Propagation of Hazelnut Stem Cuttings Using *Agrobacterium rhizogenes*

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Abstract. Hazelnut (*Corylus avellana* L.) softwood cuttings of the cultivars Ennis and Casina were propagated under mist during June and July 1987 and 1988. Rooting of stem cuttings was stimulated by both *Agrobacterium* and IBA treatment; however, IBA caused nearly complete bud abscission. Better rooting and bud retention were observed in 'Casina' than in 'Ennis' in 1988. Bud retention on *Agrobacterium*-inoculated cuttings improved as the cuttings approached the semi-hardwood stage. Six months after transplanting, *Agrobacterium*-inoculated hazelnut cuttings had an extensive root system, characteristic of hairy root. Although the mechanism remains unclear, strains of *Agrobacterium rhizogenes* are effective rooting agents in hazelnut and may cause less bud abscission than IBA. Chemical name used: 1 *H*-indole-3-butyric acid (IBA).

Corylus avellana is propagated primarily by the use of simple layers. Layerage is used because of the difficulties inherent in other methods of propagation that would otherwise be preferred (Lagerstedt, 1970). Propagation of hazelnut by stem cuttings is limited either by poor root initiation (Bergougnoux et al., 1976; Falaschi and Loreti, 1969) or by abscission of the vegetative buds on otherwise well-rooted cuttings (Lagerstedt, 1970, 1982). One of our approaches to this problem was to study the effects of *Agrobacterium rhizogenes* on rooting and bud retention.

A. rhizogenes induces formation of adventitious roots at sites of infection, resulting in a hairy-root phenotype. This extensive root formation is caused by expression of *A. rhizogenes* genes encoded by T-DNA, a fragment of DNA originating from a root-inducing plasmid (Ri) (Moore et al., 1979). The T-DNA is transferred to the host, stably integrated in the plant genome, and expressed (Zambryski, 1988; Zambryski et al., 1989). The genetically transformed plant produces indoleacetic acid and usually opines, as well as adventitious roots (Chilton et al., 1982; Willmitzer et al., 1982).

Shoots of 'Ennis' and 'Casina' hazelnut were collected from lo-year-old trees in July 1987 and June and July 1988. Cuttings consisted of shoot terminals with the apex, one or two expanding leaves, one expanded leaf, and a leafless node at the base. Cuttings were stored overnight at 5°C. The following day, cuttings were dipped for 5 sec into a 50%

ethanol solution of IBA (12.5 mM in 1987, 5 mM in 1988) or in 50% EtOH. Treated cuttings were allowed to air dry and then dipped in water or bacterial suspension and placed in a medium consisting of 5 coarse quartz sand : 1 peat (v/v). Mist frequency was regulated by a light-regulated controller (Solatrol, General Scientific Equipment, Hamden, Conn.) and time switch.

The bacterial strains used in this study were from the collection of L. Moore. *A. rhizogenes* strains were used singly and in combination (A2 + 23, A7 + 22) to inoculate hazelnut cuttings and carrot slices. Strain X-64 resulted from curing strain A4 of its Ri plasmid and served as an avirulent control. The inoculum was applied in the form of a bacterial suspension comprising 1.2 to 3.1 × 10⁸ colony-forming units/ml.

Each treatment consisted of 30 cuttings, two replicates of 15 cuttings each in randomized complete blocks. Cuttings were evaluated in October for root formation and bud retention. Where analysis of variance indicated significant treatment effects, Student's

t test was used to compare means of treatments or groups of particular interest.

Four strains of *A. rhizogenes* were tested in 1987 for their effect on rooting and bud retention on 'Ennis' hazelnut stem cuttings with and without IBA treatment. The bacteria alone generally stimulated more rooting than treatments containing IBA, but this difference was not statistically significant (Tables 1 and 2). All inoculations with virulent bacteria induced greater rooting than water and avirulent bacteria. The difference in rooting percentage between the treatment with bacteria and the water control was significant at *P* < 0.03. Treatments with bacteria plus IBA also induced greater rooting than the controls, although *P* < 0.06. Bud retention was significantly lower on all IBA-treated cuttings than on cuttings not treated with IBA. As a result, all treatments but one with virulent bacteria alone had a higher percentage of cuttings with both roots and buds than the corresponding bacteria/IBA treatment. Averaged over all treatments, this effect was highly significant. Although 43% of the water-treated and 60% of the avirulent-treated cuttings had buds, only 3.3% of the cuttings in these treatments had both roots and buds. None of the cuttings treated with IBA alone had buds.

