Genetic variation in rooting ability of loblolly pine cuttings: effects of auxin and family on rooting by hypocotyl cuttings

MICHAEL S. GREENWOOD¹ and ROBERT J. WEIR²

¹ University of Maine, 5755 Nutting Hall, Orono, ME 04469-5755, USA
² North Carolina State University, Box 8002, Raleigh, NC 27695-8002, USA

Summary After about 20 days, hypocotyl cuttings from 20-day-old loblolly pine (Pinus taeda L.) seedlings rooted easily in the presence of the auxin indole-3-butyric acid (IBA), with roots forming directly from xylem parenchyma. In contrast, woody cuttings from 1–2-year-old hedged seedlings formed roots indirectly from callus tissue in 60–90 days, but IBA had little effect on rooting. Variation in rooting among hypocotyls from both half- and full-sib families was highly significant in response to IBA, and rooting did not occur within 20 days unless IBA was applied. Hypocotyls from poor rooting families tended to produce fewer roots per cutting than hypocotyls from good rooting families. Rooting by woody cuttings and hypocotyl cuttings from the same nine full-sib families was weakly correlated, raising the possibility that at least some common genetically controlled processes were affecting rooting by both types of cutting. The phytotropin N-1-naphthylphthalamic acid (NPA), supplied at 1 µM with 10 µM IBA, significantly inhibited rooting by hypocotyl cuttings from both good and poor rooting families, but there was no significant family × treatment interaction. Family variation in rooting ability may be a function of the frequency of occurrence of auxin-responsive cells in the hypocotyls.

Keywords: indole-3-butyric acid, N-1-naphthylphthalamic acid, Pinus taeda, Pinus elliottii, phytotropin, slash pine.

Introduction

Each year, over one billion loblolly pine seedlings are grown from extensively genetically tested orchard seed and planted in the southeastern USA (Dougherty and Duryea 1991). As a result of genetic testing, which in some cases includes third generation progeny, full-sib families have been identified as candidates for mass propagation (Williams and Lambeth 1993). However, multiplication by tissue culture has not proven cost effective (Greenwood et al. 1991), and supplemental mass pollination is subject to large amounts of contamination (Bridgewater et al. 1993). For these reasons, there is much interest in propagating full-sib families of loblolly pine by means of adventitious root formation on stem cuttings. Although loblolly pine cuttings from older plants are difficult to root, cuttings from young hedged stock plants sometimes root relatively well if environmental conditions during rooting are carefully controlled (Greenwood et al. 1991). Foster et al. (1987) reported that rooted cuttings from 1- to 2-year-old stock plants grow as well as seedlings. However, rooting success varies markedly with genotype, and 5-fold differences among full-sib families have been observed (Foster 1990, Anderson et al. 1993). In order to obtain improved and more consistent rooting, an understanding of the physiological basis for genetic variation in rooting ability is desirable.

Here we describe a relatively simple experimental system based on young seedlings of loblolly pine (Pinus taeda L.) in which results are obtainable within 40 days from germination. Several studies have shown that cuttings, including the hypocotyls of young pine seedlings, root well and are responsive to exogenously applied auxin (Hyun and Hong 1968, Smith and Thorpe 1975, Groonroos and von Arnold 1988). Unlike older woody cuttings, cuttings from hypocotyls regenerate root meristems directly from xylem parenchyma without prior formation of callus tissue (Groonroos and von Arnold 1988). This partially explains why hypocotyl rooting occurs in less than a month, whereas rooting in woody cuttings may take as long as 3 months. These observations are also of interest in determining whether conclusions about the rooting process based on results from hypocotyl cuttings can be extrapolated to older woody cuttings. Because hypocotyl cuttings root quickly without mist, they are an attractive model system for investigating the basis for genetic variation in rooting.

We also describe the relationship between the rooting ability of both hypocotyl and woody stem cuttings from seedlings from nine full-sib families of loblolly pine. In addition, we have screened 44 half-sib families of loblolly pine for rooting ability in order to examine the patterns of genetic variation in rooting. A subset of these families was used to test the hypothesis that rooting differences among families are associated with varying sensitivity to the concentration of the auxin indole-3-butyric acid (IBA), or to the phytotropin N-1-naphthylphthalamic acid (NPA), which inhibits polar auxin transport (e.g., Brunn et al. 1992).
Materials and methods

Plant material

Seeds from loblolly pine families (obtained from the North Carolina State Tree Improvement Cooperative) were soaked in tap water for 24 h and then stratified in loosely closed plastic bags at 4 °C for about 30 days. The stratified seeds were sown on fine vermiculite (covered with a 1-cm layer of coarse vermiculite) and germinated in a growth chamber at the NC State Phytotron in a 16-h photoperiod at a light intensity of 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), provided by fluorescent and incandescent lamps, and a day/night temperature of 27/20 °C. The seedlings were watered daily with deionized water to saturation, and after about 20 days, the hypocotyls were fully elongated and the epicotyls had just started to develop.

