Micropropagation of *Weigela florida* ‘Tango’ through In Vitro Shoot Culture

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Abstract. *Weigela florida* (Bunge) A. DC. is a popular flowering shrub adapted to a wide range of environmental conditions. Efficient methods for micropropagation of this species have not been well developed. The present study established a protocol for in vitro shoot culture of *W. florida* ‘Tango’ after a systematic evaluation of different culture media, cytokinins, and auxins on axillary shoot induction. Single-node stems were cultured on Driver and Kuniyuki Walnut (DKW) medium for initial production of axillary shoots. The shoots were used as explants and cultured on DKW medium supplemented with 8.88 μM 6-benzylaminopurine (BA) and 0.27 μM naphthaleneacetic acid (NAA), resulting in the production of more than six axillary shoots per explant. The axillary shoots could either be used as explants for additional shoot production or be cultured on $\frac{1}{2}$ DKW medium supplemented with 0.25 μM indole-3-butyric acid (IBA) for rooting. Plantlets were transplanted into a substrate with 99% survival rate in a shaded greenhouse. This established method could be used for rapid propagation of *W. florida* to speed the introduction of new hybrids or cultivars for commercial production.

Weigela Thunb. is a genus of 12 recognized species of deciduous shrubs in the family Caprifoliaceae (Yokoyama et al., 2002). All species are native to eastern Asia and generally hardy and easily grown (Huxley, 1992). They have decorative flowers in spring and early summer varying from white through pink to red. Among them, *W. florida* (Bunge.) A. DC. is the most commonly produced species (Touchell et al., 2006). It was collected by Robert Fortune from North China in 1845 and commonly known as old fashioned weigela. This species has a dense and rounded canopy that typically grows to 1–3 m high and may spread over time to up to 4 m wide. It is pest resistant, tolerant to a wide range of environmental conditions, and cold hardy to USDA zone 4–5 (Touchell et al., 2006). It was from this species that most hybrids or cultivars have been developed (Duron and Decourtye, 1990).

Weigelas are no longer old fashioned plants; they have regained popularity in the ornamental plant industry during the last

20 years. More than 180 weigela hybrids or cultivars with different foliar and flower colors, growth forms, and reblooming characteristics are available (Wood, 2016). They are propagated mainly through stem cuttings (Weigle and Stephens, 1991). Cutting propagation of newly bred hybrids or cultivars; however, could be hampered by the availability of appropriate stems. Additionally, cutting propagation may carry and spread diseases, such as crown gall caused by *Agrobacterium tumefaciens* and gray mold caused by *Botrytis cinerea* (Jones and Benson, 2001). In vitro micropropagation could be a solution to provide disease-free propagules of the new hybrids or cultivars year-round to the ornamental plant industry (Chen and Henny, 2008).

The first in vitro culture of *Weigela* dates back to 1975, when meristems of five cultivars were cultured by Duron (1975), followed by stem internode culture (Duron, 1981), and bud culture by Calvert and Stephens (1986). All these cultures used Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium; however, details of multiplication rates were not reported. Weigle and Stephens (1991) briefly mentioned that *W. florida* ‘Red Prince’ could be micropropagated using MS medium. Ochatt (1993) regenerated plantlets of *W. florida* ‘Bristol Ruby’ using protoplasts as explants. The MS medium was also used for shoot

culture of *W. florida* ‘Red Prince’, but vitrification was a problem (Wang et al., 2000). Additionally, callus was induced from ‘Red Prince’ (Yuan and Zhang, 2006), and somatic embryos were produced from ‘Red Prince’ pollen (Wang et al., 2012); but plant regeneration from either calluses or embryos was not documented. As far as is known, reliable methods for in vitro propagation of *Weigela* have not been well established.

The objective of this study was to develop a method for in vitro propagation of *W. florida* using cultivar Tango as a model plant. Tango has a compact size (0.6 to 1 m tall), produces profuse spring flowers and purplish foliage, and is considered one of the most commonly produced cultivar. Different culture media, cytokinins, and auxins for axillary shoot induction were evaluated and microcuttings were rooted in vitro and ex vitro. A reliable protocol for in vitro shoot culture of *W. florida* ‘Tango’ was developed.

