MULTIPLICATION OF *SYRINGA* SPECIES AND CULTIVARS IN TISSUE CULTURE

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Abstract. A micropropagation method for *Syringa* × *hyacinthiflora* cv. Excel is described involving cytokinin control of shoot growth in tissue cultures Shoot tips and nodes from rooted cuttings are decontaminated in 0 5% sodium hypochlorite and transferred to nutrient medium supplemented with 2 2 μM thidiazuron Under defined conditions of light intensity, photoperiod, and temperature, shoots elongate from preformed buds to produce monopodial axes, and then lateral buds form in the axils of leaves After 6 weeks incubation, shoots can be used as sources of bud explants for further shoot multiplication, or as cuttings Plantlets produced after induction of roots on cuttings are then gradually acclimated to the greenhouse environment Shoots of other members of Oleaceae, such as additional species and cultivars of *Syringa*, and species of *Fraxinus*, *Forsythia*, and *Ligustrum* also respond in tissue cultures to these treatments, suggesting that the method may be generally applicable For *S* × *hyacinthiflora* cv Excel it was shown that the inhibitory effect of 2,4-dichlorophenoxyacetic acid on shoot growth in tissue cultures was not mediated via stimulated production of ethylene

INTRODUCTION

The standard methods for multiplying *Syringa* cultivars are by grafting and softwood cuttings (5). A problem with the latter technique is the fact that cuttings responding to auxin treatments can be obtained only during a few critical weeks of every year (1). Because of this, the success of the methodology is often unpredictable and the number of plants obtained, even in ideal circumstances, is small.

By contrast, tissue culture overcomes the shortcomings of conventional technology. Once vigorously growing shoots are obtained in culture, plant multiplication can continue throughout the year. Moreover, the scale of propagation is limited only by the space and supplies for tissue culture. We estimate that theoretically one million plants could be produced in a year starting from a single growing shoot if tissue culture methodology were utilized.

The fundamental principle involved in tissue culture propagation of *Syringa* is the control of shoot growth by cytokinin. In this respect, the technology is essentially the same as the method used for micropropagation of most other woody plants, especially species in the Rosaceae and Ericaceae (8). In what has now become the standard methodology, one uses cytokinin to promote growth and to overcome apical dominance in cultured shoots. The resulting cluster of growing

shoots is then subdivided and used for further shoot multiplication or for the production of plantlets after inducing root formation in cuttings by treatment with auxin.

We are currently screening a large number of woody plants at the Arnold Arboretum to determine whether cytokinin manipulation can be applied to plant taxa that have previously been unstudied (3). Based on research with over 100 different woody species representing more than 30 families, we find that: 1) cytokinins in combination with the usual mixture of inorganic (9) and organic nutrients (7) can sustain shoot growth in explants of many, but not all, species tested and 2), when it is found, the 'classical' cytokinin response in shoot explants is localized in defined systematic groupings; i.e. families (e.g. Ericaceae, Rosaceae), orders (e.g. Ericales) and superorders (e.g. Asteridae). As we obtain results with a larger number of species, the framework relating cytokinin response to systematic botany and horticulture will become more clearly defined.

This report summarizes methodology for propagating *Syringa* using tissue culture techniques, which are similar to the methods utilized by Hildebrandt and Harney (6) for *S. vulgaris* cv. Vesper. Most of the procedures were devised with *S.* × *hyacinthiflora* cv. Excel but they are also applicable to other cultivars and species of *Syringa*, as well as additional members of the family Oleaceae, including species of *Forsythia*, *Ligustrum*, and *Fraxinus*. *Syringa* exhibits dual control of shoot growth via auxin and cytokinin; i.e. auxin inhibits shoot growth in tissue cultures and cytokinin stimulates it. In view of the hypothesis of Burg and Burg (2) that the inhibitory effect of auxin is mediated via induced ethylene synthesis, we have also examined ethylene production in cultures. The results strongly suggest that auxin inhibition is not a consequence of ethylene.