'Ennis' and 'Casina' were propagated in Summer 1988. Although rooting percentages were similar in both cultivars, there were marked differences in bud retention. 'Ennis' stem cuttings were propagated on seven dates in 1988. Rooting of bacteria- or IBA-treated cuttings remained >50% throughout the sampling period, whereas rooting of the untreated cuttings declined significantly by 6 July (*P* < 0.001) (Table 3). Over this entire period, cuttings inoculated with bacteria alone rooted significantly better than with bacteria plus IBA (*P* < 0.001). In contrast, bud retention on 'Ennis' cuttings in all treatments was < 15% on all dates sampled. There was a small increase in bud retention on the water- and bacteria-treated cuttings on the last date, which was the same date as the 1987 experiment. This increase was statistically significant, because bud retention on all IBA-treated

Table 1. Effect of *Agrobacterium rhizogenes* and IBA on rooting percentage, percentage of cuttings with one or more buds, and percentage of rooted cuttings with one or more buds of hazelnut 'Ennis' stem cuttings propagated on 26 July 1987.

Treatment	Roots (%)	Buds (%)	Roots + buds (%)
Water	16.7	43.3	3.3
IBA, 12.5 mM	10.0	0	0
X-64	6.7	60.0	3.3
X-64 + IBA	20.0	0	0
A2	36.7	50.0	23.3
A2 + IBA	26.7	13.3	6.7
23	46.7	63.3	43.3
23 + IBA	40.0	6.7	3.3
A2 + 23	46.7	36.7	16.7
A2 + 23 + IBA	40.0	23.3	20.0
A7	30.0	63.3	16.7
A7 + IBA	26.7	13.3	6.7
22	30.0	53.3	13.3
22 + IBA	46.7	16.7	10.0
A7 + 22	56.7	53.3	30.0
A7 + 22 + IBA	46.7	10.0	6.7

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Table 2. Significance of comparisons, determined by *t* test, between treatment effects on rooting percentage (Roots), percentage of cuttings with one or more buds (Buds), and percentage of rooted cuttings with one or more buds (Roots + buds) of hazelnut 'Ennis' propagated in 1987.

Treatment	Treatment		
	Water	IBA	Bacteria
	<i>Roots</i>		
IBA	NS		
Bacteria	*	**	
Bacteria + IBA	NS	*	NS
	<i>Buds</i>		
IBA	***		
Bacteria	NS	***	
Bacteria + IBA	**	NS	***
	<i>Roots + buds</i>		
IBA	NS		
Bacteria	**	**	
Bacteria + IBA	NS	NS	***

NS, ***, ***Nonsignificant and significant difference at *P* = 0.05, 0.01, and 0.001, respectively.

Fig. 1. Phenotype of roots treated with IBA (A) or *Agrobacterium rhizogenes* (B). Note difference in density of root systems.

cuttings was zero. Since bud retention was low, the number of cuttings with both buds and roots was also low ($\geq 10\%$), with similar statistical significance. On 'Casina', bacteria-, IBA-, and bacteria/IBA-treated cuttings rooted significantly better than water-treated cuttings (Table 4), with $P < 0.001$ in each case. Bud retention on cuttings treated with IBA was significantly lower than the water- ($P < 0.001$) or bacteria-treated ($P < 0.001$) cuttings without IBA averaged over all sampling dates. As a consequence, the percentage of cuttings with both roots and buds was significantly greater over all sampling dates in the water- ($P < 0.01$) and bacteria-treated ($P < 0.001$) cuttings than in treatments with IBA. By the last sampling date (6 July), the two treatments with bacteria only resulted in a mean of 65% of the cuttings having both roots and buds, significantly greater ($P < 0.001$) than the water inoculation on that date. Averaged over the three sampling dates, the percentage of cuttings with both roots and buds in the water and bacterial treatments was not significantly different.

Table 3. Effect of *Agrobacterium rhizogenes* and IBA on rooting percentage, percentage of cuttings with one or more buds, and percentage of rooted cuttings with one or more buds of hazelnut 'Ennis' stem cuttings propagated in 1988.

