Studies of indole-3-butyric acid to indole-3-acetic acid conversion in hazelnut shoot tissue

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Abstract

Indole-3-butyric acid (IBA) is an endogenous compound that appears to regulate both lateral and adventitious root formation in many plant species and is also the auxin most available commercially for application to promote rooting. IBA is converted to indole-3-acetic acid (IAA) by β-oxidation in the peroxisomes. This process has been observed in a number of plant species and has been shown to be critical for normal root development in response to treatment with IBA. In this thesis, the process was investigated in hybrid hazelnuts (*Corylus americana* x *C. avellana*) and American elm (*Ulmus americana*), in which adventitious rooting is a major bottleneck for vegetative propagation and the efficacy of IBA treatment is highly variable across different cultivars. Using differentially stable isotope labeled IBA and IAA tracer and internal standard, respectively, and using gas chromatography coupled with selected reaction monitoring mass spectrometry, IBA-derived IAA was measured in shoot tissue treated with stable isotope labeled IBA. Variable levels of IBA-to-IAA conversion were observed across different hybrid hazelnut genotypes, which may partially explain differences in rooting ability. In elm, higher levels of IBA-to-IAA conversion were observed in cultivars which formed adventitious roots most easily in softwood stem cutting trials. High rates of root formation is a key trait for establishment of large-scale production systems. Screening for optimal rates of IBA-to-IAA conversion may facilitate selection against genotypes which respond poorly to exogenous IBA. Such genotypes are difficult to propagate using hormone treatment and thus can be eliminated from further evaluations.

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I. Introduction

Because of the problems associated with water quality and soil stability, there is a need to diversify Minnesota's agricultural landscape, which is currently dominated by corn and soybeans (covering 32% and 26% of the state's agricultural land, respectively) (USDA NASS 2013), to include crops that can boost agricultural efficiency while simultaneously providing ecosystem services. This is the goal of the Forever Green agricultural initiative (Runck *et al*. 2014) and may be achieved by developing cropping systems that cover the land year-round. Having continuous living cover on the land would protect soil quality and reduce agricultural runoff that pollutes waterways, while increasing farm productivity and benefitting rural communities. Hazelnuts are a potential alternative crop providing perennial cover on the Minnesota landscape. The American hazelnut *Corylus americana*, which has the disease resistance and cold-hardiness needed in the upper Midwest, and its hybrids with the higher-yielding, thinner-shelled European hazelnut *Corylus avellana* can be used to diversify agricultural lands and create new economic opportunities (Molnar 2011).

Like other woody perennials, hazels require tillage only during the establishment phase. Once established the production system can remain in place for twenty years without annual tillage, thus reducing potential for soil erosion, and deep roots are developed to hold soil and prevent nutrient leaching (Glover *et al.*

2010). Furthermore, woody perennials fix more carbon than annual crops because they photosynthesize at times of year when annual crops are not present in the fields and quickly establish leaf cover on established stems each spring (Rutter 1988; Glover *et al.* 2010; Post and Kwon 2000). These ecosystem services – reduced soil erosion, improved water quality, as well as wildlife habitat – are especially important on marginal land that is not suitable for row crops (Pellett *et al.* 1998). Because hybrid and American hazels grow as dense bushes they are effective at reducing wind speed when used as windbreaks and living snow fences, creating more favorable conditions for other crops downwind (Holzmueller and Jose 2012). Perennial plants like hazels can also be grown in riparian areas to protect water quality by filtering agricultural runoff before it flows into waterways and by taking up nitrogen that could otherwise leach and contribute to hypoxic zones in the Gulf of Mexico (Dinnes *et al.* 2002).

Unlike other woody species that could serve these environment-friendly functions, hazelnuts can also offer economic opportunity from a variety of marketable products. Hazelnuts can be eaten as a wholesome snack that is richer in vitamins E and B6 than most other nuts, and are also an excellent source of dietary fiber and oleic acid (Richardson 1997). Because hazelnut oil accounts for 50 to 75 percent of the dry weight of the kernels, it could be used as an alternative energy source (Xu *et al.* 2007). However, there is likely to be more profitability in selling the oil as a skin care product or as a flavorful, heart-healthy cooking oil. After pressing nuts for oil, the high-protein meal can be used as a high-value feed for livestock (Josiah 2000).

Hazels grown from seed vary considerably in bush growth habit, size, yield, maturation date, husk length, nut size, nut flavor, and resistance to Eastern Filbert Blight (EFB), a potentially lethal disease caused by the fungal organism *Anisogramma anomala* (Fischbach *et al.* 2011; Pellett *et al.* 1998; Mehlenbacher 1991). Seedlings are almost always of unknown lineage because they are obligate cross-pollinators and only the female parent can be known with certainty (Pellett *et al.* 1998). Genetic uniformity can only be ensured by vegetative propagation to produce clones of desirable plants, but existing vegetative propagation methods are not sufficiently productive to be economically viable.

Mound layering is currently the most common propagation method for hybrid hazels but its effectiveness varies greatly among different genotypes, ranging from zero to 70 new rooted stems, called layers, produced per plant per year. At this rate it is not possible to produce enough new plants to plant an orchard, much less to develop a new industry. Mound layering also has the disadvantages of being time-consuming and physically demanding (Achim *et al.* 2001; Erdogan *et al.* 2005). Although grafting is commonly used to propagate *C. avellana*, which grow as trees, it is not a practical option for bushy, surculose hybrids. In trials of softwood stem cuttings survival after rooting was found to be only about one percent (Lois Braun, pers. comm.), suggesting this is not a viable method. Trials with hardwood cuttings suggest more potential, with up to 8 new plants produced from every 10 stems collected, of which over 50 percent survived (Lois Braun, pers. comm.). Even if propagation techniques for hardwood cuttings were greatly improved, the efficiency of this method will

continue to be limited by the number of stems that can be obtained from the parent plant and the time it takes to produce them.

Because of the shortcomings of current propagation methods, widespread adoption of hazelnuts in the Midwest is limited by a lack of quality plants. This problem could potentially be solved by developing a reliable method for propagating those hybrid hazels with the most desirable characteristics by micropropagation. In places where *C. avellana* is produced on a large scale, it is often micropropagated (Hand and Reed 2014). This allows efficient production of vast numbers of genetically identical plants in a small space. Many plants can be quickly generated with micropropagation and then distributed to growers. Additionally, micropropagated plants would provide the uniformity needed for field trials to determine best management practices (Zeldin and McCown 2011). Micropropagation of hybrid hazel has not been studied as thoroughly as *C. avellana* micropropagation, but the shoot proliferation stage has been optimized for hybrid hazel (Nas and Read 2001; Nas 2004). In *C. avellana* the induction of root formation, a key step in micropropagation, has been a significant roadblock (Bacchetta *et al.* 2008) and is likely to be a major challenge in hybrid hazels as well. The goal of this thesis project is to better understand the causes of these limitations on *in vitro* rooting ability. We suspect they are due to difference across selections in hormonal processes. Identifying the hormonal roadblocks to adventitious root formation will provide knowledge that can be used to make more informed decisions on how to optimize the rooting phase and also to identify which genotypes are most likely to form adventitious roots. This could

greatly accelerate the process of propagating many genetically uniform plants that can then be strategically placed on the Minnesota landscape.

Corylus *Tissue Culture Review*

Micropropagation is the generation of new plants from small amounts of starting material using *in vitro* techniques. *In vitro* propagation can be advantageous because the researcher has control over external factors and can therefore manipulate the plant's environment in order to influence growth. The tissue is grown on media containing all the required minerals, carbohydrates, moisture, and plant hormones. Lighting and temperature can also be controlled to create optimal conditions for plant growth. However, such conditions can also promote growth of microbes that are either living on the plant tissue or that are introduced onto the media from the surrounding air or use of non-sterile tools. Microbial contaminants usually grow more quickly than plant tissue *in vitro* and can eventually weaken or kill the plant, so it is important that tissue culture be done under sterile conditions with disinfected tools and that plant material be isolated from all bacterial and fungal contaminants before being transferred to *in vitro* conditions. Micropropagation of *Corylus* is generally done by organogenesis, the stepwise formation of organs. This process involves multiple stages, which can include sterilization of plant material, a culture establishment phase, shoot multiplication, rooting, and acclimatization to *ex vitro* conditions.

Culture Establishment. During the culture establishment phase, explants are taken from parent plants, sterilized, and acclimated to *in vitro* conditions. Hazelnut micropropagation is often done with stem segments containing axillary

buds, taking advantage of existing meristems' ability to produce new growth (Dobránszki and Teixeira da Silva 2010). It is also advantageous to establish cultures with juvenile plant material which has greater morphogenic capacity than mature tissue and can be propagated more easily *in vitro* (Nas and Read 2001). It is likely that there will be less contamination during culture establishment if material is collected from plants grown in a greenhouse rather than field conditions where they are exposed to more microorganisms. Before being transferred to *in vitro* conditions, plant material must first be isolated from bacterial and fungal contaminants that could otherwise prevent the plant tissue from growing in culture. This is especially challenging with hazelnut material as it is difficult to kill all contaminants on the surface and endophytes without seriously damaging the plant material. A common approach to sterilizing hazelnut tissue is soaking explants in a sodium hypochlorite solution (Pérez *et al.* 1985; Pérez *et al.* 1987; Messeguer and Melé 1987; Díaz-Sala *et al.* 1990; Bassil *et al.* 1992; Yu and Reed 1995). This is sometimes preceded by a short soak or dip in ethanol (Pérez *et al.* 1985; Pérez *et al.* 1987; Díaz-Sala *et al.* 1990). Other chemicals that have been used for isolations include calcium hypochlorite (Jarvis *et al.* 1978), sodium merthiolate (Bacchetta *et al.* 2008), and mercuric chloride (Kosenko *et al.* 2009).