MATERIALS AND METHODS

Plant materials were obtained from the Arnold Arboretum of Harvard University at Jamaica Plain, Massachusetts. The basic components of tissue culture media were supplied as Murashige's Minimal Organics Medium from GIBCO, Inc, and as nicotinic acid and pyridoxine-HCl from Sigma Chemical Co. All cytokinins were purchased from Sigma Chemical Co. except thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) which was obtained as a gift from Nor-Am Agricultural Products, Naperville, Illinois. The 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA) were obtained from Sigma

Chemical Co., and 1-aminocyclopropane-1-carboxylic acid (ACC) was purchased from Calbiochem-Behring, Inc.

All media contained basic components consisting of the inorganic nutrients recommended by Murashige and Skoog (9); minor organics such as 0.4 mg/l thiamine-HCl and 100 mg/1 *myo*-inositol, according to Linsmaier and Skoog (7); 5 mg/l pyridoxine-HCl; 5 mg/l nicotinic acid; and 30 g/l sucrose. To sustain shoot growth in cultures, it is necessary to supplement these nutrients with any one of several different cytokinins. We then adjust the pH to 5.6 to 5.8 and add 10 g/l of agar (Phytagar, GIBCO Inc.), then heat the mixture. As soon as all of the constituents of the medium have dissolved, 20 ml volumes were dispensed to individual test tubes (20 × 150 mm). These were then covered with plastic, vented closures and autoclaved at 120°C for 15 min.

Cultures were incubated upright at appoximately 27°C (about 80°F) and under various light regimes. Vigorous growth can be obtained with cool white fluorescent lights at intensities from 15 to 75 $\mu Em^{-2}s^{-1}$ of either constant or discontinuous illumination, but we normally maintained cultures at 50 $\mu Em^{-2}s^{-1}$ using a 16 hr light and 8 hr dark photoperiod.

In determining ethylene production rates, 1 ml air samples were taken from culture tubes sealed for 24 hours with serum caps. The samples were then injected into a gas chromatograph operated isothermally at 60°C and equipped with a stainless steel column containing Porapak Q and a flame ionization detector. By interfacing the chromatograph with a minigrator, ethylene could be detected at levels as low as 0.01 ppm. Results are expressed as means of pooled data from four independent experiments, each with 4 replications per treatment.

RESULTS AND DISCUSSION

Source of explants. Shoot cultures can be obtained with varying degrees of success starting with 5 to 10 mm terminal buds or laterals from seedlings, mature plants, or rooted cuttings. Although seedling material is probably the easiest to work with, it is also the least desirable for propagation since the floral and cultural characteristics of the mature plants are unknown. Buds from mature specimens of cultivars growing outdoors probably are the most difficult to use as explants. If these buds are obtained early in the spring during the few weeks when shoots are actively growing, the tissues are especially sensitive to the hypochlorite treatment used to decontaminate explants When buds are collected at this time, even as little as 1 minute incubation in dilute hypochlorite results in greater than 98% death of explants. Later in the year after

the flush of growth has been completed, explants appear to be much more resistant to disinfectant treatment. Unfortunately, the load of microorganisms is also greatly increased.

By far the best sources of explants are rooted cuttings growing in the greenhouse. Because they represent clonal material, rooted cuttings give true-to-type propagules. In addition, they grow vegetatively during several months of the year. We take shoot tips and nodal explants from growing stems, trim the leaf blades and then wash explants with detergent for about 5 min. After this, the stem segments are soaked in disinfectant solution consisting of 0.5% sodium hypochlorite (i.e., laundry bleach diluted 10-fold with deionized water) for 5 min. and then transferred to sterilized plastic Petri plates.

Working a a laminar flow transfer hood to minimize contamination of cultures with air-borne microorganisms, each stem segment is cut into 5 to 10 mm nodal sections which contain a lateral bud in the axil of every leaf. Individual explants are then transferred to culture tubes containing nutrient medium.

