Improvements for the Micropropagation of Hybrid Hazelnut (C. americana × C. avellana)

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Abstract: The micropropagation of hybrid hazelnut (Corylus americana × Corylus avellana) has been limited in its agricultural application due to the lack of efficient procedures for tissue culture and root organogenesis. We established an efficient sterilization protocol for hazelnut micropropagation suitable for in vitro root induction that permitted us to approach the poor root organogenesis experienced with the micropropagation of hazelnut hybrids in vitro. Typically, with standard protocols, root organogenesis had yields of well under 20%. We found that cuttings grown in vitro to the four-leaf stage can be successfully rooted using a combination of indole-3-butyric acid (IBA) pretreatment, followed by a transition to continuous light. In many cases, prolific visible roots formed between 12 and 14 days.

Keywords: dark/light treatments; filbert nut; organogenesis; photomorphogenesis; root initiation

1. Introduction

Hybrid hazelnut (Corylus americana × Corylus avellana) has the potential to become a profitable and environmentally sustainable North American crop in the upper Midwestern United States and parts of Canada [1]. The hybrid hazelnut combines many of the best traits of the two species: C. avellana provides the nut quality and high yield needed for a tree nut crop, and Midwestern North American C. americana has the traits of winter hardiness and disease tolerance, allowing cultivation outside the climate range of C. avellana [2]. In addition to traditional orchard growth, the hazelnut bush can be used as part of an agroforestry system where they function as barriers for reducing wind speed, act as living snow fences, serve as erosion control structures, and be grown along with livestock on pastures, and in perennial crop system combinations [3]. They can also be grown in riparian areas to protect water quality [4]. One of the main challenges for increasing production areas of hazelnuts is a lack of uniformity and availability of high-performance germplasm. The hazels grown from hybrid seed, of course, vary considerably in bush growth habit, size, yield, maturation date, husk length, nut size, nut flavor, and disease resistance, significantly limiting their desirability for orchard establishment. Vegetative propagation is thus used to obtain lines with genetic uniformity, but the methods currently available are slow, labor-intensive, and they have not proven economically viable [2]. An alternative is to employ micropropagation that can establish plantings with high-performance elite germplasm. The micropropagation of hybrid hazelnut is not, however, widely used because several developmental and procedural ‘roadblocks’ have limited its application. Major significant challenges for hazelnut propagation are obtaining aseptic explants for micropropagation and the process of root initiation, both with vegetative cuttings [5,6] and in vitro micropropagation. A compounding issue for the development of root initiation methods is that the root induction response is not uniform among genotypes [2].

The goals of this study were to test environmental changes in order to develop more efficient protocols for hazelnut micropropagation, mainly focused on in vitro adventitious root induction.
2. Materials and Methods

The plant materials used in this study were collected from greenhouse-grown plants because they are consistently more successfully surface sterilized [7]. As rooting changes have been observed in cuttings from stock plants based on the time of year and flowering status [8–10], we used a staggered rotation period where individual plants received a 6-week cold dormancy period, and all plants received a constant photoperiod. In this growth condition, plants did not flower, and cuttings were available throughout the year. Under normal light conditions most dicotyledonous plants rarely form adventitious roots (AR) without treatment with an auxin [8,11], and our preliminary experiments showed that the application of 4.9 mM of IBA as a dip was optimal for root induction for hybrid hazelnut, similar to what has been reported for other hardwoods [12,13]. Shoot segments (2 cm) with axillary buds from potted plants were collected from the apical (in the first 15 cm) branch and surface sterilized in a laminar flow hood. A basic control sterilization protocol using antibacterial soap, 70% ethanol, and commercial bleach was not effective at reducing the surface and endophyte contaminants that were present when culturing the different genotypes. To overcome this limitation, we tested numerous other methods for tissue sterilization and settled on two that were highly efficient for essentially all genotypes. The first protocol (P1) treated the segments by placing them in a beaker and using gentle agitation in a 1% solution of antibacterial soap containing 1.33 mg/mL of benzalkonium chloride (Softsoap, Colgate-Palmolive Company, New York, NY, USA), followed by 2 rinses in sterile double distilled water (ddH$_2$O). Sections were then dipped in 70% ethanol for 5 s, again rinsed two times with sterile ddH$_2$O, and then the tissue was soaked for 20 min in a 20% solution made from commercial bleach (5.25% sodium hypochlorite; Clorox, Oakland, CA, USA) and 20 µL/100 mL of Tween-80, and rinsed two times in sterile ddH$_2$O. The last rinse was with a 0.1% solution of plant preservative mixture (PPM-0.135% 5-chloro-2-methyl-3(2H)-isothiazolone, 0.041% 2-methyl-3(2H)-isothiazolone; Plant Cell Technology, Washington, DC, USA). Before placement on the growth medium, 2-mm sections were cut from the ends of the segments. The second protocol (P2) was similar, but the segments with buds were placed in a beaker with 95% ethanol for 5 s and rinsed two times with sterile ddH$_2$O. They were then immersed in 20% Pine-Sol® (3% alkyl dimethyl benzyl ammonium chloride) plus 2% Walgreens® First Aid Antiseptic Spray (0.13% benzyl trimethyl ammonium chloride, 2.5% (diethylamino)-2′,6′-acetoxylidide monohydrochloride) solution for 8 min and rinsed 3 times with sterile ddH$_2$O. Next, they were immersed in a solution composed of 20% commercial bleach (5.25% sodium hypochlorite; Clorox, Oakland, CA, USA) with 20 µL/100 mL of Tween-80 for 10 min and rinsed 3 times with sterile ddH$_2$O. As with the P1 protocol, 2-mm sections were cut from the ends of the segments and removed.

