Vegetative propagation of putatively laurel wilt-resistant redbay (*Persea borbonia*)

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ABSTRACT

Vegetative propagation experiments were conducted on redbay (*Persea borbonia* (L.) Spreng. [Lauraceae]) to evaluate disease resistance and to conserve germplasm. We developed a primary framework for redbay vegetative propagation to address limitations of long-term seed storage and the need to preserve and screen putatively laurel wilt-resistant redbays. Healthy/asymptomatic redbay individuals were chosen from 6 field sites with high levels of disease pressure and mortality. These individuals were exposed to various IBA forms, bottom heat, and rooting media mixtures. The use of IBA was required for rooting redbay; however, we found no significant differences among IBA type or bottom heat on rooting success. Parent tree provenance significantly affected root length. A 3:1 (v:v) perlite:vermiculite mixture yielded significantly more cuttings with roots and more roots per cutting. We observed an average rooting percentage of 20 to 37%, and ramets that were still alive after 7 mo averaged 46 to 72% rooting across treatments. Therefore, we recommend treating with a 0.3% IBA gel and striking them in 3:1 (v:v) perlite:vermiculite without bottom heat under intermittent mist.

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KEY WORDS

Xyleborus glabratus, *Raffaelea lauricola*, Lauraceae, forest pathogens, restoration, rooting media

NOMENCLATURE

Plants: USDA NRCS (2013)
Foreign plants: Tropicos (2013)
Fungi: Index Fungorum (2013)
Insects: EDIS (2013)

