Micropropagation of Japanese Honeysuckle (*Lonicera japonica*) and Amur Honeysuckle (*L. maackii*) by Shoot Tip Culture[1]

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Abstract

Reliable micropropagation protocols were developed for Japanese honeysuckle (*Lonicera japonica* Thunb.) and Amur honeysuckle [*L. maackii* (Rupr.) Maxim], two horticulturally important invasive woody plants. Shoot tips were collected from mature plants and established *in vitro* on Murashige and Skoog (MS) medium and cultured in various media. Plant growth regulators and basal media had significant effects on proliferation rates. Proliferation of Japanese honeysuckle was highest on Driver and Kuniyuki walnut (DKW) medium with 5 µM (1.1 ppm) 6-benzyladenine (BA), yielding an average of 14 axillary microshoots in 12 weeks, whereas Amur honeysuckle produced 38 axillary microshoots in 12 weeks on MS medium containing 2.5 µM (0.6 ppm) BA with or without 1.25 µM (0.3 ppm) indole-3-butyric acid (IBA). Microcuttings of Japanese honeysuckle were rooted *ex vitro* with or without treatment with 980 µM (200 ppm) IBA, whereas microcuttings of Amur honeysuckle required treatment with 980 µM (200 ppm) IBA to root. These protocols can be used to continuously produce plant materials for various biotechnological modifications, such as *in vitro* mutations and genetic engineering for noninvasiveness.

Index words: invasive plants, honeysuckle, propagation, tissue culture, *in vitro*, *ex vitro* rooting.

Significance to the Nursery Industry

Japanese and Amur honeysuckle are easily propagated and have many good attributes for use in the landscape. Unfortunately, some of those attributes and their ease of propagation are what make them so invasive. In addition to vegetative invasiveness, honeysuckles produce copious quantities of seeds that are spread long distances by various methods of dispersal. In the United States, billions of dollars have been spent annually in controlling many species of invasive plants, and the costs associated with the associated economic and environmental damage are enormous. One of the strategies that can be used to halt the spread of invasive species is to generate seedless plants with fruits that birds can still eat but which have no viable seeds to facilitate undesirable spread. An efficient micropropagation system that can be used to produce consistent plant materials for developing a genetic engineering system in a biotechnological approach for producing female sterility and seedlessness in Japanese and Amur honeysuckles has been developed, allowing these species to continue to be useful landscape plants.

Introduction

Spread of invasive plants is the second greatest threat, behind habitat loss, to biological diversity and ecosystem function in the United States (16, 20). Invasive plants have infested over 100 million acres of land and have been estimated to increase at 10% annually (20). Approximately $35 billion are spent annually in controlling invasive plants and their associated environmental damage in the United States (20, 22). Many invasive plants produce massive amounts of seeds that are dispersed long distances by animals, wind, and water. Other invasive plant species invade nearby areas by vegetative means.

Japanese honeysuckle (*Lonicera japonica*) is a climbing vine and was initially introduced to North America from Asia as an ornamental plant for its appealing fragrance and flowers, and later, it was found to be effective for erosion control (22). Several exotic bush honeysuckles, such as Amur honeysuckle (*L. maackii*), Morrow's honeysuckle (*L. morrowii* A. Gray), Tatarian honeysuckle (*L. tatarica* L.), Sweet-breath-of-spring (*L. fragrantissima* Lindl. & Paxt.), and Belle honeysuckle [*L.* × *bella* Zab., (*L. morrowii* × *L. tatarica*)], have also been used for landscaping, wildlife habitats, and control of soil erosion.

Most honeysuckle species (*L.* sp.) are prolific seed producers and birds readily spread the seeds. In addition, a vigorous vegetative growth habit, wide adaptability, ease of establishment, and shade-tolerance are some of the reasons many honeysuckle species are aggressive competitors. Because of these traits, honeysuckle is difficult to eradicate by conventional weed-control strategies such as hand removal and herbicide application. The genus competes with native flora over a wide range of sites, posing a significant threat to many ecosystems (22). Moreover, these plants are still being used for landscaping and are sold in many nurseries across the country because of their seemingly favorable traits, which are actually responsible for their invasiveness (22).