Shoot Multiplication. Shoot formation can be induced by placing the explant on a medium containing a relatively high concentration of cytokinins. 6-Benzylaminopurine (BAP) is a cytokinin that has successfully been used to promote shoot development in numerous studies involving tissue culture of *C.*

avellana and other species. It is generally included in media for the shoot proliferation phase at concentrations ranging from 0.1 to 5 mg/L, depending on the explant source and other factors, as shown in **Table 1**. Pérez *et al.* found 0.1-2 mg/L BAP promoted the most shoot formation from lateral buds from adult trees while a much higher concentration (8.0 mg/L) was optimal for shoot formation from embryos (Pérez *et al.* 1987). Messeguer and Melé (1987) found that media containing zeatin (2E-methyl-4-(9H-purin-6-ylamino)-2-buten-1-ol), another cytokinin, resulted in higher shoot production, longer shoots with more leaves, and higher foliar quality from adult explants of *C. avellana* compared to the cytokinins BAP (6-(γ,γ-dimethylallylamino)purine, 2iP (N6-[2-Isopentyl]adenine), and kinetin (6-Furfurylaminopurine). Media used in the shoot proliferation phase may also contain low concentrations of auxins, such as naphthalene-1-acetic acid (NAA) (Al Kaï *et al.* 1984) or indole-3-butyric acid (IBA) (Yu and Reed 1995; Kosenko *et al.* 2009). Pérez *et al.* (1985) experimented with including gibberellins in the media but found this generally resulted in thin or bent shoots.

Media Composition. *C. avellana* has been successfully propagated on media containing Murashige & Skoog salts (MS) (Murashige and Skoog 1962) or modifications of MS media (Al Kaï *et al.* 1984; Messeguer and Melé 1987; Díaz-Sala *et al.* 1990; Bassil *et al.* 1992; Bacchetta *et al.* 2008), as well as *C. colurna* (Kosenko *et al.* 2009). Bassil *et al.* (1992) found that shoots grew healthier on a modified MS medium with a higher calcium concentration and sodium ferric ethylenediamine BIS-(2-hydroxyphenylacetate (Fe-sequestrene) instead of Fe-

ethylenediaminetetraacetic acid (Fe-EDTA) as an iron source. Al Kaï *et al.* (1984) also observed better shoot multiplication when Fe-Sequestrene was used instead of Fe-EDTA as an iron source. Díaz-Sala *et al.* (1990) had the most success with a modified MS medium with half-strength nitrates, double-strength calcium chloride and magnesium sulfate (plus other nutrients). Other types of media that have been used for *in vitro* propagation of *C. avellana* have been Keller (K) (Pérez *et al.* 1985; Pérez *et al.* 1987) and Knoxfield 2 (Thomson and Deering 2011). Yu and Reed (1995; 1993) and Caboni *et al.* (2009) found modified Driver Kuniyuki Walnut (DKW) media to work well for *C. avellana*. **Table 1** describes the types of media that have been used for successful culture establishment and shoot proliferation of *Corylus*. Sucrose is most commonly used as a carbon source for *Corylus* tissue culture, but Yu and Reed (1993) showed that glucose results in better shoot multiplication, elongation, and quality than either sucrose or fructose as a carbon source for *C. avellana*.

Table 1: Explant sources and media composition used for shoot formation in *Corylus*

Species	Explant Source	Culture Establishment		Shoot Elongation and Multiplication				Reference
		Media Type	Plant Hormones	Media Type	Cytokinins	Gibberellins	Auxins	
C. avellana	Axillary buds			MS	5 mg/L BAP	0.1 mg/L GA	0.01 mg/L NAA	Al Kaï *et al.* 1984
C. avellana	Shoot and cotyledonary node segments from 20-day-old seedlings	Half-strength K	25 µM BAP	Half-strength K	0.1 or 0.6 mg/L BAP			Pérez *et al.* 1985
C. avellana	Embryos and single lateral buds			Half-strength K composed of Cheng's mineral salts with 0.25 mg/L thiamine and 0.25 g/L inositol	8.0 mg/L BAP			Pérez *et al.* 1987
C. avellana	Segments containing one vegetative bud from seedlings from sterilized seeds grown on MS medium			Half-strength MS	2.5-10 mg/L zeatin	2 mg/L GA	0.01 mg/L IAA	Messeguer and Melé 1987
C. avellana	Axillary buds from newly developed shoots from forced outgrowth of axillary buds on field-grown branches submitted to three months cold storage (0-4 degrees C)			Double-phase culture system of MS with half-strength nitrates but double strength CaCl and magnesium sulfate, plus 100 mg/L myo-inositol, 0.5 mg/L nicotininc acid, 0.5 mg/L pyridoxine HCl, 2 mg/L glycine, 0.4 mg/L thiamine, and 2 mg/L ascorbic acid	5 mg/L BAP	0.1 mg/L GA3	0.01 mg/L IAA	Díaz-Sala *et al.* 1990
C. avellana	Buds of shoots from mature trees (collected May-October)	MS	5 µM BAP	MS	1.1 mg/L BAP			Bassil *et al.* 1992
C. avellana	Nodal segments, either from dormant branches (collected in March) grafted onto rootstocks or from forced outgrowth of field-grown suckers (collected in July).	NCGR-COR (a modified DKW medium)	22.2 µM BA, 0.04 µM IAA	NCGR-COR	1.5 mg/L BAP		8 µg/L IBA	Yu and Reed 1995

Hybrid	single axillary buds	WPM	4 mg/L BA, 0.01 mg/L IBA	NN or WPM liquid with synthetic foam to keep tissues from being submerged medium with, 30 g/L sucrose, 200 mg/L sequestrine 138	5 mg/L BAP		0.01 mg/L IBA	Nas and Read 2001
Hybrid	apical shoot tips and nodal shoot segments containing 1 or 2 axillary buds	DKW supplemented with polyamines: 0.4 mM putrescine, 0.4 mM spermidine, 0.1 mM spermine	22.2 µM BA, 0.04 µM IBA	DKW supplemented with polyamines: 0.2 mM putrescine, 0.2 mM spermidine, 0.05 mM spermine	1.5 to 3.5 mg/L BAP			Nas 2004
C. avellana	uninodal shoot explants (1 cm in length), collected from 2-year-old potted plants that came from rooted suckers.	HM (Hazelnut revised medium) induction medium		HM	1.5 mg/L BAP or 1 mg/L zeatin			Bacchetta *et al.* 2008
C. avellana	*In vitro* grown shoots originally from axillary buds of mature plants	MOLT (modified DKW medium)	2.0 mg/L BA, 0.03 mg/L GA3, 0.01 mg/L IBA	Temporary immersion system in liquid MOLT	2.0 mg/L BAP	0.03 mg/L GA3, 0.01 mg/L IBA		Caboni *et al.* 2009
C. colurna	2-3 cm grafts, each with one axillary bud			Half-strength MS	0.1 mg/L BAP	1.0 mg/L GA3 OR no GA3	0.01 mg/L IBA	Kosenko *et al.* 2009
C. avellana	Complete shoots 10-20 mm in length, from cultures that had been maintained *in vitro*	Knoxfield 2		Knoxfield 2	5 mg/L BAP			Thomson and Deering 2011

Root Induction. Shoot formation is followed by a rooting phase which is induced by application of auxins, usually IBA. Basal ends of *in vitro*-grown shoots can be soaked in an IBA solution for a brief period of time before being transferring to a hormone-free medium (Pérez *et al.* 1987; Díaz-Sala *et al.* 1990; Bassil *et al.* 1992; Caboni *et al.* 2009) since while auxin induces adventitious root formation it also inhibits subsequent root growth. Alternatively, shoots are placed on IBA-containing media for several days and then transferred to a hormone-free medium (Pérez *et al.* 1985). Yu and Reed (1995) compared an *in vitro* rooting approach, in which the shoots were transplanted onto media containing 4.9 µM IBA for four weeks and then transplanted to pots in a mistbed for two to three weeks, with an *ex vitro* method in which shoots were dipped in IBA solution and then immediately transplanted to pots. Both methods resulted in successful root formation, but the authors recommend using the *ex vitro* approach in order to save time. Bacchetta *et al.* (2008) attempted rooting by a method similar to the *in vitro* method of Yu and Reed (1995) by placing shoots on media containing 2 mg/L IBA for one month without transferring to an auxin-free medium; however, only a low percentage of shoots formed roots by this method. **Table 2** summarizes methods that have previously been used to root *Corylus*. Nas and Read (2001) noted that researchers may come to different conclusions about which micropropagation methods are most effective because of differences in genotype, explant source, and *in vitro* conditions used in their experiments. In this thesis, our aim was to identify the hormonal limitations restricting hybrid hazels' ability to form roots *in vitro*.

Table 2: Methods of auxin application and growing conditions for root induction

Species	Hormone Concentration in Solution or Medium	Application	Transfer	Rooting Success	Reference
C. avellana	10 mg/L IBA	Submerged basal ends of shoots for 5 days in ½ K(h) medium containing IBA	Hormone-free ½ K(h) solid medium for 15 days	80%	Pérez *et al.* 1985
C. avellana	1.5 g/L IBA	Dipped basal ends of shoots in IBA solution for 10 seconds	Hormone-free ½ K(h) solid medium	75% when shoots were obtained from embryos; 50% when shoots were from adult material	Pérez *et al.* 1987
C. avellana	0.1-1 mg/L IBA	Dipped basal ends of shoots in IBA solution for 10 seconds	Hormone-free ½ MS2 medium for 20 days	100%	Díaz-Sala *et al.* 1990
C. avellana	2 mg/L IBA	Dipped basal ends of shoots in IBA solution for 15 seconds	Auxin-free medium for four weeks	100%	Bassil *et al.* 1992
C. avellana	1 g/L IBA	Dipped basal ends of shoots in IBA solution for 1 minute	Transplanted directly into pots of 2 peat: 1 hemlock bark: 1 perlite mix	100%	Yu and Reed 1995
C. avellana	2 mg/L IBA	Placed shoots on 1/3 HM medium with IBA for one month		10-30%, depending on genotype	Bacchetta *et al.* 2008
C. avellana	80 mg/L IBA	Submerged basal ends of shoots in IBA solution for one day, in darkness	Hormone-free agarised medium with 1 g vermiculite per mL of medium	100% for 'Montebello' cultivar, 80% for 'Tonda Romana' cultivar	Caboni *et al.* 2009
C. colurna	0.1 mg/L BAP, 0.8 mg/L IBA, 1.0 mg/L GA3	Cultured on MS-10 medium containing BAP, IBA, and GA3			Kosenko *et al.* 2009

Acclimatization to* Ex Vitro *Conditions. After *in vitro* organogenesis under conditions of high humidity, with all essential nutrients supplied in the medium and no pathogens present, plantlets must undergo an acclimatization phase to prepare for harsher *ex vitro* conditions. During this phase, plantlets must physiologically adjust to reduce water loss in drier conditions and become fully dependent on photosynthesis as their source of carbon (Dobránszki and Teixeira da Silva 2010). Yu and Reed (1995) acclimatized plantlets that had formed both

shoots and roots *in vitro* to *ex vitro* conditions by loosening the lid of the Magenta boxes (Magenta, Chicago, IL) in which plantlets had been grown for one day, then transferring plantlets to pots containing a mix of 1 vermiculite: 1 perlite in a mist bed for 2 to 3 weeks, and then transferring to a greenhouse bench for another two weeks before transplanting a third time into their final, larger pots. Caboni *et al.* (2009) acclimatized *in vitro*-grown plantlets by transplanting them into a mix of 60% peat and 40% perlite in plastic tunnel conditions with 80% humidity for two weeks and then gradually reducing the level of humidity. Pérez *et al.* (1987) transferred plantlets to pots containing a mix of 1 peat: 1 perlite and maintained the same light and temperature conditions that the plants had experienced *in vitro*. The acclimatizing plants were watered with 10 mL sterile water every two days and with a diluted solution of Cheng's minerals (Cheng 1975) once per week.