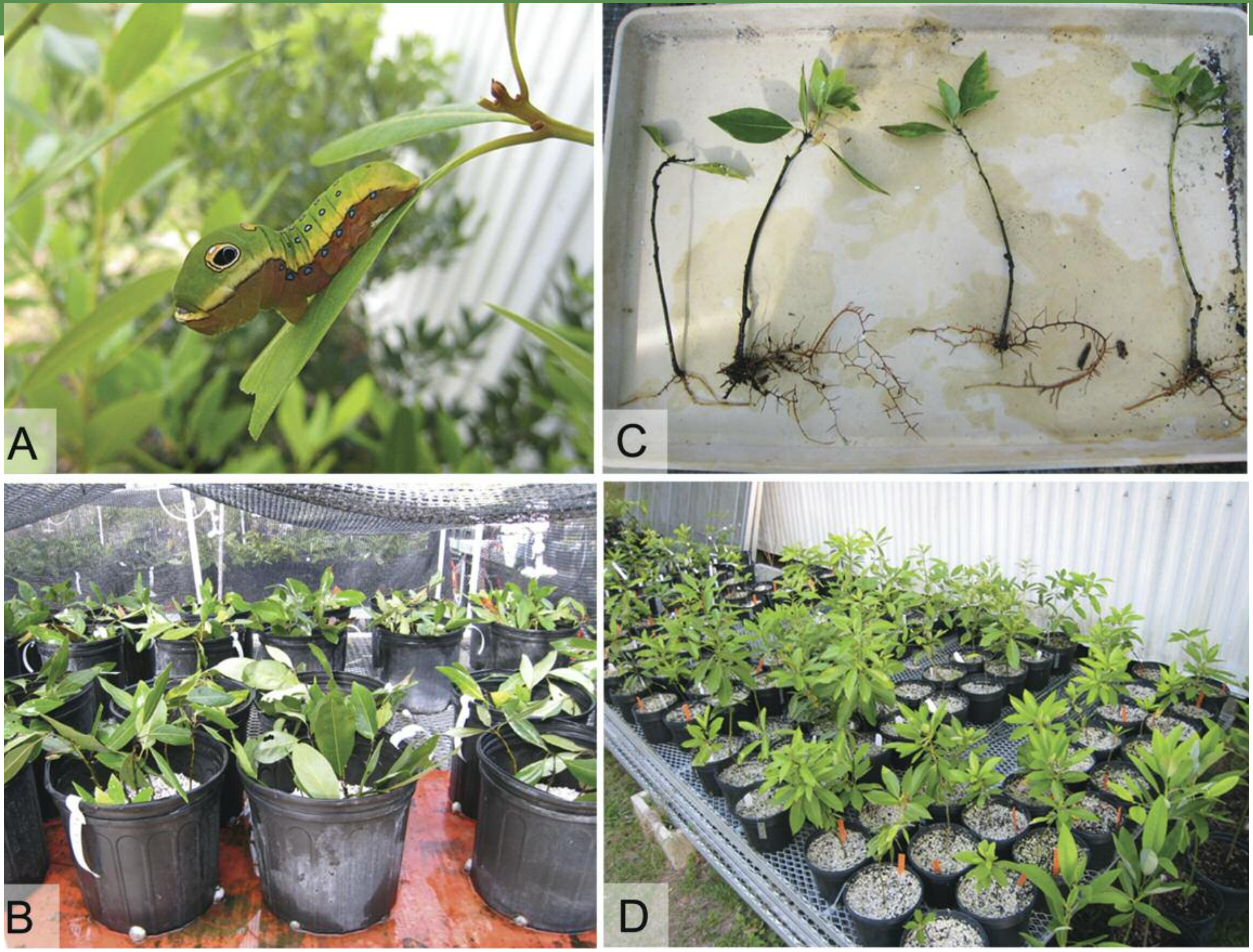

Figure 1. The propagation process: spicebush swallowtail larva feeding on redbay leaf (A); Experiment 1 containers on mist bench, containers in foreground have bottom heat provided by propagation mat (B); rooted redbay cuttings after removal from propagation medium (C); and stecklings of redbay 2 mo after re-potting in open nursery area (D). Photos by Marc A Hughes

Redbay (*Persea borbonia* (L.) Spreng. [Lauraceae]) is a common hardwood tree of the coastal lowlands and plains of the southeastern US. This attractive medium-sized tree is known for having a dense and often rounded crown of drooping branches packed with single layers of leathery, evergreen leaves (Coder 2007). Depending on the site, this aromatic member of the Lauraceae can grow to a height of approximately 18 to 21 m (59–69 ft) with a trunk diameter from 60 to 90 cm (23–35 in) in the forest (Brendemuehl 1990; Coder 2007; FNAI 2010). Redbay, along with live oak (*Quercus virginiana* Mill. [Fagaceae]) and cabbage palm (*Sabal palmetto* (Walter) Lodd. ex Schult. & Schult. f. [Arecaceae]), forms the canopy of maritime hammock forests in the southeastern US (FNAI 2010). Native American tribes in Florida have many medicinal uses for this tree. Redbay serves as the larval host for the palamedes swallowtail (*Papilio palamedes* Drury [Lepidoptera: Papilionidae]) and spicebush swallowtail (*Papilio troilus* Linnaeus) butterflies (Figure 1A), and its fruit is a nutritional source for a variety of birds, rodents, and other mammals (Brendemuehl 1990; Coder 2007; Hall and Butler 2007; Hall and Butler 2010; Steelman 2013).

In 2002, the exotic redbay ambrosia beetle (*Xyleborus glabratus* Eichhoff [Coleoptera: Curculionidae]) was discovered in monitoring traps at Port Wentworth, Georgia (Haack 2006; Rabaglia and others 2006). By 2003, mass mortality of redbays in and around Savannah, Georgia, and the southern

South Carolina area became apparent, and the responsible pathogen was determined to be one of the beetle's fungal symbionts, *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva (Ophiostomataceae) (Fraedrich and others 2008; Harrington and others 2008). This newly discovered disease became known as laurel wilt, which is spread by the deposition of *R. lauricola* spores during the wood-boring activities of the redbay ambrosia beetle. Once introduced into a redbay host, the pathogen rapidly causes complete crown wilt and death. In the past few years, the coastal forests and their redbay populations have been devastated by laurel wilt, with the mortality of large redbays reaching more than 90% in some affected sites (Fraedrich and others 2008; Goldberg and Heine 2009; Shields and others 2011).

