Rooting Cofactors in Five Acer Species

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Abstract. Methanolic extracts from leaves, young stems, and old stems of five Acer (maple) spp. were tested for their effects on adventitious root initiation in mung bean (Vigna radiata ‘Wilcox’) cuttings. An extract from the leaves of A. ginnala strongly stimulated root initiation, and the active compounds in this fraction were not synergistic with IAA. This extract was more stimulatory than IAA on mung bean cuttings and stimulated root initiation in softwood cuttings of A. saccharinum and A. griseum. Preliminary characterization of this extract indicates that it is a phenolic compound and/or a weak acid. Chemical name used: 1H-indole-3-acetic acid (IAA).

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The genus Acer includes many species that differ in ease of asexual propagation by cuttings, both among and within species. Differences in rooting of stem cuttings of woody plants have been attributed to various hormone and rooting cofactor levels (7, 9, 17). The mung bean bioassay (8, 16) has shown numerous differences that influence water uptake and seed quality in hard seed cotton. Agron. J. 52:163–167.


29.
have increased rooting when applied to difficult-to-root cuttings when auxin is also applied (13).

This paper reports studies of promoters and inhibitors of root initiation in extracts from three plant parts of five *Acer* species. Extracts were taken from an easy-to-root species and used to stimulate rooting in cuttings of easy- and difficult-to-root *Acer* spp.

**Materials and Methods**

*Application of extracts to cuttings of Vigna radiata.* Leaves, current season stems (young stems), and previous season stems (old stems) from 15- to 20-year-old plants were collected from specimens located on the Univ. of Illinois campus on 20 June, 1979. Shoots (7 to 12 cm) were collected from the lower branches of trees located in full sun, packed in ice, transported to the lab, freeze-dried, and ground in a Wiley mill. A 50-mg sample (16) was extracted with two 25-ml volumes of methanol, and the combined extracts were filtered and concentrated in vacuo for application to strips of washed (80% isopropanol) Whatman 3MM paper. The papers were dried and inserted into shell vials containing five mung bean cuttings and 5 ml of $5 \times 10^{-6}$ M IAA (2, 8, 16). Vials were placed in a growth room at 25$^\circ$ ± 3$^\circ$C with a 16-hr photoperiod of 65 to 70 $\mu$mol·s$^{-1}$·m$^{-2}$·PPF at the plant canopy supplied by a 1000-W metal halide (Sylvania Metal Arc) lamp. The number of emerged roots on each cutting was counted after 6 days. The experiments were completely randomized designs with two replications of five cuttings each.

Crude extracts were fractionated by descending paper chromatography with 80% isopropanol (16). Developed chromatograms were cut into 15 equal segments and inserted into shell vials containing 4 ml of $5 \times 10^{-6}$ M IAA and three mung bean cuttings. The number of roots was counted after 6 days. The experiments were randomized complete block designs with three replications of three cuttings each.

Effects of extract concentration on the mung bean bioassay were determined with two active fractions (fraction 0.7 with $R_f$ values of 0.67 to 0.73, and fraction 0.9 with $R_f$ values of 0.87 to 0.93) extracted from leaves of *A. ginnala*. Fraction 0.7 was applied at concentrations of 0x, 0.06x, 0.13x, and 0.25x concentrations, with a 1x concentration representing all material that Rf value from a 50-mg tissue extract. Fraction 0.9 was applied at 1x and 2x. These concentrations were based on preliminary experiments to determine the optimum levels for stimulating root initiation. Chromatographic segments were cut into appropriate sizes to obtain proper dilution factor. A 2x concentration was obtained by using two 1x paper segments. Experimental design was a randomized complete block with four replicates of three cuttings each.

The mung bean bioassay was used to determine if fractions 0.7 and 0.9 were interacting with IAA to stimulate root initiation. A 2 x 2 factorial combination of fraction 0.7 (0x and 0.13x) and IAA (0 and $5 \times 10^{-6}$ M) was used. A similar experiment was conducted with 0x and 1x concentrations of fraction 0.9. The experiments were randomized complete block designs with four replicates of three samples each.

*Application of extracts to cuttings of Acer spp.* Because fractions 0.7 and 0.9 showed strong stimulation of root initiation in mung bean cuttings, these fractions were tested on cuttings of *A. saccharinum* (14). Fractions 0.7 and 0.9 were applied in two experiments at 0.5x and 2.0x concentrations based upon preliminary experiments. The roots visible on each cutting were counted after 21 days. Each experiment was completely randomized with five replicates of two samples each.