Plant growth regulators

Stock solutions of 0.1 M IBA (Sigma Chemical Co., St. Louis, MO) and 0.005 M NPA (Schweizerhall Inc., Piscataway, NJ) in absolute ethanol were diluted with distilled water to provide rooting solutions of varying concentrations of both growth regulators. To determine if the extra ethanol needed to solubilize the NPA affected rooting, the rooting response to 10 \( \mu \text{M} \) IBA was observed in the presence of 0.01 and 0.07% ethanol, which spans the range of ethanol concentrations used to solubilize IBA and NPA in all combinations used in the experiments. The additional ethanol did not significantly affect rooting response to IBA. Roots were counted 18 to 20 days after sticking when differences among families were pronounced. Some additional rooting occurred after this time, but after sticking when differences among families were pronounced. Some additional rooting occurred after this time, but the overall family ranking for number of roots per cutting did not change. In all experiments, data from individual cuttings were used for statistical analysis.

Experiment 1: Rooting of woody cuttings and hypocotyl cuttings

To determine if genetic variation in rooting was similar between woody cuttings and hypocotyl cuttings, we compared rooting ability of woody cuttings and hypocotyl cuttings in nine full-sib families. The Woody cuttings were taken from seedlings hedged periodically to a height of 50–70 cm, beginning when the cuttings were 5 months old from seed. The rooting data are based on data pooled over three separate experiments, using cuttings collected in August 1989, February 1990 and May 1990 when the hedges were 19, 21 and 29 months old, respectively. Each family was represented by 160 cuttings, the number of half-sib families, we compared the rooting of hypocotyls from four half-sib families of both loblolly pine and slash pine (Pinus elliottii Engelm.) were investigated by means of the procedures described for Experiment 1. Each IBA concentration was represented by a single tray (ANOVA had revealed that the tray (replicate) effect in Experiment 1 was not significant), each containing family plots of 15 cuttings from all eight families. The ANOVA, based on a model with treatment and family as main effects, was performed separately for each species on root counts made 18 days after the cuttings were stuck.

Experiment 2: Auxin concentration and rooting

To compare rooting response to auxin among families, the effects of 0, 5, 10 and 50 \( \mu \text{M} \) IBA on rooting by hypocotyl cuttings from four half-sib families of both loblolly pine and slash pine (Pinus elliottii Engelm.) were investigated by means of the procedures described for Experiment 1. Each IBA concentration was represented by a single tray (ANOVA had revealed that the tray (replicate) effect in Experiment 1 was not significant), each containing family plots of 15 cuttings from all eight families. The ANOVA, based on a model with family and origin as main effects, was performed separately for each species on root counts made 18 days after the cuttings were stuck.

Experiment 3: Patterns of genetic variation

To characterize the genetic variation among a large number of half-sib families, we compared the rooting of hypocotyls from 44 half-sib families of loblolly pine in the presence of 10 \( \mu \text{M} \) IBA. Each family was represented by 30 cuttings in plots of 10 divided among each of three blocks. Each block consisted of three PVC trays, and each tray contained 14–15 families with 10 cuttings each. Roots were counted 18 days after the cuttings were stuck, and ANOVA was performed based on a model with family and block as main effects. Narrow sense heritability \( (h^2) \) was calculated by the formula:

\[
h^2 = \frac{4 \sigma_t}{\sigma_{f}^2 + \sigma_{tr}^2 + \sigma_{w}^2}.
\]

Variance components for family (\( \sigma_f^2 \)), the family by replication interaction (\( \sigma_{fr}^2 \)) and within plot error (\( \sigma_w^2 \)) were calculated by the SAS variance components estimation procedure.

Experiment 4: Auxin transport and rooting

The sensitivities of the five best rooting and five worst rooting families from Experiment 3 to both 10 \( \mu \text{M} \) IBA and 10 \( \mu \text{M} \) IBA + 1 \( \mu \text{M} \) NPA were tested by means of the procedures described in Experiment 1. Because NPA inhibits auxin transport by competing with auxin for binding sites on auxin transport proteins, it should inhibit rooting by affecting either auxin transport or some other process related to rooting that requires auxin binding. Each of the two treatments was administered in a single tray with all 10 families represented by 12 tree plots
in each. Root counts were made 19 days after the cuttings were stuck, and ANOVA was performed based on a model with treatment and family as main effects.

