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To direct, fund, promote and communicate horticultural research, which increases the quality and value of ornamental plants, improves the productivity and profitability of the nursery and landscape industry, and protects and enhances the environment.

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Generation and Identification of New Viburnum Hybrids[1]

W.A. Hoch, E.L. Zeldin, J. Nienhuis, and B.H. McCown[2]

Department of Horticulture

University of Wisconsin, Madison, WI 53706

Abstract

The controlled crossing of fragrant flowered viburnums (*V. carlesii* and its hybrids) with the hardier, rugose foliaged *V. lantana* is unattainable through traditional breeding methods due to incompatibilities within the developing seed. Hybrid plants from these crosses have now been obtained using embryo rescue techniques. Embryos were removed from developing seed 17 days following pollination and placed on WPM medium to promote continued maturation. Genotypes from two crosses, *V. lantana* 'Mohican' x *V. carlesii* 'Aurora' and *V. lantana* 'Mohican' x *V.* x *juddii*, were obtained and verified using RAPD markers. These plants are presently being evaluated for ornamental characteristics and cold hardiness.

Index words: embryo rescue, RAPD.

Species used in this study: Wayfaring tree viburnum (*Viburnum lantana* L. 'Mohican'); Koreanspice viburnum (*Viburnum carlesii* Hemsl.'Aurora'); Judd viburnum *Viburnum* x *juddii* Rehd. (*V. bitchiuense* Mak. x *V. carlesii* Hemsl.).

Significance to the Nursery Industry

The development of new woody landscape plants with improved disease and insect resistance, hardiness and landscape characteristics can be greatly constrained by traditional breeding methods. Embryo rescue techniques can be used to overcome some of these limitations, as demonstrated here with viburnums.

These new viburnums, along with future developments from coupling biotechnology with plant breeding and selection, will help fulfill the need for low maintenance yet highly ornamental plants useful in low input, sustainable landscape plantings.

Currently a significant tool in agronomic and vegetable breeding, Random Amplified Polymorphic DNA (RAPD) analysis offers tremendous potential for ornamental plant breeding and development. Cultivar identification through RAPD analysis provides the advantages of speed, low cost, and technical simplicity (5). In addition, RAPD analysis promises to be an efficient tool useful in plant patent protection (9).

Introduction

The genus *Viburnum* represents a diverse group of highly useful shrubs native to temperate and subtropical regions in Asia, Europe, North America, and South America. Within the genus, fragrant flowers, attractive foliage, ornamental fruit, and vibrant fall color are common (2, 3). However, no

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[2]Graduate Student, Senior Researcher, Associate Professor and Professor, resp. Department of Horticulture, University of Wisconsin—Madison.

single viburnum selection embodies all of these qualities. One hybrid cross that would ostensibly blend these qualities is *V. carlesii* (fragrant flowers, compact habit, and good fall color) x *V. lantana* (dark green rugose foliage, brilliant red fruit display, disease resistance, and cold hardiness).

The use of traditional breeding methods to combine desirable traits in viburnums is limited to closely related species. Wide crosses are plagued by disturbed embryo, endosperm and pericarp growth within the developing seed (1).

Three to 4 weeks after the controlled cross *V. lantana* x *V. carlesii* is performed (both species 2n = 18) (8), normal seed development is interrupted, likely due to the antagonism between embryo and surrounding tissue described above. The result is embryo starvation and eventual abortion. This sequence represents one level of incompatibility characteristic to many wide crosses (7).

Using embryo rescue, the immature embryo is removed prior to initiation of the abortion processes and placed on nutrient medium to promote continued embryo development. It is imperative that the embryo be taken before onset of the abortion process. Although the seed will appear normal for several weeks following abortion initiation, if the embryo is taken after this time, the senescence process is difficult to reverse (10).

Materials and Methods

The controlled crosses *V. lantana* 'Mohican' x *V. carlesii* 'Aurora' and *V. lantana* 'Mohican' x *V.* x *juddii* were performed in mid May. Although emasculation of the pistillate parent is unnecessary due to self sterility prevalent in viburnums, stamen emasculation was performed to facilitate access to the pistils as a result of the prominent position of the anthers above the pistil. Pollen removed from the staminate parent was placed in No. 00 gelatin capsules and applied to the pistils using a camel hair brush. Storage of the pollen was unnecessary as the flowering period of the earlier flowering species, *V. carlesii*, overlapped the start of the flowering of *V. lantana*.

In early June, embryos from both crosses were removed under sterile conditions and placed into petri dishes on Woody Plant Medium (WPM) (4) and sealed with Para-film. After two weeks embryos were moved to jars with B-caps containing either WPM without hormone, or supplemented with 1µm, 10 µm, or 40 µm of a cytokinin hormone, 6-(y,y-dimethylallylamino) purine (2iP), or zeatin. Cultures were grown in rooms with 24-hour cool-white fluorescent lighting (20 $\mu Em^{-2}sec^{-1}$) and a temperature that averaged 24C (75F).

By early December, plants began to develop and were removed from culture and placed in a rooting box under 24 hour light without rooting hormone treatment.