Treatment	Roots (%)	Buds (%)	Roots + buds (%)
<i>3 June</i>			
Water	96.7	10.0	10.0
IBA, 5 mM	90.0	0	0
A2 + 23	66.7	3.3	0
A2 + 23 + IBA	53.3	0	0
A7 + 22	100.0	0	3.3
A7 + 22 + IBA	56.7	0	0
<i>14 June</i>			
Water	56.7	3.3	3.3
IBA, 5 mM	96.7	0	0
A2 + 23	96.7	0	0
A2 + 23 + IBA	76.7	0	0
A7 + 22	90.0	0	0
A7 + 22 + IBA	63.3	0	0
<i>23 June</i>			
Water	73.3	0	0
IBA, 5 mM	76.7	0	0
A2 + 23	76.7	3.3	3.3
A2 + 23 + IBA	80.0	0	0
A7 + 22	96.7	0	0
A7 + 22 + IBA	93.3	0	0
X-64	56.7	0	0
X-64 + IBA	66.7	0	0
<i>6 July</i>			
Water	26.7	3.3	3.3
IBA, 5 mM	86.7	0	0
A2 + 23	86.7	0	0
A2 + 23 + IBA	86.7	0	0
A7 + 22	86.7	0	0
A7 + 22 + IBA	76.7	0	0
<i>15 July</i>			
Water	46.7	0	0
IBA, 5 mM	83.3	0	0
A2 + 23	83.3	0	0
A2 + 23 + IBA	53.3	0	0
A7 + 22	76.6	0	0
A7 + 22 + IBA	50.0	0	0
X-64	10.0	0	0
X-64 + IBA	66.7	0	0
<i>26 July</i>			
Water	16.7	10.0	6.7
IBA, 5 mM	53.3	0	0
A2 + 23	86.7	13.3	10.0
A2 + 23 + IBA	56.7	0	0
A7 + 22	76.7	10.0	3.3
A7 + 22 + IBA	70.0	3.3	3.3
X-64	36.7	0	0
X-64 + IBA	56.7	0	0

Once graded, the rooted cuttings were transplanted and grown. The following spring, the root systems of the cuttings inoculated with bacteria were uniformly much more dense than those of the controls, a characteristic of hairy root (Fig. 1). The roots on the IBA- and water-treated cuttings appeared normal and lacked the extensive development of secondary roots found on plants originally inoculated with bacteria. Samples of homogenized roots were streaked on manitol-glutamate-yeast extract and biovar-2 plates to test for the presence of *Agrobacterium*. After two days of incubation at 25C, *Agrobacterium* colonies appeared on both plates streaked from samples of *Agrobacterium*-inoculated tissue, but not from untreated tissue. Therefore, there was no or very little cross-contamination in the beds.

As reported by Lagerstedt (1982), bud abscission is a serious obstacle to propagation of hazelnut stem cuttings, even though the rooting percentage may be acceptable. Our results demonstrated that *A. rhizogenes* inoculation can mitigate this problem, stimulating root initiation to a degree comparable to IBA without increasing bud abscission. The bacteria did not, however, ameliorate bud abscission induced by IBA treatment. The avirulent strain of *Agrobacterium* failed to induce rooting, evidence that T-DNA is required for this response. The effects on cutting development were influenced by both cultivar and date. 'Ennis' was more prone to bud abscission than 'Casina' and varied markedly in the 2 years tested. Bud retention in both cultivars improved as the shoot matured.

Table 4. Effect of *Agrobacterium rhizogenes* and IBA on rooting percentage, percentage of cuttings with one or more buds, and percentage of rooted cuttings with one or more buds of hazelnut 'Casina' stem cuttings propagated in 1988.

Date of sampling	Treatment	Roots (%)	Buds (%)	Roots + buds (%)
14 June	Water	26.7	20.0	20.0
	IBA, 5 mM	80.0	0	0
	A2 + 23	90.0	13.3	13.3
	A2 + 23 + IBA	60.0	0	0
	A7 + 22	100.0	43.3	43.3
23 June	A7 + 22 + IBA	73.3	3.3	3.3
	Water	50.0	20.0	20.0
	IBA, 5 mM	93.3	23.3	23.3
	A2 + 23	73.3	16.7	16.7
	A2 + 23 + IBA	86.7	0	0
	A7 + 22	80.0	0	0
	A7 + 22 + IBA	80.0	0	0
6 July	X-64	60.0	16.7	16.7
	X-64 + IBA	90.0	0	0
	Water	20.0	20.0	20.0
	IBA, 5 mM	80.0	0	0
	A2 + 23	90.0	70.0	70.0
	A2 + 23 + IBA	70.0	5.0	5.0
	A7 + 22	95.0	60.0	60.0
	A7 + 22 + IBA	75.0	0	0

There are several possible reasons for the lower bud retention on 'Ennis' cuttings propagated in 1988 than in 1987. Differences in development may account for the different rooting each year. Determining whether bud retention improves as the shoot matures, without concomitant loss of rooting potential, would help assess the practicality of *A. rhizogenes* inoculations in propagating hazelnut. Another difference between 1987 and 1988 was the change in IBA concentration from 12.5 mM to 5 mM, the latter recommended by Lagerstedt (1982). Lowering the

IBA concentration, however, likely did not account for the observed effects on bud abscission.

Whether *A. rhizogenes* transformed filbert tissue is unclear. Our initial molecular analyses for T_R- and T_L-DNA and the presence of opines were equivocal. Regardless of the mechanism of root initiation, our results demonstrate that strains of *A. rhizogenes* can improve propagation of hazelnut stem cuttings. In itself, the bacterium is an effective rooting agent. Furthermore, the effect of *A. rhizogenes* can be distinguished from IBA

by reduced bud abscission, the factor limiting propagation of hazelnut.

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