

Node position influences viability and contamination in hazelnut shoot explants

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Abstract Initiation of shoot cultures is difficult in many woody plants due to internal microbial contaminants and general lack of juvenility in material from the source plants. Hazelnuts (*Corylus avellana* L.) are generally difficult to initiate into culture for these same reasons. This study was designed to determine the effects of collection and surface disinfection techniques and nodal position on the viability and contamination of shoot explants. In addition, culturable bacteria were identified on samples from surface-disinfested explants. Explants were collected from scion wood grafted onto seedling rootstocks and grown in a greenhouse. Single-node explants, excluding the shoot tip, were collected and the node location documented. After surface disinfection, explants were held in liquid contaminant detection medium for 1 wk and the effect of this treatment on explant viability was evaluated. Node position was important for obtaining viable contaminant-free explants. Bacterial and fungal contaminations both increased with the distance from the shoot tip. The use of contaminant detection medium as a part of the initiation procedure did not affect viability. Explant-derived

bacteria were identified as belonging to *Brevundimonas* sp. and *Pseudomonas* sp. through 16S rRNA sequence and API® tests. The best procedure for collecting axenic, viable hazelnut explants was to collect from the first three apical nodes, excluding the shoot tip, of actively growing greenhouse plants, place them in individual tubes for washing and surface disinfection, and use contaminant detection techniques to identify contaminant-free cultures at initiation.

Keywords Bacteria · *Corylus avellana* · Hazelnut · Micropropagation · Surface disinfection

Introduction

Initiation of actively growing and contaminant-free explants into culture is important for successful micropropagation (Debergh 1988; Von Aderkas and Bonga 2000). For hazelnuts, grafted trees grown in the greenhouse were shown to be the most successful source of explants and produced more active growth, higher viability, and reduced contamination than other methods (Messeguer and Mele 1983; Yu and Reed 1995). This was confirmed by Bacchetta *et al.* (2008). Hazelnut (*Corylus avellana* L.) mother plants grown in a protected greenhouse also are less likely to harbor high population sizes of epiphytic bacteria, including plant pathogens (Messeguer and Mele 1983; Yu and Reed 1995). Yu and Reed (1995) found that the most viable explants of ‘Barcelona’ and ‘Gassaway’ were nodal axillary segments of grafted greenhouse-grown plants collected in March, May, or July or suckers of field-grown trees collected in July (46–80% successful shoots), when compared to field trees and forced dormant shoots. Perez *et al.* (1987) used lateral buds taken from 1-yr-old greenhouse-grown ‘Negret’ plants and achieved 70% success with growing shoot explants. The node location

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also affected *in vitro* establishment. Sampling explants close to the apical meristem produced more viable shoots than from distal nodes for hazelnut (Yu and Reed 1995), *Rosa* spp. (Hsia and Korban 1996), *Azadirachta indica* (Arora *et al.* 2010), and *Viburnum tinus* (Nobre *et al.* 2000).

Care of the mother plant is very important in limiting both fungal and bacterial contamination. Healthy, fast growing plants in a sheltered environment are less likely to harbor bacteria or fungal spores (Yu and Reed 1995). Woody plants in the field may be heavily contaminated with fungal spores and often contain internal bacteria depending on the environment and the season (Yu and Reed 1995). Messeguer and Mele (1983) found that contamination of explants ranged from 35 to 38% and varied with the season. Bacterial contamination can be a serious threat to *in vitro* plants, reducing viability both at the explant stage as well as later in the culture cycle (Debergh and Vanderschaeghe 1988; Cassells 1991; Leifert and Cassells 2001). Bacteria can survive, even after standard surface disinfection procedures, in biofilms or aggregates on plant surfaces (Morris and Monier 2003) or as internal contaminants present in the vascular system or in intercellular spaces (Buckley *et al.* 1995). Reed *et al.* (1998) identified several internal bacterial contaminants from long-term hazelnut shoot cultures.

Explants are often indexed for microbial contamination during or following *in vitro* establishment. Once established *in vitro*, samples of the explants can be screened for microbial contaminants with nutrient broth or nutrient agar (NA). Pre-screening can be done before establishment using liquid-index medium where shoots are submerged in a neutral pH, half-strength MS basal salts medium for 1 wk (Reed and Tanprasert 1995). Species of plants respond differently to submersion in liquid-index medium; mint plants survived well (Buckley *et al.* 1995; Reed *et al.* 1995), while strawberry shoot tips died when completely submerged in the medium for 1 wk (Tanprasert and Reed 1997).

The identity of the bacteria isolated from explants can provide information on how to avoid or eliminate contaminants from cultures. Earlier studies using traditional microbiological tests required extensive testing to determine bacterial identities (Buckley *et al.* 1995). Molecular techniques are now readily available and bacterial DNA can be used for analysis and identification without the need for extensive metabolic tests (Reinhold-Hurek and Hurek 1998; Kuklinsky-Sobral *et al.* 2004; Rosenblueth and Martínez-Romero 2006; Quambusch *et al.* 2014).