Cytokinin control of shoot growth. The following cytokinins have been found to be effective in promoting *Syringa* shoot growth in tissue cultures: N6-isopentenyladenine (i^6Ade), N6-

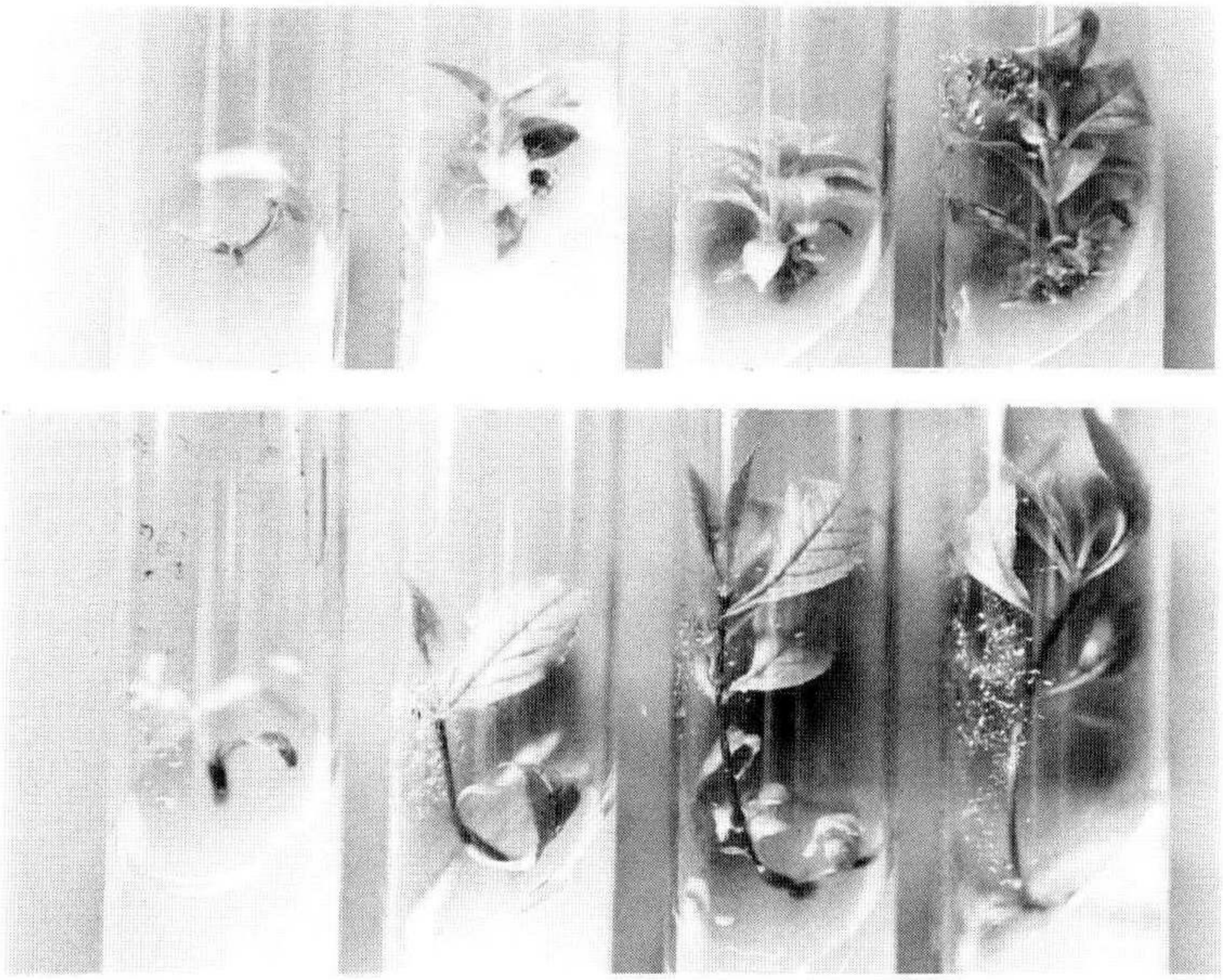

Figure 1. Effect of different i^6Ade concentrations on shoot growth in nodal of *Syringa* (top) and *Fraxinus* (bottom). Media, from left to right, contained 0, 3, 6 and 15 μM i^6Ade; respectively. Cultures were incubated for 4 weeks.