Materials and Methods

Plant materials. Young stems (10–15 cm) of *W. florida* ‘Tango’ grown at the experimental station at Hunan Academy of Forestry, Changsha City, Hunan Province, China were collected. After removing leaves, stems were washed with running tap water for 1.5 h, followed by immersion in 20% Clorox (1.2% NaOCl) solution for 20 min, and washing with sterile distilled water for 10 min. Under aseptic conditions, the stems were cut to about 5 cm, soaked in 75% ethanol for 10 s, and washed with sterile distilled water three times. They were further sterilized in bottles containing 0.1% HgCl₂ with 2–3 drops of Tween 80 for 10 min. After pouring off the HgCl₂ solution, the stems were rinsed three times with sterile distilled water and cut as single nodes in sterile petri dishes.

The DKW medium with vitamins (Driver and Kuniyuki, 1984) (Product ID: D2470, PhytoTechnology, Shawnee Mission, KS) was supplemented with 3% sucrose and 0.8% agar. The medium, after its pH was adjusted to 5.8 using 1 M KOH, was autoclaved at 121 °C for 20 min and aliquoted to 250 mL tissue culture vessels (Shanghai Zeshine Equipment Co., Shanghai, China) at 20 mL each. Sterilized nodes were cultured on the DKW and maintained in a culture room described below for initial induction of axillary shoots.

Selection of basal media. Four culture media were tested, including MS, $\frac{1}{2}$ MS, DKW, and B₅ (Gamborg et al., 1968). Each medium contained 3% sucrose and 0.8% agar with pH being adjusted to 5.8 using 1 M KOH before autoclaving at 121 °C for 20 min. When the temperature dropped to about 50 °C, filter-sterilized stock solutions of BA and NAA were added to the medium resulting in a final concentration of BA at 8.88 μM and NAA at 0.27 μM . The medium was aliquoted to 300-mL erlenmeyer flasks at 20 mL each. Three axillary shoots (about 2 cm) resulting from initial shoot induction were cultured on the media and placed in a culture

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room. Shoot numbers and shoot heights were recorded after 5 weeks of culture.

Selection of cytokinins. Autoclaved DKW medium supplemented with N-(2-chloro-4-pyridyl)-N'-phenylurea, kinetin, N-isopentenylaminopurine, N-phenyl-N'-1, 2, 3-thiadiazol-5-ylurea, zeatin, or BA, each at 8.88 μM with 0.27 μM NAA, was aliquoted to erlenmeyer flasks at 20 mL each. Three shoot explants were cultured on the medium and placed in a culture room. Shoot numbers and shoot heights were recorded after 5 weeks of culture.

Selection of auxin. 3-indoleacetic acid (IAA), IBA, or NAA were added to autoclaved DKW medium, each at a concentration of 0.27 μM with 8.88 μM BA. Three shoot explants were cultured on the medium and placed in a culture room. Shoot numbers and shoot heights were recorded after 5 weeks of culture.

Optimization of multiplication. Based on the above results, BA at concentrations of 4.44, 8.88, and 13.32 μM in a factorial combination with NAA at 0.05, 0.27, and 0.54 μM were added to autoclaved DKW medium, resulting in nine growth regulator combinations. Three shoots were cultured on the medium and maintained in a culture room. The number of axillary shoots was recorded after 5 weeks of culture. After identifying the optimal BA and NAA concentrations, this combination was used to produce more axillary shoots for the following rooting experiment.

Rooting. Microcuttings derived from axillary shoots with a height about 2.5 cm were excised and rooted in $\frac{1}{2}$ MS, $\frac{1}{2}$ DKW, and $\frac{1}{2}$ B₅ media containing 0.25 μM IAA, IBA, and NAA, respectively, five microcuttings per flask. Rooting percentage, root numbers, and mean root length were recorded after four weeks of culture. The most appropriate medium for rooting was identified.

Culture conditions. All cultures used for evaluation of media, growth regulators, and rooting were maintained in a culture room under a 12-h photoperiod provided by cool-white fluorescent lamps with a photon flux density of 30 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and temperature of $25 \pm 2^\circ\text{C}$.

Experimental design and data analysis. All experiments were arranged as a completely randomized design with 10 replications. Each culture vessel was considered an experimental unit, and there were three shoots per culture vessel except for the rooting tests that had five microcuttings. Collected data were analyzed using SPSS 13.0 for Windows (SPSS, Chicago, IL). When significant differences ($P < 0.05$) occurred, means were separated using Fisher's protected least significant differences at $P < 0.05$ level.