The objective of this study was to investigate the influence of surface disinfection techniques and nodal position on explant viability and on microbial contamination of surface-disinfested explants. In addition, the effect of liquid-indexing medium on explant viability was investigated, and some common contaminants of hazelnut explants were identified.

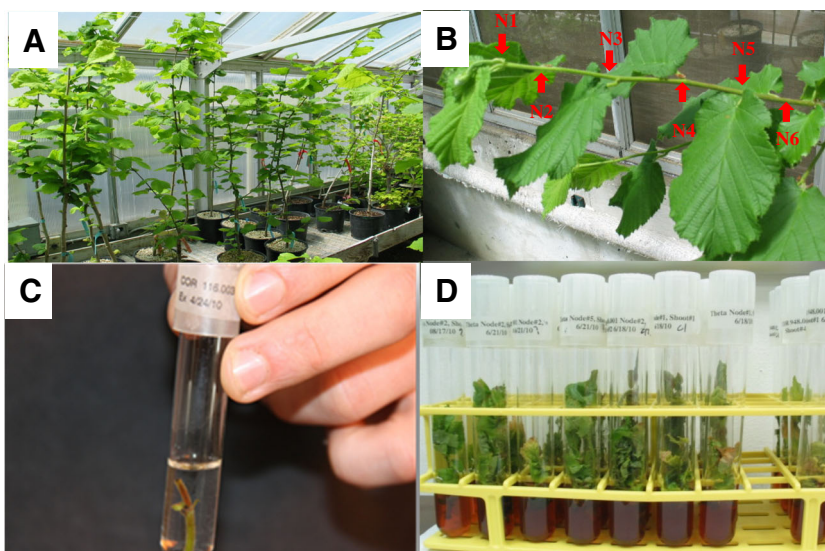
Materials and Methods

Plant materials *C. avellana* ‘Theta,’ OSU 1180.036, and ‘Zeta’ were used for initiation studies for the 2010–2011 growing seasons. Hazelnuts ‘McDonald,’ ‘Wepster,’ and OSU 918.045 were sampled for the indexing study in 2012. Plants of each genotype from grafted or layered plants were grown in 2-gal pots in the greenhouse under natural light (Fig. 1A). Dormant plants were cut to one or two branches at a height of 1 m. Plants were watered at the base and fertilized weekly during the growing season with 20N-20P-20K at 1 g L⁻¹ (Jack’s Professional, J. R Peters Inc., Allentown, PA).

Collection and surface disinfection All leaves and excess plant material were removed from the explants, the shoot apex was removed, and the explants were cut into 4–5-cm single-node sections. Collecting tools were dipped in 20% (v/v) bleach (Clorox, The Clorox Company Oakland, CA; 6.0% active chlorine) followed by deionized (DI) water between each cut. Explant node, branch, and plant number were documented throughout the process of establishment. Explants were placed in DI water when collected, rinsed in running tap water for 10 min, and surface disinfested in 10% bleach with 4 drops L⁻¹ (~80 µL) of Tween 20 polysorbate (Sigma-Aldrich®, St. Louis, MO) and shaken at 250 rpm for 10 min on a platform shaker. Explants were then rinsed twice in sterile DI water and placed into 100 × 15-mm glass test tubes containing one half strength liquid medium (Murashige and Skoog 1962), pH 6.9 (designated detection medium), to detect culturable microorganisms (Reed *et al.* 1995). Explants were grown in the detection medium for 1 wk under growth room conditions, and then non-contaminated shoots were transferred to tubes of solidified National Clonal Germplasm Repository *Corylus* medium (NCGR-COR) medium described below. Experimental conditions are noted below. Chemicals were obtained from Sigma-Aldrich® unless otherwise mentioned. All media were made in house.

Culture medium and growth conditions Explants were grown on National Clonal Germplasm Repository *Corylus* (NCGR-COR) medium (Yu and Reed 1995), composed of DKW medium salts (Driver and Kuniyuki 1984) with 30 g L⁻¹ glucose, 200 mg L⁻¹ sequestrene 138 Fe, 2 mg L⁻¹ thiamine, 2 mg L⁻¹ nicotinic acid, 2 mg L⁻¹ glycine, 1 g L⁻¹ myo-inositol, 22.2 µM BA, 0.049 µM indole-3-butyric acid, and 0.5% (w/v) agar (A111, PhytoTechnology Laboratories, Shawnee Mission, KS). Medium (5 mL), pH 5.2 was dispensed into 100 × 15-mm borosilicate glass tubes (Kimax, Kimble-Chase, Rockwood, TN) with plastic caps (VWR International, Radnor, PA) and autoclaved at 121°C for 20 min. Shoots were transferred to 150 × 20-mm tubes (10 mL medium) after 4 wk and then transferred again each 4 wk for a total culture period of 13 wk. Growth room conditions were 25°C with a 16-h