IBA-derived IAA

Indole-3-acetic acid (IAA) is the most abundant auxin naturally occurring in plants and is involved in many processes controlling plant development and environmental responses. Elongation of the acetic acid side chain of IAA by two carbons produces indole-3-butyric acid (IBA), which is an endogenous compound occurring in many plant species (Epstein and Ludwig-Müller 1993; Ludwig-Müller 2000). IBA is widely available commercially for application to promote rooting, and has long been known to be more effective at promoting adventitious root formation than IAA. Recent research suggests IBA serves as an auxin storage form (Strader *et al.* 2011) that can be converted to free IAA by peroxisomal β-

oxidation, a process is similar to β-oxidation of fatty acids. β-oxidation of IBA was first observed by Fawcett *et al.* (1960) using paper chromatography, and since then IBA-to-IAA conversion has been observed in many species including *Pinus sylvestris, P. tremula, P. communis, Malus pumila, Vitis vinifera, Olea europaea,* and *Malus domestica* (Epstein and Ludwig-Müller 1993).

Expression of several proteins involved in peroxisomal β-oxidation is required for full responsiveness to IBA, suggesting that IBA-derived IAA may be responsible for inducing adventitious root formation rather than direct action of IBA itself (Zolman *et al.* 2000; Bartel *et al.* 2001). These include PXA1, a peroxisomal ATP-binding cassette transporter that imports IBA into the peroxisomes (Zolman *et al.* 2001); several proteins encoded by the peroxisome biogenesis *PEROXIN (PEX)* genes: PEX5 and PEX7, which are peroxisomal matrix protein receptors (Zolman *et al.* 2000; Woodward and Bartel 2005(b)) and PEX6 which maintains PEX5 levels (Zolman and Bartel 2004). Enzymes directly involved in β-oxidation of IBA include the enoyl-CoA hydratase ECH2 (Strader *et al.* 2010) and proteins encoded by the *IBA Resistance* (*IBR*) genes: IBR1, which belongs to the short-chain dehydrogenase/reductase family (Zolman, *et al.* 2008); IBR3, a peroxisomal acyl-CoA dehydrogenase-like protein (Zolman *et al.* 2007); and IBR10, an enoyl CoA hydratase-isomerase.

Strader *et al.* (2011) demonstrated that mutant *Arabidopsis* seedlings unable to convert IBA to IAA display a number of developmental defects including decreased root meristem size, reduced formation of lateral roots, decreased cotyledon size, and shortened root hairs. These mutants also displayed strong

resistance to the effects of exogenous IBA on hypocotyl elongation, root growth, and initiation of lateral root primordia.

Research Objective

Because induction of adventitious root development in hazels has been a challenge in hardwood and softwood stem cuttings, mound layering, and micropropagation, the goal of this project is to better understand hormonal limitations on rooting ability. Identifying the hormonal roadblocks to adventitious root formation will provide knowledge which may be used to make informed decisions on how to most effectively induce rooting and also to identify which genotypes are most likely to form adventitious roots. This could greatly accelerate the process of propagating many genetically uniform plants which can then be strategically placed on the Minnesota landscape or used in research. Our goals are to define specific easily assayed traits that allow for the rapid identification of plant materials and offer potential for breeding cultivars with enhanced propagation responses. Because conversion of exogenous IBA to IAA seems to be critical for normal response to IBA, we hypothesize that low rooting abilities observed in some hazelnut genotypes could be partly due to lower levels of IBA β-oxidation. The ability to rapidly analyze this biochemical process related to rooting ability will enable using rooting potential in conventional and micropropagation to be used as a breeding trait for development of elite germplasm on which to build large-scale commercial operations.

II. Conversion of indole-3-butyric acid to indole-3-acetic acid in shoot tissue of hazelnut (*Corylus*) and elm (*Ulmus*)

Auxins are a class of plant hormones that include indole-3-acetic acid (IAA), the most abundant auxin, and play critical roles in a number of developmental processes in plants. Since first recognized in the 1930's (Thimann and Went 1934), auxins have been recognized for their especially important role in the formation of adventitious roots (Bellini 2014) and have proven to be of significant value in the propagation of woody species (Thimann and Behnke-Rogers 1950). Indole-3-butyric acid (IBA) in particular has found common use to promote adventitious rooting in vegetative propagation of a variety of species (de Klerk *et al*. 1999). Numerous studies on the biology of adventitious root formation have shown that it is possible to delineate distinct and successive phases of adventitious root formation: induction, initiation and expression (Kevers *et al*. 1997). Auxin has a specific effect on the early stages of induction in plants in which rooting is induced by cutting alone. Transient increases in auxin are noted in the induction period and decline at the appearance of the first cytological events (Moncousin *et al*. 1988; Blakesley 1994). These results are consistent with the observation that auxin induces the formation of root primordia yet inhibits subsequent root growth, suggesting that the proper timing would be important for auxin induced rooting to have optimal effect.

IBA can be converted to IAA by the removal of two methylene units from the side chain by β-oxidation, and can therefore function as a storage form of IAA that is transported in a manner distinct from that of free IAA (Woodward and Bartel 2005(a); Strader and Bartel 2011; Liu *et al.* 2012(a)). This conversion was first reported by Fawcett *et al.* (1960) and has since been observed in a number of plant species (reviewed in Epstein and Ludwig-Müller 1993). Differences in IBA-to-IAA conversion have been observed between easy-to-root and difficult-to-root cultivars of several species, and it has been suggested that this process may be partly responsible for differences in rooting ability (Epstein and Lavee 1984; Baraldi *et al.* 1995).

Free IAA is biosynthesized from IBA through the multistep process of β-oxidation in the peroxisomes (Bartel *et al.* 2001) in a process similar, but not identical to that of fatty acid β-oxidation. Strader *et al.* (2011) observed numerous developmental defects in *Arabidopsis* seedlings that were unable to convert IBA to IAA due to mutations in genes required for β-oxidation of IBA, thus showing that IBA-derived IAA has specific functions in plant development. Such mutants did not respond to exogenous IBA, but IBA responses could be partially induced by application of other auxins, demonstrating that IBA activity is dependent on its conversion to IAA. This metabolic relationship places unique significance on the peroxisomal process of β-oxidation of IBA in specific aspects of plant development, particularly in lateral and adventitious root initiation.

Adventitious root formation is a critical and limiting step in vegetative propagation of both hybrid hazelnut (*Corylus americana* x *C. avellana*) and American elm (*Ulmus americana*). In both species variability of rooting in response to hormone application, such as IBA treatment, has been observed across different genotypes. In this study, we developed an assay to measure conversion of exogenous IBA to IAA in small samples of plant tissue using stable isotope-labeled compounds and gas chromatography-tandem mass spectrometry (GC-MS/MS). We then used this assay to quantify differences in IBA-to-IAA conversion in hazelnut and elm genotypes.

Materials and Methods

Elm softwood stem cutting rooting trials Six elm genotypes were analyzed in this study, including five American elm genotypes: 'Saint Croix' (SC), MNT-0356 (AM), MNT-0345 (AS), 'Valley Forge' (VF) and 'Princeton' (PR) as well as one Asian hybrid elm 'Cathedral' (CA). Elm trees were five to ten years old and were grown in field plots on the University of Minnesota - Saint Paul campus. Softwood material was collected from one tree of each genotype in the second and fourth weeks in June (here on referred to as "week 1" and "week 3," respectively).

Softwood stems were collected and soft tissue at the tip and excessively woody material at the base of the stems were removed. The stems were sectioned into 10-15 cm long cuttings each containing three to four nodes. Leaves were

removed from the lower portion, leaving only two or three leaves near the top of each cutting. Leaf blades were cut in half and the tip was discarded. Basal ends of cuttings were dipped for 5 seconds into one of two hormone treatments: 1000 ppm IBA (MP Biomedicals Inc., Solon, OH) with 500 ppm 1-naphthaleneacetic acid (NAA) (Calbiochem, San Diego, CA), designated as treatment 1, or 3000 ppm IBA with 1500 ppm NAA, designated as treatment 2. Hormone treatments were based on past softwood stem cutting trials that had shown a 2:1 ratio of IBA: NAA to be optimal for adventitious root formation. Cuttings were then placed in 32-cell propagation trays (RootMaker®, Huntsville, AL) containing a 5:1 perlite: peat mix drenched in water. A total of 16 replicates per genotype × hormone combination were prepared on each of the two dates of collection. Cuttings were kept in tents with one flap left open to allow air flow, and were misted for four seconds every four minutes by Netafim foggers that were placed at two locations per each 2×1 m zone. Medallion®, a broad-spectrum fungicide, (Syngenta, Greensboro, NC) was applied to each set of cuttings to control root rot pathogens. After seven weeks, basal ends of cuttings were inspected for rooting. Roots forming from shoot material were counted as new roots, while roots originating from other roots were not counted.

Elm auxin conversion assay material Softwood stems approximately two millimeters in diameter were collected in the second and fourth weeks of June from the same trees from which material for softwood stem cuttings were taken. One stem was collected from each tree on each date. Shoot material was rinsed

in sterile water, cut into segments and assayed according to the same method as was used with hazelnut tissue (see below).

Hazelnut auxin conversion material Plant material used in IBA-to-IAA conversion assay came from 5- and 6-year-old hybrid hazelnut plants grown in field plots on the University of Minnesota St. Paul campus from rooted adventitious shoots. Softwood stems from branches not bearing any nuts approximately 15-20 cm in length and 2 mm in diameter were used in conversion assays.

To test whether variation in IBA-to-IAA conversion exists across different hybrid hazelnut genotypes, we assayed material from five plants of different genotypes: Heas C, Gibs 3-12, Gibs 3-26, Gibs 5-8, and Stap N5-6. Ten stem sections from each genotype were assayed.

