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ORIGINAL RESEARCH

Poison ivy hairy root cultures enable a stable transformation system suitable for detailed investigation of urushiol metabolism

Aneirin A. Lott[1,2] | **Catherine P. Freed**[3] | **Christopher C. Dickinson**[2] | **Susan R. Whitehead**[4] | **Eva Collakova**[2] | **John G. Jelesko**[2]

[1]Plant Molecular and Cellular Biology, University of Florida, Gainesville, FL, USA

[2]School of Plant and Environmental Science, Virginia Tech, Blacksburg, VA, USA

[3]Biochemistry Department, Virginia Tech, Blacksburg, VA, USA

[4]Biological Science Department, Virginia Tech, Blacksburg, VA, USA

Correspondence
John G. Jelesko, School of Plant and Environmental Science, Virginia Tech, Blacksburg, VA, USA.
Email: jelesko@vt.edu

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Abstract

Poison ivy (*Toxicodendron radicans*) is best known for causing exasperating allergenic delayed-contact dermatitis symptoms that last for weeks on persons who have contacted the plant. Urushiols are alkylcatechols produced by poison ivy responsible for causing this dermatitis. While urushiol chemical structures are well known, the metabolic intermediates and genes responsible for their biosynthesis have not been experimentally validated. A molecular genetic characterization of urushiol biosynthesis in poison ivy will require stable genetic transformation and subsequent regeneration of organs that retain the capacity synthesize urushiol. To this end, *Agrobacterium rhizogenes* was used to generate hormone-independent poison ivy hairy root cultures. Optimal conditions for hairy root formation were skotomorphic poison ivy hypocotyls prick-inoculated with *A. rhizogenes*, and preferential propagation of cultures with an atypical clumpy hairy root growth habit. The origin of the poison ivy accession used for *A. rhizogenes* prick-inoculation did not affect the initial formation of calli/hairy root primordia, but rather significantly influenced the establishment of long-term hormone-independent hairy root growth. *A. rhizogenes* harboring a recombinant T-DNA binary plasmid with an intron-containing Firefly Luciferase gene produced stable transgenic hairy root lines expressing luciferase activity at high frequency. Poison ivy hairy root lines produced significantly lower steady-state urushiol levels relative to wild-type roots, but higher urushiol levels than a poison ivy undifferentiated callus line with undetectable urushiol levels, suggesting that urushiol biosynthesis requires intact poison ivy organs. The lower urushiol levels in poison ivy hairy root lines facilitated the first identification of anacardic acid metabolites initially in hairy roots, and subsequently in wild-type roots as well. This study establishes a transformation hairy root regeneration protocol for poison ivy that can serve as a platform for future reverse-genetic studies of urushiol biosynthesis in poison ivy hairy roots.

KEYWORDS
Agrobacterium rhizogenes, anacardic acid, hairy roots, *Toxicodendron radicans*, transformation, urushiol

1 | INTRODUCTION

Poison ivy (*Toxicodendron radicans* (L.) Kuntze) is a native plant in North America that is widely known for causing irritating delayed contact allergenic dermatitis symptoms. After 12–72 hr from initial urushiol contact to human skin, allergenic dermatitis symptoms develop and are characterized by redness, swelling, oozing, with a persistent itching sensation (Epstein, 1987; Pfaff, 1897). These symptoms can last for several weeks, but will eventually resolve to normal healthy skin.

The poison ivy natural product that is responsible for the allergenic dermatitis is generically called urushiol. Urushiol is a small group of closely related alkylcatachol congeners that differ in the 3-alk(en)yl side chain (Kurtz & Dawson, 1971; Majima & Cho, 1907; Markiewitz & Dawson, 1965; Sunthankar & Dawson, 1954; Symes & Dawson, 1953; Majima, 1922). The alkyl chain can be either a pentadecyl or heptadecyl alkyl chain (i.e., C15 or C17, respectively), and the degrees of alkyl unsaturation can range from zero to three double bonds. A potential urushiol biosynthetic pathway was proposed (Dewick, 1997; Giessman & Bernfeld, 1967) in which C16 and C18 fatty acids are substrates for a polyketide synthase activity that catalyzes the formation of a tetraketide alk(en)yl compound that is eventually cyclized, aromatized, and dehydroxylated leading to an anacardic acid metabolic intermediate. This model also proposes that anacardic acid is subsequently decarboxylated and hydroxylated to yield the core urushiol molecular structure. It is noteworthy that, until only very recently, none of the proposed urushiol metabolic intermediates or enzyme activities responsible for forming those intermediates were experimentally validated in poison ivy, nor in any other urushiol-producing members of the *Toxicodendron* genus. We demonstrated that poison ivy nascently germinated seedlings accumulate cardanol congeners and isomers corresponding to their hypothesized role as penultimate metabolites leading to observed urushiol congeners and isomers (Lott, Baklajian, Dickinson, Collakova, & Jelesko, 2019).

Plant hairy root cultures produced by *Agrobacterium rhizogenes* (*A. rhizogenes*) provide manifold assets for a molecular investigation of most root physiological processes. *A. rhizogenes* is a phytopathogenic bacterium that induces galls characterized by prolific root production. Hairy root induction results from the insertion of two segments of *A. rhizogenes* root inducing-DNA (Ri-DNAs) containing genes that reprogram the plant cells to develop as hairy roots, as well as produce opines that can be metabolized by free living *A. rhizogenes* (Chilton et al., 1982; Willmitzer, Sanchez Serrano, Bushfeld, & Schell, 1982). A unique feature of hairy roots is hormone-independent sustained root growth in tissue culture. Thus, axenic clonal hairy roots can grow indefinitely, so hairy root cultures are ideally suited for the investigation of plant natural products synthesized in roots (Flores, Vivanco, & Loyola-Vargas, 1999). In some cases, hairy root cultures (or shoots regenerated thereof) accumulate higher levels of the natural product of interest (Behera, Jena, Das, Thirunavoukkarasu, & Chand, 2016; Chaudhuri, Ghosh, Tepfer, & Jha, 2006; Misic, Siler, & Skoric, 2013; Oksmancaldentey, Kivela, & Hiltunen, 1991; Piatczak, Krolicka, & Wysokinska, 2006; Pradel, DumkeLehmann, Diettrich, & Luckner, 1997; Rawat, Rawat, & Mehrotra, 2013; Wang, Zheng, Yuan, & Wang, 2013; Yoshimatsu, Sudo, & Kamada, 2004), yet in other cases produce lower natural product levels relative to wild-type roots (Benjamin, Roja, & Heble, 1993; Celma, Palazon, Cusido, Pinol, & Keil, 2001; Tsuro & Ikedo, 2011). In addition, *A. rhizogenes* transformed callus or hairy root cultures can also produce Ri-DNA containing shoots/plantlets exhibiting the so-called "hairy root" or "Ri" syndrome manifesting as one or more of the following: diminished apical dominance, reduced internode length, extensive root branching, and distorted leaves (Spena, Schmulling, Koncz, & Schell, 1987). *A. rhizogenes* strains containing recombinant T-DNA binary plasmids concomitantly process and transfer *Agrobacterium tumefaciens*-derived recombinant T-DNA segments into plant cells in addition to Ri-DNA (Christey & Sinclair, 1992; Kumar, Jones, & Davey, 1991; Lodhi & Charlwood, 1996; Nenz et al., 1996; Shin, Podila, Huang, & Karnosky, 1994). Thus, *A. rhizogenes* has many attributes pertinent for developing stably transformed poison ivy hairy root cultures suitable for molecular genetic characterization of metabolic intermediates involved in urushiol biosynthesis. The first objective in this study was to develop a reproducible method using *A. rhizogenes* to establish independent poison ivy hairy root lines, and evaluate the impacts of nascent hairy root grafts on the host seedling vigor. The second objective was to create transgenic poison ivy hairy root lines expressing a stably integrated recombinant luciferase transgene. The third objective was to characterize poison ivy hairy root cultures for the urushiol production. The latter objective fortuitously resulted in the first identification of anacardic acid in poison ivy, a proposed metabolic intermediate in urushiol biosynthesis.