Fractions 0.7 and 0.9 were applied in combination with IAA to softwood stem tip cuttings of 2-year-old seedling *Acer griseum* (14). Fraction 0.7 was applied at concentrations of 0x, 0.13x, and 0.50x, and fraction 0.9 was applied at 1x as described previously (14). All vials also contained 4 ml of $5 \times 10^{-6}$ M IAA. The number of roots emerged was determined after 42 days.

*Characterization of fractions 0.7 and 0.9.* The $R_f$ values of fraction 0.9 (1x) and IAA (88 $\mu$g) were compared in two solvent systems, 80% isopropanol and 8 isopropanol : 1 NH$_4$OH : 1 H$_2$O (by volume), with descending paper chromatography. The experiments were randomized complete blocks with three replicates of three cuttings each. A standard concentration curve for the effect of IAA in the mung bean bioassay was made with seven concentrations ranging from $10^{-9}$ to $10^{-3}$ M, plus a control. All solutions contained 4 ml of ethanol/liter.

The Folin–Denis procedure (12) was used to determine the total phenolic content of fractionated extracts from leaves of *A. ginnala*. Methanolic extracts were applied to Whatman 3MM paper, developed in 80% isopropanol, and cut into 15 segments as previously described. Each segment was extracted with 5 ml of methanol and two 5-ml volumes of 50% methanol. The combined extracts were made up to 25 ml with water, and a 0.5-ml aliquot was used in the Folin–Denis procedure. Absorbance at 725 nm was read after 45 min.

Paper chromatograms containing fractionated extracts from leaves of *A. ginnala* were sprayed separately with diazotized p-nitroaniline and ammoniacal silver nitrate (3) for detection of phenolic compounds. Intensity of colors formed as a result of these reactions was recorded by $R_f$ value.

Column chromatography was used to separate the active compounds in fractions 0.7 and 0.9. Leaves of *A. ginnala* were extracted as described, except that water was used as the solvent. The extract was applied to a 25-mm-diameter glass column containing 28.2 g (42% hydrated) of Amberlite XAD-4, a polystyrene adsorbent designed for the removal of phenolic compounds in the extraction of proteins (15). Amberlite XAD-4 also binds weak acids and some terpenes, quinones, and organic isothiocyanates (15). Total bed volume after full hydration was 47 ml. The column was prepared for use by backwashing with distilled deionized water for 15 min, followed by a downflow wash with five bed volumes of methanol and 20 bed volumes of water.

The sample was introduced to the Amberlite column and three bed volumes of water were passed through the column followed by three bed volumes of methanol. Aqueous and methanolic eluates were separately collected, concentrated, and applied to Whatman 3MM paper. Chromatograms were developed in 80% isopropanol. Chromatographic segments with $R_f$ values of 0.7 (range of 0.67 to 0.73) and 0.9 (0.87 to 0.93) were tested in the mung bean bioassay. Fraction 0.7 was applied to mung bean cuttings at concentrations of 1.0x, 0.25x, and 0.13x. Fraction 0.9 was applied at a 1x concentration. Additional chromatograms were examined with ultraviolet light, diazotized p-nitroaniline, and ammoniacal silver nitrate. Total phenolic content of the chromatographic segments was determined by the Folin–Denis procedure.

**Results**

*Application of extracts to Vigna radiata.* Crude methanolic extracts from the leaves of *A. ginnala* and *A. rubrum* inhibited adventitious root initiation in the mung bean bioassay (Table 1). Crude extracts from the young and old stems of *A. ginnala,*
Fig. 1. Effects of fractionated methanolic extracts from three plant parts of five *Acer* spp. on adventitious root initiation in mung bean cuttings. Horizontal line (control) extending through the vertical axis represents the mean number of roots on control cuttings. Light shaded areas above control line represent stimulation of root initiation and dark shaded areas below control line indicate inhibition. Significance (LSD 0.05) is indicated by vertical line extending up from control line at an *Rf* of 0 to 0.1.
A. nibrum, and A. griseum - 1.3 + A. rubrum - 0.1 + 9.9 + - 1.9 + A. saccharinum were not significant (F-test, 0.05 level).