Transplantation and acclimatization. After washing off the rooting medium with tap water, plantlets were transplanted to plug trays containing a substrate comprised of 20% clay soil, 40% carbonized rice hull, 20% perlite, and 20% coarse sand based on volume. Transplants were grown in a shaded

Fig. 1. In vitro shoot culture of *Weigela florida* 'Tango'. (A) Stem explants were cultured on Driver and Kuniyuki Walnut (DKW) medium devoid of growth regulators for initial induction of axillary shoots. (B) Shoot proliferation from shoot explants cultured on DKW medium supplemented with 8.88 μM 6-benzylaminopurine and 0.27 μM naphthaleneacetic acid. (C) Axillary shoots or microcuttings were rooted in $\frac{1}{2}$ DKW medium supplemented with 0.25 μM indole-3-butyric acid. (D) Plants were acclimatized in a shaded greenhouse.

greenhouse under a maximum photosynthetic active photon flux density of 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, temperature range of 20 to 28 $^\circ\text{C}$, and a relative humidity from 70% to 100%. Plants were watered through intermittent mist at 10 s per 30 min. After 2 weeks of growth in the shaded greenhouse, plugs were transplanted to containers and fertigated weekly with a nutrient solution with N at 100 $\text{mg}\cdot\text{L}^{-1}$ prepared from a 30N-10P₂O₅-10K₂O fertilizer. Containerized plants were grown under 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and survival rates of plants were recorded after 2-month growth.

Results

Medium and growth regulator selection. Axillary budbreaking occurred 1 week after a single node was cultured on DKW medium without supplementation of growth regulators (Fig. 1A). Two weeks after breaking, the axillary shoots were used as explants for screening of culture media. The DKW medium induced significantly more axillary shoots than MS, $\frac{1}{2}$ MS, and B₅ ($P < 0.05$). Shoots produced from DKW were also longer than those produced in the other media ($P < 0.05$) (Table 1). DKW thus was selected for subsequent experiments.

Among the cytokinins evaluated, BA induced significantly more axillary shoots than the others ($P < 0.05$), and shoots induced by

Table 1. Effects of different culture media on the induction of axillary shoots from stem explants of *Weigela florida* 'Tango'.^z

Medium ^y	Shoot number	Shoot ht (cm)
MS	2.10 $\pm$ 0.32 c ^x	2.14 $\pm$ 0.26 c
$\frac{1}{2}$ MS	4.23 $\pm$ 0.57 b	3.03 $\pm$ 0.38 b
DKW	5.67 $\pm$ 0.61 a	3.97 $\pm$ 0.21 a
B ₅	2.37 $\pm$ 0.56 c	2.94 $\pm$ 0.24 b

MS = Murashige and Skoog; DKW = Driver and Kuniyuki Walnut; B₅ = Gamborg B₅ medium.

^zData were collected after 5-week culture of single-node stems.

^yEach medium was supplemented with 8.88 μM BA and 0.27 μM naphthaleneacetic acid.

^xDifferent letters within a column represent significant difference at $P < 0.05$ by Fisher's protected least significant difference test.

BA were longer than those produced with other cytokinins ($P < 0.05$) (Table 2). When DKW medium supplemented with 8.88 μM BA was used for selecting auxins, NAA induced more shoots than IAA and IBA, and shoots induced by NAA were also longer than the other auxins ($P < 0.05$) (Table 3). Therefore, BA and NAA were selected to be appropriate cytokinin and auxin for inducing axillary shoots of *W. florida* 'Tango'.

To optimize shoot induction, three BA concentrations in a factorial combination with three concentrations of NAA were evaluated. Results showed that BA at 8.88 μM

with 0.054, 0.27, or 0.54 μM NAA produced the highest numbers of shoots (Fig. 1B) than the other combinations ($P < 0.05$) (Table 4). However, shoot heights induced by 8.88 μM BA with either 0.27 or 0.54 μM NAA were significantly greater than the other combinations ($P < 0.05$).