2 | MATERIALS AND METHODS

2.1 | Bacteria, plant origins, and poison ivy callus

Agrobacterium rhizogenes strains R1000 and ATCC15834 were cultivated on Nutrient Agar (NA) at 28°C, or NA supplemented with 50 μg/ml kanamycin sulfate to select for plasmid pJGJ411 (Dickinson, Weisberg, & Jelesko, 2018). This plasmid was introduced into electro-competent *A. rhizogenes* cells (McCormac, Elliott, & Chen, 1998). Poison ivy (*Toxicodendron radicans* (L.) Kuntze) drupes used in this study were collected from six states: two locations in Virginia

(RoaCo1 (Benhase & Jelesko, 2013) and MontCo1 (37°13'42.79"N 80°25'58.56"W)), Iowa, Michigan, and Texas (Jelesko, Benhase, & Barney, 2017). Drupes were also obtained from single locations in New Jersey (39°54'14"N 74°54'4"W) and Pennsylvania (39°54'14" N 74°54'4"W). Poison ivy spontaneous tissue culture callus was generated from excised axenic RoaCo1 hypocotyl explants placed on 0.5 X Woody Plant media basal salts, 3% sucrose, 0.3% phytagel, 0.5 mg/L Thidizuron (TDZ), and 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D). Callus formed from cut edges of excised hypocotyl segments and was readily propagated on the same media.

2.2 | Optimizing hairy root initiation and establishment

Poison ivy drupes were scarified, surface sterilized, and germinated in the dark (Benhase & Jelesko, 2013). After at least 2 weeks, the germinated seedlings showed a typical etiolated seedling triple response phenotype with short roots, elongated hypocotyls often with apical hooked cotyledons. The hypocotyls of etiolated poison ivy seedlings were prick-inoculated with *A. rhizogenes* strains using a sterile 27 ½ gauge needle to transfer bacterial cells into hypocotyls of intact seedlings. Mock prick inoculation of hypocotyls omitted *A. rhizogenes* cells on the needle. Inoculated intact seedlings were immediately transferred to Magenta boxes with Gamborgs B5 basal synthetic solid media and placed in an environmental growth chamber as previously described (Benhase & Jelesko, 2013). Hypocotyl segments with emerging callus and hairy roots were excised and transferred to hairy root selection media (0.5X Gamborgs media, 2% sucrose, 1X Gamborgs vitamins, 0.6 mg/ml Cefotaxime, and 0.3% Phytagel). After several weeks on selection media, elongating hairy root segments were transferred again to hairy root selection media to confirm both subsequent hormone-independent hairy root growth and clearance of residual *A. rhizogenes* bacteria. Growing hairy roots were passaged at least two times on hairy root selection media and then transferred to hairy root propagation media (same as selection media, except Cefotaxime was omitted).

To investigate both the physiological state of the host plant on hairy root initiation/growth as well as the reciprocal impacts of hairy root grafts on the host seedling vigor, a fully orthogonal three-factor random design was used to evaluate the effects of: (a) *A. rhizogenes* strain ATCC15834 treatment (mock or *A. rhizogenes*), (b) number of prick inoculations (two or six prick inoculations), and (c) the photomorphic state of the host seedling (one week of photomorphogenesis or age-match skotomorphogenesis) at the time of *A. rhizogenes* inoculation on eventual graft and host seedling development. There were eight replications of each combination of the three treatments. At the end of the experiment four response variables were measured on a per seedling basis: (a) the number of initiated hairy roots, (b) hairy root fresh weight upon immediate removal from the host, (c) seedling leaf number, and (d) total seedling fresh weight biomass—less excised hairy roots. The number of initiated hairy root sites per seedling data required natural logarithm (Ln) Ln(X + 1) transformation to better conform to the assumption of a Gaussian distribution. The addition of a constant equal to one to all hairy root sites per seedling values was required to avoid the calculation of the indeterminant value of Ln(0) for seedlings that did not produce any hairy root organs. The hairy root fresh weight biomass values were Ln transformed to achieve a better fit to a Gaussian distribution. The program JMP Pro v14 (SAS Institute Inc.) was used to perform parametric linear regression modeling and backward model selection for all main effects and their interactions (by optimizing the Akaike Information Criterion corrected (AICc) values, indicating the best fit of the parametric linear regression model to the data) were used to identify the most parsimonious parametric linear regression model accounting for each hairy root graft or seedling vigor response. The ANOVA models produced residuals that approximated a Gaussian distribution. Statistically significant differences were defined with $\alpha \leq 0.05$.

To evaluate the poison ivy accession-level effects on hairy root culture establishment 10–12 plants from seven different poison ivy accessions were prick inoculated (on average seven times) with *A. rhizogenes* strain ATCC15834 containing plasmid pJGJ411, a T-DNA binary plasmid containing a *CaMV35S* promoter driving a Firefly Luciferase gene interrupted by an artificial intron (*LUC-INT*) (Dickinson et al., 2018). The *LUC-INT* reporter gene was ideally suited for identifying T-DNA transformed hairy root plant lines because it requires plant mRNA splicing of the artificial intron to yield an intact *LUC* coding sequence suitable for translation of an active luciferase enzyme. This insures that all luciferase activity results from transformed plant cells, rather than potentially luciferase activity from free living *A. rhizogenes* bacteria harboring pJGJ411. Hairy roots were imaged using a cell phone digital camera. Bioluminescent transgenic poison ivy hairy roots were imaged using a single-photon counting video imaging system, as previously described in detail (Jelesko, Harper, Furuya, & Gruissem, 1999). To test how passage number on hairy root selection media affected hairy root accession establishment, we used regression models with number of total hairy root lines transferred as the response and passage number on hairy root selection media as the predictor variable. The data from the first hairy root passage fit the assumption of a Gaussian distribution; however, the data from the second hairy root passage required a Ln(X + 1) transformation to adequately fit a Gaussian distribution. The hairy root data from the third passage had markedly lower total numbers spread over seven treatment levels, and therefore was better modeled by a generalized linear regression model utilizing a Poisson distribution.

2.3 | Evaluating steady-state urushiol and anacardic acid accumulation levels

Established poison ivy hairy root cultures, wild-type roots from various poison ivy accessions (Iowa, Michigan, New Jersey, Pennsylvania, Texas, and two from Virginia MontCo1 and RoaCo1) grown in Magenta boxes, and two samples of the poison ivy undifferentiated

tissue callus line 1–6 were harvested, frozen at −80°C, ground to a frozen powder in liquid nitrogen using mortar and pestle, lyophilized, then urushiols and anacardic acids were extracted from three to five mg of lyophilized plant material and quantified as selective ion counts (SIC)/mg dry plant weight as previously described for urushiol congeners (Aziz et al., 2017). Wild roots and hairy root lines were collected on either one or two sampling dates. In several cases more than one sample of the same poison ivy line/accession was harvested on the same date, in such cases the urushiol levels were averaged to avoid subsampling artifacts. Given this uneven sampling approach poison ivy accession was modeled as a random categorical effect, whereas root type (i.e., hairy root or wild-type root) was modeled as a fixed effect. These two factors were modeled in a mixed-model ANOVA analyses selecting for the optimal fit model using AICc values. The accession-independent variable was included in the C17-urushiol ANOVA model because this nonsignificant term nevertheless resulted in substantially lower AICc values than models that omitted the accession-independent variable, and thus yielded a better overall model fit to the data. Authentic C15:0 anacardic acid standard (Cayman Chemical, Ann Arbor, MI) was used in GC-MS analyses to validate at least one of the suspected anacardic acid congeners as an authentic anacardic acid.

3 | RESULTS

3.1 | Hypocotyl-specific poison ivy hairy root formation

Poison ivy (*Toxicodendron radicans* (L.) Kuntze) showed a pattern of organ-specific susceptibility to *A. rhizogenes* hairy root induction. Three separate pilot experiments in which *A. rhizogenes* ATCC15834 and R1000 were used to prick-inoculate harvested axenic poison ivy leaves failed to produce either callus or hairy roots. On the other hand, the hypocotyls of intact etiolated axenic poison ivy seedlings were readily susceptible to *A. rhizogenes*-induced callus with subsequent hairy root formation, or only hairy root emergence without apparent callus formation (subsequently referred to as just hairy root formation). This approach for primary poison ivy hairy root formation is very similar to the initial steps in producing "composite plants" comprised of transgenic hairy roots on an otherwise intact wild-type seedling (Hansen, Jorgensen, Stougaard, & Marcker, 1989). Using intact poison ivy seedlings as the host tissue for initiating hairy root formation more closely resembled a natural *A. rhizogenes* infection and consistently yielded primary hairy root cultures.