Advances in genetic engineering and molecular biology have opened new avenues to potentially solve many biological and ecological problems. For example, with male and female sterility genes, genetic engineering has been proposed to neutralize the seed-mediated invasiveness of invasive plants (16). Manipulation of gene expression by environmental cues, such as temperature, has led to self-destruction of winter cover grasses in the spring (24). Generation of sterile plants would allow wildlife access to fruit that has nonviable seeds and could halt the undesirable spread by seeds. It may also be possible to manipulate suckering or underground shoot

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characteristics through molecular techniques (10). Regardless of the genetic engineering approach, a dependable tissue culture protocol is the first step and necessary for providing consistent experimental plant materials for developing a genetic engineering system and for growing the transgenic plants.

Micropropagation of honeysuckles has mostly been reported with blue honeysuckle (*Lonicera caerulea* L.) (11, 12), box honeysuckle (*L. nitida* Wils. cv. 'Maigrün') (2, 3), and woodbine (*L. periclymenum* L. cv 'Serotina') (1). Georges et al. (9) described plant regeneration from callus tissues of Japanese honeysuckle and indicated the regenerated shoots grew well in Woody Plant Medium [(WPM) (17)] with 6-benzyladenine (BA) and could be acclimatized. Whether this was the optimal growth medium was unclear. Micropropagation of Amur honeysuckle has not been reported, while reports of Japanese honeysuckle are very limited (9, 13). Therefore, the objective of this research was to develop an efficient, reliable micropropagation protocol for both Japanese and Amur honeysuckle.

Materials and Methods

Initial in vitro establishment. Vigorously growing 5 cm (2 in) long shoot tips of Japanese and Amur honeysuckle were collected on April 2 from single plants of each species in Knoxville, TN. The explants were soaked sequentially with gentle swirling in a 1% (v/v) Tween-20 (Fisher Scientific, Pittsburg, PA) solution for 5 min, 70% ethanol for 1 min, and in a 0.525% sodium hypochlorite solution (v/v) (10% Clorox®) for 15 min. They were then rinsed with sterile water three times, each for 5 min. After surface-disinfestation, the explants were trimmed from the bases to 2 cm (0.8 in) long and the lower leaves were removed. For each species, four trimmed explants per jar were inserted into each of the ninety 175-mL (5.9 oz) 98.5 × 59 mm (3.9 × 2.3 in) culture jars (Sigma-Aldrich, St. Louis, MO) that contained 30 mL (1.01 oz) of establishment medium. Establishment medium consisted of Murashige and Skoog [MS (18)] medium (PhytoTechnology Laboratories, Shawnee Mission, KS) supplemented with 100 mg·liter^{-1} (ppm) myo-inositol (Sigma-Aldrich), 3.0% (w/v) sucrose, 2.5 µM (0.6 ppm) BA, and 1.25 µM (0.3 ppm) indole-3-butyric acid (IBA). The medium was adjusted to pH 5.8, autoclaved at 120C and 103.5 kPa (15 lb·in^{-2}) for 20 min, and solidified with 0.7% (w/v) granulated agar (Fisher Scientific). For maintenance, explants were subcultured every 4 weeks to fresh medium of the same composition. Cultures were maintained in a growth room at 25C (77F) under a 16-h photoperiod provided by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 µmol·m^{-2}·s^{-1}as measured by a Licor LI-250 light meter (LI-COR Inc., Lincoln, NE), at the top of the culture vessels. The aseptic cultures of Japanese and Amur honeysuckle were maintained for 8 months on establishment medium, then used for three subsequent experiments to determine the most suitable combination(s) of plant growth regulators (PGRs) and basal medium for *in vitro* axillary microshoot proliferation and subsequent *ex vitro* rootability.