Figure 1. Plant materials used for initiation and bacterial contamination studies: (A) Two-yr-old *Corylus* mother plants. (B) Explants were sampled from nodes one to six starting below the apical meristem (cv. Zeta). (C) Surface-disinfested explant in contamination detection medium. (D) Explants growing on NCGR-COR medium (Yu and Reed 1995) medium after 13 wk.



photoperiod. The average illumination measured at the top of the vessels was $70\text{--}90\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ provided by half warm-white and half cool-white fluorescent bulbs (GE, Worcester, MA). Only shoots without obvious contamination were transferred and each shoot was indexed in $100 \times 15\ \text{mm}$ (VWR International, Radnor, PA) Petri dishes on nutrient agar (NA) composed of nutrient broth (Difco Nutrient Broth, Becton Dickinson, Franklin Lakes, NJ) with 1% (*w/v*) glucose and 0.8% agar (Phytotechnology A111). All culturable bacterial contaminants were isolated and characterized.

Influence of node position and surface disinfestation technique Collection and sample processes varied as noted below. Explants were collected from five branches for each genotype, from nodes one to six, where the first node location was closest to the apical meristem (Fig. 1B). Upon each transfer, the quality of the explant and any contamination were recorded. Explants were categorized as follows: alive (healthy growing shoots, indexed and without visible contaminants), contaminated (bacteria or fungi), or non-viable (no detectable shoot growth, indexed, and without visible contaminants). Final data was recorded 13 wk after explanting. Each treatment was replicated at three time periods with five explants per treatment for each node.

For bulk surface-disinfested experiments, explants were collected on June 16, 18, and 21, 2010. All explants from each node position were grouped in 250-mL beakers for processing. The beakers were covered with cheese cloth and placed under running tap water for 10 min and then surface disinfested as previously described and rinsed with sterile water in the same beakers.

For individually surface-disinfested experiments, explants were collected on April 26, 27, and June 1, 2011. All explants were collected and surface disinfested in individual small test tubes ($100 \times 16\ \text{mm}$) as described previously but without the running tap water rinse.

Effect of bacterial indexing on explant viability Explants were collected on May 23 and June 29, 2012. Single node sections were placed into individual $150 \times 20\text{-mm}$ test tubes containing distilled water with 4 drops L^{-1} ($\sim 80\ \mu\text{L}$) of Tween 20. Individual explant tubes were shaken with a vortex mixer (Cole-Parmer, Chicago, IL) for 3 s, the solution was decanted, and explants were surface disinfested in the individual tubes at 22°C . Explants were vortexed again for 3 s while in the first rinse. Explants were divided into three detection-medium duration groups of 0, 5, or 7 d with six explants from each node in each treatment. All groups were held in tube racks under growth room conditions previously described for the treatment duration. After the required duration in detection medium (Fig. 1C), the explants were initially transferred to $100 \times 15\text{-mm}$ glass tubes of NCGR-COR medium (Fig. 1D). Shoots were transferred to $150 \times 20\text{-mm}$ tubes of new medium at 4 wk intervals for a total of 13 wk. Upon each transfer, each shoot was indexed on NA and the viability of the explant and any contamination were recorded.

Experimental design and statistical analysis Explant collection was set up as a randomized design for each time period. There were three replicates for each time period. Because the three collection periods showed no significant differences and the genotype effects were not significant, the data were pooled for final analysis using SAS (SAS Institute Inc., Cary, NC). Paired *t* tests were used to determine significant differences between means.

Bacterial identification: molecular Bacteria were isolated from samples collected during the 2010 explant study from both the liquid detection medium and the NA plates used at each subculture. Contaminants were sampled with a sterile loop and placed in 5 mL nutrient broth and also plated on NA. Single-colony bacterial isolates were streaked to purity

on NA. Isolated bacterial colonies were grouped based on morphological characteristics. Bacteria from each morphological group were identified using the 16S ribosomal RNA (rRNA) sequence (Weisburg *et al.* 1991; Hall 1999). Colonies were suspended in sterile MilliQ water (EMD Millipore, Billerica, MA), pelleted, and 25 μ L of lysis solution (1 mL sterile MilliQ water, 10 μ L 5 M NaOH, 25 μ L 10% sodium dodecyl sulfate [SDS]) was added. The suspension was heated to 100°C for 10 min and then diluted with 175 μ L of sterile MilliQ water. Three microliters of the colony lysate was added to 22 μ L of a PCR mastermix composed of 15.5 μ L water, 0.5 μ L of KOD polymerase (EMD Millipore), 2.5 μ L of 10 \times buffer, 1.5 μ L of 25 mM magnesium sulfate, 2.5 μ L of 2 mM dNTP, and 0.75 μ L of 10 μ M primers specific for 16S rRNA (16S forward: 5'-AGAGTTTGATCCTG GCTCAG-3'; 16S reverse: 5'-ACGGCTACCTTG TTACGACTT-3'; Weisburg *et al.* 1991). PCR conditions consisted of 1 cycle of 95°C for 2 min; 29 cycles of 95°C for 20 s, 55°C for 20 s, and 68°C for 20 s, and a final cycle at 68°C for 5 min. Three microliters of each PCR product was visualized on a 0.8% agarose gel and stained with ethidium bromide. The PCR single-band product was treated with ExoSAP-IT and sequenced at the Center for Genome Research and Biocomputing at Oregon State University. A consensus sequence was developed from aligning the forward and reverse nucleotide sequence for each bacteria isolate with BioEdit Sequence Editor Version 7.2.0 (Hall 1999). The consensus sequences were compared with NCBI GenBank using the BLAST database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for similarities.