Limited data was available from field rooting trials with mound layering and greenhouse softwood stem cutting trials which had suggested some varieties showed somewhat better adventitious root formation (Erick 3-4, Erick 5-13, and Stap N7-6) than the other selections (Erick 8-9 and Stap N2-7). Two cloned plants each of Stap N2-7 and Stap N7-6, and three cloned plants each of Erick 3-4, Erick 5-13, and Erick 8-9 were sampled. Four stems were collected from each plant during the third week in July; one sample from each stem was assayed.

To demonstrate that IBA is converted to IAA by hybrid hazelnut shoot tissue in the absence of endophytes or other microbial contamination, we also assayed shoot material from *in vitro*-grown cultures under sterile conditions. Shoot

cultures were established from axillary bud explants taken from rooted adventitious shoots grown in pots in a greenhouse in a 4:1 mix of MetroMix MM852 perlite supplemented with Osmocote 15-9-12 fertilizer for one year. Shoot segments approximately two centimeters in length and 0.5 cm in diameter containing one axillary bud were sterilized by shaking in a solution of 0.1% Tween 80 (National Biochemical Corporation, Cleveland, OH) for five minutes, rinsing twice with water, soaking in a 0.9% sodium hypochlorite solution (made from diluting regular commercial bleach (Clorox, Oakland, CA), 8.25% sodium hypochlorite, with deionized water) for 20 minutes, rinsing three times with water, and soaking in a 5% solution of Plant Preservative Mixture (0.135% 5-chloro-2-methyl-3(2H)-isothiazolone, 0.041% 2-methyl-3(2H)-isothiazolone; Plant Cell Technology, Washington, DC) for eight hours. In a laminar flow hood, 2-3 mm of the basal end of each explant was removed before explants were transferred to GA7 culture vessels (Magenta, Chicago, IL) containing a modified Lloyd & McCown woody plant media (Phytotechnology Laboratories, Overland Park, KS) containing 200 mg/L calcium nitrate tetrahydrate (Fisher Scientific, Fair Lawn, NJ), 5 μM zeatin (Phytotechnology Laboratories), 2% grade II sucrose (Sigma-Aldrich, St. Louis, MO), 1 g/L Gelrite (Research Products International, Mt. Prospect, IL), 3 g/L phytoagar (Phytotechnology Laboratories), and 0.2% Plant Preservative Mixture. Media was adjusted to pH 5.8 with KOH before autoclaving. Shoots were grown in culture for six months, transferring regularly to fresh media, under a 24 h photoperiod with 60 μmol m^{-2} s^{-1} PAR at 23° C.

IBA-to-IAA conversion assay [$^{13}C_8{}^{15}N_1$]IBA was synthesized essentially as described (Cohen and Schulze 1981; Sutter and Cohen 1992). Because the [$^{13}C_8{}^{15}N_1$]IBA solution was found to contain trace levels of [$^{13}C_8{}^{15}N_1$]IAA, the solution was further purified by preparative HPLC. 400 µL of the 0.63 mg/mL [$^{13}C_8{}^{15}N_1$]IBA stock solution in 50% isopropanol was injected onto a 21.2 × 250 mm ZORBAX Eclipse XDB-C18 Prep HT column (Agilent Technologies, Santa Clara, CA) with a 7 µm particle size. A gradient starting with 10% methanol, increasing to 90% methanol over 20 minutes, and holding at 90% methanol for two minutes with a constant flow rate of 20 mL/min was used to separate IBA from IAA with an Agilent 1200 Series G1361A preparative pumping system. Absorbance at 280 nm was monitored with an Agilent 1200 series G1365B multiple wavelength detector to detect elution of indolic compounds; [$^{13}C_8{}^{15}N_1$]IBA eluted at approximately 16 minutes and was collected in an acid-washed glass bottle. HPLC purification was repeated several times to purify approximately 10 mL of the original stock solution. [$^{13}C_8{}^{15}N_1$]IBA fractions were pooled and reduced to a volume of approximately 50 mL on a SpeedVac vacuum concentrator (Thermo). Concentration of the final purified solution was determined by measuring the absorbance of [$^{13}C_8{}^{15}N_1$]IBA in 1% methanol at 265 nm and an extinction coefficient of 16,982 (Acharya and Sengar 2013) using a HP 8453 UV-Visible spectrophotometer (Agilent).

Elm and hazelnut stems were rinsed with sterile deionized water, cut into 7-11 mg internodal segments, weighed, and placed basal end-up into wells of an untreated 384-well plate each containing 40 µL of 10 µM [$^{13}C_8{}^{15}N_1$]IBA in distilled

water. After incubating in the 10 µM [$^{13}C_8{}^{15}N_1$]IBA solution at room temperature for 6 hours, shoot segments were removed from wells, blotted on a laboratory tissue, transferred to 1.5 mL microcentrifuge tubes, frozen by dipping in liquid nitrogen or dry ice in isopropanol and stored at -80° C until extraction.

In vitro-grown shoots were assayed under sterile conditions. Plant material was cut and weighed in a laminar flow hood using sterile technique. [$^{13}C_8{}^{15}N_1$]IBA solution was filter sterilized with an Acrodisc 13 mm syringe filter with a 0.2 µM PTFE membrane (Pall Life Sciences, Ann Arbor, MI) and a sterile 384 well plate with a lid (Thermo Fisher, Rochester, NY) was used.

Extraction and GC-MS/MS analysis Frozen plant tissue was homogenized with 0.85 ng of [$^{13}C_6$]IAA internal standard (Cambridge Isotope Laboratories, Tewksbury, MA) in 20 µL of 65% isopropanol buffer with 0.2 M imidazole, pH 7.0, by placing two 3 mm tungsten-carbide beads into the microcentrifuge tube containing plant material and buffer and homogenizing for ten minutes at 25 Hz using a TissueLyser model MM 300 ball mill (Qiagen, Germantown, MD). Labeled IAA was extracted from plant tissue by micro solid phase extraction using NH_2 and polymethylmethacrylate epoxide (PMME) resins, essentially as described by Liu *et al.* (2012(b)). Samples were derivatized with diazomethane prior to GC-MS analysis to form IAA methyl ester, using ethereal diazomethane synthesized as described (Cohen 1984; Barkawi and Cohen 2010).

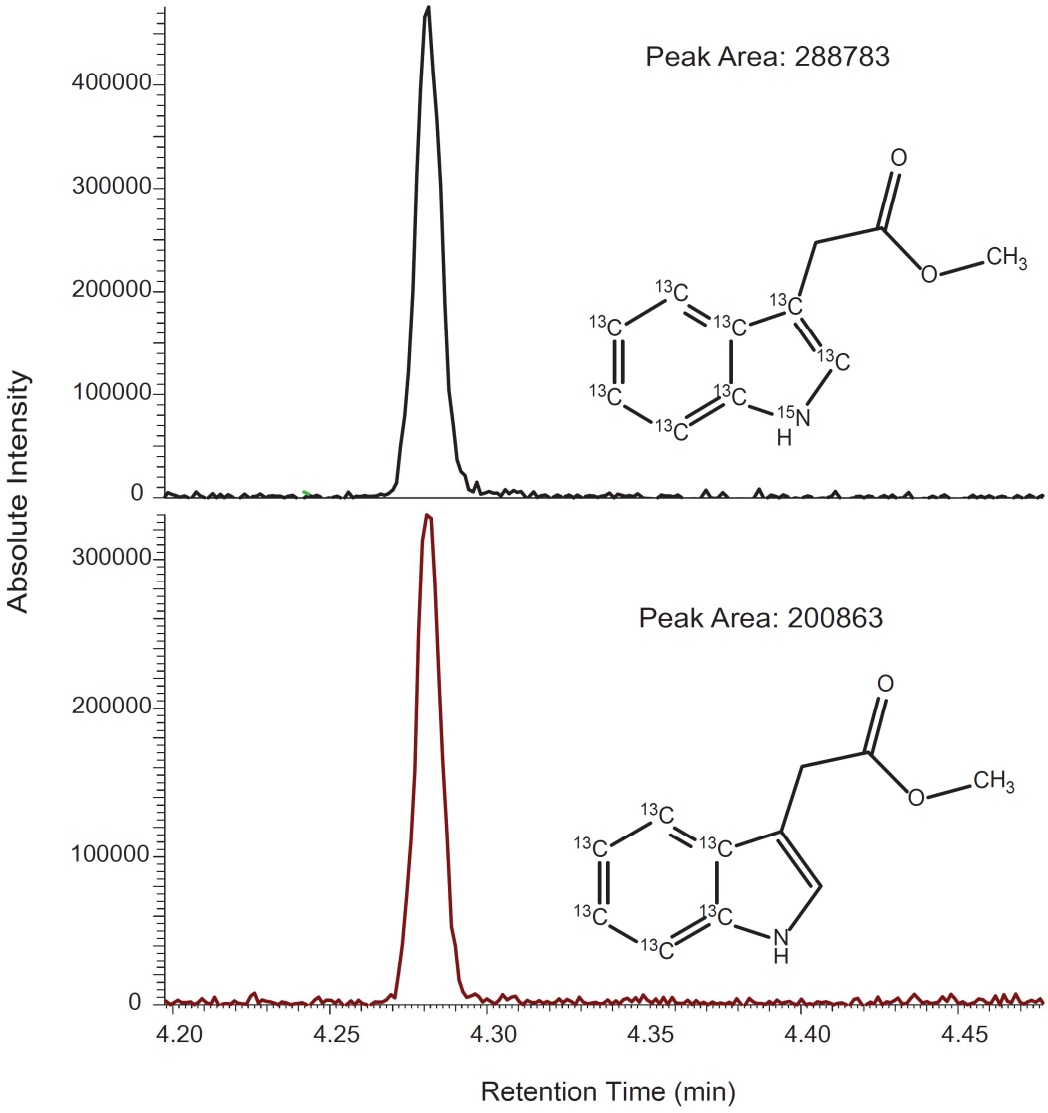
Samples were analyzed on a Thermo Trace GC Ultra gas chromatograph with a 5 m Zebron Z-Guard GC guard column connected to a 15 m Zebron ZB5MS

analytical column with a 0.25 mm diameter and 0.25 µm film thickness (Phenomenex, CA), coupled with a Thermo TSQ Quantum XLS mass spectrometer. The GC temperature profile was set to 70^{o} C for 1 minute, then increased by 50^{o} C per minute to 240^{o} and was held at 240^{o} for 1.5 minutes. Compounds eluted from the GC column were ionized by electron impact with an emission current of 100 µA. Selected reaction monitoring (SRM) acquisition mode was used to select the molecular ions of m/z 195 and 198 and detect quinolinium product ions of m/z 136 and 139 of [$^{13}C_6$]IAA and [$^{13}C_8{}^{15}N_1$]IAA methyl esters, respectively. Quinolinium product ions were produced by collision with argon gas using a collision energy of 10 V and 1.5 mTorr collision gas pressure and detected using scan times of 0.025 s. Peak areas were calculated using Qual Browser in Xcalibur software, and were then used to determine concentration of [$^{13}C_8{}^{15}N_1$]IAA in treated tissue by isotope dilution (Cohen *et al.* 1986, Barkawi *et al.* 2010).

