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Induction, Identification, and Characterization of Tetraploids in Japanese Privet (*Ligustrum japonicum*)

Mohammed I. Fetouh[1]
Horticulture Department, Faculty of Agriculture, Tanta University, Tanta, Egypt; and Institute of Food and Agricultural Sciences, Gulf Coast Research and Education Center, University of Florida, 14625 County Road 672, Wimauma, FL 33598

Abdul Kareem[2]
Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan; and Institute of Food and Agricultural Sciences, Gulf Coast Research and Education Center, University of Florida, 14625 County Road 672, Wimauma, FL 33598

Gary W. Knox
Department of Environmental Horticulture, West Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 155 Research Road, Quincy, FL 32351

Sandra B. Wilson
Department of Environmental Horticulture, Institute of Food and Agricultural Sciences, University of Florida, Fifield Hall, Gainesville, FL 32611

Zhanao Deng[3]
Department of Environmental Horticulture, Gulf Coast Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 14625 County Road 672, Wimauma, FL 33598

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Abstract. **A number of privet species (*Ligustrum* spp.) that are important to the nursery and landscape industry have escaped cultivation and become invasive or weedy in the United States and other countries. Induced tetraploids in these species may produce new selections or cultivars with reduced or eliminated invasive potential. Applying drops of semisolid agar containing 0.1% to 0.3% colchicine and 0.2% dimethyl sulfoxide (DMSO) to newly emerged seedlings of japanese privet (*Ligustrum japonicum* Thunb.) resulted in 15.6% to 22.6% tetraploid induction. The nuclear DNA content of tetraploids was 5.31 pg/2C, 101.9% higher than that of diploids. Compared with diploid plants, tetraploids were more compact, with an average of 31.0% shorter plant height and 33.1% smaller canopy width. Tetraploids had 29.2% thicker internodes, and their leaves were 39.5% larger and 33.8% thicker, resulting in 42.1% to 24.1% greater fresh or dry leaf weights (per leaf) in tetraploids compared with diploids. Without indole-3-butyric acid (IBA) treatment, cuttings from tetraploids showed 28% lower rooting than diploids. IBA treatments improved the rooting of tetraploid cuttings, resulting in 65% rooting success. These results indicate that tetraploids can be readily induced in japanese privet and induced tetraploids show significant changes in plant growth and size, shoot growth, leaf morphology, and rooting of cuttings. The modified tetraploid induction method and the induced tetraploids are expected to be useful for producing new selections or cultivars with reduced invasive potential in japanese and other privets.**

Exotic plant invasions are considered one of the main causes of the degradation of ecosystems and the loss of biodiversity globally (Theoharides and Dukes, 2007). Ornamental horticulture has been recognized as the main source of plant invaders (Bell et al., 2003; Niemiera and Von Holle, 2009; Reichard and White, 2001; Rejmánek, 2014). The economic impacts of invasive plant species in the United States are estimated at nearly $35 billion (Pimentel et al., 2005). To help mitigate this huge impact and meet the nursery and landscape industry's need for plant materials, horticulturists have been searching for cultivars with reduced invasive potential (Knox and Wilson, 2006; Trueblood et al., 2010; Wilson and Mecca, 2003; Wilson et al., 2004, 2012). Multiple ornamental breeding programs in the United States have initiated breeding projects to develop new cultivars with reduced or eliminated invasive potential in major invasive ornamental shrubs or trees (Anderson, 2007; Czarnecki et al., 2012; Freyre et al., 2012; Leonhardt and Shi, 2009; Olsen, 2007; Phillips et al., 2015; Ranney et al., 2007, 2010; Thammina et al., 2011; Vining et al., 2012). Developing genetic tools and generating germplasm resources that can be used for this objective have become essential and important for current and future ornamental plant breeding (Anderson, 2007; Li et al., 2004; Olsen, 2007; Thammina et al., 2011; Vining et al., 2012).

Several species of *Ligustrum* L. (privet) are commonly used as ornamentals in many parts of the world (Dirr, 1998; Wilson et al., 2014). In the landscape, these plants are valued for their evergreen leaves, white flowers, adaptability to a range of landscape conditions, tolerance to pruning, resistance to diseases, and wide availability (Dirr, 1998). Some *Ligustrum* species have escaped cultivation and become naturalized in natural areas (Munger, 2003). For example, 16 countries report naturalization of chinese privet (*Ligustrum sinense* Lour.) (Morris et al.; 2002; Munger, 2003). In the United States, chinese privet has escaped in 20 states and is considered invasive in many states (FLEPPC, 2015; Munger, 2003). Glossy privet (*Ligustrum lucidum* W.T. Aiton) has escaped cultivation in 10 states in the United States (Munger, 2003; Wilson et al., 2014) and is also a weed in Australia (Panetta, 2000), New Zealand (Miller and Henzell, 2000), and Argentina (Hoyos et al., 2010). Japanese privet (*L. japonicum* Thunb.) is native to Japan and eastern Asia and was introduced to the United States from Japan and Korea in 1845 as an ornamental landscape plant (Munger, 2003; Wilson et al., 2014). Since then, this species has been widely used in the landscape in the southeastern, southern, and western United States. It has escaped cultivation and become naturalized in 12 southeastern states in the United States (Munger, 2003). Japanese privet commonly forms dense thickets in the field or forest understories, shading and displacing many native species. Once established, it is difficult to eradicate.

The invasiveness of many ornamentals is attributed to a number of factors including prolific production of viable seeds. Thus, making plants sterile or seedless can reduce, even eliminate, their invasive potential (Anderson, 2007; Ranney, 2004, 2006). Several genetic tools have been used to reduce seed production, viability, and/or germination, including natural mutations, artificial mutagenesis, interspecific hybridization, ploidy manipulation (Czarnecki et al., 2012; Freyre et al., 2012; Trueblood et al., 2010), endosperm culture (Thammina et al., 2011), and transgenics (Li et al., 2004; Vining et al., 2012). So far, ploidy manipulation has resulted in considerable success and yielded multiple sterile, noninvasive cultivars or breeding lines in several important ornamental plants, such as

Hypericum androsaemum L. (Trueblood et al., 2010), *Lantana camara* L. (Czarnecki et al., 2012), and *Ruellia simplex* Wright (Freyre et al., 2012).

