Adventitious Root Initiation in De-bladed Petioles from the Juvenile and Mature Phases of English Ivy

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Abstract. An in vitro system has been developed to study adventitious root initiation in the juvenile and mature phases of English ivy (Hedera helix L.). The system uses de-bladed petiole explants cultured in a defined liquid medium. Adventitious roots are visible macroscopically after 18 days. Juvenile petiole explants show a dose-response to auxin application with optimal root initiation at 100 μM NAA or IAA. With optimal auxin concentration, root initials form in juvenile petiole explants directly from cortical parenchyma cells, which involves induction (1–6 days), meristem organization (6–9 days), and root elongation stages (9–18 days). Sucrose is required for outgrowth of root primordia but not for initiation of primordia. Mature petiole explants respond to auxin with random cell divisions in cortical parenchyma cells; root initials form at a low frequency from callus resulting from this cortical cell division. Distribution of 14C at various times after administration of 14C-labeled NAA is similar in juvenile and mature petioles. Because of their difference in rooting potential, coupled with similarity in anatomical organization, distribution of 14C from NAA, and identical genotype, juvenile and mature petioles provide an excellent experimental system for analyzing the morphogenetic, physiological, and genetic basis of rooting potential. Chemical names used: 1-naphthaleneacetic acid (NAA); 1H-indole-3-acetic acid (IAA).

During the life cycle of a woody plant, two distinct phases of development are observable (17). The gradual transition from a juvenile to a mature state is termed phase change (3) or ontogenetic aging (8). The juvenile phase of development begins during embryo formation and proceeds through vegetative growth after germination. The juvenile phase is described as a period of development in a woody plant when flowering does not occur or cannot be induced by normal floral initiation conditions or treatments (13). The juvenile phase is characterized by ease of root initiation during cutting propagation (15).

The inability to vegetatively propagate superior clones selected during the mature phase of development seriously limits the introduction of superior clones into production forestry and horticulture. Many researchers have documented a loss or decrease in root initiation potential for mature cuttings when compared to juvenile cuttings of the same species (5, 9, 20, 21, 23). Systems have been developed to study and compare easy-to-root and difficult-to-root stages of the same species (5, 6, 12, 19).

The importance of inclusion of an easy-to-root juvenile and a difficult-to-root mature phase for studying the physiology of root initiation was demonstrated in vitro by Hackett (12) using mature shoot apex cuttings of English ivy. He found that co-factors extracted from juvenile English ivy tissue promoted rooting in easy-to-root juvenile ivy shoot apex cuttings as in mung bean hypocotyl cuttings (16), but mature English ivy shoot apices did not root in response to these co-factors (12). Thus, difficult-to-root tissue is needed as a rooting assay when studying factors controlling root initiation in such tissue.

An in vitro experimental system to study adventitious root initiation has been developed using de-bladed petioles from a clone of English ivy. This system is advantageous for studying root initiation because it compares root initiation in cuttings taken from juvenile and mature phase plants representing a single (presumably genetically uniform) clone expressing two phenotypes with distinctly different morphology and morphogenetic potential.

Materials and Methods

Stock plants

Stock plants from the mature and juvenile phase of an English ivy clone were maintained under greenhouse conditions. Juvenile stock plants were grown in 15-mm clay pots and trained to a single stem. Mature stock plants were maintained as multiple stemmed plants in 20-liter containers. Stock plants were pruned back periodically to maintain vegetative vigor. Fertilizer was applied in the irrigation water as a half-strength Hoagland's solution. Stock plants were shaded with Saran cloth from May through October to equalize seasonal light intensity at about 700–800 μmol·s−1·m−2.

Petiole rooting system

Petioles from mature and juvenile stock plants were selected for use when the new lamina was determined to have just completed expansion. Upon removal from the stock plant, the lamina was immediately detached from the petiole and the de-bladed petioles were maintained in deionized water until prepared for assay use. After rinsing in deionized water, petioles were surface-sterilized by agitation for 10 min in a solution of 0.5% sodium hypochlorite and 0.1% Alconox detergent. They were then rinsed three times with autoclaved deionized water. Each petiole was aseptically trimmed by removing the flanged petiole base plus a section from the blade end to give a standard 23-mm length. Trimmed petioles were aseptically cultured in a 25-ml Erlenmeyer flask on filter paper saturated with the nutrient medium. Each flask was fitted with a translucent plastic retainer to hold the petioles upright. The plastic retainer was made by

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cutting a 40 x 20 mm segment from polypropylene bottles (Nalgene). Five 3-mm-diameter holes were drilled into the central portion of the plastic segment. The plastic segment was folded at a right angle 7 mm from each end of its long dimension to form a bridge shape (Fig. 1). The plastic retainer was forced into the 25-ml Erlenmeyer flask. Whatman #1 filter paper was cut into 250-mm² pieces and three pieces were inserted under the retainer in each Erlenmeyer flask. A 1-ml aliquot of Romberg medium as modified by Hackett (12), including the test components (NAA, IAA, sucrose), was added to each flask. Each Erlenmeyer flask was closed with an aluminum foil cap and autoclaved at 121°C. De-bladed petioles were aseptically inserted into the retainer with the morphological base of the petiole contacting the medium-saturated filter paper.