Bacterial identification: API® tests Bacterial cultures were transferred to NA plates and incubated in the dark at 30°C for 18–24 h before use for diagnostic tests. Gram stains and oxidase tests were performed according to the methods described by Goldman and Green (2009). The API® 20NE test (bioMérieux, Durham, NC) for identification of gram-negative, non-Enterobacteriaceae was used following the test instructions. Briefly, a bacterial colony was suspended in 0.85% saline, adjusted to 0.5 McFarland density, and transferred to the first five test cupules (NO₃ to PNPG). A 200- μ L sample of the remaining suspension was added to API AUX medium and transferred to the remaining cupules (GLU to PAC). Mineral oil was added to the glucose fermentation, arginine dihydrase, and urease cupules to cover the openings. API strips were placed in the plastic box and incubated for 24–48 h at 22°C. After the first 24 h, the NO₃ test and tryptophan (TRP) test were performed. Nitrate reagent A, B, and zinc powder were used for the reduction of nitrate test, and Kovacs solution was used to detect indole production in the TRP test. All the other test results were obtained after 48 h of incubation.

Results

Node position and surface disinfection protocol influence viability and incidence of contamination of explants The node position was significant for both viability and total contamination (bacteria and fungi) ($p < 0.003$). There were no significant differences among the collection dates for each of the techniques, so the data were pooled (Fig. 2). There were significant differences between the collection techniques for viability at nodes two and three where the individual explants were surface disinfested was best ($p < 0.005$). Total contamination was significantly different only at node six where the surface disinfested bulked explants had a greater incidence of contamination ($p < 0.02$). Viability was highest for the first three nodes for the individually surface disinfested explants and the first two for the bulked surface disinfested explants (Fig. 2A). There were differences with surface disinfection technique depending on the node, but the overall trend was a decrease in viability with an increasing distance from the shoot apex.

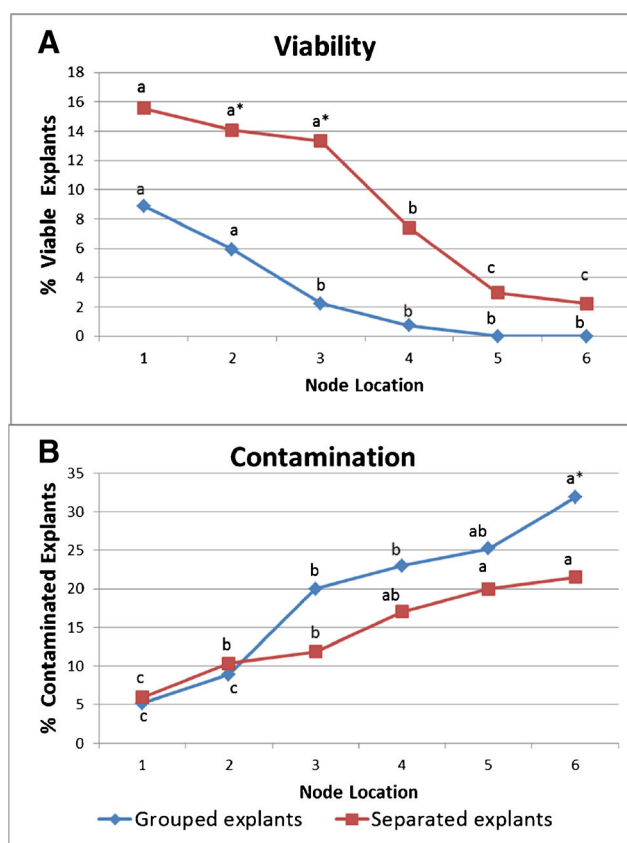


Figure 2. Surface disinfection method and node effects on explant viability and contamination. (A) Incidence of viable and (B) contaminated hazelnut explant shoots sampled by two disinfection protocols from each branch location after 13 wk. Node one was closest to the apical meristem. $n = 45$ at each node. Means within a treatment followed by the same letters are not significantly different, $P \leq 0.05$. Means with an asterisk are significantly different from the other treatment mean at that node.