Currently, almost all commercially available redbays are grown from seed, which is readily available from mature fruiting trees in September to October (Bonner 2008). Because of the rapid loss of mature redbays from the coastal plain, researchers have begun to locate and monitor healthy redbay individuals with putative resistance. Clonal propagation is essential in order to 1) develop a laurel wilt-resistance screening program for redbays; 2) better understand the possible mechanisms for this putative resistance; 3) establish breeding populations with potentially resistant individuals; and 4) restore affected areas with resistant germplasm. An established method for vegetatively propagated redbay was absent, and based on what is known about their close relatives, swamp bay (*Persea palustris* (Raf.) Sarg.) and avocado (*Persea americana* Mill.), vegetative propagation was considered problematic (Platt 1976; Dehgan and Sheehan 1991). The purpose of this study was to investigate factors influencing the vegetative propagation of putatively laurel wilt-resistant redbays. The investigation was divided into 2 experiments: the first evaluated the effects of rooting hormones and bottom heat on rooting of cuttings; the second investigated the effects of various potting media on redbay rootability.

METHODS AND MATERIALS

Field Sites

Six field locations were chosen along the Atlantic coastal plain and barrier islands of Florida, Georgia, and South Carolina, with 2 sites selected per state. The sites were chosen based on their prior abundance of redbays, which had been decimated by laurel wilt disease. Selecting sites that already had high levels of redbay mortality facilitated the selection for resistance to this disease (data not presented here). The sites in Florida were Ft George Island near the Kingsley Plantation (lat 30°24′35″N, long 81°25′50″W) and Ft Clinch State Park (lat 30°40′05″N, long 81°26′03″W). Georgia sites included the Cumberland Island National Seashore Park (lat 30°51′53″N, long 81°26′59″W) and the maritime forest of St Catherines Island (lat 31°38′00″N, long 81°09′34″W). The South Carolina sites were Hunting Island State Park (lat 32°21′58″N, long 80°26’40″W) and Edisto Beach State Park (lat 32°30′41″N, long 80°18′23″W).

After an initial survey of the field location, 8 to 20 healthy, asymptomatic trees >7.5 cm (3 in) diameter at breast height were selected for monitoring and propagation per site. Selected trees were visually tagged, and global positioning system (GPS) coordinates were recorded. Because cuttings were collected at various times of the year as field locations and research permits became available, collection time was excluded as an experimental variable; however, special care was taken to collect only semi-hardwood cuttings. Large branch cuttings were taken using a pole pruner and then sectioned into smaller segments by hand pruner. Plant material was placed in plastic bags, labeled, and placed on ice within coolers. After returning to the laboratory, coolers were placed in a walk-in cooler and held at 4 °C (39 °F) for no more than 3 d. For each experiment, prior to propagation the branch material was trimmed into single-leader ramets about 15 to 25 cm (6–10 in) in length, 4 to 8 mm (0.16–0.3 in) in diameter, and with 2 to 4 remaining leaves.

Experiment 1

Ten redbay trees were selected from 6 field locations for a total of 60 trees. Forty cuttings per tree were randomly assigned to 4 treatments, with 10 ramets per treatment per pot. All cuttings were placed in 12.5-l (3.3-gal) black plastic pots filled with a 3:1 (v:v) perlite:vermiculite mixture, with polyester fiber covering the drainage holes. The 4 treatments were: 1) powdered indole-3-butyric acid (IBA) 0.1% with bottom heat; 2) powdered IBA 0.1% without bottom heat; 3) IBA gel 0.3% with bottom heat; and 4) IBA gel 0.3% without bottom heat. Bottom heat was provided by heavy rubber propagation heat mats (Pro-Grow Supply Corporation, Brookfield, Wisconsin) set to approximately 10° above ambient (22 °C ± 4° [72 °F ± 4°]). Because of factors such as the scarce number of asymptomatic redbays within the region, the knowledge that wounding can increase their attractiveness to the vector (Hanula and others 2008), and previous failures to propagate redbay without a rooting compound, we used negative (non-hormone) controls, with and without bottom heat, for a random subset of only 20 trees (10 with heat, 10 without heat) in order to avoid oversampling from the putatively resistant germplasm.