This hairy root induction method was repeated three times to assess the efficiency of two *A. rhizogenes* strains to produce poison ivy hormone-independent hairy root cultures. Approximately 2 weeks after *A. rhizogenes* prick inoculation, hairy root primordia were consistently observed (Figure 1a). Hypocotyl segments containing hairy roots were excised and transferred to hairy root selection media containing Cefotaxime to both suppress the growth of the residual *A. rhizogenes* and to begin selection for hormone-independent hairy root growth. There was no significant difference between the number of initial hairy roots produced between the two *A. rhizogenes* strains (R1000 and ATCC15384) used in this study (contrast $F = 0.0708$, p-value = .7909). Poison ivy seedlings subjected to mock prick-inoculation (Table 1) occasionally formed roots from the hypocotyl, but these adventitious roots did not subsequently grow on hairy root selection media and thus did not show sustained hormone-independent root growth.

Hairy roots emanating from the excised hypocotyl explants typically grew well and formed clumps of hairy roots on selection media. However, the number of hairy roots with both sustained root tip

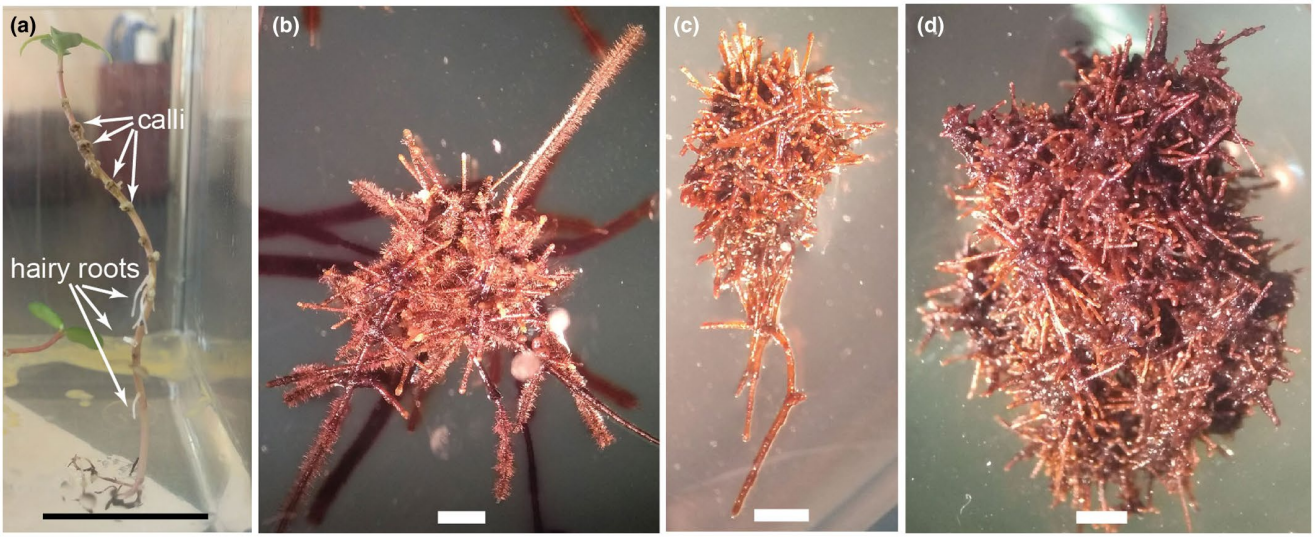

FIGURE 1 Poison ivy hairy root induction, and hormone-independent clumped hairy root growth habit. (a) poison ivy seedling prick inoculated with *A. rhizogenes*. Arrows indicate either emergent calli or hairy roots on hypocotyl inoculation sites. Scale bar 20 mm. (b) hairy root line HR7-5C, scale bar 2 mm. (c) hairy root line HR29-2A, scale bar 2 mm. (d) hairy root line HR29-3B, scale bar 2 mm

TABLE 1 Hairy roots readily initiated at hypocotyl inoculation sites, but few established into cultures with sustained hormone-independent growth

A. rhizogenes strain	Seedlings	Inoculation sites	Initial hairy roots	First passage*	Second passage*	Third passage	Perpetual passage	Establishment efficiency
R1000	32	251	222	214	72	32	1	0.45%
ATCC15834	33	275	250	213	76	28	2	0.80%
mock	20	151	6	4	0	0	0	0.00%

Note: Aggregated values from three independent experimental replications using poison ivy accession RoaCo1 seedlings. Asterisks indicate cefotaxime selection. Perpetual passage refers to hairy root lines that had undergone repeated passages on hairy root propagation media with sustained growth.

growth and root branching activity dramatically decreased during subsequent passage on selection media (Table 1). Cessation of hairy root tip elongation was most apparent with the transfer of isolated unbranched hairy root tips (~1 cm in length). Eventually, only three phenotypically distinct poison ivy hairy root lines showed sustained hormone-independent growth on hairy root propagation media, representing an overall efficiency of only 0.7% of the initial hairy root explants transferred to the first passage on selection media. One hairy root line grew slowly as a single-thickened root, producing few lateral branches. The other two poison ivy root lines grew as dense clumps of roots that could be repeatedly sectioned and passaged as hormone-independent dense clumps of growing roots. One of these two lines, HR7-5C, showed dense clumps of roots with root hairs (Figure 1b). The other hormone-independent root line grew as dense clumps of roots lacking root hairs. Representative examples of this root hair-less clumpy hormone-independent poison ivy root growth phenotype are shown in Figure 1c,d. Regardless, of the presence or absence of visible root hairs, all *A. rhizogenes*-induced root lines showing a hormone-independent growth habit were subsequently referred to as poison ivy hairy root lines as is customary for *A. rhizogenes*-induced hormone-independent root cultures. This limited sampling suggested that the densely clumped root growth habit might be the preferred growth habit of poison ivy hairy roots.

3.2 | Reciprocal impacts between hairy root grafts and their poison ivy host seedlings

The formation of hairy root organs on intact poison ivy seedlings amount to transgenic grafts that are both dependent upon and could reciprocally negatively affect host seedling vigor. Such graft-host interactions are germane toward optimizing the number of hormone-independent hairy root cultures produced, as well as the future utility of composite whole poison ivy plants with rDNA-transformed hairy roots. To this end, the relative contributions of: *A. rhizogenes*, number of prick inoculation sites, and the photomorphic physiological state of the host seedling (at the time of prick inoculation) on metrics of final hairy root initiation and/or host seedling vigor were evaluated in an orthogonal three factor random design experiment. To evaluate the relative additive contributions of each main effect and/or synergistic interactions among them, the results were analyzed using parametric linear regression modeling.

The number of hairy roots initiated per seedling was were significantly positively affected by *A. rhizogenes* treatment, the number of prick inoculation sites, and skotomorphic seedling development (Figure 2a and Table 2). Moreover, there were significant interactions for all pairwise and three-way interactions of these three treatments (Table 2). Many significant interaction terms suggest there were complex physiological interactions between the bacterium, number of wound sites, and the physiological state of the host that contributed to the eventual number of initiated hairy roots. Such complexity was not observed with hairy root biomass. Hairy root biomass was significantly positively affected by skotomorphic development of the host seedling at the time of prick inoculation (Table 2, Figure 2b).