**Results**

In Experiment 1, 94% of all hypocotyl cuttings from 20-day-old loblolly pine seedlings rooted in less than 25 days, but only in response to exogenously supplied IBA (Figures 1 and 2). Without auxin, less than 10% of the hypocotyls rooted after a period of more than 50 days. In contrast, 34% of the woody cuttings from older loblolly pine from the same full-sib families rooted after 16–20 weeks and showed little or no response to exogenously applied IBA (Greenwood et al. 1980). There were significant differences among families both for number of roots per cutting (hypocotyls, \( P < 0.001 \)) and percent rooting (woody cuttings, \( P < 0.05 \)). Family \( \times \) replication effects on hypocotyl rooting were not significant (\( P < 0.24 \)). The number of roots per cutting was not counted on the woody cuttings, and there was not sufficient replication by block within family to perform ANOVA on percent rooting for hypocotyls. Both percent rooting and number of roots per cutting by hypocotyls were positively correlated with percent rooting by woody cuttings (Pearson correlation, \( r = 0.35 \) and 0.70, respectively, and only the latter correlation was significant at \( P < 0.036 \), see Figure 1).

In Experiment 2, all four families of loblolly pine and slash pine showed similar responses to varying concentrations of IBA: an increase in rooting with increased IBA concentration (Figure 2) and no rooting without IBA. The effects of family, treatment and their interaction were all significant at \( P < 0.01 \). When the interaction was used as the error term, the effect of IBA treatment was significant at \( P < 0.003 \) for loblolly pine and at \( P < 0.002 \) for slash pine. We were unable to detect a significant family effect for either species (\( P < 0.2 \) for loblolly pine, \( P < 0.3 \) for slash pine). Variation in rooting between families increased with increasing IBA concentration, and family ranks changed. Slash pine appeared to form more roots per cutting in response to all concentrations of IBA than loblolly pine.

There was a highly significant difference in rooting ability among the 44 half-sib families (\( P < 0.001 \)), but the effect of replication was not significant (\( P < 0.2 \)). The family \( \times \) replication interaction was used as the error term for both effects.
Although the growth chamber environment was uniform, the family × replication effect was significant at $P < 0.001$ because, as a result of mortality, there was an uneven distribution of some families among the three replications. Some of the best and worst rooting families were retested in four subsequent trials, and the means ranged from 1.5 to 4.4 roots per cutting with the good and poor rooting families averaging 4.3 and 2.3 roots per cutting, respectively. The results of one such trial are shown in Table 1. The variation in rooting frequency within a family was high, and poor rooting families exhibited a higher proportion of cuttings with no roots than good rooting families (Figure 3).

Poor rooting families were characterized by a high proportion of cuttings that formed no roots at 18 days, whereas cuttings from the good rooting families rooted relatively quickly, and formed many roots per cutting.

<table>
<thead>
<tr>
<th>Families</th>
<th>10 µM IBA</th>
<th>10 µM IBA+ 1 µM NPA</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Good rooters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.6</td>
<td>2.3</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>5.1</td>
<td>2.8</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>3.4</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>4.2</td>
<td>51</td>
</tr>
<tr>
<td>5</td>
<td>4.2</td>
<td>2.8</td>
<td>33</td>
</tr>
<tr>
<td>Means</td>
<td>5.2</td>
<td>2.9</td>
<td>41</td>
</tr>
<tr>
<td><strong>Poor rooters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.6</td>
<td>1.7</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>1.9</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>2.2</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>4.5</td>
<td>2.2</td>
<td>51</td>
</tr>
<tr>
<td>Means</td>
<td>3.3</td>
<td>2.0</td>
<td>34</td>
</tr>
</tbody>
</table>

Although more cuttings from the poor rooting families rooted after 18 days, the number of roots per cutting remained relatively low. A few individuals among the six worst rooting families produced as many as seven roots per cutting, whereas about 10% of the cuttings among the six best rooting families produced 8–10 roots each. A narrow sense heritability of 0.35 for rooting based on family means (number of roots per cutting) was calculated using the variance components from Experiment 3 with 44 half-sib families.

The phytotropin NPA inhibited rooting in almost all the families regardless of whether they were good or poor rooters (see Table 1). N-1-Naphthylphthalamic acid significantly delayed rooting by hypocotyl cuttings from most good and poor rooting families. If 10 µM NPA (equimolar with IBA) was used, rooting was completely inhibited (unpublished data). The effects of both family and NPA treatment were significant at $P < 0.01$ and $P < 0.0001$, respectively, whereas the family × treatment interaction was not significant ($P = 0.24$).