Experiment 2

A total of 25 redbay trees were selected from the Ft Clinch and the St Catherines Island field sites. Forty cuttings per each selected tree were randomly assigned to 4 treatments, with 10 ramets per treatment per pot. The 4 media mixtures tested were the following: 1) 3:1 (v:v) perlite:vermiculite; 2) 1:1:1

(v:v:v) perlite:Canadian peat moss:cypress sawdust; 3) 1:1 (v:v) perlite:Canadian peat moss; and 4) 2:1:1 (v:v:v) perlite:cypress sawdust:seedling soilless mix (Super-Fine Germinating Mix, Sun Gro Horticulture, Agawam, Massachusetts). All rooting media mixtures were placed in 12.5-l (3.3-gal) black plastic pots with polyester fiber covering the drainage holes.

Ramet Treatments, Care, and Sampling

In both experiments, the ends of all cuttings were freshly cut with hand shears, quickly dipped in a 1:1 (v:v) 8.25% sodium hypochlorite:water solution, followed by a freshwater rinse. Cuttings were then dipped into powdered IBA 0.1% or IBA gel 0.3% for Experiment 1, and only powdered IBA 0.1% for Experiment 2, for 5 s and placed into holes dibbled within 12.5-l pots, with 10 cuttings per pot. Pots were arranged on a bench in a completely randomized design under an automated mist system in a climate-controlled greenhouse (21.1 °C day/18.3 °C night [70 °F/64 °F]) at the University of Florida, Gainesville (Figure 1B). Mist was run for 20 s every 16 min. Dead cuttings were removed and plants were treated for scale insects and mealy-bugs as needed with 3-in-1 Insect, Disease & Mite Control (Bayer CropScience, Durham, North Carolina). After 7 mo, plants were removed from mist for processing. To free the cuttings from their rooting media, pots were floated in a water tub and flooded until cuttings were released (Figure 1C). The following variables were recorded: number of cuttings rooted per pot, average number of roots per cutting for each pot, and average longest root per cutting for each pot. Cuttings were then transplanted into 3.8-l (1-gal) black plastic pots and returned to the greenhouse to acclimate for 4 to 6 w. After that, pots were moved to outdoor tables (Figure 1D).

Statistical Analysis

For some clones, because of disease all cuttings died or showed remarkable disease symptoms on the mist bench; these were removed and not considered in the data set. The experimental units in these experiments were pots, not individual cuttings. Data were analyzed using SAS 9.3 for Windows (SAS Institute, Cary, North Carolina) using the GLIMMIX procedure. Percentage variables were analyzed using logistic mixed models to negate the effects of any correlations due to factors other than treatments (for example, time of year and spatial genetic clumping). All other models were analyzed using linear models. Type III tests of fixed effects were used to analyze the effects of treatments and for the presence of treatment interactions, with a $P = 0.05$. Tukey's Honestly Significant Difference (HSD) test was used for multiple comparisons in all models with a $P = 0.05$.