The results for experiments with bulk surface-disinfested explants are as follows: At the first node, 9% of explants were viable and free of culturable contaminants and 5% were contaminated (Fig. 2). Most of the contamination was bacterial in the grouped explants (Fig. 3A); however, there was a low level of fungal contamination (Fig. 3B) and approximately 30% were non-responsive (Fig. 3C). The basal nodes were less viable and had a higher incidence of contamination than the upper nodes

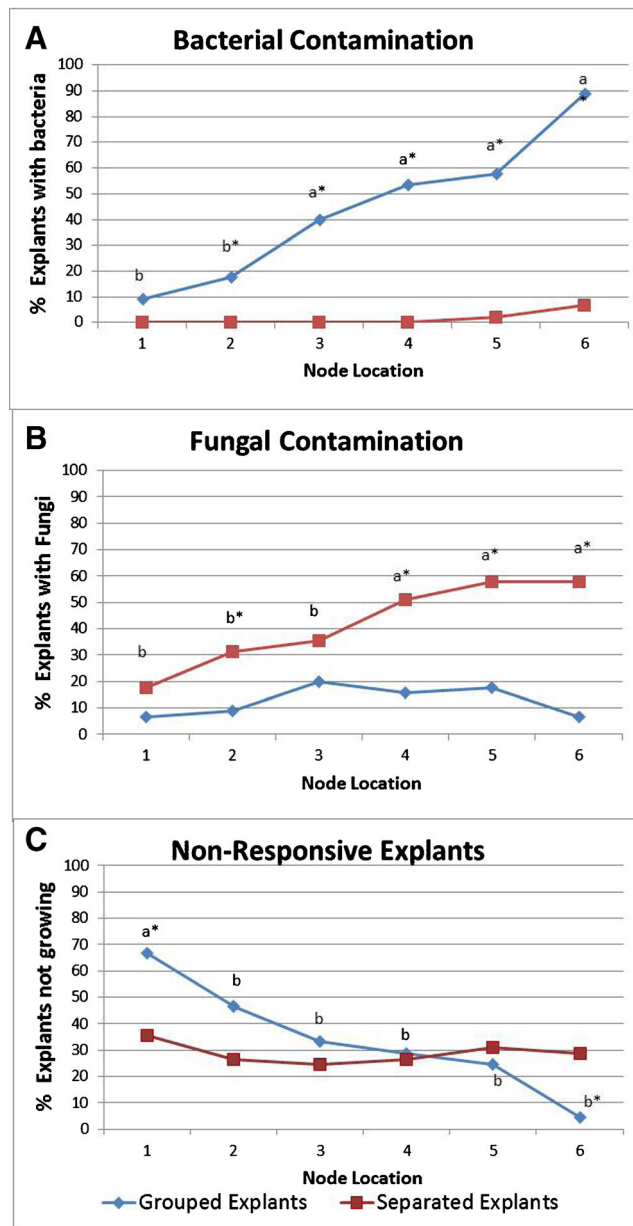


Figure 3. Surface disinfection method and node effects on *A* bacterial contamination, *B* fungal contamination, and *C* non-responsive hazelnut explants sampled by two disinfection protocols from each branch location after 13 wk. Node one was closest to the apical meristem. $n = 45$ at each node. Means within a treatment followed by the same letters are not significantly different, $P \leq 0.05$. Means with an asterisk are significantly different from the other treatment mean at that node.

(Figs. 2 and 3). Examination at the individual node level showed that most clean viable shoots were obtained from the first two nodes. The greatest incidence of contamination occurred at nodes five and six where most nodes were contaminated and none were viable (Fig. 2B).

The results for experiments with individually surface-disinfested explants are as follows: Shoots from 16% of the explants were alive and viable. Total contamination was not significantly different from the grouped explants except at the sixth node (Fig. 2B). Bacterial contaminants were not common (Fig. 3A), but fungi were frequent contaminants on these explants with significantly more at nodes four to six (Fig. 3). Non-responsive shoots accounted for about 30% of explants at all nodes (Fig. 3C). As with the grouped explants, the basal nodes had fewer viable, clean shoots, and the incidence of contamination increased with distance from the shoot tip (Figs. 2 and 3).

Incubation in liquid-index medium was not deleterious to survival of explants There were no significance differences in viability of explants for duration of exposure to detection medium among the three treatments (data not shown). The 7-d treatment was useful for contaminant indexing and did not reduce viability or growth of the plants (Fig. 1C, D).

Bacterial contaminants were identified as members of *Brevundimonas* and *Pseudomonas* Isolates were grouped into eight morphological groups based on shape, color, and other colony characteristics (Table 1). Colony colors ranged from bright orange, to light orange, dark yellow, light yellow, and cream on NA medium (Fig. 4). Based on molecular analysis, six isolates were identified as members of the genus *Brevundimonas* from BLASTN analysis of the 16S rRNA consensus sequences (Table 1 and Supplement 1). The 16S rRNA sequence of two other isolates (7.01 and 10.01) indicated that they were members of the genus *Pseudomonas* (Supplement 1). The *Brevundimonas* species were formerly considered to be *Pseudomonas* but were reclassified (Krieg and Holt 1984; Segers *et al.* 1994).