Table 3. Effects of fractions 0.7, 0.9, and IAA on adventitious root initiation in mung bean cuttings.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean difference in number of roots from the control*</th>
<th>No. roots/cuttingy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Young stems</td>
</tr>
<tr>
<td>A. ginnala</td>
<td>-10.6*</td>
<td>+5.4*</td>
</tr>
<tr>
<td>A. griseum</td>
<td>-1.3</td>
<td>+0.1</td>
</tr>
<tr>
<td>A. rubrum</td>
<td>-10.4*</td>
<td>+9.9*</td>
</tr>
<tr>
<td>A. saccharinum</td>
<td>-1.9</td>
<td>+8.0*</td>
</tr>
<tr>
<td>A. saccharatum</td>
<td>+0.2</td>
<td>-2.0</td>
</tr>
</tbody>
</table>

*Values reported are the mean number of roots greater or less than the control. Control cuttings averaged 10.6, 8.5, and 8.5 roots each for leaves, young stems, and old stems, respectively. Positive values indicate root numbers in excess of the control, negative numbers less. Significant (LSD 0.05) means are indicated by an asterisk.

Table 3. Effects of fractions 0.7, 0.9, and IAA on adventitious root initiation in mung bean cuttings.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration</th>
<th>No. roots/cuttingy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>0.25 x</td>
<td>12.0 d*</td>
</tr>
<tr>
<td>0.7</td>
<td>0.13 x</td>
<td>51.6 a</td>
</tr>
<tr>
<td>0.7</td>
<td>0.06 x</td>
<td>33.2 ab</td>
</tr>
<tr>
<td>0.9</td>
<td>2.00 x</td>
<td>32.9 abc</td>
</tr>
<tr>
<td>0.9</td>
<td>1.00 x</td>
<td>16.7 bcd</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>12.7 d</td>
</tr>
</tbody>
</table>

Table 2. Effects of concentration of two fractions extracted from leaves of A. ginnala on adventitious root initiation in mung bean cuttings.

Table 4. Effects of two extracts from leaves of A. ginnala on the number of roots initiated in cuttings of A. saccharinum in two experiments*.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>2.0 x</td>
<td>0.5 c</td>
<td>14.8 b</td>
</tr>
<tr>
<td>0.7</td>
<td>0.5 x</td>
<td>17.5 a</td>
<td>35.5 a</td>
</tr>
<tr>
<td>0.9</td>
<td>2.0 x</td>
<td>10.5 b</td>
<td>20.0 b</td>
</tr>
<tr>
<td>0.9</td>
<td>0.5 x</td>
<td>13.0 ab</td>
<td>16.3 b</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>11.5 b</td>
<td>17.8 b</td>
</tr>
</tbody>
</table>

*See Table 2 for definition and concentration of fractions.
†Mean of 10 cuttings in each experiment.
‡Mean separation using LSD 0.05 level. Means are for comparison within each experiment only.

Fraction 0.7 was most effective at a concentration of 0.13 x (Table 2), stimulating the initiation of 51.6 roots per cutting. Fraction 0.9 was the most effective at the highest concentration (2 x) used (Table 2).

Fractions 0.7 and 0.9 both stimulated root initiation in mung bean cuttings (Table 3); however, there was no significant interaction of either fraction with IAA.

A. rubrum, and A. saccharinum stimulated rooting. Crude extracts from all other tissues and species had no significant effect. All three plant parts of all five species, with the exception of young stems of A. rubrum, contained fractions with Rf values of 0.8 to 1.0, which strongly stimulated root initiation in mung bean (Fig. 1). Stimulatory fractions with other Rf values were found in other species and tissues, but with no consistent trend.

Leaf extracts of A. ginnala and A. rubrum contained fractions with Rf values of 0.60 to 0.73, which completely inhibited root initiation. Leaves of A. saccharinum appeared to show inhibition at the same Rf values, but differences were not significant.

When fraction 0.7 was diluted, it strongly stimulated rooting. Fraction 0.7 was most effective at a concentration of 0.13 x (Table 2), stimulating the initiation of 51.6 roots per cutting. Fraction 0.9 was the most effective at the highest concentration (2 x) used (Table 2).

Fig. 2 Relative phenolic content of fractionated methanolic extracts from leaves of A. ginnala in absorbance units at 725 nm using Folin-Denis reagent. A 0.05-mg tannic acid standard produced an absorbance of 0.49.

When fraction 0.7 was diluted, it strongly stimulated rooting. Fraction 0.7 was most effective at a concentration of 0.13 x (Table 2), stimulating the initiation of 51.6 roots per cutting. Fraction 0.9 was the most effective at the highest concentration (2 x) used (Table 2).