Results

GC-SRM-MS/MS assay: The basis for the conversion assay is illustrated in the GC-MS/MS selected reaction monitoring ion chromatograms shown in Figure 1. The selected reaction product quinolinium ions produced from both labeled forms (i.e. the [$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA and the [$^{13}C_6$]IAA used as the internal standard) resulted in chromatographic peaks with the same retention times of 4.28 minutes but with distinct mass differences.

Figure 1. Example GC-MS results from auxin conversion assay. [$^{13}C_8{}^{15}N_1$]IAA produced in the assay and [$^{13}C_6$]IAA internal standard were analyzed as their methyl esters using gas chromatography coupled with selected reaction monitoring (SRM) on a triple quadrupole mass spectrometer. Retention time of isotope-labeled IAA-methyl esters was 4.28 minutes.



Elm rooting trials: Formation of adventitious roots in elm softwood stem cuttings varied greatly by hormone treatment and by cultivar (Figure 2). During week 1, a higher percentage of VF and CA cuttings formed adventitious roots in response to hormone treatment 1 (1000 ppm IBA and 500 ppm NAA) compared to treatment 2 (3000 ppm IBA, 1500 ppm NAA), while more AS, PR and SC cuttings rooted in response to treatment 2 (Figure 2B). AM cuttings had equally high rooting success with either treatment. AS, VF, and CA formed on average a higher number of roots in response to treatment 1, while genotypes PR, SC, and AM formed more roots in response to treatment 2.

During week 1, the AM genotype had the highest rooting success of all the cultivars tested, in terms of percentage of cuttings that rooted as well as number of adventitious roots formed per cutting. Both hormone treatments resulted in a rooting rate of 94 percent (roots formed on fifteen out of sixteen cuttings), making AM the highest rooting genotype in response to either treatment. AM also formed the highest average number of adventitious roots in response to both treatments: 6.6 ± 1.3 roots per cutting in response to treatment 1 and 7.9 ± 1.4 roots per cutting in response to treatment 2 (Figure 2C). Conversely, AS had the overall poorest root formation of the cultivars tested. 25% of AS cuttings rooted in response to treatment 1, making it the lowest rooting genotype along with PR which also had 25% rooting in response to treatment 1. Treatment 2 resulted in 42% rooting in AS, with only the CA cultivar having fewer rooted cuttings at 31%.

Adventitious root formation generally decreased for cuttings collected in week three (Figure 2E). VF failed to root at all and AS formed roots in only one

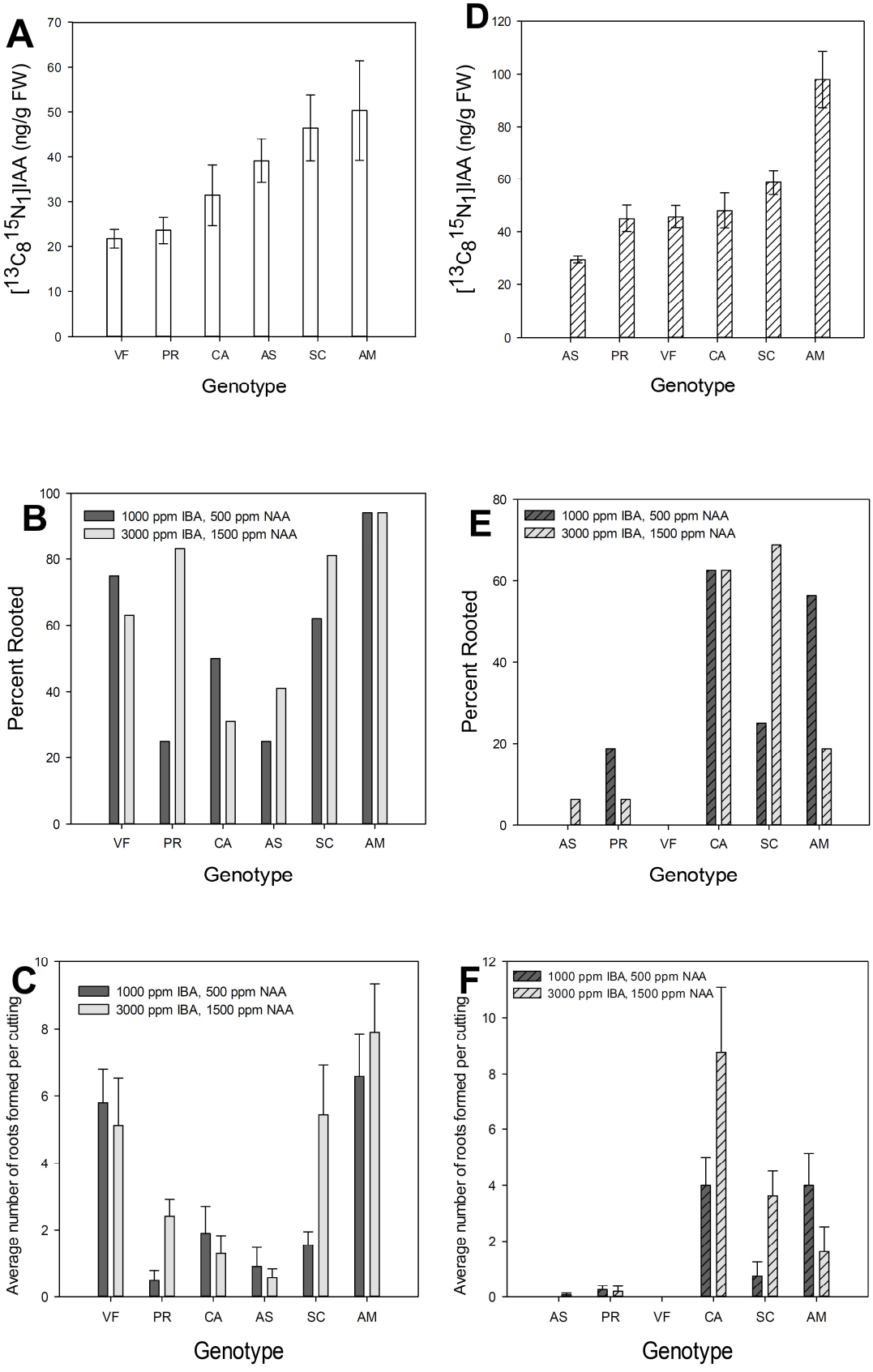
replicate in treatment 2. Formation of adventitious roots in the AM genotype also decreased in week 3 with 56% rooting success in treatment 1 and 19% success in treatment 2. The PR genotype showed a similar response, but with lower overall rooting success with 19% rooting success in treatment 1 and 6% success in treatment 2.

SC experienced lower overall rooting in week three but showed a positive rooting response with the addition of higher levels of applied auxin in treatment 2 at 69% versus 25% in treatment 1, similar to the trend observed in this genotype for week 1. CA cuttings had a higher percentage of rooting in week 3 than in week one with both treatments rooting at 62%. The number of roots formed in week three varied between genotypes and auxin treatment (Figure 2F). All genotypes except AS formed more roots in treatment 1 than treatment 2. Overall root number was highest in the CA genotype with a two to three-fold increase in root number when compared to week 1.

Elm auxin conversion assays: The stable isotope assay revealed considerable variation in IBA-to-IAA conversion in the elm cultivars tested (Figure 2A and C). Notably higher levels of [$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA were observed in AM tissue than any other cultivar. In AM tissue assayed in the first week, we observed 50.3 ± 11.1 ng [$^{13}C_8{}^{15}N_1$]IAA per gram of fresh plant tissue. In tissue from week 3, we observed 98.0 ± 10.9 ng [$^{13}C_8{}^{15}N_1$]IAA/g, which was 67% higher than the genotype with the next highest level of conversion. The lowest levels of conversion in material collected week 1 were observed in VF and PR, at 21.7 ±

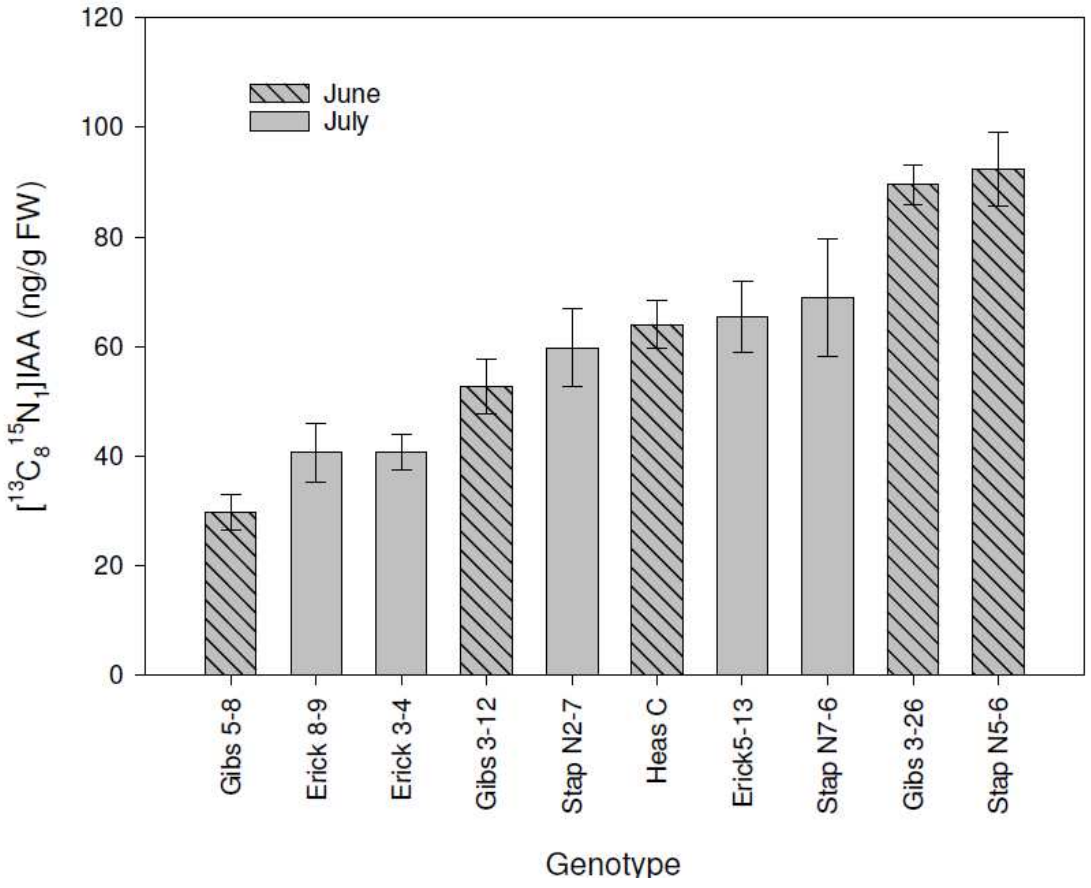
2.0 and 23.6 ± 3.0 ng [$^{13}C_8{}^{15}N_1$]IAA/g, respectively. AS had the lowest level of conversion in week 3 at 29.4 ± 1.5 ng/g.