The key step in ploidy manipulation is chromosome doubling and induction of stable tetraploids, which are the gateway to obtain other ploidy levels (triploids, pentaploids, hexaploids, octoploids, etc.) through interploid crosses. Several antimitotic agents have been used to inhibit the separation of chromosomes at the anaphase of cell division and achieve chromosome doubling in ornamental plants (Contreras, 2012; Contreras et al., 2010; Nadler et al., 2012; Vining et al., 2012). Colchicine has been one of the widely used agents to induce tetraploids in numerous ornamental plants (Abdoli et al., 2013; Henny et al., 2009; Lehrer et al., 2008; Leonhardt and Shi, 2009). Several types of plant tissues or organs, such as seeds, seedlings, axillary buds or shoot tips, embryos, and cultured cells or tissues, have been used as the target for colchicine treatment with various rates of successful tetraploid induction and mixoploidy (Habbard et al., 2016; Henny et al., 2009; Jones et al., 2007; Lehrer et al., 2008; Leonhardt and Shi, 2009; Vining et al., 2012). The availability of an effective and efficient chromosome doubling and tetraploid induction protocol is critical for successful ploidy manipulation and development of sterile, noninvasive cultivars in invasive ornamental plants.

Our literature searches and quick surveys of privet cultivars indicated a lack of tetraploids in glossy, chinese, and japanese privet. Preliminary work on treating axillary buds of privet plants with colchicine resulted in few solid tetraploids. Thus, the main objectives of this study were to use japanese privet as a model to evaluate the effectiveness and efficiency of the semisolid agar method for induction of stable tetraploids in privet and to characterize the effects of chromosome doubling and tetraploidy on shoot growth and leaf morphology.

Materials and Methods

Plant materials. Open-pollinated seeds were collected from mature shrubs of *L. japonicum* 'Texanum' grown at the University of Florida (UF) North Florida Research and Education Center, Quincy, FL, in Feb. 2010 and shipped to the UF Gulf Coast Research and Education Center (GCREC), Wimauma, FL. Dry seeds were sown onto a commercial potting mix (Metro Mix 200; Sun Gro Horticulture, Agawam, MA) in plastic containers (15 cm in diameter) in Feb. 2011. Seeds were covered with a layer of horticultural grade vermiculite (≈1 cm thick) and germinated in a growth room at ≈24 °C with 16 h of light (120 to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 8 h of dark. About 5 weeks after sowing, seedlings emerged with fully expanded cotyledons.

Induction, identification, and confirmation of tetraploids. The semisolid agar method of Jones et al. (2007) was used with some modifications. Colchicine was applied to the growing points of newly emerged seedlings at the cotyledonary stage to induce tetraploids. A 1% colchicine stock solution was made by dissolving colchicine power (Sigma-Aldrich, St. Louis, MO) in water. The colchicine stock solution was added to melted semisolid agar containing one-fourth-strength Murashige and Skoog (MS) basal salts, 0.55% agar, and DMSO (Sigma-Aldrich; final concentration 0.2%) to a final concentration. Three colchicine concentrations were used: 0.1%, 0.2%, and 0.3%. The colchicine-containing agar solution was kept at 40 °C and then 30 µL were pipetted onto the growing point of each privet seedling. Treated seedlings were placed in a high humidity (≈100% relative humidity) growth chamber at 24 °C in dark to minimize colchicine degradation by light. Control seedlings received semisolid agar without colchicine. All treatments were repeated during three consecutive days.

After treatment, seedlings were grown in a greenhouse, and fertigated with 50 to 100 ppm nitrogen as needed. Seedlings were kept in community containers until they produced shoots with several true leaves and later transplanted individually into new containers (10 to 15 cm in diameter). Seedlings were grown in these containers for ≈1 year, and then transplanted into ground beds at the UF's GCREC in Mar. 2013. All transplanted plants were grown in full sun, irrigated through drip tapes, and fertilized with 15 g of 15N–3.9P–12K controlled-release fertilizer (Osmocote; Scotts, Marysville, OH).

To identify tetraploids, multiple fully expanded young leaves were collected from each plant, and each leaf was analyzed separately for ploidy level in Jan. and Feb. 2013. The Partec PA Ploidy Analyzer (Partec, Münster, Germany) was used as described by Czarnecki and Deng (2009). The CyStain Ultraviolet Ploidy Precise P dye (Partec) was amended with 1.25% (w/v) polyvinylpyrrolidone (PVP) and 0.125% (v/v) β-mercaptoethanol (ME) to improve the sharpness of the peaks indicating ploidy levels. Nontreated seedlings (controls) were used as the diploid reference. Identified tetraploids were confirmed by analyzing additional leaves on ≈4-year-old plants in Mar. 2015 using a CyFlow® Cube 6 flow cytometer (see below).

Nuclear DNA content determination and ploidy confirmation. Nuclear DNA contents of identified tetraploid plants were determined on a CyFlow® Cube 6 flow cytometer (Sysmex; Partec GmbH Otto-Hahn-Strasse 32 D-48161, Münster, Germany) using the procedure described by Doležel et al. (2007) and modified by Cao et al. (2014). Four plants with known nuclear DNA contents (Doležel et al., 2007) were tested as potential internal references. Radish (*Raphanus sativus* 'Saxa') with a nuclear content of 1.11 pg/2C was selected as the internal reference for use in this study. Using a sharp razor blade, fresh tender leaves were cochopped with a similar amount of leaf tissue of radish in 1 mL of cold LB01 lysis buffer to release the nuclei. The lysis buffer contained 15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, and 0.1% (v/v) Triton X-100. The buffer was adjusted to pH 7.5 and filter-sterilized. Before use, ME was added to the lysis buffer to a final concentration of 15 mM. The collected nuclei (in ≈1 mL of lysis buffer) were stained with 50 µL of DNA fluorochrome propidium iodide stock solution (1 $\mu g \cdot \mu L^{-1}$) (Sigma-Aldrich) and 50 µL of RNase stock solution (1 $\mu g \cdot \mu L^{-1}$) (Sigma-Aldrich) for 2 to 5 min at ambient temperatures and in dark conditions. The stained nuclei suspension was then analyzed by flow cytometry. At least three runs were performed for each privet sample, and at least 3000 nuclei were counted in each run. The nuclear DNA content of the japanese privet samples was calculated according to Doležel et al. (2007): sample nuclear DNA content (pg/2C) = internal reference nuclear DNA content (1.11) × (mean fluorescence value of sample/mean fluorescence value of internal reference).