Total host seedling biomass was significantly affected by only two main effects. Host biomass was positivity affected by prior seedling photomorphic growth (Table 2 and Figure 2e) and negatively affected by *A. rhizogenes* treatment (Table 2 and Figure 2f). Thus, the *A. rhizogenes* and photomorphic main effects were influencing total seedling biomass independent of each other. The negative effects of *A. rhizogenes* treatment were not likely due to the bacteria per se, but rather due to the hairy root metabolic sink initiated by the bacteria. Indeed, regression analysis of total seedling biomass versus hairy root biomass demonstrated a significant negative correlation (Figure S1, R^2 = .253, and p-value = .0333). Thus, *A. rhizogenes* and photomorphic main effects were influencing total seedling biomass independent of each other. Interestingly, seedling leaf number was also significantly affected by these same two treatments, but in a different way. In contrast to total seedling biomass where *A. rhizogenes* treatment was a significant main effect, for total leaf number there was a significant interaction of *A. rhizogenes* with photomorphic development (Table 2) that negatively impacted total leaf number (Figure 2d). This synergistic interaction between of *A. rhizogenes* treatment with photomorphogenesis is evident in Figure 2d because the mock-inoculated (blue) line crosses the *A. rhizogenes* (red) line. In addition, there was no significant difference in final leaf number between mock-inoculated seedlings beginning as either skotomorphic or photomorphic developmental states, so total light exposure alone did not result in differential total leaf number at the end of the experiment (connecting "B" letters on Figure 2d blue line). It is noteworthy that the number of *A. rhizogenes* prick inoculation sites did not significantly affect the final host leaf number nor final seedling biomass. In

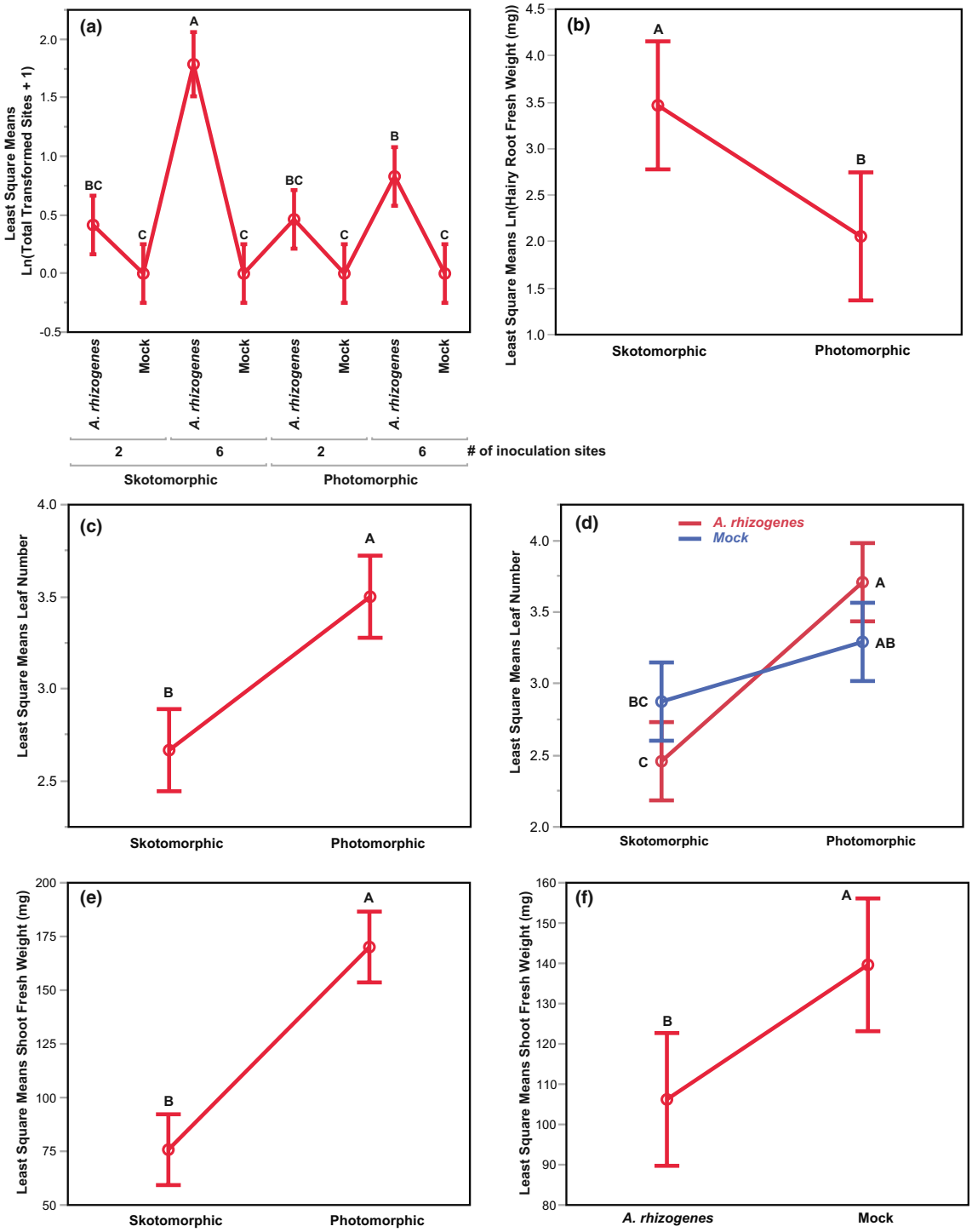

FIGURE 2 Seedling photomorphic state and inoculation site number significantly affected hairy root establishment and host seedling vigor. Least square means illustrated with circles, and error bars indicate associated 95% confidence limits. Skotomorphic or Photomorphic indicates seedling physiological state at the time of *A. rhizogenes* prick inoculation, Mock indicates prick inoculation with a sterile needle, and *A. rhizogenes* indicates prick inoculation with a needle covered with ATTC15384. Numbers 2 and 6 indicate the number of prick inoculation sites on seedling hypocotyls. Same connecting letters indicate non-significant effects, and non-shared letters indicate statistically significant effects with $\alpha \leq 0.05$. (a) Effects of *A. rhizogenes* treatment, inoculation number, and seedling photomorphic state on hairy root induction. (b) Effect on hairy root fresh weight by seedling physiological state. (c) Effect on leaf number by seedling photomorphic state. (d) Interaction of *A. rhizogenes* and seedling photomorphic state on leaf number. (e) Effect of seedling photomorphic state on shoot fresh weight. (f) Effect of *A. rhizogenes*-treatment on shoot fresh weight

summary, the parametric linear regression modeling demonstrated that depending upon the final (graft or host) trait measured, there were complex interactions between *A. rhizogenes*, *A. rhizogenes*-induced hairy root grafts, and the host seedling. These interactions

TABLE 2 *A. rhizogenes*, inoculation site number, and seedling photomorphic physiological state showed both additive and synergistic effects on poison ivy hairy root induction and host seedling vigor

	Main effect *p*-values			
Dependent variable	*A. rhizogenes* treatment	Inoculation number	Photomorphic development at time of inoculation	*p*-values of Interactions between main effects
Ln (hairy root sites per seedling)	<.0001	<.0001	.0141	A*I (<.0001), I*P (.0071), A*P (.0141), A*I*P (.0071)
Hairy root biomass per seedling	N.S.	N.S.	.0161	N.S.
Leaf number per seedling	N.S.	N.S.	<.0001	A*P (.0108)
Total seedling biomass	.0059	N.S.	<.0001	N.S.

Note: *p*-values for statistically significant main effects and interactions between main effects in the optimal linear model. Interaction terms are abbreviated by the first letter of the primary effect: A, *Agrobacterium rhizogenes* ATCC15834 treatment; I, inoculation site number; and P, photomorphic developmental state (i.e., photomorphic vs. skotomorphic) at the time of seedling inoculation. Not significant (N.S.) indicates either the main effect or an interaction between main effects were not significantly contributing to the model and thus removed during model selection.