Basal medium and plant growth regulator effects on proliferation. To determine the effect of basal medium type on axillary microshoot proliferation, three different basal media, MS (18) medium, Driver and Kuniyuki walnut [DKW (8)] medium, and WPM (17) (all purchased from PhytoTechnology Laboratories), were tested, each with the addition of 100 mg·liter^{-1} (ppm) myo-inositol, 3.0% (w/v) sucrose, 2.5 µM (0.6 ppm) BA, and 1.25 µM (0.3 ppm) IBA. To determine the most suitable PGR combination(s) for axillary microshoot proliferation, five combinations of BA and IBA were tested: (1) 0 BA + 0 IBA, (2) 2.5 µM (0.6 ppm) BA + 0 IBA, (3) 5.0 µM (1.1 ppm) BA + 0 IBA, (4) 2.5 µM (0.6 ppm) BA + 1.25 µM (0.3 ppm) IBA, and (5) 5.0 µM (1.1 ppm) BA + 2.5 µM (0.5 ppm) IBA. The basal medium used for Japanese honeysuckle was DKW (8) and that for Amur honeysuckle was MS (18). These media were supplemented with 100 mg·liter^{-1} (ppm) myo-inositol and 3.0% (w/v) sucrose. Prior to being autoclaved, all media were adjusted to pH 5.8 and solidified with 0.7% (w/v) granulated agar. At the onset of both experiments, each treatment consisted of five replicate jars and each jar contained four microshoots. Every 4 weeks for 12 weeks the number of microshoots were recorded then subcultured, four per jar, to fresh medium. The experiments were conducted two times.

Rooting of microcuttings. Microcuttings of Japanese and Amur honeysuckle harvested from *in vitro* proliferation on establishment medium were rooted directly *ex vitro*. The basal 5 mm (0.2 in) of each microcutting was dipped for 5 sec in either distilled water or a 980 µM (200 ppm) IBA solution prepared by dissolving IBA in seven drops of 1N KOH then adjusting to volume with distilled water. Each individual microcutting was placed into a 55 mL (1.9 oz) pot filled with autoclaved soilless potting medium (Premier Pro-Mix, Premiere Hort. LTD, Red Tail, PA). Each of the two rooting treatments contained 23 individually-potted microcuttings randomly placed into one of two trays (Pactiv Corp, Lake Forest, IL), which measured 23 × 33 × 5 cm (9 × 13 × 2 in) and were covered with a clear plastic snap-on top (Pactiv Corp). The trays were maintained in the same growth room conditions as the sterile cultures. The potted microcuttings were misted with tap water twice daily. After 1 week, the microcuttings were gradually acclimatized to ambient conditions by opening the lid and progressively increasing the size of the opening while reducing the mist over a 2 week period. At the end of 1 month, the microcuttings were removed from the pots to record the number of primary roots per plantlet.

Experimental design and statistical analysis. All data were subjected to analysis of variance using the mixed procedure of the Statistical Analysis System (21). The experimental design for the two proliferation studies was completely randomized (CRD) with repeated measures to analyze treatment effects on proliferation over time (four, eight, and twelve weeks). The normal logs of the means were analyzed using Fisher's least significant difference (LSD) at $P < 0.05$. The statistical design of the rooting experiment was a CRD and the means were analyzed using Fisher's LSD at $P < 0.05$.

Results and Discussion

Both Japanese and Amur honeysuckle were established *in vitro*, yielding 100% contamination-free microshoots (data not presented). After experimenting with different basal media, Japanese honeysuckle produced statistically more microshoots on DKW basal medium than on MS or WPM (Table 1). When cultured on DKW medium, the original four explants proliferated into an average of 11 microshoots at

Table 1. Effect of basal media on the mean number of microshoots produced per original explant after four, eight, and twelve weeks of treatment.[z]

Medium[y]	Shoot number		
	4 weeks[x]	8 weeks[x]	12 weeks[x]
	Japanese honeysuckle		
DKW	5a	7a	11a
MS	4a	4b	4b
WPM	4a	4b	5b
	Amur honeysuckle		
DKW	8a	16a	33a
MS	7a	15a	30a
WPM	5a	7b	5b

[z]All media were supplemented with 2.5 µM (0.6 ppm) BA + 1.25 µM (0.3 ppm) IBA. Each treatment consisted of five replicate jars and each jar contained four explants. The experiments were conducted two times. Data are means of two experiments.
[y]DKW = Driver and Kuniyuki walnut medium, MS = Murashige and Skoog medium, and WPM = Woody Plant Medium.
[x]Means comparisons within columns were conducted using Fisher's LSD at $P < 0.05$ for each species.

the end of 12 weeks (three subculture cycles), while nearly no increase was observed on MS and WPM media (Table 1). The explants growing on DKW medium also appeared greener, were more branched, less vitrified, and exhibited less shoot tip browning (Fig. 1A). Explants of Amur honeysuckle proliferated more on DKW and MS medium (33 and 30 microshoots in 12 weeks, respectively) than on WPM (5 microshoots in 12 weeks) (Table 1).