benzyladenine (bzl^{6}Ade), kinetin, and thidiazuron. Figure 1 shows the effects of different i^{6}Ade concentrations on *Syringa* and *Fraxinus* shoots. The results demonstrate the essentiality of cytokinin for growth as well as the range of i^{6}Ade concentrations that have been found to be adequate. Based on several tests, 5 to 30 μM i^{6}Ade seems to be about equally effective for *Syringa* and *Fraxinus* tissue cultures, but we routinely utilize 30 μM i^{6}Ade which gives continued, vigorous growth in subcultures. An interesting feature of *Syringa* and *Fraxinus* tissue cultures, shared by shoot cultures of other Oleaceae such as *Ligustrum* and *Forsythia*, is the tendency of shoots to grow as unbranched monopodial axes even in the presence of elevated cytokinin concentrations. This physiological characteristic limits the range of strategies that can be used for tissue culture propagation.

Figure 2 illustrates the control of shoot growth in *Syringa* and *Fraxinus* using different cytokinins, plus or minus IAA. As indicated, 0.5 mg/l (i.e. 2.2 μM) thidiazuron stimulates tissue culture growth of both *Syringa* and *Fraxinus* shoots and, on the basis of nearly three years of tests, this has been adopted as the standard medium for species in the family Oleaceae.