Morphological characterization. Four tetraploid and four diploid plants (≈4 year old) were selected in Feb. 2015 for morphological characterization. Plant heights were measured from the ground level vertically to the highest point of the plant canopy; plant widths were measured by projecting the canopy of individual plants to the ground and measuring the widths of the projected area on the ground in two perpendicular directions. Four straight terminal shoots (≈40 cm in length) on each of the selected tetraploid and diploid plants were selected for determining shoot characteristics. The selected shoots were cut off the plants and all leaves were removed from the shoots at the base of the leaves. The bare shoots were used to measure the internode length, internode diameter, and shoot fresh weight. The shoots were then oven dried at

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Mohammed I. Fetouh confirmed the ploidy levels, determined nuclear DNA contents, characterized plant morphological changes and growth characteristics, conducted rooting experiments, collected and analyzed data, prepared figures, and drafted the manuscript. Abdul Kareem germinated seeds and treated seedlings to induce tetraploids. Gary W. Knox and Sandra B. Wilson grew parental plants, collected and processed seeds. Zhanao Deng designed the experiments, supervised the project, reviewed experiments and data, and revised and finalized the manuscript.
[1]Visiting scholar.
[2]Visiting graduate student.
[3]Corresponding author. E-mail: zdeng@ufl.edu.

70 °C to a constant mass before shoot dry weight determination.

To measure relative stem elongation rates (RSER), branches with expanding leaf buds (≈1–2 cm) were selected at random and labeled in Mar. 2015. The lengths of the selected branches were measured weekly for 5 weeks during March and Apr. 2015 from the bud scale scars to the tip of the branches when the shoots were elongating. RSERs were calculated according to Morris et al. (2002).

$$\text{RSER} = \frac{\log_e L2 - \log_e L1}{t2 - t1}$$

where $L1$ is the shoot length at the start of the time interval $t1$, $L2$ is the shoot length at the end of the time interval $t2$, and RSER is expressed as cm·week^{-1}.

To determine leaf characteristics, mature leaves were harvested from each of the randomly selected stems, and the number, area, thickness, and fresh weight of leaves were recorded. The harvested leaves were then oven dried at 70 °C to a constant mass to obtain dry weights. Leaf areas were measured according to Matthew et al. (2002); digital images of individual leaves were obtained from a flatbed scanner at a resolution of 300 dots per inch and then their area was determined using Scion Image (version 4.0.2; Scion Corp., Frederick, MA). Leaf area and dry mass data were used to derive the specific leaf area (SLA) and specific leaf mass (SLM). SLA was calculated according to Cornelissen et al. (2003): leaf area was divided by the dry mass of the leaves and expressed in mm^2·mg^{-1}. SLM was calculated according to Larcher (2003): leaf dry mass was divided by the one-side area of fresh leaves and expressed in mg·mm^{-2} (thus SLM is the inverse of SLA).

Fig. 1. Seedlings of 'Texanum' japanese privet shortly after their growing points between cotyledons were exposed to colchicine (final concentration 0.1% to 0.3%) in droplets of semisolid agar containing 0.2% dimethyl sulfoxide and one-fourth-strength Murashige and Skoog basal salts.

Rooting of tetraploid cuttings and effect of IBA concentration. Semi-hardwood terminal shoots, ≈10 cm long and consisting of four to five nodes, were taken from tetraploid and diploid plants on 1 Mar. 2015 and brought back to the laboratory. Shoots were re-cut at ≈0.5 cm below the third node (from tip) at a 45° angle. Then, leaves at the lower two nodes were removed, and the leaf at the apical tip was kept. Prepared cuttings were arranged in groups of 40 with a rubber band and completely soaked in water for 24 h to leach out potential rooting inhibitors (Wang and Zhong, 2012). Soaked cuttings were then briefly immersed in a fungicide solution (Banrot 40WP, Everris NA, Inc., Dublin, OH; 0.53 g·L^{-1}) followed by a 5-s dip of their lower ends in a root-promoting solution to a depth of 2 cm. Three different concentrations of IBA were used (1000, 2500, or 5000 mg·L^{-1}) for the root-promoting solution. The control was water without any IBA. Cuttings were then stuck into 40-cell propagating trays (cells 3 × 3 × 3 cm) filled with potting mix (Fafard® 3B; Sun Gro Horticulture). Trays were placed on a metal bench in a glasshouse under an automatic mist system run for 3 s at 5-min intervals during the day. Air temperatures in the glasshouse were 18 to 28 °C, relative humidity was 70% to 90%. After 8 weeks, cuttings were harvested and the number and fresh weight of roots on each cutting, the length (cm) and diameter (mm) of the longest root on each cutting, and the percentage of cuttings rooted were recorded. The rooting experiment was repeated in May 2015.

Statistical analysis. Plant, shoot, and leaf morphological data were analyzed using a randomized complete block design with the ploidy level as the main factor. The rooting experiment was analyzed using a completely randomized design with ploidy level as factor A

Table 1. Effect of colchicine concentrations on seedling survival and tetraploid induction in japanese privet (*Ligustrum japonicum*).

Colchicine concn (%)	Individuals treated (no.)	Survival (%)	Tetraploids (no.)	Avg tetraploid induction rate ± SD (%)	Mixoploids, no. (%)
Control	10	100.0	0	0	0
0.1	67	64.2	9	20.9 ± 6.5	8 (11.9)
0.2	49	71.4	10	22.6 ± 12.0	8 (16.3)
0.3	66	68.2	7	15.6 ± 10.6	7 (10.6)