RESULTS

Experiment 1

Rooting percentages for cuttings were similar between treatments, indicating that neither bottom heat nor form of IBA product contributed significantly to rooting ability. Cuttings that received a treatment regime rooted at a rate of 24 to 27%, while all non-hormone control plants failed to root, regardless of the presence of heat. If the cuttings stayed green and viable during the 7-mo period on the mist bench, then 60 to 68% of those cuttings produced roots. A heat × location interaction was detected for the rooting percentage of all cuttings and the cuttings that were viable after 7 mo (Table 1): propagules from Edisto Beach and Ft Clinch State Parks preferred bottom heat,

TABLE 1

Experiment 1: P *values for provenance, IBA level, and presence or absence of heat and their interactions on rooting response of redbay cuttings.*

Source of variation	df[z]	Rooting (%)	Rooting of live cuttings only (%)	Roots per cutting (mean)	Longest root per cutting (mean in cm)
Provenance (P)	5	0.193	0.559	0.225	0.002
IBA (I)	1	0.515	0.084	0.078	0.181
Heat (H)	1	0.457	0.932	0.563	0.492
H x I	1	0.548	0.718	0.693	0.857
H x P	5	0.019	0.010	0.164	0.211
P x I	5	0.412	0.113	0.720	0.852
H x P x I	5	0.584	0.731	0.879	0.709

[z] df = degrees of freedom; *n* = 54; number of observations = 216.

while propagules from the other locations rooted better without supplemental heat. The treatments that produced roots longer than 0.5 cm (0.2 in) had an average of 2.7 to 3.6 roots per cutting without significant differences between treatments and without interactions (Table 1). The average longest root was 11.3 to 13.3 cm (4.4–5.2 in) in length and was unaffected by treatment (Table 1). The average longest root per cutting was significantly different, however, when tree provenance was compared (Table 1). Average longest root from the Edisto Beach State Park trees (19.9 cm [7.8 in]) was significantly longer than were roots from trees from Cumberland Island National Seashore Park (7.6 cm [3.0 in]), St Catherines Island (10.2 cm [4.0 in]), Hunting Island State Park (11.0 cm [4.3 in]), and Ft Clinch State Park (11.3 cm [4.4 in]) (Figure 2).

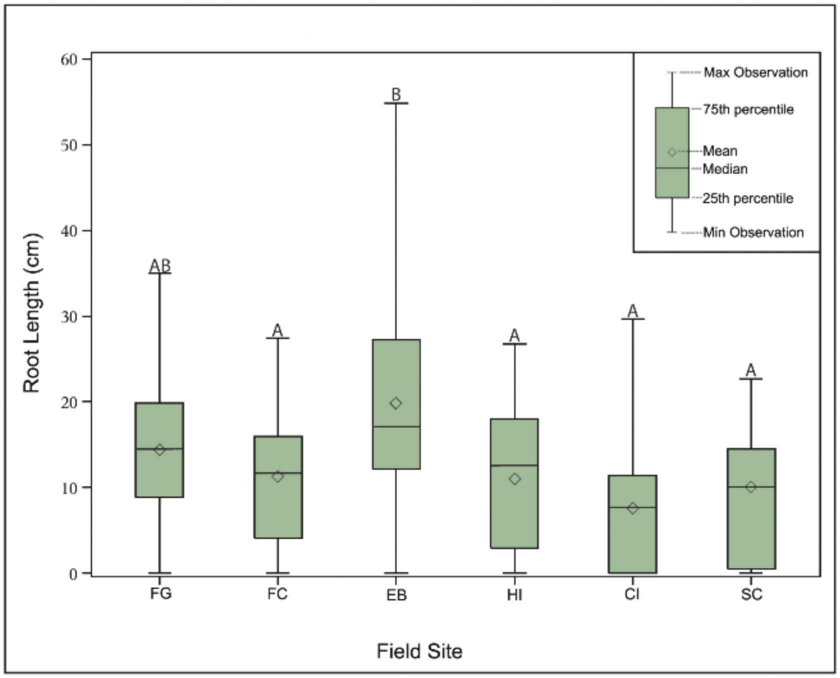

Figure 2. Experiment 1. Comparison of the mean longest root of the propagated cuttings per field collection site. Boxes labeled with different letters are significantly different (*P* = 0.05) using Tukey's HSD test for mean longest root. Means = (FG) Ft George Island, FL = 14.4 cm; (FC) Ft Clinch, FL = 11.3 cm; (EB) Edisto Beach, SC = 19.9 cm; (HI) Hunting Island, SC = 11.0 cm; (CI) Cumberland Island, GA = 7.6 cm; (SC) St Catherines Island, GA = 10.2 cm. Conversion note: 1 cm = 0.4 in.