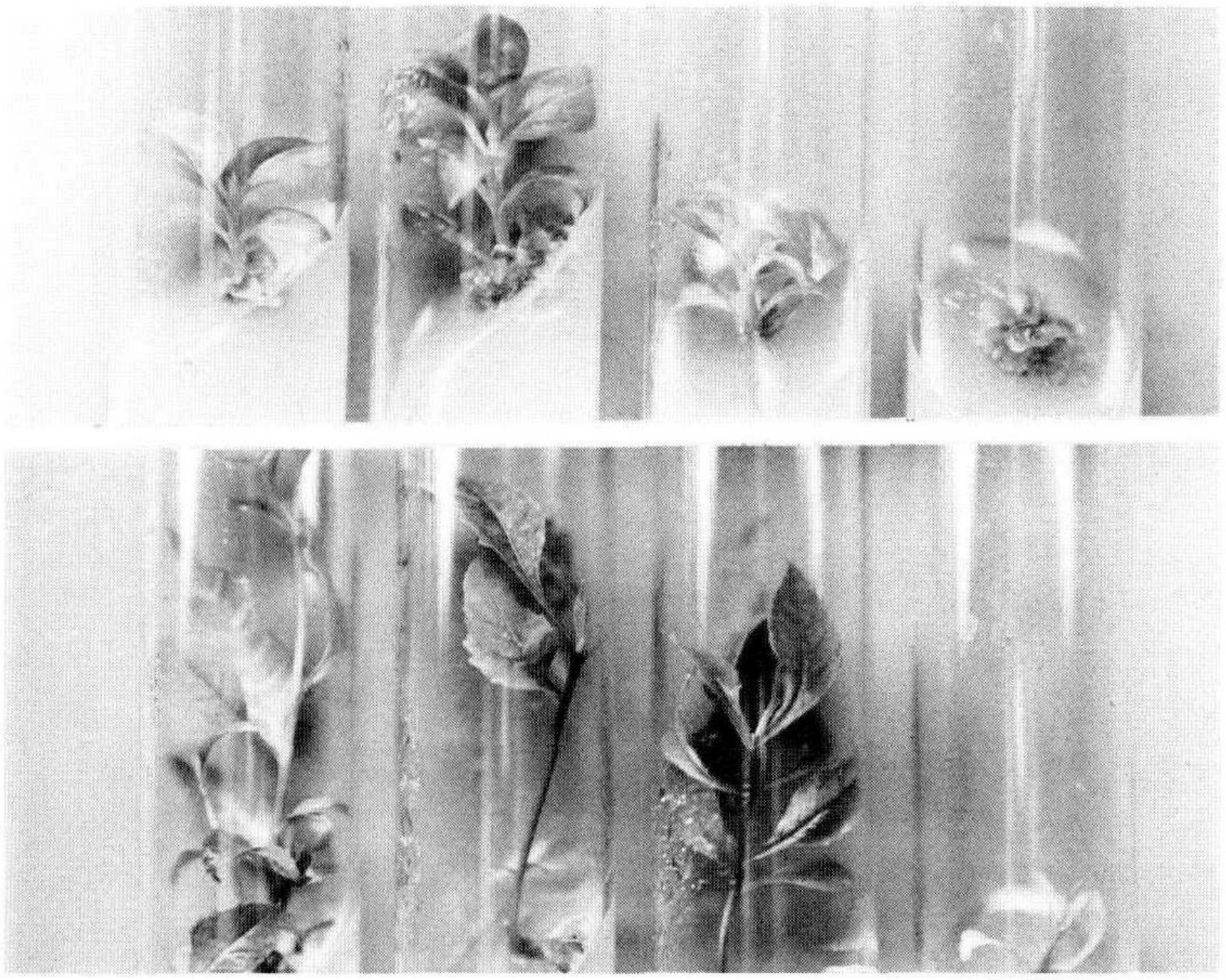

Figure 2. Control of shoot growth in nodal explants of *Syringa* (top) and *Fraxinus* (bottom) by cytokinin and auxin. Media, from left to right, contained 2.2 μM thidiazuron, 33 μM bzl^{6}Ade plus 0.6 μM IAA, 15 μM i^{6}Ade plus 35 μM IAA, respectively. Cultures were incubated for 4 weeks.

Nevertheless, other cytokinins can also be used with about the same effectiveness. Thus, i^6Ade and bzl^6Ade both will also promote shoot growth. The medium containing 7.5 mg/l (33 μM) bzl^6Ade and 0.1 mg/l (0.6 μM) IAA was recommended by Hildebrandt and Harney (6) for *Syringa* tissue cultures.

Multiplication of propagules. Under the defined conditions of incubation, *Syringa* lateral buds and shoot tips proceed through the normal phases of vegetative growth characteristic of intact plants (4); that is, a phase of shoot elongation during which preformed leaf primordia and internodes rapidly increase in size to produce a shoot axis consisting of 3 to 12 internodes followed by a phase of axillary bud development when elongation growth stops and miniature shoot axes form in the axils of leaves. For S. × *hyacinthiflora* cv. Excel, the duration of the shoot elongation and subsequent bud development phases is 6 weeks.

When shoots have completed growth, they are removed from the culture tubes and cut into nodal segments, plus shoot tips, using a scalpel. We then trim leaf blades, transfer the segments to tubes containing fresh media and incubate the cultures as described. On the average, each shoot grown in culture produces 4 propagules for the subsequent passage. At 6 weeks per passage, this rate of multiplication corresponds to more than one million shoots obtained from one in a little over a year. Thus, the methodology can be scaled up to practically any level of production.

Rooting and hardening. To prepare plantlets for the greenhouse, elongated shoots are first removed from the culture tubes and a fresh cut is made at the base of each. These cuttings are dipped in a commercial rooting powder containing 0.4% indolebutyric acid, plus thiram as a fungicide, and then transferred to enclosed plastic boxes containing horticultural vermiculite. After 2 weeks incubation at 27°C and 50 $\mu Em^{-2}s^{-1}$, greater than 95% of the cuttings form at least 2 roots each. At this point, individual plantlets can be transferred to soil in separate containers which are also kept in the culture room for 2 weeks before moving them to the greenhouse. As is the case with all micropropagation procedures, success in producing greenhouse plants depends on a gradual hardening of plantlets to the lower humidity and increased light intensity of the greenhouse environment.

Shoots of plants of other Oleaceae species. Table 1 lists the species and cultivars that have been grown successfully as shoot cultures with all of the following different media: 1) 2.2 μM thidiazuron, 2) 33 μM bzl^6Ade plus 0.6 μM IAA, or 3) 30 μM i^6Ade. Based on this, it appears that the method for multi-

plication of *Syringa* can probably be applied to other members of the family Oleacaea. Although it may prove to be difficult to establish shoots of certain species in tissue cultures, it is expected that the problems in these cases will involve factors such as explant sensitivity to hypochlorite, or microorganism contamination, rather than the inherent responses of shoots to cytokinin.