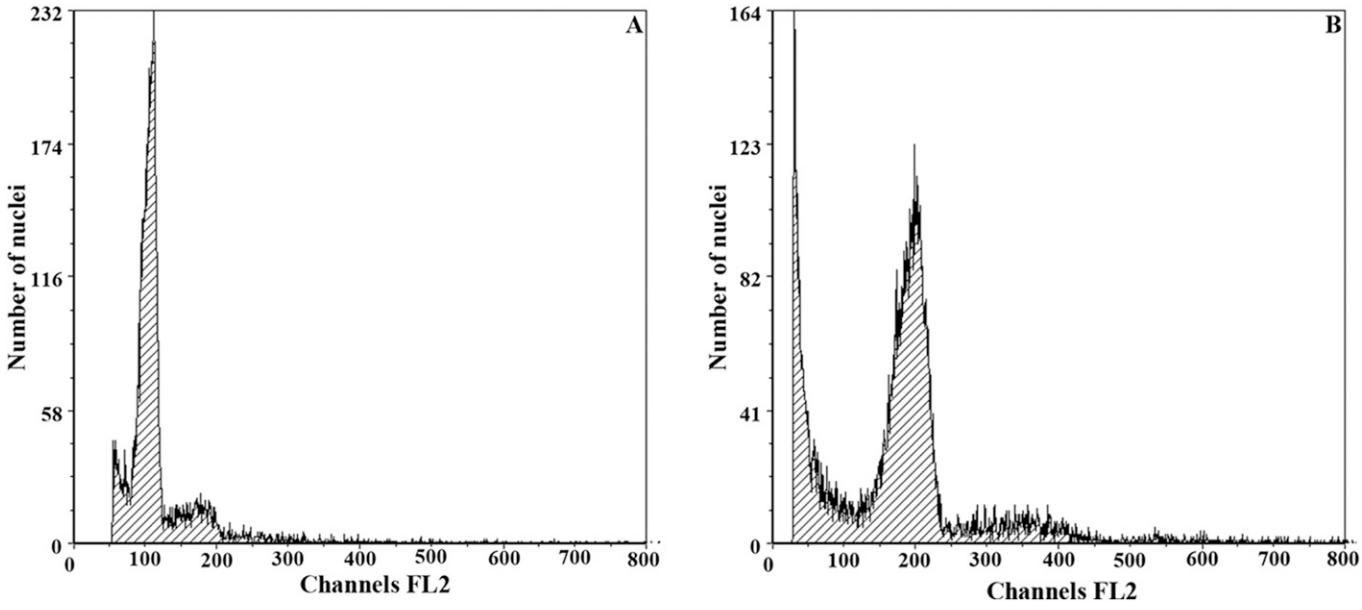

Fig. 2. Flow cytometry histograms of (**A**) a typical diploid and (**B**) an introduced tetraploid) of japanese privet generated by a CyFlow® Cube 6 flow cytometer. Diploid and tetraploid leaf cells were stained with propidium iodide and the relative fluorescence of individual nuclei was observed on a CyFlow® Cube 6. The horizontal axes (Channels FL2) show the relative fluorescence of individual nuclei and the vertical axes show the number of nuclei.

and IBA concentration as factor B. The rooting experiment was replicated four times and with 10 cuttings treated per combination of ploidy level and IBA concentration. The experiment was repeated once and similar results were obtained. Thus, both results were combined and analyzed using the MSTAT-C program (Michigan State Univ., East Lansing, MI) for variance and mean differences (least significant difference test at $P = 0.05$).

Results

Induction of tetraploids. Seeds of 'Texanum' japanese privet germinated readily with an average of 42% emergence within 5 weeks. By this time, the majority of seedlings (≈80%) had two cotyledons with a very small growing point in between them, which is the ideal stage for the semisolid agar method. After three applications of the semisolid agar-containing colchicine, the growth of some seedlings was inhibited, but others resumed growth and produced shoots (Fig. 1). Multiple leaves were collected from each of the treated seedlings for ploidy analysis. On average, 16% to 23% of treated seedlings were solid tetraploids, and 11% to 16% were mixoploids containing diploid and tetraploid cells, or tetraploid and octoploid cells in the same plants (Table 1). Mixoploid plants were discarded before transplant to the ground beds. Two years after the initial analysis, all solid tetraploids were confirmed by analyzing the ploidy levels of leaves from multiple new shoots. There were no significant differences among the three colchicine concentrations used (0.1%, 0.2%, and 0.3%) in terms of survival rate, tetraploid induction rate, and frequency of mixoploids (Table 1).

Nuclear DNA contents. Sharp histograms of relative fluorescence and small coefficients of variation were obtained for the samples analyzed (Fig. 2). Based on 'Saxa' radish as an internal reference, the average nuclear DNA contents of diploid and tetraploid japanese privet were 2.63 and 5.31 pg/2C, respectively (Table 2). Induced tetraploids contained 102% more nuclear DNA than the diploids.

Morphological comparisons. Significant differences were observed between tetraploid and diploid japanese privet seedling in a number of morphological characters (Table 3). The most obvious difference was the smaller stature of the induced tetraploids. On average, the induced tetraploid plants were 31.0% shorter and their canopy width was 33.1% smaller than the diploid plants. The internodes of the tetraploids were significantly thicker (29.3% increase). Tetraploids and diploids had similar fresh and dry shoot weights. During the growing season, the shoots of tetraploids and diploids elongated by an average of 0.32 to 0.44 cm per week.

Leaves of tetraploids were much larger and thicker, with 39.5 and 33.8% increase in leaf area and leaf thickness, respectively, compared with the leaves of diploids (Fig. 3; Table 1). These larger and thicker leaves also resulted in greater leaf fresh and dry weights in tetraploids. The average fresh and dry weight of individual leaves were 1.11 and 0.35 g in tetraploids, 42.1% and 24.1% greater than the fresh and dry leaf weight of diploids, respectively.

Table 2. Nuclear DNA contents of japanese privet (*Ligustrum japonicum*) diploids and induced tetraploids determined by flow cytometry.

Ploidy level	Individuals analyzed (no.)	Nuclear DNA contents (pg/2C)	Mean nuclear DNA content ± SD (pg/2C)
2*x*	7	2.52–2.77	2.63 ± 0.06
4*x*	5	5.07–5.43	5.31 ± 0.13

Table 3. Morphological comparison between diploid and induced tetraploid plants of japanese privet (*Ligustrum japonicum*).

	Samples analyzed (no.)	Traits	Diploids	Tetraploids (percent change over diploids)
Trees	8	Plant height (cm)	155.63	107.33 (–31.0%)*
	8	Canopy width (cm)	122.00	81.67 (–33.1%)
Shoots	60	Internode length (cm)	2.52	3.17 (+25.8%)^{NS}
	60	Internode diameter (mm)	3.57	4.62 (+29.3%)*
	60	Fresh weight (g)	6.95	7.20 (+3.6%)^{NS}
	60	Dry weight (g)	3.49	3.59 (+3.0%)^{NS}
	32	RSER (cm·week^{-1})	0.44	0.32 (–27.7%)^{NS}
Leaves	240	Leaves per shoot (≈40 cm long)	32.50	25.25 (–22.3%)^{NS}
	240	Leaf area (cm^2)	12.81	17.88 (+39.5%)*
	240	Leaf thickness (mm)	0.62	0.82 (+33.8%)*
	240	Fresh leaf weight (g)	0.78	1.11 (+42.1%)*
	240	Dry leaf weight (g)	0.28	0.35 (+24.1%)*
	240	Specific leaf area (mm^2·mg^{-1})	45.43	51.19 (+12.7%)^{NS}
	240	Specific leaf mass (mg·mm^{-2})	0.02	0.02 (0.0%)^{NS}

RSER = relative stem elongation rate (cm·week^{-1}).
^{NS}, *Nonsignificant or significant at $P \leq 0.05$, respectively.