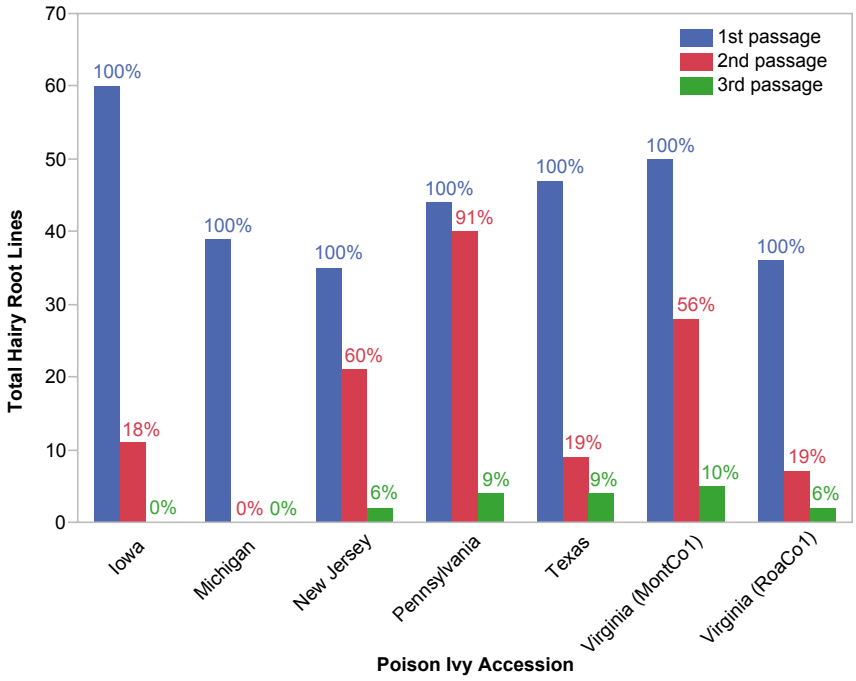

FIGURE 3 Reduced hairy root viability from first to third passages on hairy root selection media. Total number of growing hairy root lines at the end of specified passage number on selection media. Color-coded values above each column indicate the percentage of actively growing hairy root accessions relative to the original number of accession-specific initial hypocotyl-hairy root explants placed on hairy root selection media

are important considerations depending upon the desired utility of the poison ivy hairy root (e.g., isolated hormone-independent hairy root cultures, or as composite poison ivy plants with recombinant DNA-modified hairy root grafts).

3.3 | Accession-level differential hairy root culture establishment

The previous experiments utilized a reference accession of poison ivy commonly used in this laboratory, Virginia RoaCo1 (Benhase & Jelesko, 2013). To investigate the potential role of poison ivy accession on the *A. rhizogenes*-induced hairy root formation and establishment, a randomized experimental design using 12 individuals from seven geographically distinct poison ivy accessions was performed. *A. rhizogenes* strain ATCC15834 harbored a T-DNA binary plasmid pJGJ411 (Dickinson et al., 2018) with a Firefly Luciferase gene containing an artificial intron (*LUC-INT*) was used for the prick-inoculation of the seven poison ivy accessions. In contrast to previous experiments that had an inherent (but not exclusive) bias for the preferential transfer of unbranched single-root tips, this experiment preferentially selected a clumpy hairy root growth habit at each of the three passages on selective media (Figure 3). Poison ivy accession did not have a significant effect on the initial number of hairy root explants that were suitable for initial transfer to selection media (One-way ANOVA, $F = 1.23$, p-value = .3028). Poison ivy accession did have a significant effect (One-way ANOVA, $F = 5.04$, p-value = .0002) on the number of growing hairy roots suitable for a second passage on selection media. Likewise, poison ivy accession was a significant factor (a One-way generalized linear model using a Poisson distribution $X^2 = 13.24$, p-value = .0393) affecting the number of growing hairy root cultures surviving to the third passage

on selection media. The selection bias for clumpy hairy root growth habit resulted in an overall hairy root efficiency of 5.3% proliferating hairy root lines after the third passage on selection media (Figure 3). This was a 7.6-fold improvement in hairy root formation efficiency over the study described in Table 1. It is noteworthy that the hairy root cultures transferred to the first passage on selection media included underlying and adjacent hypocotyl tissue from which the initial hairy roots emerged from. However, hairy root cultures transferred during the second and third passages typically did not include any hypocotyl tissues, and it was in these stages that significant accession-level differences in hairy root viability were observed. These results demonstrated that the poison ivy accession effect did not influence the initial formation of hairy roots on the hypocotyl, but rather the accession-level effect somehow influenced the capacity of these lines to establish as hormone-independent perpetual hairy root cultures. Lastly, the overall low frequency (~5.3%) of hairy root accessions showing hormone-independent growth suggests that most established poison ivy hairy root accessions were likely composed of unique Ri-DNA transformation events (i.e., single clones).

3.4 | High frequency of Ri-DNA and Ti-DNA co-transformation

To assess the frequency of co-transformation of both Ri-DNA and Ti-DNA into hairy root cultures, hairy roots lines were analyzed for in vivo luciferase bioluminescence activity. Hairy root sub-samples were obtained from arbitrarily selected 34 clumpy hairy root lines completing two passages on selection media (five from RoaCo1, six from MontCo1, ten from PA, thirteen from NJ). Luciferase activity was readily observed from transparent/opaque hairy root tips emanating from hairy root clumps (representative luciferase imaging shown in Figure 4b). Nineteen of 34 accessions yielded both Ri-DNA (i.e., hairy root growth phenotype) and T-DNA (i.e., luciferase activity) co-transformation phenotypes, resulting in an estimated co-transformation frequency of 55.8%. The observed luciferase bioluminescence was not due to luciferase expression from residual *A. rhizogenes* bacteria because the artificial intron in the *LUC-INT* gene abrogates expression of the luciferase enzyme in *A. rhizogenes* (Dickinson et al., 2018; Mankin, Allen, & Thompson, 1997). Given the observed low overall hairy root establishment rate (5.3%), the relatively high frequency (55.8%) of luciferase positive hairy root accessions is most parsimoniously explained as originating from single-plant cells that were simultaneously co-transformed with both Ri-DNA and T-DNA, rather than the much less probable scenario of two different plant cells at the same prick inoculation site each separately co-transformed with both Ri-DNA and T-DNA resulting in chimeric hairy root lines (estimated dual transformation frequency of $0.053 \times 0.053 = 0.0028$, or 0.28%).

3.5 | Reduced urushiol steady-state accumulation levels in hairy roots

To the best of our knowledge, steady-state urushiol accumulation levels in the roots of any *Toxicodendron* species have not been previously reported. To evaluate if poison ivy hairy root lines accumulated wild-type urushiol levels, both wild-type roots from axenic poison ivy seedlings and independent hairy root lines were harvested, then total penta- and heptadecyl(en)yl-urushiol (i.e., C15- and C17-urushiols, respectively) congeners were extracted and quantified. Figure 5 illustrates that C15-urushiol levels were greater than C17-urushiol levels (26-fold higher, averaged over all accessions) in wild-type roots. In contrast, an undifferentiated poison ivy tissue culture callus line 1–6 (derived from a Virginia RoaCo1 seedling) did not produce detectable urushiol levels above the limit of detection in the GC-MS assays (Figure 5). Hairy root C15-urushiol accumulation levels were also consistently larger (8.7-fold) than the corresponding

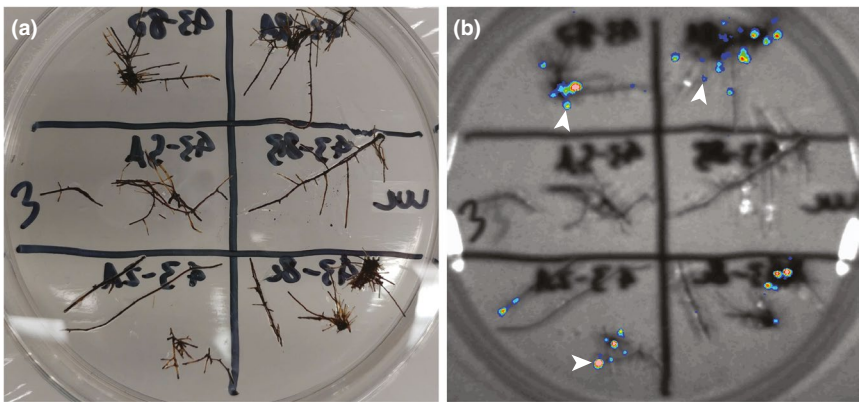

FIGURE 4 High frequency of *LUC-INT* T-DNA cotransformation associated with Ri-DNA-induced poison ivy hairy root establishment. (a) shows a color digital image of a representative plate of subsampled hairy root branches taken from independent hairy root lines. (b) shows a pseudo-color photon counting image superimposed on a reflected light image of the same plate. Blue color indicates low photon emission (i.e., luciferase bioluminescence activity), whereas yellow to red color indicates increasing levels of photon emission. Three white arrowheads indicate relative bioluminescence levels ranging from low, to medium, to high photon emission