Figure 2. IBA-to-IAA conversion and rooting in elm. Softwood stem samples were collected from mature field-grown trees the second week ("week 1," A-C) or fourth week ("week 3," D-F) in June. **(A and C)[$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA in treated elm shoot tissue.** 7-10 mg shoot segments from field-grown elms of six different cultivars were assayed for IBA-to-IAA conversion by treating with 10 µM [$^{13}C_8{}^{15}N_1$]IBA for six hours at room temperature. Tissue was assayed the second and fourth weeks in June (A and C, respectively), with ten samples per genotype per week. After [$^{13}C_8{}^{15}N_1$]IBA treatment, plant tissue was homogenized with [$^{13}C_6$]IAA internal standard, purified by solid phase extraction, and derivatized with diazomethane. [$^{13}C_8{}^{15}N_1$]IAA-methyl ester was then quantified by GC-MS using selected reaction monitoring to select parent ions of m/z 195 and 198 and monitor quinolinium product ions of m/z 136 and 139 generated from [$^{13}C_6$]IAA-methyl ester and [$^{13}C_8{}^{15}N_1$]IAA-methyl ester molecules, respectively. Concentration of [$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA was calculated from peak areas using isotope dilution. **(B and E) Rooting of softwood stem cuttings from elm.** Softwood material was collected from the same six plants that were assayed for IBA-to-IAA conversion. Stems were cut into approximately 15-20 cm sections each containing at least three nodes and dipped for five seconds in a solution of either 1000 ppm IBA and 500 ppm NAA or 3000 IBA and 1500 ppm NAA. Cuttings were placed in trays containing a 5:1 perlite:peat mix in a mist tent and were assessed after seven weeks for root formation. **(C and F) Mean number of roots per cutting.** Number of adventitious roots forming directly from shoot tissue were counted after seven weeks


A
[13C8 15N1]IAA (ng/g FW)
70
60
50
40
30
20
10
0
VF
PR
CA
AS
SC
AM
Genotype
D
[13C8 15N1]IAA (ng/g FW)
120
100
80
60
40
20
0
AS
PR
VF
CA
SC
AM
Genotype
B
1000 ppm IBA, 500 ppm NAA
3000 ppm IBA, 1500 ppm NAA
Percent Rooted
100
80
60
40
20
0
VF
PR
CA
AS
SC
AM
Genotype
E
1000 ppm IBA, 500 ppm NAA
3000 ppm IBA, 1500 ppm NAA
Percent Rooted
80
60
40
20
0
AS
PR
VF
CA
SC
AM
Genotype
C
1000 ppm IBA, 500 ppm NAA
3000 ppm IBA, 1500 ppm NAA
Average number of roots formed per cutting
10
8
6
4
2
0
VF
PR
CA
AS
SC
AM
Genotype
F
1000 ppm IBA, 500 ppm NAA
3000 ppm IBA, 1500 ppm NAA
Average number of roots formed per cutting
12
10
8
6
4
2
0
AS
PR
VF
CA
SC
AM
Genotype

Hazelnut auxin conversion assays: Variability in IBA-to-IAA conversion was also observed across genotypes of hybrid hazelnut assayed in June, ranging from 29.7 ± 3.2 [$^{13}C_8{}^{15}N_1$]IAA/g in Gibs 5-8 to 92.4 ± 6.8 ng [$^{13}C_8{}^{15}N_1$]IAA/g in Stap N5-6 (Figure 3). Of the hazelnut genotypes assayed in July , Stap N7-6 and Erick 5-13 had the highest levels of [$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA at 69.0 ±10.8 ng/g and 65.5 ± 6.5 ng/g, respectively; the two genotypes with the lowest levels of [$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA were Erick 8-9 at 40.7 ± 5.3 ng/g and Erick 3-4 at 40.8 ± 3.3 ng/g. Hazelnut material assayed under sterile conditions produced [$^{13}C_8{}^{15}N_1$]IAA at levels similar to non-sterile tissues (data not shown).

Figure 3. [$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA in hazelnut genotypes of unknown rooting ability. 7-10 mg shoot segments from field-grown hybrid hazelnut plants were assayed for IBA-to-IAA conversion by treating with 10 µM [$^{13}C_8{}^{15}N_1$]IBA for six hours at room temperature. For each of the five genotypes assayed in June, eight replicate samples from the same shoot were assayed (shown by hatched bars); for samples assayed in July (shown by solid bars), 2-4 samples from separate stems were collected from each of 2-3 clonal propagules of the same parent. After [$^{13}C_8{}^{15}N_1$]IBA treatment plant tissue was homogenized with [$^{13}C_6$]IAA internal standard, purified by solid phase extraction, and derivatized with diazomethane. [$^{13}C_8{}^{15}N_1$]IAA-methyl ester was quantified by GC-MS using selected reaction monitoring to select parent ions of m/z 195 and 198 and monitor quinolinium product ions of m/z 136 and 139 generated from [$^{13}C_6$]IAA-methyl ester and [$^{13}C_8{}^{15}N_1$]IAA-methyl ester molecules, respectively. Concentration of [$^{13}C_8{}^{15}N_1$]IBA-derived [$^{13}C_8{}^{15}N_1$]IAA was calculated from peak areas using isotope dilution.



Discussion

The development of a facile stable isotope-based mass spectrometry assay allowed the qualitative measurement of the level of IAA in plant tissues resulting from the conversion of IBA to IAA in a number of different cultivars of two woody plant species. IBA-to-IAA conversion has previously been monitored and quantified in woody species by employing [^{14}C]IBA (Epstein and Lavee 1984; Baraldi *et al.* 1995) and [4-^{3}H]IBA (Van der Krieken *et al.* 1993), or by observations of increased free IAA levels after treatment with unlabeled IBA (Alvarez *et al.* 1989, Nordström *et al.* 1991). Use of [$^{13}C_8$,$^{15}N_1$]- and [$^{13}C_6$]-labeled compounds in this assay provided not only a quantitative measurement of IBA-derived IAA, but also provided analytical assurance that the compound measure was in fact IAA derived from the labeled IBA and not a secondary treatment effect or a labeled product with similar chromatographic behavior. More importantly, the method is facile, allowing the potential for automation and, even manually, large numbers of plants can be screened.

IBA functions primarily as an IAA precursor (Strader *et al.* 2011), meaning that deficiencies in conversion of IBA to IAA would result in a reduced response to IBA application. Thus, for IBA to be an effective inducer of root initials, it would be expected that it must first be converted to the active hormone IAA (Baraldi *et al.* 1995). Both the capacity to carry out this reaction and the timing of the IAA production appear to be important aspects of the rooting process (Ludwig-Müller *et al.* 2005; Pop *et al*. 2011). Although root initiation is a complex process involving hormonal, developmental and environmental factors (Pijut *et al*. 2011),

the ability to convert the applied IBA to the active hormone IAA is a critical step without which hormone treatment is inevitably ineffective. We reasoned that when looking at field selected elite germplasm of a new potential crop, its suitability for propagation might be evaluated for this biochemical trait before further development of the selected material. This approach should thus eliminate the more intractable plant materials that would be ill-suited for mass propagation regimes, either by conventional or micro-propagation approaches. Because specific selected lines and suitable datasets for hybrid hazelnut have as yet not become available, we first evaluated elm as a model system to confirm a possible relationship between IBA to IAA conversion and rooting ability. We also examined a number of hybrid hazelnut selections to ascertain if variability in this biochemical trait was present in these plant materials. The elm cultivar AM, which contained the highest level of [$^{13}C_8$,$^{15}N_1$]IAA after treatment with [$^{13}C_8{}^{15}N_1$]IBA, also had the highest percentage of rooted cuttings and produced the most adventitious roots per cutting in the week 1 rooting trial. Elm cultivars that were found to produce lower levels of [$^{13}C_8{}^{15}N_1$]IAA in this assay, such as AS, generally did not root as well. These data suggest the ability to produce higher levels of IBA-derived IAA may contribute to greater rooting ability in these genotypes. This is consistent with what Alvarez *et al.* (1989) reported, observing significantly higher levels of free IAA in a relatively easy-to-root apple genotype compared to a more difficult-to-root genotype after treatment with IBA. The authors suggested this may be due to a higher level of conversion of IBA to free IAA. We (Baraldi *et al.* 1993; Baraldi *et al.* 1995) previously also observed

differences in IBA-to-IAA conversion between easy- and difficult-to-root pear cultivars, with the difficult-to-root cultivar requiring a tenfold higher concentration of IBA treatment in order to produce a detectable level of IBA-derived IAA. The rate of conversion is likely important not simply related to supplying the IAA necessary for the induction of adventitious roots. Slow and protracted IBA-to-IAA conversion could result in a secondary auxin effect inhibiting root growth (Eliasson *et al.* 1989) or causing the inhibition of root initials by auxin that occurs after an extended period of time (Pop *et al.* 2011).