Two bacterial cultures (IDs 1.01 and 14.01) were tested by the API system and confirmed as members of *Brevundimonas*, specifically *Brevundimonas vesicularis*. The only differences observed in bacterium 1.01 and bacterium 14.01 were the colony color and oxidase tests results (Table 2). Bacterium 1.01 had orange-colored colonies (Fig. 3A) and a weak positive reaction for oxidase; and bacterium 14.01 (and 15.01) had cream-colored colonies (Fig. 3B) and a solid positive reaction for oxidase. Bacterium 14.01 had the same BLAST output as bacterium 4.02, 12.01, and 15.01 which suggests that all of these isolates were *B. vesicularis*.

Table 1. Bacterial colony ID, morphology, and identity of colonies isolated from hazelnut cultures

Colony	Shape	Elevation	Edge	Color	Closest organism in GenBank	API® test
1.01	Circular	Convex	Entire	Orange	<i>Brevundimonas</i> sp.	<i>Brevundimonas vesicularis</i>
4.02	Circular	Convex	Entire	Orange	<i>Brevundimonas</i> sp.	–
6.01	Circular	Convex	Entire	Lt Orange	<i>Brevundimonas</i> sp.	–
7.01	Irregular	Irregular	Undulate	Lt Orange	<i>Pseudomonas</i> sp.	–
10.01	Irregular	Raised	Undulate	Yellow	<i>Pseudomonas</i> sp.	–
12.01	Circular	Convex	Entire	Yellow	<i>Brevundimonas</i> sp.	–
14.01	Circular	Convex	Entire	Cream	<i>Brevundimonas</i> sp.	<i>Brevundimonas vesicularis</i>
15.01	Circular	Raised	Entire	Cream	<i>Brevundimonas</i> sp.	–

Results are the alignment of 16S rRNA sequences of the bacteria to the closest sequences in GenBank BlastN. API® tests were done on bacterium 1.001 and 14.01 for further identification

Discussion

The initiation of viable and contamination-free explants is important to establish healthy cultures of any plant. In hazelnut, contamination and viability are both issues that reduce the number of shoots that develop and can be micropropagated. If contamination rates are high, it is also difficult to determine the actual viability rate. The environment that the source plants are exposed to has been shown previously to influence the incidence of contamination (Yu and Reed 1995). Field

plants are sometimes used as an explant source, but often, forced shoots are grown in protected conditions to reduce fungal contaminants. The current study followed the techniques of earlier studies that employed greenhouse-grown plants to reduce contaminants, and grafted shoots on seedling rootstocks or layered shoots to improve juvenility (Yu and Reed 1995). This study focused on the method of collection and surface disinfection as well as the importance of the nodal location of explants to both the viability and the incidence of contamination in the cultured explants.

Figure 4. Examples of bacteria detected in hazelnut shoot cultures: (A) Orange-colored bacteria (ID 1.01) *Brevundimonas vesicularis*; (B) cream-colored bacteria (ID 15.01) *B. vesicularis*; (C) light-orange-colored bacteria (ID 7.01) *Pseudomonas* sp.; (D) cream-colored bacteria (ID 10.01) *Pseudomonas* sp. All bacteria were grown on nutrient agar amended with glucose and identified with 16S rRNA sequence analysis. Isolates in A and B also were evaluated with an API-20NE panel.