Japanese honeysuckle produced the smallest number of new microshoots when cultured on medium without PGRs, averaging 5.2 new microshoots in 12 weeks, compared with those cultured on medium with PGRs, averaging 8.6 to 14.2 new microshoots in 12 weeks (Table 2). Addition of IBA did not enhance the effect of BA (Table 2). Although the medium containing 5 µM (1.1 ppm) BA alone did not yield significantly more microshoots than other media [except medium with 2.5 µM (0.6 ppm) BA + 1.25 µM (0.3 ppm) IBA] at the end of 12 weeks, this treatment was considered the best because the microshoots exhibited less shoot tip browning and vitrification and appeared healthier and greener than those in other treatments.

Explants of Amur honeysuckle remained alive in medium without PGRs, but the proliferation rate was low (Table 3). The greater number of microshoots were produced on medium containing 2.5 µM (0.6 ppm) BA with or without 1.25 µM (0.3 ppm) IBA (Table 3). At the end of 12 weeks, the four original microshoots in each jar proliferated into 38 new microshoots (Table 3). Further increase of BA to 5 µM (1.1 ppm) and IBA to 2.5 µM (0.5 ppm) caused a proliferation rate decline (Table 3). Of the PGR combinations tested, 2.5 µM (0.5 ppm) BA + 1.25 µM (0.3 ppm) IBA was considered the best for proliferation of Amur honeysuckle because the microshoots cultured on this medium appeared larger and healthier (Fig. 2).

Microcuttings rooted differently depending upon the treatment and species (Fig. 3). Eighty-seven percent of the Japanese honeysuckle microcuttings treated with 980 µM (200 ppm) IBA rooted, which was insignificantly greater than microcuttings treated with water (78%). However, 15% of the Amur honeysuckle microcuttings rooted with the water treatment, which was significantly less than those microcuttings treated with 980 µM (200 ppm) IBA which rooted at 76% (Fig. 3). For both treatments, the number of primary roots on each microcutting ranged from one to seven with the majority being two to three (data not presented). Although the survival rate of Japanese honeysuckle was 100% for both treatments, and that of Amur honeysuckle was 87% for water and 91% for IBA (data not presented), the rooted microcuttings were not transplanted because they were anticipated to have high survival rates due to their invasiveness and broad adaptability.

Japanese and Amur honeysuckle have many desirable attributes for use in the landscape. However, these same attributes contribute to their invasiveness and subsequent environmental damage. These plants are commonly sold in the nursery trade, therefore making them highly controversial. Our long-term goal is to produce seedless and suckerless honeysuckle through genetic engineering so that they will no longer be invasive yet remain useful as ornamentals. In this study, reproducible protocols for micropropagation of Japanese and Amur honeysuckle were developed. These protocols

Fig. 1. The best performance of (A) Japanese honeysuckle on DKW medium with 2.5 µM (0.6 ppm) BA and 1.25 µM (0.3 ppm) IBA and (B) Amur honeysuckle on MS medium with 2.5 µM (0.6 ppm) BA and 1.25 µM (0.3 ppm) IBA. The photographs were taken at the conclusion of the experiment.