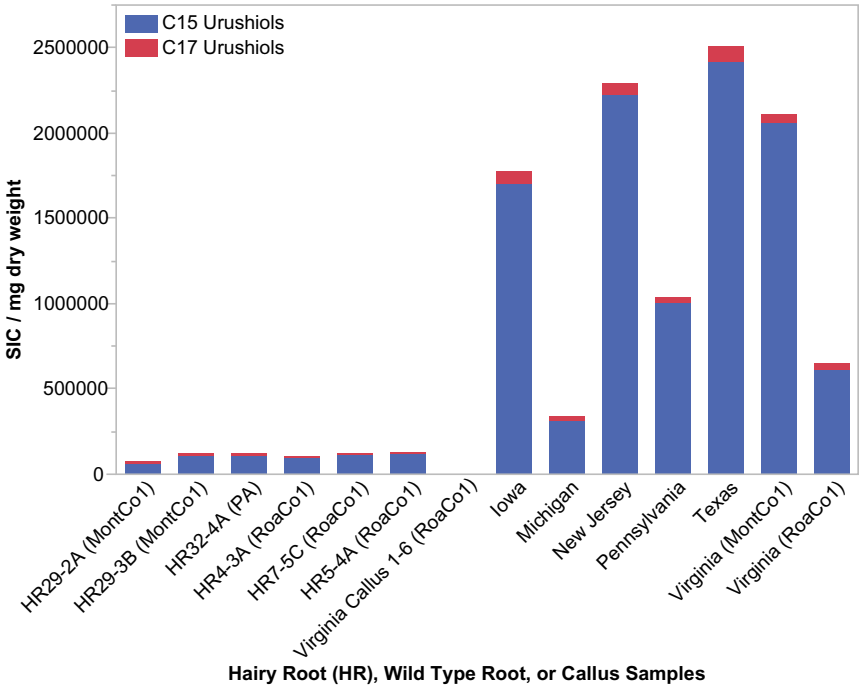

FIGURE 5 Lower urushiol accumulation levels in poison ivy hairy root cultures. Hairy root samples have HR prefix. Wild-type roots are indicated by the state that the drupe was collected from. Dedifferentiated callus sample was derived from a wild-type Virginia RoaCo1 hypocotyl (see Materials and Methods). Selective ion current (SIC) for *m/z* 179 fragmentation ion corresponding to all congeners of either C15-urushiols (blue) or C17-urushiols (red)

C17-urushiol accumulation levels (Figure 5). However, both hairy root C15- and C17-urushiol levels were significantly lower than corresponding wild-type root urushiol levels (Figure 5; and a statistical contrast comparing all wild-type accession to all hairy root lines (C15-urushiol contrast $F = 15.5$, p-value = .0022; C17-urushiol contrast $F = 15.5$, p-value = .0024). Thus, established poison ivy hairy root cultures accumulated significantly lower C15- and C17-urushiol levels compared to wild-type poison ivy roots.

3.6 | Identification of anacardic acid congeners in poison ivy hairy root lines

In some hairy root lines, both total ion chromatograms (TIC) and selective ion chromatograms (SIC) in the region where C17-urushiols eluted (between 13 and 14 min elution time) showed two peaks that did not correspond to expected C17-urushiols (i.e., absence of the *m/z* 179 common urushiol fragmentation ion) (Figure 6a). A search of the NIST chemical library suggested substantial similarity to ginkgolic acid and hydro-ginkgolic acid. These compounds are synonymous with C15:1 anacardic acid and C15:0 anacardic acid (respectively) originally isolated from cashew shells (Gellerman, Anderson, & Schlenk, 1976; Symes & Dawson, 1953; Trevisan et al., 2006). Similar to urushiol, anacardic acid is comprised of four congeners that differ in their degree of unsaturation of the C15-alk(en)yl chain (Trevisan et al., 2006). The fragmentation patterns of trimethylsilyl (TMS)-derivatized anacardic acids isolated from cashew are published (Trevisan et al., 2006) (incorporated into Table 3), and they are distinct from TMS-derived urushiol parent and fragmentation ions. The C15:0- and C15:1-anacardic acid parent ions (*m/z* = 492 and 490, respectively) were observed at expected low levels relative to their more abundant respective major fragmentation ions *m/z* 477 and 475, respectively (Figure 6a, Table 3, and Figure S2). Indeed, when commercially available authentic C15:0-anacardic acid was spiked into urushiol extractions of a hairy root line, authentic C15:0-anacardic acid eluted with the same retention time as the purported C15:0-anacardic acid in the poison ivy hairy root extracts (Figure 6b,c) and showed comparable mass spectra (Table 3 and Figure S2). Two distinct C15:1a- and C15:1b-anacardic acid isomers with a *m/z* of 475 were observed (Figure 6a). Two peaks with *m/z* of 477 fragmentation ions indicated a probable C15:0a* (M+2 ion of a more abundant C15:1a-anacardic acid), and an authentic C15:0b-anacardic acid congener (Figure 6a and Figure 7a,b). Asterisks were subsequently used to indicate probable M+2 artifactual anacardic acid congener species. The presumed C15:0b-anacardic acid eluted with the same retention time as authentic C15:0-anacardic acid standard, and demonstrated similar *m/z* 492 parent and 477 and 219 fragmentation ions observed with the authentic C15:0 anacardic acid (Table 3, Figure 6b, and Figure S2). Hairy root accession HR32-4A also showed C15:2a-anacardic acid accumulation. Hairy root lines HR29-2A and HR29-3B likewise predominantly accumulated C15:0b- and C15:1a-anacardic acids (Figure 7a,b). Control wild-type roots from poison ivy accessions obtained from widely disbursed states all produced major fragmentation ions corresponding to C15:2a-, C15:2b*-, C15:2c-, and C15:3a-anacardic acid congeners (Figure 7d and Figure S3), whereas the hairy root lines produced predominantly the more saturated C15:0b- and C15:1-anacardic acid congeners. So, both wild-type and *A. rhizogenes*-induced hairy root cultures accumulated C15-anacardic acid congeners, previously hypothesized as precursor metabolites leading to urushiol biosynthesis. The identification of both urushiol and anacardic acid metabolites in poison ivy hairy root cultures validates the utility of such hairy