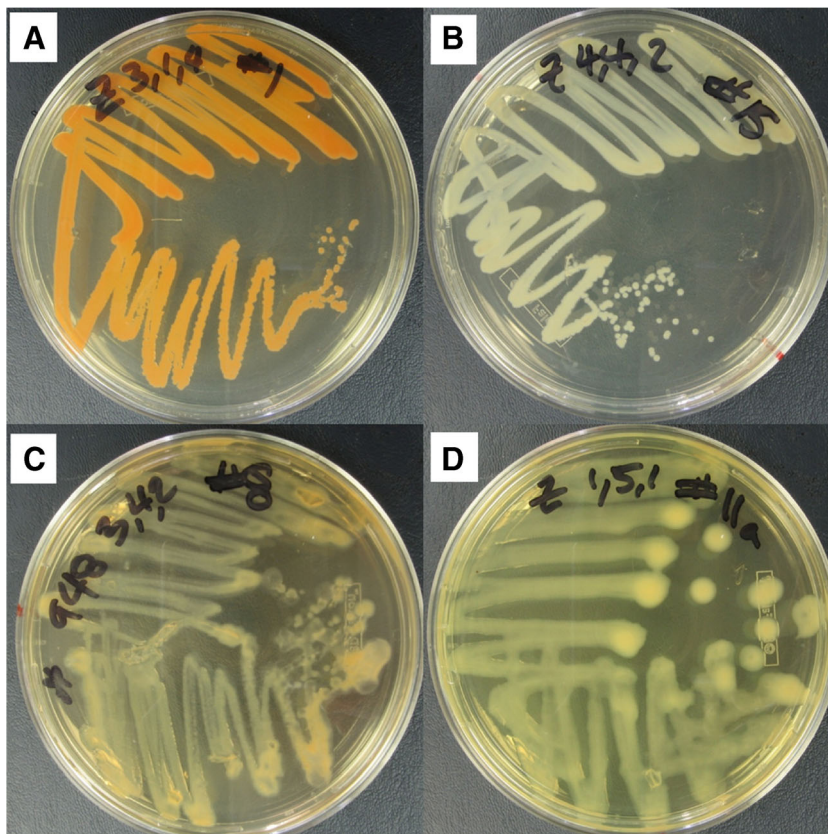


Table 2. Morphological characteristics of *Brevundimonas* spp. (colony ID 1.01, 14.01) from API 20NE identification system and a description of the species

		1.01	14.01	<i>Brevundimonas vesicularis</i>	
Morphology:	Colony color	Orange	Cream	Yellow or orange	
	Cell shape	Rod	Rod	Rod	
API 20NE	Gram stain	–	–	–	
Reduction of:					
	NO ₃ → NO ₂	–	–		
Production of:	NO ₃ → N ₂	+	+		
Fermentation of:	Indole	–	–		
Enzyme activity:	D-Glucose	–	–		
	Arginine dihydrase	–	–		
	Urease	–	–		
	Esculin hydrolysis	+	–		
	Gelatin hydrolysis	–	–		
	β-Galactosidase	–	–	–	
	Assimilation of:	Oxidase (addition to API)	w	+	
		D-Glucose	+	+	+
	L-Arabinose	–	–	–	
	D-Mannose	–	–	–	
	D-Mannitol	–	–		
	<i>N</i> -Acetyl-glucosamine	–	–		
	D-Maltose	+	+	+	
	Potassium gluconate	–	–		
	Capric acid	+	+		
	Malic acid	+	+		
	Trisodium citrate	–	–		

w - weakly positive

The method of collection and surface disinfection of the explants had a significant effect on the number of viable explants and the type of contaminants noted. Keeping individual explants separated during collecting and surface disinfection produced almost double the viability with fewer non-responsive explants overall, and much less bacterial contamination, than when the explants were grouped (Figs. 2A and 3A, C). The explants kept separate throughout the process did have more fungal contamination, probably because they were not washed in running water prior to bleach treatment (Fig. 3B). The converse was true for the grouped explants where the running water wash significantly reduced fungal contamination at four of the six nodes, but grouping during collection probably resulted in the spread of bacteria to otherwise uncontaminated explants (Fig. 3). The reason for differences in the amount of non-responsive explants is not clear but probably is confounded by contamination rates.

This study demonstrated that nodal position had a significant influence on the success of explant establishment of *C. avellana*. Across the two protocols used for surface disinfection, the viability of explants was significantly higher for the first two or three nodes compared to more basal positions (Fig. 2). Other studies documented that

nodal position influences explant *in vitro* establishment. Hsia and Korban (1996) found that *Rosa* spp. explants closer to the apex produced the most shoots per explant. They sampled nodes one through six and found that node two produced the most shoots per explant; sampling further from the apex produced taller shoots with larger leaves per explant. Size, diameter, and length of *Rosa* spp. segments influenced *in vitro* success and nodal segments with large diameter and longer internodes (1.5 cm) did better regardless of the nodal position. Arora *et al.* (2010) found that explants of a 40-yr-old *A. indica* (Neem tree) had maximum bud break (78.6–81%) at the third or fourth node from the apex. They concluded that larger size and thickness of nodal stem segments improved explant survival and proliferation. The shoot tips of *C. avellana* cultivars were found to be not viable as explants for establishment, but lower nodes were suitable for culture (Yu and Reed 1995). Douglas *et al.* (1989) also reported that longer stem segments (>2 cm) of mini roses improve shoot proliferation. In the current study, hazelnut explants were cut to lengths of 4–5 cm; in general, the diameter of the explant increased further down the branch, and contamination increased. It was difficult to correlate

C. avellana diameter of branch to viable explant shoot growth because contamination was such a large factor for the lower buds.

Moura *et al.* (2009) studied the micropropagation of *Viburnum treleasei* and found that explants sampled as shoot tips had the lowest contamination compared to single-node segments or isolated meristems. Shoot tips of *V. tinus* were less contaminated than single node cuttings (Nobre *et al.* 2000). Although the shoot tips of hazelnuts will not grow in culture, the current data agrees with these studies, as the first two to three nodes below the apex had a low incidence of contamination. The two surface disinfestation techniques did not differ much in the incidence of total contamination for any node except for the sixth (Fig. 2); however, there were significant differences in the bacterial and fungal contamination (Fig. 3).