**Discussion**

In pine hypocotyls, the roots form directly from vascular parenchyma centripetal to the resin canal associated with each vascular pole, with little or no callus formation prior to the organization of the root meristem. This is called direct rooting (Smith and Thorpe 1975, Stickney 1987, Groonroos and von Arnold 1988). In contrast, woody cuttings and epicotyls (and hypocotyls that receive no auxin) root with a much lower frequency, and callus formation usually precedes the organization of the root meristem (Flygh et al. 1993). The observation that exogenous auxin does not promote rooting by woody cuttings suggests that endogenous auxin is not limiting, whereas endogenous auxin is limiting for rooting by hypocotyl cuttings.

We observed a weak, but significant, positive correlation between the number of roots per hypocotyl and percent rooting by woody cuttings from the same full-sib families, although the number of families tested was minimal to demonstrate a significant correlation (see Figure 1). Percent rooting and number of roots per cutting are highly correlated in woody cuttings (Foster et al. 1984, Pounders and Foster 1992). Although Flygh et al. (1993) suggest that hypocotyls from Scots pine ($Pinus sylvestris$ L.) can be used as a model system to study auxin-induced rooting in conifers, further experiments with larger numbers of families are needed to clarify the nature of the relationship between rooting by hypocotyls and rooting by woody cuttings from older plants.

We observed a highly significant variation in hypocotyl rooting among half-sib families of loblolly pine, and further testing of selected good and poor rooting families yielded similar results to Experiment 1 (Table 1). Foster (1990) and Anderson et al. (1993) calculated narrow sense heritabilities of 0.46 and 0.31, respectively, based on family means for percent rooting for woody cuttings from hedged families of loblolly pine. These values are similar to the value of 0.35 reported here for number of roots per hypocotyl cutting. These heritability values are moderate because of large within-family variation.
in rooting ability. Thus, the degree of genetic control over family variation in rooting by both hypocotyl and woody cuttings appears to be approximately the same.

Greenwood and Goldsmith (1970) showed that the phytotropin triiodobenzoic acid (TIBA) inhibits rooting as well as both polar auxin transport and basal accumulation of $^{14}$C-indole-3-acetic acid (IAA) applied to the cotyledons of sugar pine (Pinus lambertiana Dougl.) hypocotyl cuttings. We do not know whether the inhibitory effects of NPA reported here are due to a reduction in the amount of auxin reaching the base of the cutting. However, because the base of the cuttings was continually exposed to the auxin solution, and phytotropins promote tissue uptake of auxin (Faulkner and Rubery 1992), we conclude that it is unlikely that auxin concentration in the tissues of the base of the cutting was limiting for rooting. The hypothesis that NPA may inhibit some aspect of the rooting process directly, perhaps by competing with auxin for binding sites, is being tested.

The genetic variation in rooting that we observed is clearly a function of variation of the response of each family to auxin. The six best and worst rooting half-sib families all came from second generation seed orchards. The clones that produced the seed for both groups did not show any common first generation parents. Both good and poor rooting families showed similar response patterns to auxin, although there was a significant family $\times$ treatment interaction (Figure 1) that appeared to be due to rank changes in family response with increasing IBA concentration. None of the families rooted without auxin, but several families of both loblolly and slash pine changed rank between 10 and 50 $\mu$M IBA. Family variation in endogenous production of auxin is an unlikely cause of family variation in rooting, because auxin was limiting in both poor and good rooting families. Hypotheses to account for family variation in rooting include: (1) the presence of differential amounts of either rooting inhibitors or non-auxin rooting promoters among families; (2) the possibility that poor rooting families may have fewer groups of cells that can respond to auxin by forming a root meristem directly; or (3) the concentration of rooting-specific receptor sites for auxin within these cells, which are confined to the parenchyma centripetal to the xylem, may vary among families. Furthermore, such receptor sites may be lacking in the cells of woody cuttings, where endogenous auxin concentrations do not appear to be limiting for root formation. In woody cuttings, these receptors may be regenerated in callus tissue that forms at the wound surface. Another hypothesis is that the greater responsiveness of hypocotyl cells is a function of processes that occur after auxin is bound to a receptor. These processes decline with maturation, but can be partially restored in the callus that forms at the base of woody cuttings.

Acknowledgments

We thank Steve McKeand for help with the statistical analysis, and Chris Hunt and Tom Gilmour for their technical assistance. Maine Agricultural Experiment Station Paper No. 1860.

References