The Erlenmeyer flasks were placed in a single growth chamber maintained at a constant day/night temperature of 21°C and =80% RH. A daylength of 16 hr light and 8 hr dark was maintained with cool-white fluorescent lights giving a photon flux density of 200 μmol·s⁻¹·m⁻² at the level of the flasks. The experiments were terminated after 18 days, when adventitious roots were visible through the epidermis in juvenile petioles.

### Distribution of NAA or its metabolites and root initiation after exposure to NAA for various time periods

Juvenile and mature petioles were incubated on a medium with 100 μM NAA for 0, 1, 3, 5, 7, 9, and 18 days and then transferred to a medium without NAA. Petioles were evaluated for root initiation after 18 days. To evaluate distribution of NAA or its metabolites, juvenile and mature petioles were incubated for various times on a medium containing 100 nmol per flask of radiolabeled NAA (α-naphthaleneacetic acid-carboxyl-¹⁴C) with a specific activity of 2.5 mCi/mmol (1 Ci = 37 GBq; Pathfinder Lab, St. Louis). The petioles were removed from the medium and rinsed in deionized water to remove residual media on the surface of the petiole. The petioles were cut into three equal sections (7 mm) and dried for 48 hr at 50°C. The dried samples were combusted in a Packard Tri-Carb 306 sample oxidizer and the collected ¹⁴CO₂ was counted in a Beckman LS 9000 liquid scintillation system.

A nested classification experimental design was used in conjunction with the analysis of variance and means were separated with the LSD test. Data are presented as the mean number of roots per cutting for 10 petioles from two flasks of each treatment. The results are representative of replicated experiments.

### Histological preparation

Mature and juvenile petioles were harvested at 1, 3, 6, 9, and 12 days after incubation in the assay medium with and without 100 μM NAA. The lower 10 mm of petiole was fixed in FAA and dehydrated with 2,2-dimethoxypropane (18) and an acetone/chloroform dehydration series. The petioles were embedded in Tissueprep and 10-μm sections were cut with a standard microtome. Sections were stained with safranin and aniline blue.

### Dose response to auxin

#### Juvenile

Both NAA and IAA stimulated adventitious rooting in juvenile petioles (Table 1). The average number of roots increased with increasing concentration of auxin from 10 to 100 μM. There was no stimulation of rooting at 0, 0.1, or 1 μM NAA or IAA. Concentrations >100 μM of both IAA and NAA depressed rooting and 1000 μM prevented rooting. NAA was more stimulatory to rooting than IAA at any given concentration from 10 to 100 μM.

#### Mature

Mature petioles did not form roots in response to auxin (Table 1). During the course of repeated experiments with mature petioles treated with NAA at 100 μM, only 15 of 815 petioles (1.8%) showed any capacity to form adventitious roots and, in these cases, only one or two roots formed per petiole.

### Root initiation in response to sucrose concentration

For juvenile petioles treated with 100 μM NAA, the concentration of sucrose in the medium significantly affected the average number of visible roots per petiole (Table 2). Of the concentrations tested, the optimal concentration of sucrose was 2%. No visible roots were apparent in petioles incubated on a medium without sucrose. Microscopic observation revealed that root primordia had developed without sucrose and at all sucrose concentrations. However, root primordia failed to elongate on a medium without sucrose (Fig. 2). Sucrose concentration did not affect root initiation in mature petioles (data not shown).

### Table 1. Rooting response of juvenile petioles treated with IAA and NAA cultured on a modified Romberger medium (2% sucrose).

<table>
<thead>
<tr>
<th>Auxin concn (μM)</th>
<th>No. roots per petiole</th>
<th>Juvenile</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IAA</td>
<td>NAA</td>
<td>IAA</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.5 c</td>
<td>1.2 c</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>1.3 c</td>
<td>7.3 b</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>6.4 b</td>
<td>9.4 a</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>4.6 b</td>
<td>0.8 c</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not significantly different at the 0.05 level.

Fig. 1. The de-bladed petiole rooting system. Illustrated is the plastic retainer bridge holding rooted juvenile petioles treated with 100 μM NAA.
Table 2. Rooting of juvenile petioles treated with 100 μM NAA in response to sucrose concentration.