Low viability of explants is a problem for many woody plants (Yu and Reed 1995; Ercisli *et al.* 2000). Besides the effects of node and surface disinfestation techniques on explant viability and contamination as seen in this study (Figs. 2 and 3), other factors must be involved. Tests with various surface disinfestation techniques show that excessive exposure to bleach reduces explant viability, but those with higher viability were often contaminated (Kitamura *et al.* 2008). Due to high levels of endogenous contamination in hazelnut explants, indexing for contaminants is important and is another stress that might reduce the number of viable explants. The current study found no deleterious effects of exposure to liquid-indexing medium prior to growth on normal growth medium (data not shown). One factor not studied was the initiation medium (Mohammed and Dunstan 1986). Over 30% of uncontaminated explants were non-responsive in this study and these generally occurred at all six nodes (Fig. 3C). The initiation medium used in this study was probably not optimal and could be a factor in the low response (Hand *et al.* 2014). Use of suboptimal growth media may reduce the growth of explants (Nas and Read 2004; Bacchetta *et al.* 2008). It is very likely that a growth medium that is optimal for promoting growth could greatly improve viability. This was shown for *Pyrus* where an improved growth medium increased initiation success from 43 to 82% (Reed and DeNoma 2011). Higher concentrations of cytokinins might also be useful for initiating bud growth.

For a micropropagation system to be successful, clean initiation of explants and maintenance of clean cultures is important and this process can be improved if the type of contaminant is known. Bacteria may have growth promoting effects or detrimental impacts on explant materials. Difficult-to-propagate *Prunus* genotypes had significantly different bacterial populations compared to genotypes that were easier to culture (Quambusch *et al.* 2014). Kitamura *et al.* (2008) used molecular techniques (16S rRNA) to identify the bacterial flora on *Hydrangea* shoot tips. They identified 12 bacterial species including several *Pseudomonas* sp. This information was used to develop a more effective protocol for sterilizing *Hydrangea*

shoot tips that included a 30-min 0.05% available chlorine concentration disinfestation solution. The number of uncontaminated, viable *Hydrangea* explants ranged from 0 to 95% depending on cultivar. The procedure of the current study used 0.06% available chlorine with a surfactant for 10 min. This may not be optimal; however, the low number of viable shoots after the treatment might be further reduced if exposed to bleach for a longer period. The differences in the procedures concerning grouping of cultures and rinsing in running water before surface disinfestation were significant in the level of contamination (Fig. 3). As contamination is reduced in the procedure, many of those explants would likely be viable.

Earlier studies of hazelnut cultures used traditional bacteriological tests to determine the genus and species of contaminants. Some of the bacteria found in hazelnut cultures are *Agrobacterium radiobacter* B, *Pseudomonas fluorescens*, *Xanthomonas* spp., *Enterobacter asburiae*, *Flavobacterium* spp., and *Alcaligenes* spp. (Reed *et al.* 1998). The organisms ranged from white and beige to yellow and pink to red. The current study found bacterial contaminants that were white, beige, light, and deep orange (Table 1). The molecular analysis and API tests in the current study identified two major groups, *Brevundimonas* with two color forms, and two isolates of *Pseudomonas*. *Brevundimonas* was formerly classified as *Pseudomonas*, and both groups are common on plants and in soil (Krieg and Holt 1984). The low bacterial diversity of these explants also highlights the positive impact of a sheltered growing environment for mother plants allowing for fewer bacterial colonizers and lower contamination rates for the explants. It might also be possible to treat the greenhouse plants with an antibacterial spray prior to explant collection to reduce the amount of bacteria present on the plant surface. The running water rinse was very effective for reducing fungal contaminants.

Detection of contaminants at the explant stage is critical for producing clean cultures. The influence of the index medium on survival of explants varies among plant species. With a higher pH and altered nutrient content, the index medium is favorable to the growth of contaminants and is easy to visually inspect without causing harm to the explant (Fig. 1C, D). Strawberry explants responded poorly to being completely submerged in index medium (Tanprasert and Reed 1997), while mint could be totally submerged (Reed *et al.* 1995). The results of the current study indicated that *C. avellana* can be completely submerged in index medium for up to 7 d without harming explant shoot growth, thus allowing better detection of microorganisms.

Conclusions

The present study demonstrated that the surface disinfestation technique was important and that the choice of nodes used as explants influenced propagation success and contamination rates. The best procedure for

collecting viable axenic hazelnut explants was to collect from the first three nodes below the apex of fast-growing greenhouse plants, use individual containers, rinse in running water before surface disinfection, and use contamination-detecting indexing techniques to identify and discard contaminated cultures. Molecular techniques in combination with traditional techniques were helpful to identify some of the bacterial flora present on or within *C. avellana* tissue. Despite reducing the contamination rates, low viability remains an important issue with hazelnut explants. Surface disinfection techniques significantly affected the success of obtaining viable explants or reducing the incidence of contamination only at certain nodes. Low viability of explants may be due to nutritional deficiencies and could possibly be reduced by improving the growth medium. Additional initiation studies on improved growth medium may increase the viability of non-contaminated explants (Hand *et al.* 2014; Akin *et al.* 2016).

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