Table 1. Plants in the family Oleaceae that have been grown as shoot cultures At least 48 shoots per species and cultivar were used

Species and cultivar	Source of explant: Seedlings	Source of explant: Mature shoots
Forsythia *mandshurica*	+	
F *ovata*	+	+
Fraxinus pennsylvanica	+	
Ligustrum obtusifolium	+	
Syringa × *diversifolia*		
cv William H Judd		+
cv Excel		+
cv Louvois		+
S *reticulata*	+	+
cv Hippolyte Maringer		+
cv Madame Abel Chatenay		+

Ethylene production. On the basis of studies with excised pea shoots, Burg and Burg (2) hypothesized that the inhibitory effect of auxin on shoot growth results from induced ethylene. To support their hypothesis they showed that auxin treatment stimulated ethylene production and that, even in the absence of auxin, ethylene could inhibit shoot growth.

Because the subject of shoot growth regulation is fundamental to micropropagation techniques, we conducted studies to determine whether auxin inhibition in *Syringa* tissue cultures is mediated via ethylene. Figure 3 summarizes a series of experiments in which shoot growth and ethylene production were measured in the presence of different media It shows that a medium with 30 μM i^6Ade plus 2 μM 2,4-D stimulated ethylene production 18 to 80 fold over a medium with i^6Ade alone. This medium also inhibited shoot growth completely. Nevertheless, it seems unlikely that ethylene is responsible for the inhibition caused by auxin, based on the observation that vigorous growth was obtained on a medium containing i^6Ade plus the ethylene precursor ACC As shown in Fig. 3, 50 μM ACC gave ethylene production rates comparable to a medium supplemented with 2,4-D, but shoot growth essentially as vigorous as i^6Ade alone Rather than being an inhibitor of growth, ethylene appeared to be a normal consequence of it