Fractions 0.7 and 0.9 both stimulated root initiation in mung bean cuttings (Table 3); however, there was no significant interaction of either fraction with IAA.
Table 6. Effects of two solvents on elution of active compounds in fractions 0.7 and 0.9 from an Amberlite XAD-4 column, as measured by the mung bean bioassay.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Solvent</th>
<th>Concentration</th>
<th>Amberlite eluant</th>
<th>No. roots/cutting</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>Water</td>
<td>1.00×</td>
<td>-</td>
<td>15.0 de</td>
</tr>
<tr>
<td>0.7</td>
<td>Water</td>
<td>0.25×</td>
<td>-</td>
<td>17.5 dc</td>
</tr>
<tr>
<td>0.7</td>
<td>Water</td>
<td>0.13×</td>
<td>-</td>
<td>17.5 dc</td>
</tr>
<tr>
<td>0.9</td>
<td>Water</td>
<td>1.00×</td>
<td>-</td>
<td>31.7 abc</td>
</tr>
<tr>
<td>0.7</td>
<td>Methanol</td>
<td>1.00×</td>
<td>-</td>
<td>6.8 e</td>
</tr>
<tr>
<td>0.7</td>
<td>Methanol</td>
<td>0.25×</td>
<td>-</td>
<td>42.1 a</td>
</tr>
<tr>
<td>0.7</td>
<td>Methanol</td>
<td>0.13×</td>
<td>-</td>
<td>34.7 ab</td>
</tr>
<tr>
<td>0.9</td>
<td>Methanol</td>
<td>1.00×</td>
<td>-</td>
<td>37.2 ab</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15.0 de</td>
</tr>
</tbody>
</table>

*See Table 2 for definition and concentration of fractions.

*Mean Rf values (see Table 2) with 60% isopropanol in descending paper chromatography following elution from Amberlite column.

*Mean separation by Duncan's multiple range test, 0.05 level. Each value a mean of nine cuttings.

Table 7. Solvent elution and reaction of compounds from fractions 0.7 and 0.9 to phenolic detection tests.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amberlite eluant</th>
<th>Phenolic content</th>
<th>Ultraviolet fluorescence</th>
<th>Diazotized p-nitroaniline</th>
<th>Ammoniacal silver nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>Water</td>
<td>0.003</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.9</td>
<td>Water</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.7</td>
<td>Methanol</td>
<td>0.286</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.9</td>
<td>Methanol</td>
<td>0.005</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*See Table 2 for definition of fractions.

*Aqueous extracts from leaves of A. ginnala eluted from column of Amberlite XAD-4.

*Phenolic content determined by the Folin-Denis procedure is expressed in absorbance units at 725 nm.

*Strength of reaction to detection tests as follows: - = none, + = weak, ++ = strong.

Discussion

Crude extracts from leaves of A. ginnala strongly inhibited root initiation at the concentration used. When the crude extract was fractionated with paper chromatography, the inhibitory activity occurred at an Rf of 0.7 (Fig. 1). When diluted, it markedly stimulated root initiation (Table 2). Stimulatory compounds with an Rf of 0.7 may also be present in the other Acer spp., as evidenced by the strong inhibition of rooting from leaves of A. rubrum and the apparent inhibition from leaves of A. saccharinum (Fig. 1). If the stems of these five Acer species contain the same active compounds as fraction 0.7, they are present at lower levels than in the leaves, as indicated by the lack of inhibition of rooting at an Rf of 0.7 in the stem histograms (Fig. 1). Presence and concentration of the active components in fraction 0.7 also could account for the effects of crude extracts of these species (Table 1). Active components of fraction 0.7 could occur in decreasing concentration in leaves, young stems, and old stems, which would explain the strong inhibition in some of the leaf extracts and the stimulatory effects of old stems and young stems. Crude extracts from the young stems of the easy-to-root Acer species (4, 5) were stimulatory to root initiation, whereas those from the difficult-to-root species (A. griseum and A. saccharum) had no effect. At present, it is unknown if there is any direct relationship between rooting and the occurrence of stimulatory compounds in the crude extracts. Additional tests would be required to substantiate this.

Of particular interest is the finding that fraction 0.7 was more stimulatory than any of the IAA concentrations tested in the mung bean bioassay. Fraction 0.7, at a concentration of 0.13×, stimulated the initiation of 52 roots per cutting (Table 2), as compared to 30 roots for 10×4 M IAA, the optimum IAA concentration in our tests. The activity of fraction 0.7 becomes more apparent when one considers that a concentration of 0.13× represents the equivalent of the total methanolic extract from only 6.5 mg of dry tissue.