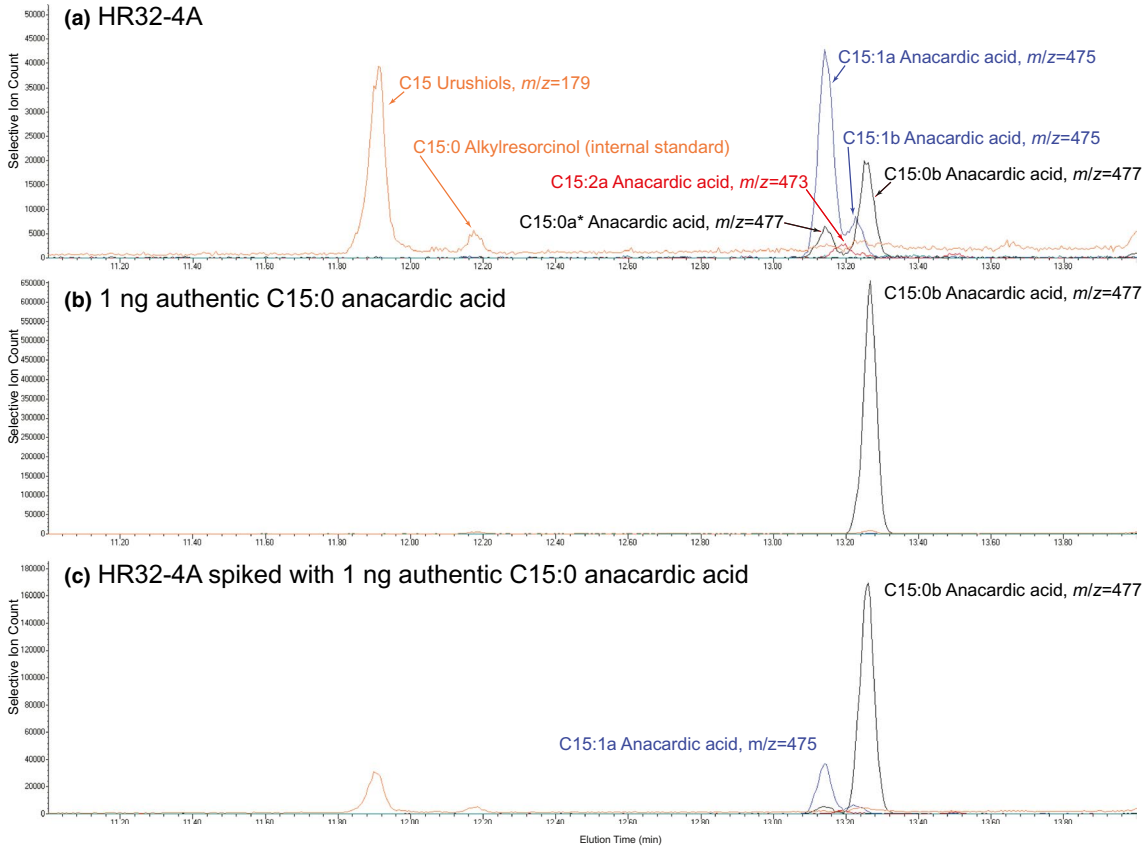

FIGURE 6 GC-MS selective ion scans identified anacardic acid congeners in poison ivy hairy roots. 179 m/z fragmentation ion (brown) indicates total C15- or C17-urushiol congeners, and part of C15:0 alkylresorcinol internal standard. 477 m/z ion indicates an artifactual C15:0a*- and an authentic C15:0b-anacardic acid fragmentation ions (black). 475 m/z indicates two unique C15:1-anacardic acid isomers (blue). 473 m/z fragmentation ion indicates an authentic C15:2a anacardic acid congener (red). Multiple peaks with the same *m/z* ratio are differentiated by a sequential lower-case letter, beginning with the letter "a" for the first eluting peak. (a) hairy root line HR32-4A; (b) 1 ng authentic C15:0 anacardic acid, and (c) HR32-4A extract spiked with 1 ng authentic C15:0 anacardic acid

roots for detailed molecular biology-oriented studies in urushiol biosynthesis.

4 | DISCUSSION

Agrobacterium rhizogenes-induced hairy root formation is a valuable resource that enables a variety of molecular-oriented investigations of root physiology. Moreover, *A. rhizogenes* can be used to concomitantly produce hairy roots containing recombinant transgenes. Similar to previous studies of hairy root induction on woody plants (Cseke, Cseke, & Podila, 2007; Diouf et al., 1995; Han, Keathley, Davis, & Gordon, 1993; Phelep, Petit, Martin, Duhoux, & Tempe, 1991; Shin et al., 1994; Tzfira, Yarnitzky, Vainstein, & Altman, 1996; Yazawa, Suginuma, Ichikawa, Kamada, & Akihama, 1995), the optimal poison ivy target tissue for *A. rhizogenes* transformation was the hypocotyl (Diouf et al., 1995; Han et al., 1993; Phelep et al., 1991; Shin et al., 1994; Tzfira et al., 1996; Yazawa et al., 1995). With that said, hypocotyls on etiolated poison ivy seedlings resulted in significantly more hairy root tissue than hypocotyls on previously light-adapted seedlings. The observed negative effect of photomorphogenesis on poison ivy hairy root formation is in contrast to *Brassica napus* seedlings in which light treatment promotes *A. rhizogenes*-induced hairy root formation (Damgaard & Rasmussen, 1991). The reasons for photomorphic poison ivy seedlings showing reduced hairy root initiation were not investigated, but may be due to increased plant defense processes upon photomorphogenesis. Along this line, photomorphic poison ivy leaves syringe-infiltrated with *Agrobacterium tumefaciens* produce a brown phytochemical/phytoalexin and relatively low transient T-DNA transformation rates (Dickinson et al., 2018). Consistent with *A. rhizogenes'* life history as a plant pathogen (Chilton et al., 1982; Willmitzer et al., 1982) more prick-inoculation sites resulted in more hairy roots that in turn imparted a metabolic sink tissue, resulting in significantly reduced shoot biomass (Figure S1). To date, none of the hairy root lines kept on propagation media have spontaneously produced shoots. Thus, these specific conditions for producing poison ivy hairy roots were not suitable for the regeneration of poison ivy shoots or plantlets.

Poison ivy hairy root growth had several aspects that were markedly different from this laboratory's previous experience

TABLE 3 Validation of C15:0- and C15:1-anacardic acid congeners in a poison ivy hairy root culture

	TMS-derivatized anacardic acid parent and fragmentation ions:			
Sample:	**C15:0**	**C15:1**	**C15:2**	**C15:3**
Anacardic acid isolated from Cashew (Trevisan et al., 2006)	492 (1) **477** (100) 402 (1) 219 (19) 147 (6)	490 (7) **475** (100) 400 (9) 219 (35) 147 (16)	488 (8) **473** (100) 398 (52) 219 (58) 147 (39)	486 (3) **471** (100) 396 (46) 219 (68) 147 (44)
HR32-4A	492 (2) **477** (100) 402 (6) 219 (26) 147 (32)	490 (5) **475** (100) 400 (11) 219 (36) 147 (27)	B.L.D.	B.L.D.
C15:0 Anacardic acid authentic standard	492 (1) **477** (100) 402 (2) 219 (19) 147 (7)	N.A.	N.A.	N.A.
HR32-4A with Anacardic Acid spike	492 (1) **477** (100) 402 (4) 219 (20) 147 (4)	490 (5) **475** (**100**) 400 (14) 219 (41) 147 (37)	B.L.D.	B.L.D.

Note: Underlined *m/z* indicates parent ion. Bold indicates most abundant fragmentation ion. Numbers between parentheses are percentage relative to the most abundant fragmentation ion level.

Abbreviations: B.L.D., below the limit of detection; N.A., not applicable.

with tobacco hairy root cultures. Tobacco hairy roots were easily sub-cultured from single-short hairy root tips that would readily elongate and branch into a dense network of hairy roots (Hildreth et al., 2011). In contrast, isolated poison ivy root tips showed both slow growth rates and reduced lateral root branching activity (Table 1 and Figure 3). This was especially evident over the course of the three sequential passages of hairy root lines on selection media when single-root tips were typically, but not always transferred. In contrast, when clumps of hairy roots were preferentially passaged on selective media (Figure 3) the frequency of hormone-independent root growth increased substantially. The clumpy growth habit with reduced lateral root formation was a consistent characteristic of poison ivy hairy root lines produced in this study, and is in marked contrast to most *A. rhizogenes*-induced hairy root reports that typically describe extensive hairy root elongation with concomitant lateral branching (Benjamin et al., 1993; Daimon & Mii, 1995; Mano & Matsuhashi, 1995; Ohara, Akasaka, Daimon, & Mii, 2000; Suginuma & Akihama, 1995). With that said, the overall efficiency of establishing true hormone-independent hairy root lines was still rather low at approximately five percent. Nevertheless, these poison ivy hairy root lines yielded new metabolic insights into urushiol metabolism.

