0_b	36.8_a
30	12.8_a	16.8_a	24.0_a	36.0_a
(BAP + IAA)/GA3	<0.001			
CW	<0.001			
(BAP + IAA)/GA3 × CW	<0.001			

No. of shoots standard error: 0.05; length standard error: 0.25. Means followed by the same letter in a column are not statistically different at $p \leq 0.05$.

per culture (5.0 and 2.7, respectively) and for the length of shoots (32.8 mm and 10.7 mm, respectively) (Table 2).

4. Discussion

4.1. Establishment of in vitro culture

Plant material from different locations of the same tree showed different sprouting ability (topophysis). Suckers proved to be the more suitable explants source, in respect to crown material that failed to respond, showing a direct relationship between juvenile characteristic of the plant material and ontogenetic ability (Zalewska et al., 2010). Yu and Reed (1995) observed the same behavior in branches of 'Tonda Gentile delle Langhe' which showed a high percentage of unsprouted buds or buds with leaf expansion but slight elongation and development of shoots.

The use of different types of sucker explants (apical and axillary buds) gave different performance in terms of *in vitro* reactivity, with a rapid sprouting of terminal buds unlike of nodal sections. Similar results were obtained for apices and nodes in culture establishment of basal sprouts of *Castanea sativa* Mill. (Vieitez et al., 1983), highlighting the topophysis effect on the rate of propagation. Different results were obtained by Yu and Reed (1995) for hazelnut explants from shoot tips, which showed initial leaf expansion, followed by explant death without shoot development.

Tissue darkening and the release of exudates in the culture medium are the most frequent problems found at the beginning and during the maintenance of hazelnut and generally in woody plant tissue grown *in vitro* (Laukkanen et al., 2000; Tang and Newton, 2004). Some authors argue that darkening of the explants (or oxidative stress) is related to the activation of physiological disorders such as recalcitrance, hyperhydricity, genetic and epigenetic effects (Cassells and Curry, 2001; van Staden et al., 2006). Tissue browning was observed mainly in the few explants from crown material even with the use of ascorbic acid, in agreement with Yu and Reed (1995). They noted that hazelnut explants from juvenile materials with juvenile traits (grafted greenhouse plants) had less oxidation than explants from mature materials (field-grown tree) and generally lower phenolic compound production. The release of exudates was indeed partially solved with the use of activated charcoal that allows the irreversible adsorption of inhibitory compounds in the culture medium decreasing the toxic metabolites, phenolic exudation and brown exudate accumulation (Thomas, 2008).

Table 2
Effect of horizontal and vertical position on number of shoots per explant and length of shoots (evaluation of three subcultures).

Orientation	No. of shoots per explant	Length (mm) of shoots
Horizontal	5.0	32.8
Vertical	2.7	10.7
p	*	*

Shoots standard error: 0.15; length standard error: 0.53.
* Significantly different at $p \leq 0.05$.

4.2. Proliferation of explants

From our data and previous studies (Díaz-Sala et al., 1990; Bassil et al., 1992; Yu and Reed, 1995; Nas and Read, 2004; Bacchetta et al., 2008; Garrison et al., 2013), several factors appear to significantly affect the success of hazelnut micropropagation: genotype, culture medium composition (including growth regulators and their combinations), collection time, explants developmental stage and explants orientation in culture.

In particular, the growth regulators play a key role in the qualitative and quantitative development of shoots. BAP, alone or with other plant growth regulators, is the most effective inducer of explants proliferation in hazelnut (Anderson, 1984; Bassil et al., 1992; Díaz-Sala et al., 1990; Pérez et al., 1987). The presence of BAP is essential for explants development, in combination with GA_3 and CW.

Coconut water is a complex additive which contains many nutritional and hormonal substances. The liquid of the coconut endosperm contains amino acids, organic acids, nucleic acids, vitamins, carbohydrates, plant regulators with high levels of zeatin, and minerals (Ge et al., 2005; Yong et al., 2009). The promotory effect, with regard to morphogenesis, is related to its growth regulator content specially cytokinins (Chugh et al., 2009). In the last years, there has been a significant increase in the use of coconut water in micropropagation protocols of economically important species such as passion fruit (Hall et al., 2000), coffee (Ismail et al., 2003) and orchids (Santos-Hernandez et al., 2005), often as an alternative to zeatin, which is very expensive (Peixe et al., 2007), to induce plant cells to divide and grow rapidly. This is the first time that its use has been described in hazelnut micropropagation.

Coconut water alone is not normally sufficient to promote satisfactory multiplication. In this case, the use of CW alone was able to significantly improve the number of shoots per explant. As reported in Grigoriadou et al. (2002), the combination of CW with cytokinins

is the most effective way to improve rates of multiplication. The results obtained with high concentration of CW in combination with BAP and GA_3 doubled the effect of CW alone.

Increasingly, importance has been given in recent years to the effect of explant orientation in the culture medium, revealing the horizontal orientation as the most effective. In this experiment the horizontal position increased the number of shoots per explant and improved multiplication rate, probably due to larger contact of the explant tissue with the culture medium, with increased nutrient absorption. The effectiveness of this treatment may also be due to breaking apical dominance, inhibiting auxin translocation and, consequently, stimulating the growth of lateral buds (Vieitez et al., 1993).

The horizontal position enhances proliferation rates. Studies performed by San-José et al. (1988, 1990) in *Quercus robur* L. and *Camellia reticulata* L. demonstrate an increase of proliferative capacity and rooting by means of a system of re-cultivation of previously-decapitated explants in a horizontal position.

5. Conclusion

The *in vitro* establishment of explants of hazelnut is a critical phase for several cultivars, such as 'Tonda Gentile delle Langhe' (Díaz-Sala et al., 1990; Yu and Reed, 1995). The explants fail to respond or show leaf expansion and slight shoot elongation without further development. At the moment, an efficient protocol for TGL *in vitro* establishment and propagation is not available in literature. In this work, an *in vitro* proliferation system for hazelnut 'Tonda Gentile delle Langhe' cultivar was obtained using sucker shoots from adult trees and combining growth regulators with coconut water.

The juvenility and ontogenetic state of the plant material used for *in vitro* culture establishment affected bud development ability. Terminal buds of suckers proved to be the most suitable explant source. Crown explants were characterized by a poor ability to sprout, high tissue darkening and release of exudates into the culture medium.

The results highlighted a synergistic effect between growth regulators and coconut water promoting shoot elongation and proliferation. The addition of 20% coconut water in combination with 2 mg/L BAP, 0.01 mg/L IAA and 0.5 mg/L GA_3 was the most effective treatment. It is suggested to test the effect of zeatin in further studies concerning the *in vitro* proliferation of hazelnut.

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