The inhibitory effects of exogenous auxin have been previously observed in American elm, where higher levels of applied auxin in tissue culture resulted in poorer rooting (Shukla *et al.* 2012). This inhibition is further supported by results from this study showing an increase in rooting in CA after a small increase in IBA-to-IAA conversion and a decrease in rooting in AM after a major increase in auxin conversion. Not only did both AM treatments drop in their rooting success after this increase in IBA-to-IAA conversion, but rooting in treatment 2 was reduced by over 75%, suggesting greater inhibition from the applied auxin. Similarly, the lack of any rooting in week 3 for VF may be due to a lower threshold of IBA-to-IAA conversion, requiring much lower levels of conversion for optimum rooting, especially when compared to other genotypes. This suggests that the relationship between IBA-IAA conversion and rooting success may be best described as an ideal range where rooting is optimized in a specific range for each genotype. Additionally, these results show that rooting success varies

greatly between American elm genotypes and timing of cuttings collection remains a critical factor in determining overall success in propagation.

Propagation of hybrid hazelnut does not have a long history as it is a new potential crop adapted well to cold climates. Unlike elm, pear, apple and other long-cultivated tree crops, hybrid hazelnut has selections that are very difficult to propagate by stem cuttings and others that respond somewhat better. By traditional approaches we have not found cultivars that would be truly classified as 'easy-to-root'; however, previous propagation work in hybrid hazelnuts has shown nevertheless that adventitious rooting is highly variable across different genotypes and environmental conditions. We observed variability in IBA-to-IAA conversion in different hazelnut genotypes, which may account for some of the differences in rooting ability. We assayed five genotypes that were suspected to be generally easier or more difficult-to-root based on limited rooting data provided by other researchers from softwood stem cutting and mound layering propagation trials. However, the greenhouse trials were not well controlled for time-of-harvest and mound layering trials were conducted in the field over the growing season. While no obvious correlation was observed between the level of IBA-derived IAA and previous scoring in these genotypes; it seems likely that various environmental and developmental dissimilarities may have been responsible for some of the differences in rooting and better controlled rooting trials are necessary once a more limited number of promising lines are identified. However, the noted differences in IBA to IAA conversion do suggest that cultivars with poor ability to carry out this process may be candidates to be eliminated

from the set of plant materials that are likely to respond well to IBA treatments to induce adventitious rooting.

IBA-to-IAA conversion may be an important factor regulating formation of adventitious roots; however, it is not the only factor at play in this complex process. Easy-to-root and difficult-to-root cultivars may display differences in transport of applied IBA, as shown in Krisantini *et al.* (2009). Other influences on adventitious root formation include, but are not limited to: nutrient availability, light conditions, age of the plant, cross talk between endogenous hormones, and interactions with microorganisms that may alter nutrient uptake or secrete signaling molecules (reviewed in Davis *et al*. 1988; Bellini *et al.* 2014). Thus, it is expected that analysis of unknown plant materials for their ability to convert IBA to IAA can serve to exclude those plant materials that are likely to respond poorly to IBA treatments, but such a screen will likely not assure that those plants selected will in all cases respond well by forming adventitious roots under any particular set of conditions.

The development of propagation methods can be a labor and time consuming process. Nevertheless, establishment of a woody plant cropping system is highly dependent on the ability to obtain uniform genetic materials expressing a set of desirable traits. We have shown that an important aspect of hormonally induced adventitious rooting is likely the conversion of applied IBA to IAA and have developed a rapid and facile method to screen germplasm for this trait. The assay can be used as an early screen to eliminate plant materials that are

unlikely to respond well to IBA treatment and thus can be used to further focus germplasm development efforts for new woody crop species.

III. Conclusion

As woody perennials, hazelnuts are an important piece of the Forever Green agriculture initiative (Runck *et al.* 2014), providing continuous living cover on the land to protect soil and water quality while simultaneously producing a valuable crop. Efficient vegetative propagation is necessary for the development of a profitable hazelnut industry in the Midwest, and *in vitro* micropropagation has potential to quickly produce vast quantities of genetically identical plants. However, micropropagation and other propagation techniques currently lack the efficiency needed to build the hazelnut industry, largely due to poor and unreliable rooting abilities of most hybrid hazelnut genotypes. Initiation of adventitious roots is major roadblock to propagation many other woody plant species as well; one such example is the American elm.

The aim of this project was to investigate whether conversion of IBA to free IAA could be a limiting factor in the induction of adventitious roots in hazelnut. We quantified the conversion of exogenous IBA to IAA in shoot tissue from a variety of hazelnut and elm cultivars with varying rooting abilities. We found that some genotypes converted much lower levels of exogenous IBA to IAA than others, which could at least partially explain observed differences in adventitious rooting in response to IBA treatment. Our methods of measuring IBA β-oxidation with stable isotope-labeled compounds and GC-MS/MS could be used in the future to quickly identify hazelnut genotypes that are likely to show poor root initiation, which can then be excluded from further breeding efforts. To confirm that assaying for IBA-to-IAA conversion will serve as an effective screen for low

rooting abilities, rooting trials with stem cuttings treated with IBA under controlled conditions can be performed to identify genotypes that show exceptionally good and poor rooting ability, which can then be assayed for IBA-to-IAA conversion to determine whether there are significant differences between the two groups. Clearly, however, plants that cannot effectively form the active auxin IAA from its precursor IBA will not be good candidates to respond efficiently to IBA application.

Developing a deeper understanding of auxin regulation of adventitious root formation may provide insights that could improve propagation efficiency in woody plants. Future directions of this work should include investigating changes in IBA metabolism during adventitious root formation, particularly conversion of IBA to IAA, formation of IBA conjugates, and homeostasis of free IBA levels. Such work will require analysis of these processes in small tissue samples at the site of adventitious root initials, and will improve our knowledge of adventitious rooting which can be applied to hazelnuts as well as other plant species.

Bibliography

Acharya S, Sengar K (2013) Fluorescence spectral studies on indole-3-butyric acid (I-3-BA) in micellar media. Res J Pharm Biol Chem Sci 4:385-391

Achim G, Godeanu I, Baiciu A (2001) Research on clonal propagation of hazelnut in Valcea-Romania. Acta Hort 556:281-286

Al Kaï H, Salesses G, Mouras A (1984) Multiplication *in vitro* du noisetier (*Corylus avellana L*.). Agronomie 4:399-402

Alvarez R, Nissen S, Sutter E (1989) Relationship between indole-3-acetic acid levels in apple (*Malus pumila* Mill) rootstocks cultured *in vitro* and adventitious root formation in the presence of indole-3-butyric acid. Plant Physiol 89:439-443

Bacchetta L, Aramini M, Bernardini C, Rugini E (2008) *In vitro* propagation of traditional Italian hazelnut cultivars as a tool for the valorization and conservation of local genetic resources. HortScience 43:562-566

Baraldi R, Bertazza G, Bregoli A, Fasolo F, Rotondi A, Predieri S, Serafini-Fracassini D, Slovin J, Cohen J (1995) Auxins and polyamines in relation to differential *in vitro* root induction on microcuttings of two pear cultivars. J Plant Growth Regul 14:49-59

Baraldi R, Bertazza G, Predieri S, Bregoli A, Cohen J (1993) Uptake and metabolism of indole-3-butyric acid during the *in vitro* rooting phase in pear cultivars (*Pyrus communis*). Acta Hort 329:289-291

Barkawi L, Cohen J (2010) A method for concurrent diazomethane synthesis and substrate methylation in a 96-sample format. Nat Protoc 5:1619–1626

Barkawi L, Tam Y, Tillman J, Normanly J, Cohen J (2010) A high-throughput method for the quantitative analysis of auxins. Nat Protoc 5:1609-1618

Bartel B, LeClere S, Magidin M, Zolman B (2001) Inputs to the active indole-3-acetic acid pool: *de novo* synthesis, conjugate hydrolysis, and indole-3-butyric acid β-oxidation. J Plant Growth Regul 20:198-216

Bassil N, Mok D, Mok M, Rebhuhn B (1992) Micropropagation of the hazelnut, *Corylus avellana*. Acta Hort 300:137-140

Bellini C, Pacurar D, Perrone I (2014) Adventitious roots and lateral roots: similarities and differences. Annu Rev Plant Biol 65:639-666

Blakesley D (1994) Auxin metabolism and adventitious root initiation. In: Davis T, Haissig E (eds) Biology of Adventitious Root Formation. Springer Science, New York, pp 143-153.

Caboni E, Frattarelli A, Giorgioni M, Meneghini M, Damiano C (2009) Improving micropropagation of hazelnut Italian cultivars through temporary immersion system. Acta Hort 845:255-260

Cheng T (1975) Adventitious bud formation in culture of Douglas fir (*Pseudotsuga menziesii* (MIRB.) Franco). Plant Sci Lett 5:97-102

Cohen J (1984) Convenient apparatus for the generation of small amounts of diazomethane. J Chrom A 303:193-396

Cohen J, Baldi B, Slovin J (1986) $^{13}C_6$-[Benzene ring]-indole-3-acetic acid: a new internal standard for quantitative mass spectral analysis of indole-3-acetic acid in plants. Plant Physiol 80:14-19

Cohen J, Schulze A (1981) Double-standard isotope dilution assay. 1. Quantitative assay of indole-3-acetic acid. Anal Biochem 112:249-257

Davis T, Haissig B, Sankhla N (1988) Adventitious Root Formation in Cuttings (Vol 2 in Advances in Plant Sciences series). Dioscorides Press, Portland, pp 315

De Klerk G-J, van der Krieken W, de Jong J (1999) The formation of adventitious roots: new concepts, new possibilities. *In Vitro* Cell Dev Biol-Plant 35:189-199

Díaz-Sala C, Rey M, Rodríguez R (1990) *In vitro* establishment of a cycloclonal chain from nodal segments and apical buds of adult hazel (*Corylus avellana* L.). Plant Cell Tiss Org 23:151-157

Dinnes D, Karlen D, Jaynes D, Kaspar T, Hatfield J, Colvin T, Cambardella C (2002) Review and interpretation: Nitrogen management strategies to reduce nitrate leaching in tile-drained Midwestern soils. Agron J 94:153-171

Dobránszki J, Teixeira da Silva J (2010) Micropropagation of apple--A review. Biotechnol Adv 28:462-488

Eliasson L, Bertell G, Bolander E (1989) Inhibitory action of auxin on root elongation not mediated by ethylene. Plant Physiol 91:310–314

Epstein E, Lavee S (1984) Conversion of indole-3-butyric acid to indole-3-acetic acid by cuttings of grapevine (*Vitis vinifera*) and olive (*Olea europea*). Plant Cell Physiol 25: 697-703

Epstein E, Ludwig-Müller J (1993) Indole-3-butyric acid in plants: Occurrence, synthesis, metabolism and transport. Physiol Plantarum 88:382-389