The method of DNA extraction used for this RAPD analysis was adapted from the procedure developed by Skroch and Nienhuis(6), and is as follows: One half to 0.75 g of fresh leaf tissue was harvested, frozen using liquid nitrogen, and ground in 50 µl of extraction buffer using mortar and pestle. Extraction buffer consisted of 2% CTAB, 100 mM TRIS pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, and 1% PVP (polyvinyl-pyrolidone). After grinding, an additional 450 µl extraction buffer was added to the sample, vortexed briefly, and allowed to incubate in a water bath for 30 minutes at 65C (149F). Following soaking, the mixture was micro-centrifuged at 10,000 RPM for 10 minutes to pellet debris. To precipitate nucleic acids, supernatants were transferred to a clean test tube, a 6:1 mixture of ethanol and 7.5 M ammonium acetate was added and allowed to precipitate for 30 minutes at room temperature. Precipitated nucleic acids were pelleted by spinning in a micro-centrifuge for 5 minutes at 3000 RPM, and 300 µl TE buffer (1 mM TRIS pH 7.5, 0.1 mM EDTA pH 8.0) was added. RNAase A was added to a final concentration of 100 µg/ml and incubated at 37C (99F) for 1 hour. Tubes were spun in a micro-centrifuge at 14,000 RPM for 10 seconds, and supernatants were transferred to clean 1.5 ml micro-centrifuge tubes. DNA was precipitated by filling tubes with a 20:1 mixture of ethanol and 3M sodium acetate, mixed, and allowed to precipitate for 30 minutes at room temperature. Samples were spun for 5 minutes at 5000 RPM to pellet DNA, ethanol poured off, and the pellets washed by filling tubes with 70% ethanol and vortexing. Pellets were collected by spinning for 15 seconds at 14,000 RPM, pouring off alcohol, and drying by inverting tubes on a paper towel. Pellets were then rehydrated in 200 µl TE buffer and quantified using a fluorometer.

Operon primers AA1 through AA6, obtained from Operon Technologies Inc., Alameda, CA, were used in the RAPD reactions. The reactions were performed in an air thermal cycler, with the cycling temperature settings at 91C (196F) for denaturation, 42C (108F) for annealing, and 72C (162F) for elongation. The first 2 cycles were timed at 1 minute for denaturation, 7 seconds for annealing, and 70 seconds for elongation. During the remaining 38 cycles, denaturation was set for 1 second, annealing for 7 seconds and elongation for 70 seconds. These cycles were followed by a 4-minute holding period at 72C (162F). The reaction buffer was composed of 50 mM Tris, pH 8.5, 2 mM $MgCl_2$, 20 mM KCl, 500 µg/ml BSA, 2.5% Ficoll 400, and 0.02% (w/v) Xylene Cyanol. The reaction concentrations were 100 µM dNTPs, 2 ng/µl DNA template, 0.4 µM RAPD primer, and 0.6 unit Taq DNA polymerase in a final volume of 10 µl.

RAPD products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Gels were then placed on a UV light trans-illuminator and results recorded on Polaroid 667 film.

Results and Discussion

Embryos developed equally well on all levels of 2iP and zeatin used. For approximately 4 weeks following embryo rescue, embryos showed enlargement, chlorophyll development, and callus growth where the radicle was cut to facilitate embryo removal (in the seed, the radicle tip is embedded in the pericarp). After this period, development was highly variable, ranging from large amounts of callus growth to senescence and death. Thirteen weeks after rescue, 5% of the embryos had developed normally and began shoot and radicle elongation.

Shoots removed from culture rooted in an average of 10 weeks, uncharacteristically slow for micropropagation material. This is likely attributable to the staminate parent, *V. carlesii*, a species relatively difficult to root. Attempts at rooting in vitro were unsuccessful, as roots that had developed in vitro died when moved to rooting boxes for acclimatization.

Several morphological features suggested the hybrid nature of the progeny. Both the shape and venation of the leaves,

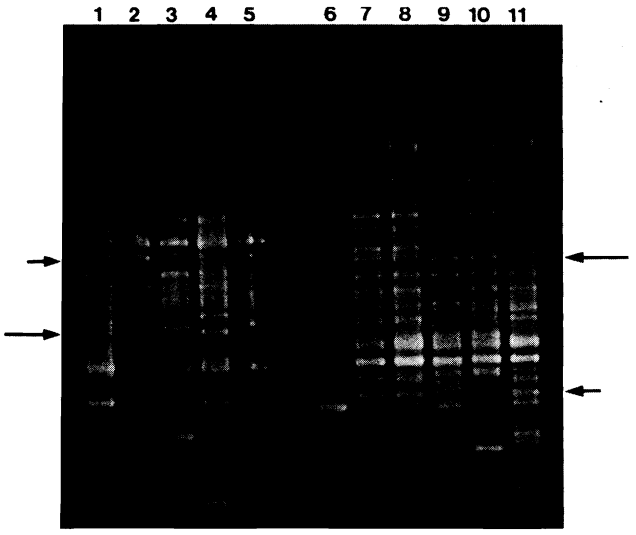

Fig.1. Two of six primers used, Operon primers AA2 lanes 1–5 and AA3 lanes 6–11. Pistilate parent *V. lantana* 'Mohican' lanes 5 and 11. Staminate parent *V.* x*juddii* lanes 4 and 10. Hybrid progeny lanes 1, 2, 7, and 8. ⟵ indicates examples of dominant genes inherited from staminate parent. ← indicates examples of dominant genes inherited from pistilate parent. Lanes 3, 6, and 9 are parents of crosses not shown in photograph.

and the tomentum on the naked buds exhibited traits intermediate between the two parents.

RAPD markers were utilized successfully to verify the desired hybrid character of the progeny. Of the six random primers selected for DNA amplification, five produced clear differences in marker banding patterns (Fig. 1). A total of 14 specific bands were identified that distinguished the hybrids from parental DNA. Eight of the 14 bands represented dominant genes inherited from the staminate parent, ruling out the possibility of the progeny being the result of apomixis, or undesired pollen.

These hybrids have been planted in diverse field test plots for continued evaluation of ornamental characteristics and cold hardiness.

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