The steady-state urushiol levels in the poison ivy hairy roots were significantly lower than urushiol levels from roots from intact axenic poison ivy seedlings. The low steady-state urushiol levels in poison ivy hairy roots were nonetheless much greater than the undetectable urushiol levels in a dedifferentiated poison ivy callus tissue culture line Virginia 1–6, suggesting that urushiol biosynthesis likely requires one or more differentiated cell types in poison ivy roots. This is consistent with a number of reports indicating that urushiol accumulates in poison ivy stem and flower organs (Aziz et al., 2017; Gillis, 1971) as well as lacquer tree bark (Zhao, Liu, Zheng, Wei, & Hu, 2013) and all of these organs have specialized resin canal/duct tissues. The spatial association of urushiol accumulation with resin canals/ducts was confirmed using high-resolution 2D-MS in poison ivy stem cross sections (Aziz et al., 2017). Although total steady-state urushiol levels were lower in poison ivy hairy roots, the relative C15- to C17-urushiol congener ratios (C15:C17 urushiol ratio) were similar to wild-type roots. This large C15:C17 urushiol congener ratio is consistent with other reports of urushiol congener levels in poison ivy (Baer, Hooton, Fales, Wu, & Schaub, 1980; Billets, Craig, Corbett, & Vickery, 1976; Craig, Waller, Billets, & Elsohly, 1978; Gross, Baer, & Fales, 1975; Lott et al., 2019). The reduced steady-state urushiol accumulation levels in five independent poison ivy hairy root lines suggest this is a consistent attribute of poison ivy hairy root formation. While the exact cause for the reduced steady-state urushiol levels in hairy roots was not determined, reduced natural product formation in hairy roots from other plant species is documented (Benjamin et al., 1993; Celma et al., 2001; Tsuro & Ikedo, 2011). Cardanols were recently confirmed in poison ivy seedlings (Lott et al., 2019). Cardanol congeners were also detected in poison ivy hairy roots, but were not elaborated on in this report in order to instead focus on the novel identification of another predicted urushiol precursor metabolite anacardic acid.

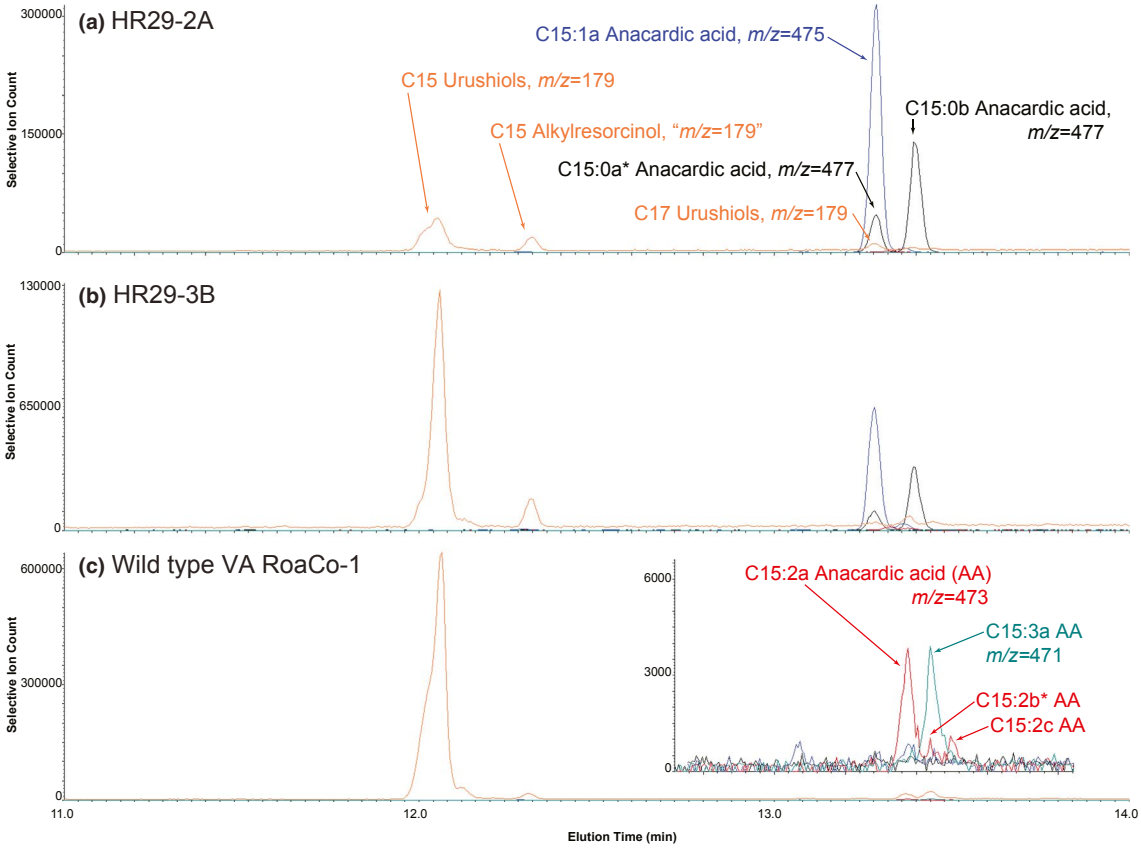

FIGURE 7 Anacardic acid congener composition in hairy roots was different than wild-type roots. The *m/z* ratios for selective ions are color coded as described in Figure 6, with the addition of a *m/z* 471 ion indicating a possible C15:3 anacardic acid fragmentation ion (green). The insert panels visualize selective ion chromatograms omitting the substantially more abundant *m/z* = 179 scan in order to visualize the much lower abundance of major anacardic acid fragmentation ions. Asterisks indicate likely artifactual M+2 anacardic acid ions. Y-axis scale is different for each graph. (a) hairy root line HR29-2A, (b) hairy root line HR29-3B, and (c) wild-type RoaCo1 roots

The greatly reduced C17-urushiol accumulation levels in poison ivy hairy roots facilitated the initial identification of several anacardic acid congeners. Anacardic acid is a proposed metabolite in urushiol biosynthesis (Dewick, 1997; Giessman & Bernfeld, 1967), but was not previously empirically validated in poison ivy. The detection of elevated C15:0- and C15:1-anacardic acids in two hairy root lines may have been due to reduced rates of one or two predicted biosynthetic steps (i.e., a predicted decarboxylation and a hydroxylation step) between anacardic acid and urushiol, resulting in presumably aberrantly elevated steady-state anacardic acid levels. This seems likely because wild-type poison ivy roots accumulated demonstrably higher total C15-urushiol levels than all hairy root cultures.

In addition to the formation of hormone-independent poison ivy hairy root growth resulting from Ri-DNA transfer, *A. rhizogenes* containing a recombinant T-DNA binary plasmid was used to stably transform the hairy roots with a *CaMV35S promoter-LUC-INT* reporter gene. Approximately, 55% of the poison ivy hairy root lines generated by ATCC15834/pJGJ411 showed luciferase bioluminescence activity due to integration and expression of the *LUC-INT* reporter gene on a T-DNA fragment. This high cotransformation efficiency of Ri-DNA (as evidenced by the formation of hairy roots) with co-expression of Luciferase bioluminescence is consistent with previous reports of *A. rhizogenes* harboring recombinant binary T-DNA plasmids transformation rates ranging from 27% to 95% co-transformation with Ri-DNA (CabreraPonce, VegasGarcia, & HerreraEstrella, 1996; Cho, Farrand, Noel, & Widholm, 2000; Cho, Widholm, Tanaka, Nakanishi, & Murooka, 1998; Sretenovic-Rajicic, Ninkovic, Miljus-Dukic, Vinterhalter, & Vinterhalter, 2006; Tsuro, Ikedo, & Kato, 2009; Tzfira et al., 1996). These results demonstrated the feasibility of stable foreign gene transformation and regeneration of transgenic poison ivy hairy root cultures that produce (albeit less) urushiol and in some cases elevated levels of the predicted metabolic intermediate anacardic acid, previously asserted to be involved in urushiol biosynthesis (Dewick, 1997; Giessman & Bernfeld, 1967). In conclusion, this study established a tractable poison ivy transformation-root regeneration protocol for creating transgenic hairy root cultures that could be used for reverse genetics-oriented investigations of putative genes and/or transcripts (Weisberg, 2017) involved in urushiol biosynthesis.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

A.A.L. and C.P.F. preformed experiments and collected data. E.C. and S.R.W. provided GC-MS instrumentation, and either ran samples or supervised C.C.D. C.C.D. ran samples on GC-MS instrumentation and contributed to data analysis. J.G.J designed and supervised all experiments, analyzed data, and wrote the manuscript. All authors read, edited, and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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