Erdogan V, Smith D (2005) Application on rooting of hazelnut layers. HortScience 40:1457-1460

Fawcett C, Wain R, Wightman F (1960) The metabolism of 3-indolylalkanecarboxylic acids, and their amides, nitriles and methyl esters in plant tissues. Proc R Soc Lon B 152:231-254

Fischbach J, Braun L, Demchik M, Wyse D (2011) Hazelnut production potential in the Upper Midwest: A report on hybrid hazelnut yields. Research Bulletin #17, University of Wisconsin Extension, Ashland and Bayfield Counties. Available from: http://www.midwesthazelnuts.org/research.html

Glover J, Reganold J, Bell L, Borevitz J, Brummer E, Buckler E, Cox C, Cox T, Crews T, Culman S, DeHaan L, Eriksson D, Gill B, Holland J, Hu F, Hulke

B, Ibrahim A, Jackson W, Jones S, Murray S, Paterson A, Ploschuk E, Sacks E, Snapp S, Tao D, Van Tassel D, Wade L, Wyse D, Xu Y (2010) Increased food and ecosystem security via perennial grains. Science 328:1638-1639

Hand C, Reed B (2014) Minor nutrients are critical for the improved growth of *Corylus avellana* shoot cultures. Plant Cell Tiss Organ Cult 119:427–439

Holzmueller E, Jose S (2012) Biomass production for biofuels using agroforestry: Potential for the North Central Region of the United States. Agrofor Syst 85:1-10

Jarvis B, Wilson D, Fowler M (1978) Growth of isolated embryonic axes from dormant seeds of hazel (*Corylus avellana* L.). New Phytol 80:117-123

Josiah S (2000) Commercial hazelnuts in Minnesota. University of Minnesota Extension Service. Available from: http://www.extension.umn.edu/food/small-farms/crops/commercial-hazelnuts-in-minnesota/

Kevers C, Hausman J, Faivre-Rampant O, Evers D, Gaspar T (1997) Hormonal control of adventitious rooting: progress and questions. J Appl Bot 71:71–79

Kosenko I, Boyko A, Opalko A, Nebykov M, Tarasenko G (2009) Micropropagation of *Corylus colurna* L. Acta Hort 845:261-266

Krisantini S, Johnston M, Beveridge C, Ross J, Williams R (2009) Rooting, quantification of indole-3-acetic acid and indole-3-butyric acid, and IBA transport in *Grevillea*. Propag Ornam Plants 9: 90-96

Liu X, Barkawi L, Gardner G, Cohen J (2012(a)) Transport of indole-3-butyric acid and indole-3-acetic acid in *Arabidopsis* hypocotyls using stable isotope labeling. Plant Physiol 158:1988-2000

Liu X, Hegeman A, Gardner G, Cohen J (2012(b)) Protocol: High-throughput and quantitative assays of auxin and auxin precursors from minute tissue samples. Plant Methods 8:31. doi:10.1186/1746-4811-8-31

Ludwig-Müller J (2000) Indole-3-butyric acid in plant growth and development. Plant Growth Regul. 32:219-230

Ludwig-Müller J, Vertocnik A and Town C (2005) Analysis of indole-3-butyric acid-induced adventitious root formation on *Arabidopsis* stem segments. J Exp Bot 56:2095–2105

Mehlenbacher S (1991) Hazelnuts (*Corylus*). Genetic resources of temperate fruit and nut crops 290:791-838

Messeguer J, Melé E (1987) *In vitro* propagation of adult material and seedlings of *Corylus avellana*. Acta Hort 212:499-503

Molnar T (2011) *Corylus*. In: Kole C (ed.), Wild Crop Relatives: Genomic and Breeding Resources, Forest Trees. Springer-Verlag, Berlin Heidelberg, pp 15-48

Moncousin C, Favre J and Gaspar T (1988) Changes in peroxidase activity and endogenous IAA levels during adventitious root formation in vine cuttings. In: Kutacek M, Bandurski R and Krekule J (eds) Biochemistry of Auxins in Plants. SPB Academic Publishing, The Hague, pp 331-338

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15:473-497

Nas M (2004) Inclusion of polyamines in the medium improves shoot elongation in hazelnut (*Corylus avellana* L.) micropropagation. Turk J Agric For 28:189-194

Nas M, Read P (2001) Micropropagation of hybrid hazelnut: medium composition, physical state and iron source affect shoot morphogenesis, multiplication and explant vitality. Acta Hort 556:251-258

Nordström A, Jacobs F, Eliasson L (1991) Effect of exogenous indole-3-acetic acid and indole-3-butyric acid on internal levels of the respective auxins and their conjugation with aspartic acid during adventitious root formation in pea cuttings. Plant Physiol 96:856-861

Pellett H, Davis D, Joannides J, Luby J (1998) Positioning hazels for large-scale adoption. A report prepared for the Minnesota Agricultural Research Institute by the Univ. of MN's Center Center for Integrated Natural Resources and Agricultural Management

Pérez C, Rodriguez A, Revilla A, Rodriguez R, Sánchez-Tamés R (1987) Filbert plantlet formation through *in vitro* culture. Acta Hort 212:505-510

Pérez C, Rodríguez R, Tamés R (1985) *In vitro* filbert (*Corylus avellana* L.) micropropagation from shoot and cotyledonary node segments. Plant Cell Rep 4:137-139

Pijut P, Woeste K, Michler C (2011) Promotion of adventitious root formation of difficult-to-root hardwood tree species. Hort Rev 38:213-251

Pop T, Pamfil D, Bellini C (2011) Auxin control in the formation of adventitious roots. Not Bot Hort Agrobot Cluj 39:307-316

Post W, Kwon K (2000) Soil carbon sequestration and land-use change: Processes and potential. Global Change Biol 6:317-327

Richardson D (1997) The health benefits of eating hazelnuts: implications for blood lipid profiles, coronary heart disease, and cancer risks. Acta Hort 445:295-300

Runck B, Kantar M, Jordan N, Anderson J, Wyse D, Eckberg J, Barnes R, Lehman C, DeHaan L, Stupar R, Sheaffer C, Porter P (2014) The reflective

plant breeding paradigm: A robust system of germplasm development to support strategic diversification of agroecosystems. Crop Sci 54:1939–1948

Rutter P (1988) Reducing Earth's greenhouse CO_2 through shifting staples production to woody plants. Proceedings of the second North American conference on preparing for climate change; Climate Institute, Washington DC. Arbor Day Foundation. Available from: http://www.arborday.org/globalwarming/paperReducingCO2.cfm

Shukla M, Jones A, Sullivan J, Liu C, Gosling S, Saxena P (2012) *In vitro* conservation of American elm (*Ulmus americana*): potential role of auxin metabolism in sustained plant proliferation. Can J For Res 42:686-697

Strader L, Bartel B (2011) Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid. Mol Plant 4:477-486

Strader L, Wheeler D, Christensen S, Berens J, Cohen J, Rampey R, Bartel B (2011) Multiple facets of *Arabidopsis* seedling development require indole-3-butyrc acid-derived auxin. Plant Cell 23:984-999

Strader L, Culler A, Cohen J, Bartel B (2010) Conversion of endogenous indole-3-butyric acid to indole-3-acetic acid drives cell expansion in *Arabidopsis* seedlings. Plant Physiol 153:1577-1586

Sutter E, Cohen J (1992) Measurement of indolebutyric acid in plant tissues by isotope dilution gas chromatography mass spectrometry analysis. Plant Physiol 99:1719-1722

Thimann K, Behnke-Rogers J (1950) The use of auxins in the rooting of woody cuttings. Harvard Forest, Petersham, pp 344

Thimann K, Went F (1934) On the chemical nature of the root forming hormone. Proc K Ned Akad Wet Ser C Biol Med Sci 37:456-459

Thomson G, Deering T (2011) Effect of cytokinin type and concentration on in vitro shoot proliferation of hazelnut (*Corylus avellana* L.). N Z J Crop Hortic Sci 39:209-13

United States Department of Agriculture National Agricultural Statistics Service (USDA NASS) (2013), Minnesota county estimates. Available from: http://www.nass.usda.gov/Statistics_by_State/Minnesota/Publications/County_Estimates/.

Van der Krieken W, Breteler H, Visser M, Mavridou D (1993) The role of the conversion of IBA into IAA on root regeneration in apple: introduction of a test system. Plant Cell Rep 12:203-206

Woodward A, Bartel B (2005(a)) Auxin: regulation, action, and interaction. Ann Bot 95:707-735

Woodward A, Bartel B (2005(b)) The *Arabidopsis* peroxisomal targeting signal type 2 receptor PEX7 is necessary for peroxisome function and dependent on PEX5. Mol Biol Cell 16:573-83

Xu Y, Hanna M, Josiah S (2007) Hybrid hazelnut oil characteristics and its potential oleochemical application. Ind Crop Prod 26:69-76

Yu X, Reed B (1993) Improved shoot multiplication of mature hazelnut (*Corylus avellana* L.) *in vitro* using glucose as a carbon source. Plant Cell Rep 12:256-259

Yu X, Reed B (1995) A micropropagation system for hazelnuts (*Corylus* species). HortScience 30:120-123

Zeldin E, McCown B (2011) Micropropagation of American hazelnut - promise and potential. Hazelnut News 2:4-5. Available from: http://www.midwesthazelnuts.org/assets/files/July%202011%20Newsletter.pdf

Zolman B, Bartel B (2004) An *Arabidopsis* indole-3-butyric acid-response mutant defective in PEROXIN6, an apparent ATPase implicated in peroxisomal function. Proc Natl Acad Sci USA 101:1786-1791

Zolman B, Martinez N, Millius A, Adham A, Bartel B (2008) Identification and characterization of *Arabidopsis* indole-3-butyric acid response mutants defective in novel peroxisomal enzymes. Genetics 180:237-251

Zolman B, Nyberg M, Bartel B (2007) IBR3, a novel peroxisomal acyl-CoA dehydrogenase-like protein required for indole-3-butyric acid response. Plant Mol Biol 64:59-72

Zolman B, Silva I, Bartel B (2001) The *Arabidopsis pxa1* mutant is defective in an ATP-binding cassette transporter-like protein required for peroxisomal fatty acid β-oxidation. Plant Physiol 127:1266-1278

Zolman B, Yoder A, Bartel B (2000) Genetic analysis of indole-3-butyric acid responses in *Arabidopsis thaliana* reveals four mutant classes. Genetics 156:1323-1337