Table 2. Effect of plant growth regulators (PGRs) on the mean number of microshoots produced per original explant of Japanese honeysuckle after four, eight, and twelve weeks of treatment.[z]

PGRs[y] [µM (ppm)]		Shoot number		
BA	IBA	4 weeks[x]	8 weeks[x]	12 weeks[x]
0	0	5a	7ab	5c
2.5 (0.6)	0	5a	6b	9ab
5 (1.1)	0	5a	10a	14a
2.5 (0.6)	1.25 (0.3)	4a	6b	9b
5 (1.1)	2.5 (0.5)	4a	8ab	9ab

[z]The basal medium was DKW medium. Each treatment consisted of five replicate jars and each jar contained four microshoots. The experiments were conducted two times. Data are means of two experiments.
[y]BA = 6-benzyladenine and IBA = indole-3-butyric acid.
[x]Means separation within columns by Fisher's LSD at $P < 0.05$.

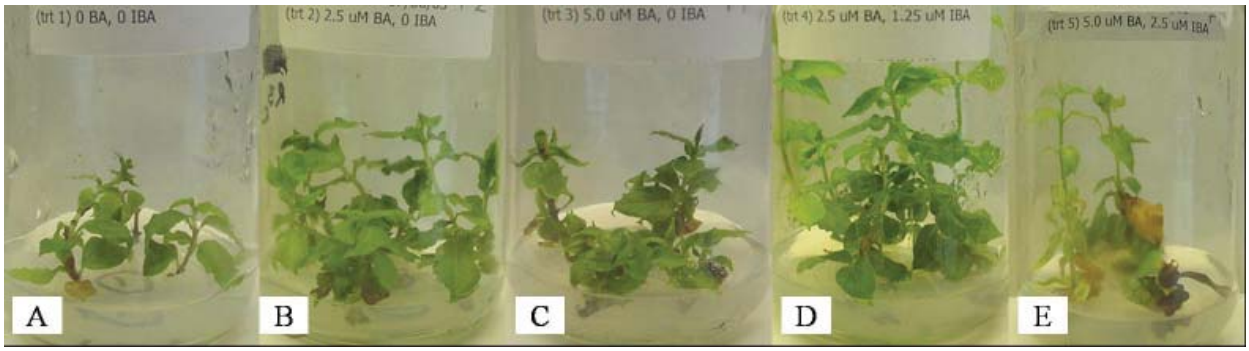

Fig. 2. Comparison of Amur honeysuckle proliferation on MS medium with various PGR combinations. (A) = 0 BA + 0 IBA, (B) = 2.5 µM (0.6 ppm) BA + 0 IBA, (C) = 5.0 µM (1.126 ppm) BA + 0 IBA, (D) = 2.5 µM (0.6 ppm) BA + 1.25 µM (0.3 ppm) IBA, and (E) = 5 µM (1.1 ppm) BA + 2.5 µM (0.5 ppm) IBA. The photographs were taken at the conclusion of the experiment.

can be used to produce aseptic plant material all year round for developing efficient plant regeneration and transformation systems and to micropropagate plants if transgenic plants are produced. Specific combinations of PGRs and basal media affected efficiency of proliferation. Such effects have been documented in other woody plant species such as Asian white birch (*Betula platyphylla* Sukaczev) (4), eastern redbud (*Cercis canadensis* L.) (7), and Siberian elm (*Ulmus pumila* L.) (5), as well as some honeysuckle species (2, 9, 19). Even though both Japanese and Amur honeysuckle belong to the same genus, the proliferation response to the PGRs was different. Japanese honeysuckle which is very invasive *ex vitro*, did not proliferate well *in vitro*, while Amur honeysuckle was much more responsive to PGRs and thus proliferated more readily *in vitro*. Genotypic variation has been observed within blue honeysuckle such as *L. kamtschatica* [Sevast.] Pojark (23) and *L. caerulea* (11). Karhu (11) found a linear relationship between the concentration of BA [ranging from 1.1 to 17.8 µM (0.25 to 4.0 ppm)] and the number of axillary microshoots produced (3 to 13 new microshoots). The difference in proliferation rates in three different media was a result of the effect of medium composition. For example, the nitrate content is much higher in MS and DKW basal media than it is in WPM basal medium. The effect of the basal media on plant growth has been widely reported, for example, in propagation of eastern redbud (7), regeneration of aspen (*Populus canescens* Aiton Sm. × *P. grandidentata* Michx. *and P. tremuloides* Michx. × *P. davidiana* Dode) (6) and some honeysuckle species (19).