Experiment 2

Significant differences were observed for some of the different rooting media used, with no detectable interactions (Table 2). The highest percentage of rooted cuttings overall occurred when the 3:1 (v:v) perlite:vermiculite medium was used, and this medium also contained the most cuttings that remained viable during the 7-mo period that rooted (Table 2). In contrast, the 2:1:1 (v:v:v) perlite:cypress sawdust:seedling soilless mix and 1:1:1 (v:v:v) perlite:Canadian peat moss:cypress sawdust mixtures contained the lowest number of rooted cuttings (Table 2). Cuttings in all mixtures produced between 1.7 to 3.1 roots on average per cutting, without significant differences or interactions among treatments (Table 2). The 1:1 (v:v) perlite:Canadian peat moss mixture produced the longest roots on average, with no significant differences or interactions between treatments (Table 2).

DISCUSSION

Data from Experiment 1 indicated that IBA was necessary for rooting redbay, although the specific formulations tested and bottom heat were statistically unimportant. Propagation trials of the related swamp bay were similar to our findings, with no significant differences between the IBA concentrations tested (0.25 and 0.5%); however, semi-hardwood cuttings of this species produced roots without the addition of IBA during the time frames before and after flowering and fruit set, when endogenous auxins and carbohydrates within the cuttings were presumed to be at their highest levels (Gaspar and Hofinger 1988; Dehgan and Sheehan 1991). Tree provenance was shown to influence the length of the longest root, with cuttings from Edisto Beach producing longer roots than were produced at 4 of the other sites. Provenance affecting root length was also the case in seaside alder (*Alnus maritima* (Marshall) Muhl. ex Nutt. [Betulaceae]), with cuttings from Oklahoma having longer roots than those from Pennsylvania, which suggests that some characteristics related to rooting may be site-associated (Schrader and Graves 2000).

Previous propagation research of other woody species illustrated that the use of auxins can generate more rooted cuttings compared with non-hormone treated controls; however, the specific type and (or) concentration needed for optimal rooting can be species-specific (Tchoundjeu and others 2002; Sharma and others 2006). Among 3 auxin types tested for initiating rooting in the medicinal plant *Pausinystalia johimbe* (K. Schum.) Pierre ex Beille (Rubiaceae), IBA performed better than IAA (indole-3-acetic acid) and NAA (1-napthalene acetic acid) at 50 µg per cutting. Similar results were obtained with *Arbutus andrachne* L. (Ericaceae) with IBA performing better than the other auxin forms tested (Al-Salem and Karam 2001; Tchoundjeu and others 2004). Auxin concentration can also affect rooting, with cherry bark elm (*Ulmus villosa* Brandis ex Gamble [Ulmaceae]) rooting better when IBA concentrations were above 0.4% (Bhardwaj and Mishra 2005). Although our study found no differences between the 2 IBA formulations tested, further work is required to elucidate the effects of dif-

TABLE 2

Experiment 2: The rooting responses (mean ± standard error) and P values of redbay cuttings in 4 rooting media.