<table>
<thead>
<tr>
<th>Sucrose concn (%)</th>
<th>No. roots per petiole&lt;br&gt;2&lt;br&gt;</th>
<th>&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1 c</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.8 b</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9.3 a</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.9 a</td>
<td></td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not significantly different at the 0.05 level.

Fig. 2. Cross section of a juvenile petiole treated with 100 μM NAA on a medium without sucrose. Root primordia (arrow) have formed but failed to elongate. Reference bar represents 200 μm.

Table 3. Root initiation in juvenile petioles exposed to 100 μM NAA for varying periods of time.

<table>
<thead>
<tr>
<th>Days of exposure to NAA</th>
<th>No. roots per petiole&lt;sup&gt;2&lt;/sup&gt;</th>
<th>&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.9 d</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.6 c</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.3 bc</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5.8 b</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>6.1 b</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>9.4 a</td>
<td></td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not significantly different at the 0.05 level.

Distribution of NAA or its metabolites and root initiation after exposure to NAA for various time periods

The number of days of exposure to 100 μM NAA affected the adventitious root initiation response of juvenile petioles. Increasing exposure time of petioles to NAA for up to 18 days increased root initiation (Table 3), but length of exposure to NAA did not affect root initiation in mature petioles (data not shown).

There was a similar distribution of 14C from NAA within the petiole of juvenile and mature petioles expressed on a per-segment basis (Table 4). The amount of 14C from NAA within the petiole increased with increasing exposure time. More than 90% of the 14C from NAA was localized in the basal one-third of juvenile and mature petioles.

Table 4. Distribution of 14C in juvenile and mature petioles at various times after administration of 14C-labeled NAA.

<table>
<thead>
<tr>
<th>Days</th>
<th>Top&lt;br&gt;nmol of 14C from NAA/segment&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Middle&lt;br&gt;</th>
<th>Bottom&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10 (0.04)</td>
<td>0.16 (0.05)</td>
<td>3.76 (1.17)</td>
</tr>
<tr>
<td>3</td>
<td>0.43 (0.14)</td>
<td>0.50 (0.21)</td>
<td>9.37 (1.13)</td>
</tr>
<tr>
<td>5</td>
<td>0.17 (0.04)</td>
<td>0.18 (0.05)</td>
<td>13.11 (0.61)</td>
</tr>
<tr>
<td>7</td>
<td>0.31 (0.09)</td>
<td>0.14 (0.07)</td>
<td>18.18 (3.43)</td>
</tr>
<tr>
<td>9</td>
<td>0.57 (0.08)</td>
<td>0.52 (0.06)</td>
<td>17.17 (1.18)</td>
</tr>
</tbody>
</table>

*Mean values with se in parentheses.

Histological observations

**Juvenile.** The first cell divisions in response to auxin occurred at day 6 and were first visible in the epithelial cells associated with ducts that were adjacent to the vascular bundles (Fig. 3b). Observations at day 9 showed localized cell division in the inner cortical parenchyma cells adjacent to the vascular bundle (Fig. 3c). By day 12, root primordia with well-defined meristems and vascular systems were observable (Fig. 3d). Cell divisions were also initiated in the outer cortical parenchyma cells basal to the plane of cell divisions involved in root initiation. These divisions were not directly involved in root primordia formation. Juvenile petioles not treated with NAA showed little or no cell division during the assay period (Fig. 3e).

**Mature.** Although mature petioles initiated root primordia at a very low frequency, they did show a substantial cell division response to auxin. The first cell division was observable at day 6 in the epithelial cells of ducts adjacent to vascular bundles (Fig. 4b). By day 9, unorganized cell division had occurred throughout the cortical parenchyma cells (Fig. 4c). The cell divisions appeared to be random in orientation and did not result in formation of an organized meristem or subsequent root initial. Little or no cell division was evident in mature petioles not treated with NAA (Fig. 4e). Histological investigation of 18-day-old mature petioles that did initiate root primordia revealed that the root initials lacked vascular connections to the vascular bundle and appeared to originate from cell divisions in cortex-derived callus (Fig. 4d).

The ducts observed in both juvenile and mature petioles occurred just exterior to each of seven vascular bundles in cortex tissue. At the base of the petioles where rooting occurred, there was a single duct adjacent to each vascular bundle.