Success of *in vitro* rooting of microcuttings has also been reported with two forms of blue honeysuckle using IBA (12). Karhu (12) reported differences of *in vitro* rooting of axillary microshoots of *L. caerulea* L. f. *caerulea* and *L. caerulea* L. f. *edulis* Regel, with rooting percentages > 90% for microcuttings of *L. caerulea* f. *caerulea*, but < 40% for microcuttings of *L. caerulea* f. *edulis* when not treated with auxin. However, continuous auxin treatments inhibited microshoot elongation, increased the diameter of primary roots, and induced callus formation at the base of the microshoots (12). A 7-day pulse with 4 µM (0.9 ppm) IBA followed by *ex vitro* rooting yielded a high rooting percentage, good root growth, and *ex vitro* survival of *L. caerulea* f. *edulis* microplants (12). In this study, it is not surprising Japanese honeysuckle rooted successfully without auxin treatment. Because of its natural vine-growing habit, it roots readily at every node when in contact with soil. Amur honeysuckle is a woody shrub and could be expected to root with more difficulty. The micro-

Table 3. Effect of plant growth regulators (PGRs) on the mean number of microshoots produced per original explant of Amur honeysuckle after four, eight, and twelve weeks of treatment.[z]

PGRs[y] [µM (ppm)]		Shoot number		
BA	IBA	4 weeks[x]	8 weeks[x]	12 weeks[x]
0	0	3c	3d	3c
2.5 (0.6)	0	8a	27a	38a
5 (1.1)	0	5bc	14b	19b
2.5 (0.6)	1.25 (0.3)	7ab	21ab	38a
5 (1.1)	2.5 (0.5)	6ab	8c	14b

[z]The basal medium was MS medium. Each treatment consisted of five replicate jars and each jar contained four microshoots. The experiments were conducted two times. Data are means of two experiments.

[y]BA = 6-benzyladenine and IBA = indole-3-butyric acid.

[x]Means separation within columns by Fisher's LSD at $P < 0.05$.

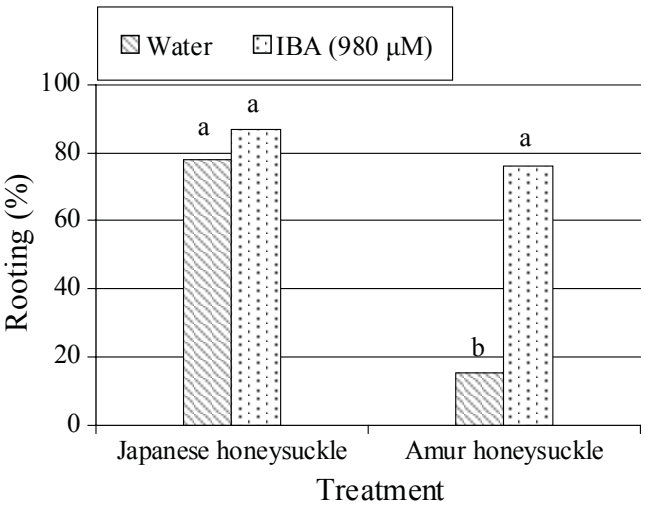

Fig. 3. Percentages of Japanese and Amur honeysuckle microcuttings that rooted after treatment with either water or 980 µM (200 ppm) IBA. Each treatment consisted of 23 microshoot replicates. Means comparisons among treatments within a species were conducted using Fisher's LSD at $P < 0.05$.

cuttings of Amur honeysuckle required a quick-dip in auxin to achieve an acceptable rooting percentage. The success of *ex vitro* rooting suggests *in vitro* rooting is not necessary, as demonstrated with some other woody plant species such as Siberian elm (5), therefore, making propagation more efficient and less labor-intensive.

In summary, efficient and reproducible protocols for micropropagating two honeysuckle species, Japanese honeysuckle and Amur honeysuckle, have been developed. These procedures can be used to produce consistent plant material of these species which can then be utilized for developing a plant regeneration system as in other honeysuckle species (1, 2, 3, 14) and for developing noninvasive plants as proposed by Li et al. (15, 16).

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