Fraction 0.7 was effective in stimulating root initiation in A. saccharinum at a concentration of 0.5× (Table 4). This treatment resulted in a doubling of the number of roots initiated as compared to control cuttings. The browning of the lower portion of the stem with a profusion of roots above the dip line observed in these cuttings indicates that a slightly lower concentration of fraction 0.7 may have been even more stimulatory.
Fraction 0.7 stimulated the number of roots and rooting percentage in *A. griseum* (Table 5). The increase in number of roots was small, but the rooting percentage was twice that of the control. In a species as difficult to root as *A. griseum*, any increase in rooting percentage or a small increase in number of roots per cutting is very beneficial, because it may determine if a species can or cannot be propagated asexually. Additional work with a purified form of fraction 0.7, applied in combination with an optimal concentration of an auxin such as IBA, might further improve the rooting percentage and the number of roots per cutting. These *A. griseum* cuttings also were rooted in solution culture, which is generally not the optimal condition for rooting difficult species. Alternate application methods might also allow for mist propagation in a standard medium.

This is one of the first reports of using extracts from an easy-to-root species, *A. ginnala* (4, 5), to stimulate root initiation in a difficult-to-root species, *A. griseum* (4, 5), in the same genus. Kawase (13) used a water soluble extract from stems of *Salix alba*, an easy-to-root species, in combination with IBA to stimulate rooting of cuttings from *Betula alleghaniensis*, a difficult-to-root species.

The chromatographic segment comprising fraction 0.7 showed a positive test for phenolics with two spray reagents, and the Folin–Denis test also showed a large phenolic content (Fig. 2). The active component(s) in fraction 0.7 can be bound to Amberlite XAD-4 resin in aqueous solution, eluted with methanol, and again produce positive phenolic tests. Thus, fraction 0.7 contains a large quantity of phenolic compounds. The ability to use this column to bind the active components in fraction 0.7 also provides information on their structural classification. Results of the various phenolic detection tests in combination with the capacity to bind to Amberlite XAD-4, suggests that active compound(s) in fraction 0.7 is/are phenolic compound(s) and/or a weak acid. Additional analytical work is required for more complete characterization and identification.

Active components of fraction 0.7 acted additively with IAA to stimulate root initiation in mung bean cuttings (Table 3). Hess (10) demonstrated that phenolic compounds with specific structural relationships acted synergistically with IAA; however, other phenolic compounds also have been reported to act additively with IAA without interaction or synergism (14). Hess (11) identified an auxin cofactor (cofactor 3) with an *Rf* of 0.60 to 0.73, as chlorogenic acid and its isomers; however, chlorogenic acid was not shown to be stimulatory to root initiation in a later study (14).

The leaves of *A. ginnala* also contain a stimulatory fraction with an *Rf* of 0.9. Stimulatory compounds with this *Rf* were present in all three tissues of all five species, with the exception of young stems of *A. rubrum* (Fig. 1). Fraction 0.9 was most stimulatory to root initiation at the highest concentration tested (2×) (Table 2). It is unknown if higher concentrations would improve its activity. Fraction 0.9 acted additively with IAA to stimulate root initiation in mung bean cuttings (Table 3), but it had no effect on root initiation in either *A. saccharinum* or *A. griseum* (Tables 4 and 5). Although the mung bean bioassay did not show the same response as the maple cuttings, its initial detection of stimulatory compounds served as a valuable screening tool for the later maple rooting tests, which would have been much more difficult to perform in the numbers required for initial screening of extracts.

Comparisons of fraction 0.9 with IAA showed that their *Rf* values in 80% isopropanol are similar. IAA, however, does not occur in high enough concentration in plants to account for the stimulatory effects of fraction 0.9. Reported auxin concentrations in leaf tissues range from 0.3 to 30 ng·g⁻¹ (fresh weight), or =1.7 × 10⁻⁹ to 1.7 × 10⁻⁷ M (18). Such low concentrations of IAA had no effect in the mung bean bioassay. Fraction 0.9 showed little to no reaction to the phenolic detection tests; therefore, it is unlikely that the active compounds in fraction 0.9 are phenolics. Hess’s cofactor 4 (11), which was found to be a group of oxygenated terpenoids (17), had an *Rf* of 0.80 to 0.93 in 80% isopropanol.

**Literature Cited**


17. Osawa, T., A. Suzuki, and S. Tamara. 1971. Isolation of chlorogenic acid and its isomers; however, chlorogenic acid was not shown to be stimulatory to root initiation in a later study (14).