Culture medium for hazelnut: Segments with axillary buds were placed in Petri plates containing Lloyd & McCown Woody Plant Basal Medium with vitamins (Lloyd and McCown 1981; PhytoTechnology Laboratories, Lenexa, KS, USA); 10 µM of zeatin (PhytoTechnology); 2% sucrose; 0.1% GELRITE gellan gum (Research Products International, Mount Prospect, IL, USA); 0.3% micropropagation grade agar (PhytoTechnology); and 0.2% PPM (Plant Cell Technology, Washington, DC, USA). The pH was adjusted to 5.8 prior to autoclaving. The segments were cultured for 7 to 10 days to allow the leaf primordia to grow and expand, at 23 ± 1 °C under cool white fluorescent tubes (~180 µmol s$^{-1}$m$^{-2}$) with a 24-h day photoperiod. Explants with expanding leaf primordia were transferred individually in the vertical position to a culture vessel (Magenta GA-7, Sigma-Aldrich, St. Louis, MO, USA) containing fresh culture medium, which was identical, except the concentration of zeatin was 5 µM.

Root induction procedure: The shoots with at least four leaves were taken from the medium and the basal end (2 mm) was removed with a sterile scalpel. The cut basal end was dipped in a 4.9 mM solution of IBA (diluted in 50% ethanol and 50% sterile ddH$_2$O) for 3 min. The shoots were then returned to the same medium but without added zeatin, since cytokinins inhibit root differentiation [14]. For the “Dark/Light” treatments, the culture vessels were placed in a tray which was double wrapped in aluminum foil as
a dark treatment and kept for 7 days in a growth chamber at 23 ± 1 °C. After 7 days of dark treatment, the tray with culture vessels was unwrapped and kept in a growth chamber at 23 ± 1 °C under cool white fluorescent tubes (~180 µmol s m⁻²) with a 24-h day photoperiod. For the “Light” treatment, the explants were dipped into 4.9 mM of IBA solution and placed directly in the growth chamber at 23 ± 1 °C under cool white fluorescent tubes (~180 µmol s m⁻²) with a 24-h day photoperiod. The average of the roots per explant (12 explants per genotype-Humble16 and Arb7-1) after IBA application in “Dark/Light” and “Light” treatments was analyzed using a two-sample Z-test with a significance level of \( p < 0.05 \).

3. Results and Discussion

To establish hazelnut in tissue culture, stem segments with an axillary bud are typically used from juvenile or adult maternal plants. There are many different sterilization protocols available for varied plant or tissue types, but establishing procedures has been especially challenging with hazelnut as it is difficult to kill all the contaminants present on the surface and endophytes without seriously damaging the plant material. In addition, the different genotypes of hybrid hazelnut have different morphologies, including more trichomes and exudates that increase the difficulty of sterilization. Therefore, different hybrid genotypes were used to test the sterilization protocols. For the “control” sterilization, barely 30% of the plants were uncontaminated; however, protocols P1 and P2 both proved to be consistently effective, with 100% of the treated plants free of microbial contamination (Table 1). PPM had previously been shown to have broad-spectrum antimicrobial activity for use in plant tissue culture [15] and was extremely effective for preventing contamination with P1. Anecdotal evidence from coworkers also suggested the potential for hazelnut tissues to harbor slow-growing endophytes. Thus, it was decided out of caution to include PPM as an addition to the culture medium.

### Table 1. Efficacy of stem bud sterilization using the two protocols, P1 and P2, for explants from hybrid hazelnut explants. The standard error is shown for the percentages calculated.

| Treatment      | Sample Size | Explants Showing Microbial Growth | Percent without Contamination |
|----------------|-------------|-----------------------------------|------------------------------|
| Control        | 50          | 35                                | 30%                          |
|                | 28          | 25                                | 11%                          |
|                | 25          | 19                                | 24%                          |
| Control mean ± SE | 23.1% ± 0.82 |                                   |                              |
| Protocol 1     | 30          | 0                                 | 100%                         |
|                | 28          | 0                                 | 100%                         |
|                | 25          | 0                                 | 100%                         |
| P1 mean ± SE   | 100% ± 0    |                                   |                              |
| Protocol 2     | 41          | 0                                 | 100%                         |
|                | 20          | 0                                 | 100%                         |
|                | 57          | 0                                 | 100%                         |
| P2 mean ± SE   | 100% ± 0    |                                   |                              |