Rooting of tetraploid cuttings. When IBA was not applied to cuttings, 45.0% of the tetraploid cuttings rooted within 8 weeks after they were stuck into the rooting substrate, whereas 62.5% of the diploid cuttings rooted (Fig. 4A). Tetraploid cuttings appeared to exhibit 26.5% to 35.5% lower rooting rates than diploid cuttings when IBA was applied at 1000, 2500, or 5000 ppm, although the differences between diploid and tetraploid cuttings in rooting percentage were not statistically significant.

When IBA was not applied, tetraploid cuttings produced new roots of 40.2% greater diameter or thickness than the roots produced by diploid cuttings (Fig. 4D and F). Diploid cuttings developed thicker roots when IBA was applied and the diameter of new roots appeared to increase with the increase of IBA concentration. On the other hand, the diameter of roots from tetraploid cuttings did not change significantly with the application of IBA or the change of IBA concentration. Thus, when 2500 and 5000 ppm of IBA were applied, tetraploid and diploid cuttings did not show any significant differences in root diameter.

In terms of the number, length, and fresh weight of roots per cutting, tetraploid and diploid cuttings did not show significant differences when IBA was not applied (Fig. 4B, C, and E). IBA treatments increased the root length of diploid cuttings (2500 and 5000 ppm) and the fresh root weight of tetraploid cuttings (Fig. 4C).

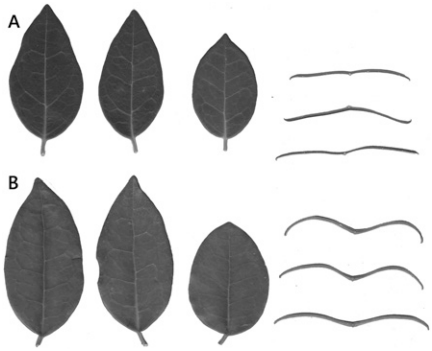

Fig. 3. Typical leaves and transverse sections of (**A**) diploid and (**B**) tetraploid japanese privet plants showing changes in leaf size and thickness.

Discussion

This study reveals that the semisolid agar method of Jones et al. (2007) can be readily applied to japanese privet and relatively high frequencies of solid tetraploids can be induced within several months using colchicine. Newly emerged privet seedlings each had a growing point between two relatively large cotyledons. This structure allowed for easy placement of droplets of colchicine-containing semisolid agar onto the growing point, and privet seedlings were tolerant of the applied colchicine. This method has been applied to two other privet species, and dozens more tetraploids have been obtained in these species (Fetouh and Deng, unpublished data). The availability of these tetraploids will make it possible to create other polyploidy levels in privets. Multiple polyploidy levels will be valuable for developing new privet cultivars or breeding lines with sterility and reduced invasive potential and for understanding the effects of polyploidy on privet growth, development, and fecundity.

Two modifications were made to the original semisolid agar method of Jones et al. (2008), including 0.2% DMSO and one-fourth-strength