Media	Rooting (%)	Rooting of live cuttings only (%)	Roots per cutting (mean)	Longest root per cutting (mean in cm)
3:1 P:V	37.0 ± 6.8 a	71.6 ± 8.4 a	3.1 ± 0.2 a	11.8 ± 1.9 a
1:1:1 P:PT:SD	19.5 ± 4.8 b	48.7 ± 10.6 bc	2.0 ± 0.2 a	12.1 ± 1.9 a
1:1 P:PT	28.9 ± 6.1 ab	66.8 ± 9.3 ab	1.7 ± 0.2 a	15.2 ± 1.9 a
2:1:1 P:SD:S	20.0 ± 4.9 b	46.4 ± 10.6 c	2.2 ± 0.2 a	11.7 ± 1.9 a

Source of variation	d.f.	*P* values			
Provenance (P)	1	0.860	0.298	0.743	0.768
Media (M)	3	0.0001	0.0009	0.153	0.291
P x M	3	0.450	0.718	0.367	0.955

Notes: 3:1 P:V = 3:1 (v:v) perlite:vermiculite media mix, 1:1:1 P:PT:SD = 1:1:1 (v:v:v) perlite:Canadian peat moss:cypress sawdust media mix, 1:1 P:PT = 1:1 (v:v) perlite:Canadian peat moss media mix, and 2:1:1 P:SD:S = 2:1:1 (v:v:v) perlite:cypress sawdust: seedling soilless mix. Means in columns followed by different letters are significantly different (*P* = 0.05) using Tukey's HSD test. *n* = 25; number of observations = 100.

ferent auxins and their combination and concentrations on redbay.

The choice of propagation medium was very important in overall rooting success, with 3:1 (v:v) perlite:vermiculite mix containing the highest percentage of rooted cuttings compared with the 1:1:1 (v:v:v) perlite:Canadian peat moss:cypress sawdust and 2:1:1 (v:v:v) perlite:cypress sawdust:seedling soilless mix. The 1:1 (v:v) perlite:Canadian peat moss mix was statistically similar to the 3:1 (v:v) perlite:vermiculite mix in regards to root production. Akin to hormone concentrations, the optimal rooting media for propagation can vary within species and cultivars (Loach 1985). Of the 9 woody plant species Giroux and others (1999) tested by using differing perlite-peat compositions, significant differences were found in 5 species for number of roots produced, and in 4 species for longest root length. At the cultivar level, distinctions in optimal rooting media were also apparent in olive (*Olea europaea* L. [Oleaceae]) (Hosseini and others 2008). Moisture retention and aeration are critical and often inversely related physical properties of a rooting medium. High moisture retention aids in the cuttings' ability to avoid water deficit and stress, while proper aeration is necessary to provide oxygen for developing root initials (Loach 1985). Media with high water-holding capacities have improved the rooting of kiwifruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson [Actinidiaceae]), African teak (*Milicia excelsa* (Welw.) C.C. Berg [Moraceae]), and others (Ofori and others 1996; Ercisli and others 2002; Tchoundjeu and others 2004). Conversely, but similar to our results from redbay, the rooting of the Leyland cypress cultivar 'Castlewellan' (×*Hesperotropsis leylandii* 'Castlewellan' (A.B. Jacks. & Dallim.) Garland & Gerry Moore (*Hesperocyparis macrocarpa* × *Callitropsis nootkatensis*) [Cupressaceae]) and *A. andrachne* was improved as the air content of the media increased (or water content decreased), suggesting that the ideal medium relies on a variety of factors (species, time of year, climate, and so on) (Loach 1985; Al-Salem and Karam 2001). In addition, Loach (1985) suggested that propagation systems may also play a role in rooting success, with "wet" systems calling for an open rooting medium. This parallels our experience with redbay under mist irrigation, with the airy 3:1 (v:v) perlite:vermiculite being the most successful medium tested.