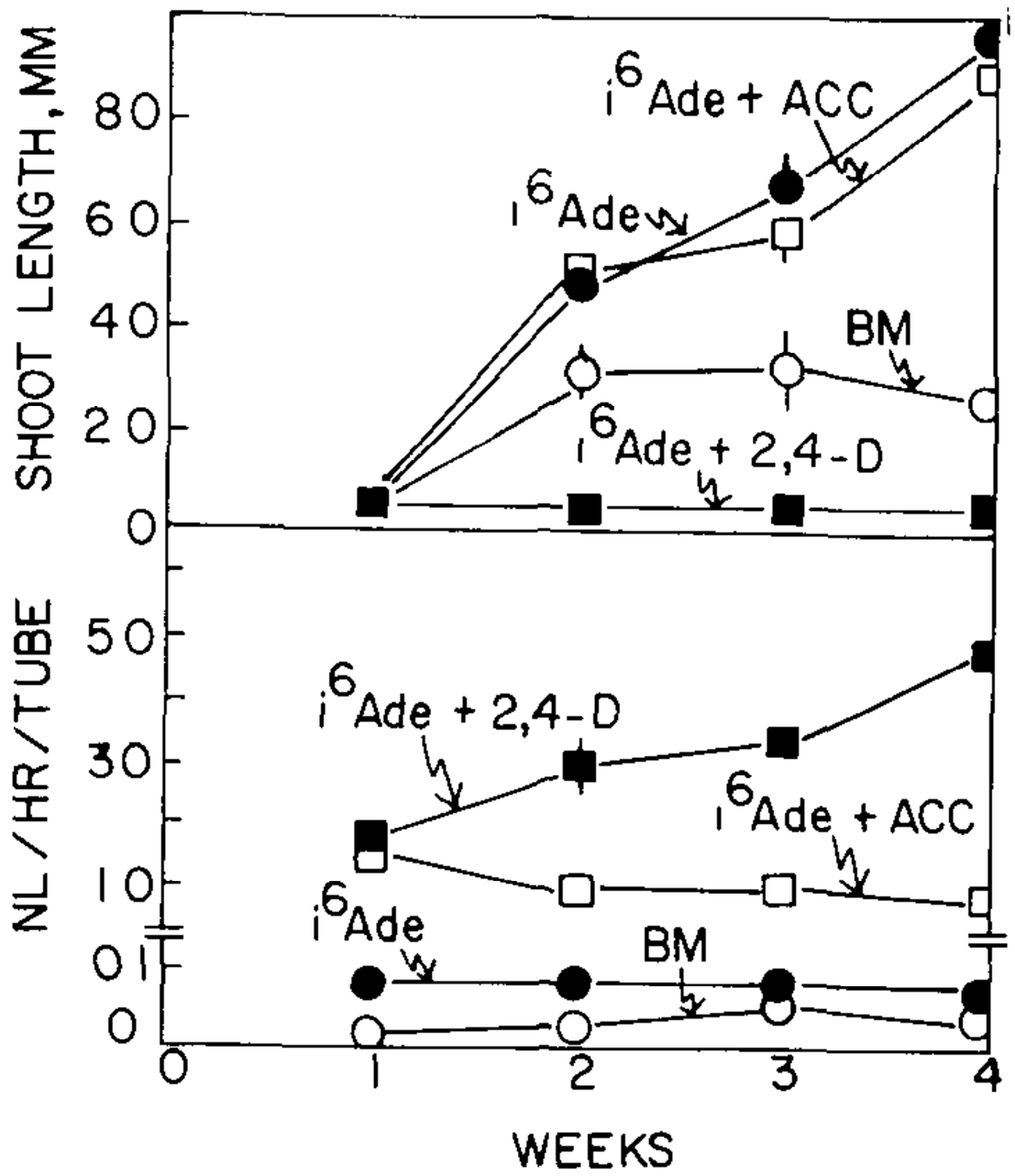

Figure 3. Shoot growth (above) and ethylene production (below) in *Syringa* tissue cultures The following concentrations of supplements were used i^6Ade, 30 μ M, 2,4-D, 2μM, ACC, 50 μM BM indicates the basal medium without supplements

based on the fact that ethylene production rates were elevated approximately 3-fold in i^6Ade cultures, compared to cultures without phytohormone.

SUMMARY

The methodology described in this report for *Syringa*, *Forsythia*, *Fraxinus* and *Ligustrum* is similar to methods for *Syringa vulgaris* cv. Vesper (6) and for several other woody plants. All of these procedures are based on the established role of cytokinin as a shoot growth regulator and the fact that shoot explants from many woody species can be grown in tissue cultures using a medium consisting of basal nutrients plus cytokinin. Our continuing research to determine the generality of this response among several taxa suggests that this methodology may be applicable to many other species of woody plants. The results to date also suggest that several groups may not be amenable to this technology. Obviously, a different methodology for micropropagation will need to be devised for unresponsive species.

The cytokinin response of excised shoots is central to the technology involved in micropropagation of Oleaceae species. In the standard procedure known as "shoot multiplication," cytokinin is used to promote growth and to overcome apical dominance in excised shoots The resulting cluster of shoots is

subdivided and individual shoots are used either for further shoot multiplication or for plantlet production. When these approaches are applied to *Syringa*, it is necessary to use nodes with inhibited laterals for shoot multiplication since an unbranched monopodial axis is produced during each passage in tissue culture. If this is done, rates corresponding to one million-fold multiplication can be obtained yearly.

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RED-FLOWERED PERENNIAL GARDEN DELPHINIUMS THROUGH INTERSPECIFIC HYBRIDIZATION

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This project was begun in 1938 while the senior author was a graduate student at the University of California, at Berkeley, California. In 1939 the project was transferred to UCLA, and in 1945 to the Missouri Botanical Garden in St. Louis, Missouri. In 1952 it was again moved to the University of Connecticut in Storrs, Connecticut.

The original reason for undertaking this work many years ago was the appearance in some California nurseries of a red