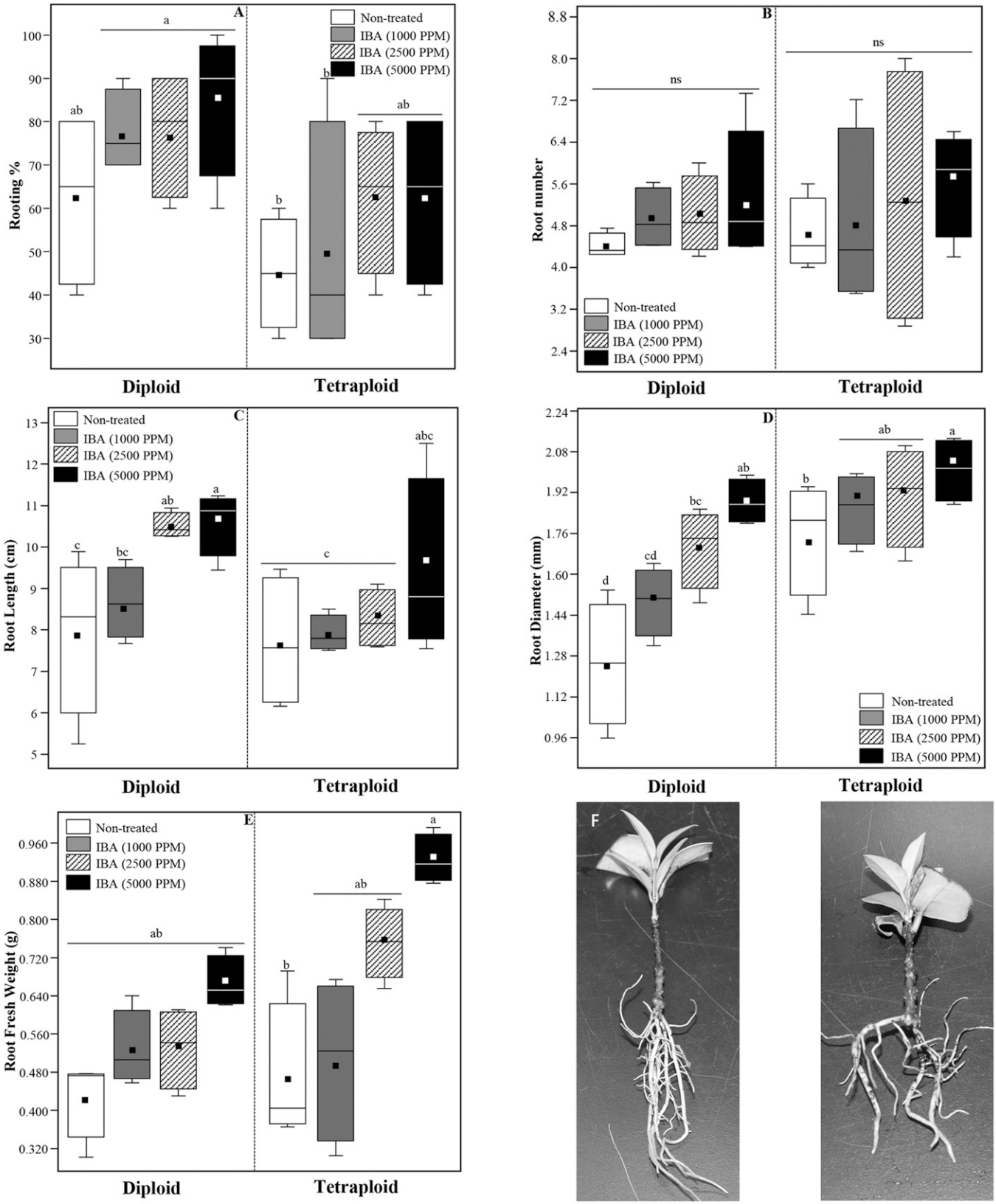

Fig. 4. Rooting and root characteristics of diploid and tetraploid cuttings with and without indole butyric acid (IBA) application. (**A**) Rooting percent, (**B**) number of roots per cutting, (**C**) total length of all roots per cutting (cm), (**D**) diameter of roots (mm), and (**E**) the fresh weight of all roots per cutting (g). Boxes show the interquartile ranges including 25% to 75% of the data, and squares indicate the mean values of the data. Boxes headed by different letters differ significantly for each other according to the least significant difference at $P = 0.05$. (**F**) Roots generated from diploid (left) and tetraploid cuttings (right).

MS basal salt mixture. DMSO has been commonly used to facilitate the penetration of chemical mutagens such as colchicine into targeted tissues and cells and increase the effectiveness of colchicine treatments (Sanders and Hull, 1970). The one-fourth-strength MS salts added in the semiagar was intended to provide some nutrients to promote cell division in the growing point. However, specific effects

of these modifications remain to be determined for the induction of tetraploids in privets. In tetraploid induction experiments, the concentration of colchicine was considered to be critical. Up to 2% colchicine was used in previous reported experiments (Habbard et al., 2016). Higher concentrations of colchicine resulted in high mortalities of treated seedlings, buds, or shoots, and deformed new growth, and/or high frequencies of mixoploids (Henny et al., 2009; Thao et al., 2003). In this study, the three concentrations of colchicine (0.1%, 0.2%, and 0.3%) used were equally effective and resulted in similar tetraploid induction rates as well seedling survival and mixoploids. To minimize the quantity of colchicine needed, we recommend the use of 0.1% colchicine and three consecutive applications to young (≈5-week old) privet seedlings over a period of 3 d.

Accurate, efficient ploidy analysis is essential for rapid identification of polyploids among chemically treated plants or shoots or polyploids from interploid crosses. When CyStain Ultraviolet Ploidy Precise P dye was used directly, privet diploid and tetraploid leaf samples yielded broad peaks on the ploidy analyzer and large coefficients of variation among samples. The inclusion of PVP and ME to the dye significantly improved the sharpness of the fluorescence peaks with excellent reproducibility among replicates. So far, there was only one record of nuclear DNA content for *Ligustrum* in the Plant DNA C-value Database (http://data.dew.org/cvalues; Bennett and Leitch, 2012), which was from *Ligustrum vulgare* L. (1.57 pg/1C; Siljak-Yakovlev et al., 2010). Data from this study provided the first record of nuclear DNA contents for japanese privet. The somatic chromosome number of *L. vulgare* is $2n = 46$ (Missouri Botanical Garden, 2016), whereas the somatic or gametic chromosome number of *L. japonicum* is not available in literature. Although the nuclear DNA content of japanese privet appears to be lower than that of *L. vulgare*, it remains to be determined if japanese privet has the same or a smaller somatic chromosome number. Several lysis buffers have been used for determination of plant nuclear DNA contents (Doležel et al., 2007). The lysis buffer LB01 of Doležel et al. (2007) worked well with several other ornamental plants in our program (Cao et al., 2014), and it worked well with japanese privet samples. In recent analysis, this buffer also worked well with chinese and glossy privet samples. Among the four plant species with known nuclear DNA contents that were tested as internal references for japanese privet samples, *R. sativus* 'Saxa' worked best for japanese privet leaf samples.

Due to the lack of privet tetraploids (natural or induced), the effects of tetraploidy on privet plant growth and ornamental value have not been examined previously. Results from this study showed that japanese privet tetraploids were more compact with lower plant heights and smaller canopy than diploids. Tetraploid shoots were thicker, and tetraploid leaves were larger, thicker, and greener, similar to morphological changes observed in induced autotetraploids in several other woody plants including crape myrtle (*Lagerstroemia indica* L.), rose (*Rosa* L.), and London plane [*Platanus acerifolia* (Aiton) Willd.] (Kermani et al., 2003; Liu et al., 2007; Ye et al.; 2010). With these morphological changes, japanese privet tetraploid plants seemed to have higher ornamental values. Studies have been initiated to further assess the beneficial effects of tetraploidy on japanese plant performance and tolerance to severe pruning in the landscape. Several studies have shown that autotetraploids tend to exhibit better tolerance to droughts or water deficits (Allario et al., 2013; Liu et al., 2011). It will be of interest to explore whether these japanese privet tetraploids show any increase in drought tolerance.

It is expected that future sterile polyploid privet cultivars or breeding lines with reduced or eliminated invasive potential will depend on cuttings for commercial propagation and production. Thus, cuttings' rootability will be important to nurseries. Few previous studies have assessed the effects of tetraploidy on the rootability of cuttings. Results from this study indicate that tetraploid cuttings have lower rootabilities, and treating tetraploid cuttings with IBA (2500 or 5000 ppm) could improve their rootability to an acceptable level. Other factors, such as light intensity and shading, are known to affect the rooting of privet cuttings (Knox and Hamilton, 1982). Manipulating these factors in propagation beds or houses may lead to higher rooting percentages from japanese privet tetraploid cuttings.

Literature Cited

Abdoli, M., A. Moieni, and H.N. Badi. 2013. Morphological, physiological, cytological and phytochemical studies in diploid and colchicine-induced tetraploid plants of *Echinacea purpurea* (L.). Acta Physiol. Plant. 35:2075–2083.

Allario, T., J. Brumos, J.M. Colmenero-Flores, D.J. Iglesias, J.A. Pina, L. Navarro, M. Talon, P. Ollitrault, and R. Morillon. 2013. Tetraploid Rangpur lime rootstock increase drought tolerance via enhanced constitutive root abscisic acid production. Plant Cell Environ. 36:856–868.