The most significant (and most difficult to quantify) aspect in the success of vegetative redbay propagation was the difference between clones (*P* = 0.0001). Propagation experiments of *Taxus globosa* Schltdl. (Taxaceae) found differences among clones in terms of rooting, and like our experiment, trees of different sizes were selected from natural stands, thus making it impossible to verify if the rooting differences among clones were attributable to genetics, age of mother tree, cutting vigor, or other factors (Muñoz-Guitérrez and others 2009). Previous propagation research on the closely related avocado showed that the genetic background of the parent tree was one of the most important factors for rooting success, with the Mexican races rooting better than both the Guatemalan and the West Indian races. Even with the general success of rootability being linked to the avocado's race, however, results were inconsistent among cultivars and individual clones (Platt 1976; Gustafson and Kadman 1969). This variability of rooting success among clonal propagation of avocado cultivars accounted for the abandonment of this system for the more efficient and productive use of grafted container trees (Platt 1976).

Interwoven with the clonal effects, tree and cutting vigor seemed to be an important factor in rooting success. Although vigor ratings were absent from our experiments, visual observations indicated that the more vigorous redbays in the field tended to yield healthier cutting material, which in turn rooted better in these experiments when compared with cuttings from weak or slowly growing trees. Dawson and King (1994) found that well-fertilized stock plants increased rooting percentage compared with nutrient-deprived plants. This may be a simplistic view, however, as more intensive studies of plant nutrition and adventitious rooting noted complex interactions in which more nutrition is not always better (Blazich 1988; Schwambach and others 2005). We observed that parent trees grown under high light levels appeared to give rise to more robust cuttings, whereas trees grown in heavy shade tended to produce weak, brittle, and often diseased cuttings. The relative health of the cuttings was also variable within the tree's canopy, with top canopy branches appearing much more robust than shaded stems from the interior and lower canopy. Research from Hackett (1988) may be correct in suggesting that upper and peripheral parts of a tree will display reduced adventitious rooting due to a mature ontogenetic state of the tissue; nonetheless, our observations show that the vitality of the cutting material plays a more important role, with the sickly, brittle, shade grown, lower canopy cuttings being unsuitable for propagation. Although easiest to collect, these lower cuttings tended to abscise leaves quickly and die, a phenomenon noted to have grave impacts in rooting avocado (Reuveni and Raviv 1980). Future rounds of propagation from our clonal germplasm will most likely yield better rooting success simply because of the increased nutrition, vitality, and high light conditions of our potted material compared to wild shade-grown redbays.

Finding a successful method for vegetative propagation of redbay is important for species preservation, reforestation efforts, and laurel wilt-resistance research programs. The current effort to collect and maintain redbay seed for conservation is an essential part of securing the diversity of the species; however, long-term seed storage is problematic with viability rapidly decreasing after 24 mo (Vankus 2009). The collection and propagation of germplasm from extant trees in the forests is a useful practice for species that cannot easily be grown from seed, such as *T. globosa* and *P. johimbe*, which are prized and overharvested for their medicinal properties (Tchoundjeu and others 2004; Muñoz-Guitérrez and others 2009). Redbay is critical to the culture and medicine of the Seminole and Miccosukee tribes in Florida, and tribal elders are exploring opportunities for propagating and maintaining trees for these uses (Smith 2013). Our goal is to use cuttings to preserve germplasm, such as that being used for black poplar, which is disappearing, in the UK (Cotrell 2004; Sussex Floodplain Forest Group 2005) and for American elm, which is ravaged by Dutch elm disease, in the US (Smalley and Guries 1993). An additional goal is to find resistance that can, like the American elm, be released as cultivars (Mittempergher and Santini 2004; Townsend and others 2005).

The ability to quickly and efficiently generate redbay clones combined with continued collection of *R. lauricola* isolates facilitates in-depth studies of disease resistance, host-parasite interactions, and the possible genetic factors that mediate these relations. Demand for a practical vegetative propagation method is valid and essential given the ever-decreasing availability of container redbay seedlings and the increasing need of plant material for laurel wilt research and replanting projects. Our study establishes a primary framework for redbay vegetative propagation involving simple and readily available materials. Future research will explore the seasonality and other propagation parameters that may increase the productivity and efficiency of our system.

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