Discussion

English ivy de-bladed petioles are an excellent experimental system to study the physiology of root initiation in an easy-to-root juvenile phase and a difficult-to-root mature phase of a woody perennial. Previous work has established that mature English ivy stem cuttings are difficult to root even in the presence of auxin (10, 16). The concentrations of NAA and IAA giving optimum rooting of juvenile petioles (Table 1), and the concentration range over which rooting is stimulated, are similar to those reported for juvenile English ivy shoot apices, indicating a similar response to auxin (12). Auxin does not stimulate...
Fig. 3. Cross sections of in vitro-cultured juvenile de-bladed petioles. (a–d) Treated with 100 μM NAA at day 0. (a) Day 0. (b) Day 6, divisions were evident in the epithelial cells surrounding a duct (arrow). (c) Day 9, root initials were evident (black arrows). Localized cell divisions occurred first in the cortical parenchyma cells adjacent to vascular bundles (white arrow). (d) Day 12, the root primordia were well-formed, with a defined meristem and a vascular system. (e) Juvenile petiole not treated with NAA showed no observable cell divisions by day 18. Reference bars represent 200 μm.

root initiation in mature petioles or shoot apices. These results provide good justification for using de-bladed petioles as an explant source having similar rooting potential as stem cuttings or shoot apices of English ivy.

Induction of rooting (Table 1) or stimulation of cell division (Figs. 3e and 4e) was not observed in mature or juvenile petioles without auxin. This result indicates a low endogenous level of auxin in de-bladed petioles. Sucrose was not required for root primordium initiation, but appeared to be necessary to support growth of the root primordium once it had been initiated (Table 2, Fig. 2).

The distribution of 14C from NAA within the petiole (Table 3) did not account for the different rooting potentials observed for the mature and juvenile phases of English ivy. The anatomies of the juvenile and mature petioles at day 1 were very similar (Figs. 3a and 4a). The first cells to divide in petioles of both phases were the epithelial cells surrounding the ducts (Figs. 3b and 4b). Smith and Thorpe (25) observed that adventitious
root initiation in *Pinus radiata* seedlings was associated with divisions of the cells of the developing resin ducts. The epithelial cells of English ivy should not be considered initial cells for the entire population of cells from which the juvenile root primordia were formed. These epithelial cells appeared to be more sensitive to the auxin treatment and divided earlier than the surrounding parenchyma cells. This rapid response may be explained by several possibilities that may or may not have casual significance in the root initiation process: 1) auxin may reach these cells prior to diffusing to adjacent parenchyma cells; 2) the epithelial cells may be more meristematic and predisposed to cell division; and 3) some material in the duct is involved in
the predisposition for early division. Early division of epithelial cells was probably not critical to root initiation since such division does not result in root formation in mature petioles.

The major difference in the responses of petioles from juvenile and mature plants to auxin treatments was that, by day 9, the juvenile petioles showed localized, anticlinal cell divisions in cortical parenchyma cells adjacent to vascular bundles leading to root primordia (Fig. 3c). The mature petioles exhibited extensive random cell divisions throughout the cortex (Fig. 4d). Root primordia were not formed in mature petioles adjacent to vascular bundles, as was the case with juvenile petioles. In a small percentage of mature petioles, roots were initiated from the callus formed from dividing cortical cells (Fig. 4d). These roots lack vascular connections to petiole vascular bundles, at least initially. This origin of roots in mature tissue was not unexpected, since Girouard (10) observed root primordia forming in callus from mature stem cuttings of English ivy. Root primordia can be initiated from callus of many difficult-to-root species (2, 4, 5, 22).

Adventitious root initiation has been described as a sequential process with several stages of development (1, 4, 7, 11, 24).

In juvenile petioles of English ivy, three stages of adventitious root initiation were evident, based on anatomical observation. These stages were similar to those presented by Hartman and Kester (14): 1) an induction period (1 to 6 days); 2) meristem organization (6 to 9 days); and 3) a root elongation period (9 to 18 days). It appears that biochemical changes were induced in localized cells during the induction period in preparation for cell division. However, induction was more than a general preparation for cell division since cell division occurred in non-rooting mature petioles treated with auxin. Meristem organization was apparent a few days after the first cell division and was characterized by localized and specifically oriented cell divisions leading to an organized root primordium. The elongation period includes the time required for the root primordia to elongate through the outer cortex and become visible through the epidermis, by day 12 in some cases. Elongation was a period of increased cell division and vascular differentiation in the developing root.

In conclusion, we have shown that de-bladed petioles from the juvenile and mature phase of English ivy have a rooting response similar to stem cuttings and excised shoot apices from English ivy. The petiole rooting system will allow a critical analysis of the morphogenetic, physiological, and biochemical basis for root initiation potential related to the phase of development in woody perennials. Direct comparisons can be made between tissue with high and low rooting potential, but having the same developmental age and identical genetic makeup. The identification of an anatomical sequence for root initiation will make it possible to relate biochemical and physiological changes to anatomical events during root initiation in future research.

**Literature Cited**