Anderson, N.O. 2007. Prevention of invasiveness in floricultural crops, p. 177–214. In: N.O. Anderson (ed.). Flower breeding and genetics. Springer, Berlin, Germany.

Bell, C.E., C.A. Wilen, and A.E. Stanton. 2003. Invasive plants of horticultural origin. HortScience 38:14–16.

Bennett, M.D. and I.J. Leitch. 2012. Plant DNA C-values database (release 6.0, Dec. 2012). 23 June 2014. <http://data.dew.org/cvalues>.

Cao, Z., Z. Deng, and M. McLaughlin. 2014. Interspecific genome size and chromosome number variation sheds new light on species classification and evolution in *Caladium* (Araceae). J. Amer. Soc. Hort. Sci. 49:449–459.

Contreras, R.N. 2012. A simple chromosome doubling technique is effective for three species of *Cupressaceae*. HortScience 47:712–714.

Contreras, R.N., J.M. Ruter, and B.M. Schwartz. 2010. Oryzalin-induced polyploidy in *Cryptomeria japonica* (Thunb. ex L.f.) D. Don (*Cupressaceae*). HortScience 45:316–319.

Cornelissen, J.H.C., S. Lavvorel, E. Garnier, S. Diaz, N. Buchmann, D.E. Gurvich, P.B. Reich, H.T. Steege, M.G.A. Van der Heijden, J.G. Pausas, and H. Poorter. 2003. A handbook of protocols for standarised and easy measurement of plant functional traits worldwide. Austral. J. Bot. 55:335–380.

Czarnecki, D.M., II, S.B. Wilson, G.W. Knox, R. Freyre, and Z. Deng. 2012. UF-T3 and UF-T4: Two sterile *Lantana camara* cultivars. HortScience 47:132–137.

Czarnecki, D.M., II and Z. Deng. 2009. Occurrence of unreduced female gametes leads to sexual polyploidization in *Lantana*. J. Amer. Soc. Hort. Sci. 134:560–566.

Dirr, M.A. 1998. Manual of woody landscape plants. Their identification, ornamental characteristics, culture, propagation and uses. 5th ed. Stipes Publ., Champaign, IL.

Doležel, J., J. Greilhuber, and J. Suda. 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nat. Protoc. 2:2233–2244.

FLEPPC (Florida Exotic Pest Plant Council). 2015. Florida Exotic Pest Plant Council's 2015 list of invasive plant species. 31 Jan. 2016. <http://www.fleppc.org/list/2015FLEPPCLIST-LARGEFORMAT-FINAL.pdf>.

Freyre, R., A. Moseley, G.W. Knox, and S.B. Wilson. 2012. Fruitless *Ruellia simplex* R10-102 ('Mayan Purple') and R10-108 ('Mayan White'). HortScience 47:1808–1814.

Habbard, J.L., A.R. Griffin, S. Foster, C. Brooker, L.D. Kha, and A. Koutoulis. 2016. Production of colchicine-induced autotetraploids as a basis for sterility breeding in *Acacia mangium* Willd. Forestry 85:427–436.

Henny, R.J., J.R. Holm, J. Chen, and M. Scheiber. 2009. In vitro induction of tetraploids in *Dieffenbachia* × 'Star Bright M-1' by colchicine. HortScience 44:646–650.

Hoyos, L.E., G.I. Gavier-Pizarro, T. Kuemmerle, E.H. Bucher, V.C. Radeloff, and P.A. Tecco. 2010. Invasion of glossy privet (*Ligustrum lucidum*) and native forest loss in the Sierras Chicas of Córdoba, Argentina. Biol. Invasions 12:3261–3275.

Jones, J.R., T.G. Ranney, and T.A. Eaker. 2007. Inducing polyploidy in *Rhododendron* seedlings. Combined Proc. Intl. Plant Propagators' Soc. 57:697–700.

Kermani, M.J., V. Sarasan, A.V. Roberts, K. Yokoya, J. Wentworth, and V.K. Sieber. 2003. Orazalin-induced chromosome doubling in *Rosa* and its effect on plant morphology and pollen viability. Theor. Appl. Genet. 107:1195–1200.

Knox, G.W. and D.F. Hamilton. 1982. Rooting of *Berberis* and *Ligustrum* cuttings from stock plants grown at selected light intensities. Scientia Hort. 16:85–90.

Knox, G.W. and S.B. Wilson. 2006. Evaluating north and south Florida landscape performance and fruiting of ten cultivars and a wild-type selection of *Nandina domestica*, a potentially invasive shrub. J. Environ. Hort. 24:137–142.

Larcher, W. 2003. Physiological plant ecology: Ecophysiology and stress physiology of functional groups. 4th ed. Springer-Verlag, Berlin, Germany.

Lehrer, J.M., M.H. Brand, and J.D. Dubell. 2008. Induction of tetraploidy in meristematically active seeds of japanese barberry (Berberis thunbergii var. *atropurpurea*) through exposure to colchicine and oryzalin. Scientia Hort. 119:67–71.

Leonhardt, K.W. and X. Shi. 2009. Production of tetraploid forms of eight tropical landscape tree species. Acta Hort. 813:133–140.

Li, Y., Z. Cheng, W. Smith, D. Ellis, Y. Chen, X. Zheng, Y. Pei, K. Luo, D. Zhao, Q. Yao, H. Duan, and Q. Li. 2004. Invasive ornamental plants: Problems, challenges, and molecular