The induction of AR formation is a critical and often limiting step in the propagation of a number of plant species [12]. Root initiation and development has been a challenge with both hardwood and softwood stem cuttings of hybrid hazelnut, using either conventional mound layering and stem cuttings, or by micropropagation [7]. Considering the observation that the efficacy of IBA treatment alone is highly variable across different cultivars of hazelnut [16] and the general need for higher rates of successful rooting, we explored environmental requirements.
As noted by Monteuuis and Bon [17], light—or, in some cases more precisely, the lack of light—can exert an influence on the process of AR [18]. While in some species a dark pre-treatment can have a decidedly negative effect on AR, this has mainly been attributed to nutritional deprevation [19,20]. For roots, light has been ascribed both as a stressor, and that its perception modifies root responses to hormonal treatments including causing a major reduction in response to IAA treatment [21]. Light treatments have also been proposed to alter endogenous auxin levels as well as response [22], or to potentiate the response to cytokinins [23], which would include the inhibition of root initiation [24]. AR initiation obviously responds to environmental clues, including light and dark periods, and it is generally agreed that plant hormones, especially auxins, play important roles in the decisive signaling network for cell fate specification and determination. However, there is also some agreement that our knowledge of that hormonal network regulating AR initiation is “fragmentary” or “is still unknown” [25,26]. Our laboratory has two parallel research tracks to understand AR initiation: a basic track using the reference species Arabidopsis and a woody plant model using hybrid hazelnut. Our basic model is based on the system outlined by Sorin et al. [27] and includes a one-week dark period. We have observed in Arabidopsis, as have many others [28], that an extended period of darkness promotes the induction of AR if followed by a re-exposure to light [29]. Based on our results in the Arabidopsis system, two hybrid lines were tested, a variety Humble16 considered easy to root and a more difficult variety, Arb 7-1. Humble16 and Arb 7-1 were tested with two treatment variations: IBA application followed by a dark period (one week) and then followed by a light regimen (24 h-photoperiod) (IBA/dark/light), and IBA application followed by a light regimen (24 h-photoperiod) (IBA/light). When genotype Humble16 was treated with IBA/dark/light the rooting success was 75%, compared with 58% for Arb7-1 in the same treatment. In contrast, Arb7-1 treated with an IBA/light regime had only 17% rooting, while Humble16 showed the same 75% rooting success. The beginning of visible roots occurred between 12 to 14 days after the transition to 24 h-light for genotype Humble16 (Figure 1a); then, after 30 days, the shoots were fully rooted (Figure 1b,c). Genotype Humble16 showed the first roots 15 days after treatment (DAT) with IBA for both IBA/dark/light and IBA/light treatments (Figure 2). The genotype Arb7-1 just started to gain roots around 30 DAT (data not shown). At 50 DAT the number of roots on Humble16 was four times higher compared with Arb7-1 at IBA/dark/light treatment, and after that Humble16 still increased the number of roots until 90 DAT for both treatments. For Arb7-1, the difficult-to-root genotype, the IBA/dark/light treatment was important in order to gain roots, otherwise, the percentage of plants with roots remained low and only increased moderately with time (Figure 2).

![Humble16 shoot with visible roots](image1a.png)

![Arb7-1 shoot with visible roots](image1b.png)

![Roots of Humble16 after 30 days](image1c.png)

**Figure 1.** (a) Hazelnut (genotype Humble16) shoot with visible roots 12 days after transition for 24-h light at the IBA dark/light treatment; (b) shoot 30 days after transfer to 24-h light at the IBA dark/light treatment; (c) roots at 30 days viewed from the bottom of the culture vessel.
A comparison of the number of roots formed on genotype Humble16 and Arb7-1 after IBA application in “Dark/Light” and “Light” treatments. For each treatment, the total number of roots formed on 12 explants for each genotype and each treatment are reported. Values are means ± standard errors. The difference between light and dark/light treatments at 50 days and at the end of the experiment (95 days) were significant at p < 0.05 using a two-sample Z-test.

The difference in root induction between hazelnut lines cannot be explained by these studies, since improvements in rooting still saw a persistent difference between lines. IBA functions primarily as an IAA precursor [30]; thus, while the conversion of IBA-to-IAA is an important aspect of the response to IBA application [31], it alone does not define rooting efficiency [14]. As discussed by Alallaq et al. [26], light and hormonal interactions during AR is complex, but auxin responses to the light environment is central to the process. Indeed, significant changes in both the levels and biosynthetic pathways of the indole auxin network occur during light-induced AR in Arabidopsis hypocotyls [29]. How changes in the endogenous metabolism interact with applied auxins is unknown, and many endogenous signal molecules can inhibit AR [28], but this fact does not necessarily imply that they suppress AR in difficult to root plant lines. Although it remains uncertain exactly how light and auxins interact to elicit an AR response, it is nevertheless useful to utilize dark/light treatment along with auxins as part of a standard protocol to propagate buds from hazelnut and thereby induce extensive root formation.

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