tools to neutralize their invasiveness. Crit. Rev. Plant Sci. 23:381–389.
Liu, S., S. Chen, Y. Chen, Z. Guan, D. Yin, and F. Chen. 2011. In vitro induced tetraploid of *Dendranthema nankingense* (Nakai) Tzvel. shows an improved level of abiotic stress tolerance. Scientia Hort. 127:411–419.
Liu, G., Z. Li, and M. Bao. 2007. Colchicine-induced chromosome doubling in *Platanus acerifolia* and its effect on plant morphology. Euphytica 157:145–154.
Matthew, E.O., A.L. Douglas, and R. Isaacs. 2002. An inexpensive accurate method for measuring leaf area and defoliation through digital image analysis. J. Econ. Entomol. 95:1190–1194.
Miller, S.A. and R.F. Henzell. 2000. Seed germination of weeds from New Zealand native plant communities. Agron. Soc. N.Z. 12:115–116.
Missouri Botanical Garden. 2016. IPCN chromosome reports. 25 Aug. 2016. <http://www.tropicos.org/NameSearch.aspx?projectid=9>.
Morris, L.L., L.W. Jeffrey, and N.H. Siti. 2002. Growth and reproduction of the invasive *Ligustrum sinense* and native *Forestiera ligustrina* (Oleaceae): Implications for the invasion and persistence of a nonnative shrub. Intl. J. Plant Sci. 163:1001–1010.
Munger, G.T. 2003. *Ligustrum* spp. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (producer), Fire effects information system. 30 May 2015. <http://www.fs.fed.us/database/feis/>.
Nadler, J.D., M.R. Pooler, R.T. Olsen, and G. Coleman. 2012. In vitro induction of polyploidy in *Cercis glabra* Pamp. Scientia Hort. 148:126–130.
Niemiera, A.X. and B. Von Holle. 2009. Invasive plant species and the ornamental horticulture industry, p. 167. In: Inderjit (ed.). Management of invasive weeds. Springer Science + Business Media B.V., Berlin, Germany.
Olsen, R.T. 2007. Utilizing polyploidy for developing improved nursery crops: Restoring fertility in wide hybrids, limiting fertility of invasive species, embryo culture of triploids, pest resistance, and inheritance of ornamental traits. North Carolina State Univ. Raleigh, PhD Diss. 30 May 2016. <http://www.lib.ncsu.edu/resolver/1840.16/3495>.
Panetta, F.D. 2000. Fates of fruits and seeds of *Ligustrum lucidum* W.T. Ait. and *L. sinense* Lour. maintained under natural rainfall or irrigation. Austral. J. Bot. 48:701–705.
Phillips, W.D., T.G. Ranney, D.H. Touchell, and T.A. Eaker. 2015. Developing non-invasive callery pears: Fertility and reproductive biology of triploid cytotypes. SNA Res. Conf. 60th Annu. Rpt. 60:205–208.
Pimentel, D., R. Zuniga, and D. Morrison. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol. Econ. 52:273–288.
Ranney, T.G. 2004. Population control: Developing non-invasive nursery crops. Combined Proc. Intl. Plant Propagators' Soc. 54:604–607.
Ranney, T.G. 2006. Polyploidy: From evolution to new plant development. Combined Proc. Intl. Plant Propagators' Soc. 56:137–142.
Ranney, T.G., D.H. Touchell, T. Eaker, J. Mowrey, N. Lynch, and J. Smith. 2010. Progress developing non-invasive nursery crops. Combined Proc. Intl. Plant Propagators' Soc. 60:422–423.
Ranney, T.G., D.H. Touchell, T.A. Eaker, N.P. Lynch, J.A. Mowrey, and J.C. Smith. 2007. Breeding non-invasive nursery crops. Proc. Southern Reg. Intl. Plant Propagators' Soc. 57:643–645.
Reichard, S.H. and P. White. 2001. Horticulture as a pathway of invasive plant introductions in the United States. Bioscience 51:103–113.
Rejmánek, M. 2014. Invasive trees and shrubs: Where do they come from and what we should expect in the future? Biol. Invasions 16:483–498.
Sanders, J. and J.W. Hull. 1970. Dimethyl sulfoxide as an adjuvant of colchicine in the treatment of *Rubus* seeds and shoot apices. HortScience 5:111–112.
Siljak-Yakovlev, S., F. Pustahija, E.M. Solic, F. Bogunic, E. Muratovic, N. Bašić, O. Catrice, and S.C. Brown. 2010. Towards a genome size and chromosome number database of Balkan flora: C-values in 343 taxa with novel values for 242. Adv. Sci. Lett. 3:190–213.
Thammina, C., M. He, L. Lu, K. Cao, H. Yu, Y. Chen, L. Tian, J. Chen, R. McAvoy, D. Ellis, D. Zhao, Y. Wang, X. Zhang, and Y. Li. 2011. In vitro regeneration of triploid plants of *Euonymus alatus* 'Compactus' (burning bush) from endosperm tissue. HortScience 46:1141–1147.
Thao, N.T.P., K. Ureshino, I. Miyajima, Y. Ozaki, and H. Okubo. 2003. Induction of tetraploids in ornamental *Alocasia* through colchicine and oryzalin treatments. Plant Cell Tissue Organ Cult. 72:19–25.
Theoharides, K.A. and J.S. Dukes. 2007. Plant invasion across space and time: Factors affecting nonindigenous species success during four stages of invasion. New Phytol. 176:256–273.
Trueblood, C.E., T.G. Ranney, P.L. Nathan, J.C. Neal, and R.T. Olsen. 2010. Evaluating fertility of triploid clones of *Hypericum androsaemum* L. for use as non-invasive landscape plants. HortScience 45:1026–1028.
Vining, K.J., R.N. Contreras, M. Ranik, and S.H. Strauss. 2012. Genetic methods for mitigating invasiveness of woody ornamental plants: Research needs and opportunities. HortScience 47:1210–1216.
Wang, X.L. and Z. Zhong. 2012. Seasonal variation in rooting of the cuttings from tetraploid locust in relation to nutrients and endogenous plant hormones of the shoot. Turk. J. Agr. For. 36:257–266.
Wilson, S.B., G.W. Knox, K.L. Nolan, and J. Aldrich. 2014. Landscape performance and fruiting of 12 privet selections grown in northern and southern Florida. HortTechnology 24:148–155.
Wilson, S.B., G.W. Knox, R. Freyre, and Z. Deng. 2012. Characterizing the invasive potential of ornamental plants. Acta Hort. 937:1183–1192.
Wilson, S.B. and L.K. Mecca. 2003. Seed production and germination of eight cultivars and the wild type of *Ruellia tweediana*: A potentially invasive ornamental. J. Environ. Hort. 21:137–143.
Wilson, S.B., L.K. Mecca, J.A. Gersony, M. Thetford, and J.S. Raymer. 2004. Evaluation of 14 butterfly bush taxa grown in western and southern Florida: II. seed production and germination. HortTechnology 14:612–618.
Ye, Y.M., J. Tong, X.P. Shi, W. Yuan, and G.R. Li. 2010. Morphological and cytological studies of diploid and colchicine-induced tetraploid lines of crape myrtle (*Lagerstroemia indica* L.). Scientia Hort. 124:95–101.