Rooting and acclimatization. The $\frac{1}{2}$ DKW medium supplemented with 0.25 μM IBA

Table 2. Effects of six different cytokinins on the induction of axillary shoots from shoot explants of *Weigela florida* ‘Tango’.^z

Cytokinin ^y	Shoot number	Shoot ht (cm)
BA	6.53 $\pm$ 0.88 a ^x	3.76 $\pm$ 0.22 a
ZT	2.30 $\pm$ 0.46 b	2.15 $\pm$ 0.28 b
KT	2.10 $\pm$ 0.52 bc	1.89 $\pm$ 0.26 c
2iP	1.83 $\pm$ 0.57 cde	1.83 $\pm$ 0.26 c
TDZ	1.93 $\pm$ 0.41 bcd	1.92 $\pm$ 0.24 c
CPPU	1.47 $\pm$ 0.32 ef	1.92 $\pm$ 0.21 c

CPPU = N-(2-chloro-4-pyridyl)-N'-phenylurea; KT = kinetin; 2iP = N-isopentenylaminopurine, TDZ = N-phenyl-N'-1, 2, 3-thiadiazol-5-ylurea; ZT = zeatin, or BA = 6-benzylaminopurine.

^zData were collected after 5-week culture of shoot explants.

^yDriver and Kuniyuki Walnut medium supplemented with individual cytokinin at 8.88 μM with 0.27 μM naphthaleneacetic acid.

^xDifferent letters within a column represent significant difference at $P < 0.05$ by Fisher's protected least significant difference test.

Table 3. Effects of different auxins on the induction of axillary shoots from shoot explants of *Weigela florida* ‘Tango’.^z

Auxin ^y	Shoot number	Shoot ht (cm)
NAA	6.73 $\pm$ 0.90 a ^x	3.57 $\pm$ 0.39 a
IBA	3.63 $\pm$ 0.66 b	2.40 $\pm$ 0.22 b
IAA	2.93 $\pm$ 0.44 bc	2.19 $\pm$ 0.32 bc

IAA = 3-indoleacetic acid; NAA = naphthaleneacetic acid; IBA = indole-3-butyric acid.

^zData were collected after 5-week culture of shoot explants.

^yDriver and Kuniyuki Walnut medium supplemented with individual auxin at 0.27 μM with 8.88 μM 6-benzylaminopurine.

^xDifferent letters within a column represent significant difference at $P < 0.05$ by Fisher's protected least significant difference test.

Table 4. Different concentrations of 6-benzylaminopurine (BA) and naphthaleneacetic acid (NAA) and their influence on shoot proliferation from shoot explants of *Weigela florida* ‘Tango’.^z

BA (μM)	NAA (μM)	Shoot number	Shoot ht (cm)
4.44	0.05	3.90 $\pm$ 0.75 c ^y	2.47 $\pm$ 0.19 de
4.44	0.27	4.27 $\pm$ 0.83 c	2.58 $\pm$ 0.22 de
4.44	0.54	4.40 $\pm$ 0.64 c	2.80 $\pm$ 0.33 d
8.88	0.05	6.37 $\pm$ 0.58 a	3.25 $\pm$ 0.27 bc
8.88	0.27	6.67 $\pm$ 0.86 a	3.78 $\pm$ 0.20 a
8.88	0.54	6.53 $\pm$ 0.57 a	3.86 $\pm$ 0.24 a
13.32	0.05	5.27 $\pm$ 0.52 b	3.16 $\pm$ 0.23 c
13.32	0.27	5.67 $\pm$ 0.65 b	3.34 $\pm$ 0.30 bc
13.32	0.54	5.40 $\pm$ 0.57 b	3.52 $\pm$ 0.40 b

^zData were collected after 5-week culture of shoot explants on Driver and Kuniyuki Walnut medium.

^yDifferent letters within a column represent significant difference at $P < 0.05$ by Fisher's protected least significant difference test.

Table 5. Rooting percentages of microcuttings of *Weigela florida* ‘Tango’ cultured on different media supplemented with different auxins.^z

Medium	Auxin ^y	Rooting percentage	Root number	Mean root length (cm)
$\frac{1}{2}$ MS	IBA	93.3 $\pm$ 0.09 ab ^x	6.2 $\pm$ 1.30 b	3.04 $\pm$ 0.29 b
$\frac{1}{2}$ MS	NAA	83.3 $\pm$ 0.12 bc	4.0 $\pm$ 1.41 cde	1.16 $\pm$ 0.30 d
$\frac{1}{2}$ MS	IAA	16.7 $\pm$ 0.12 f	2.6 $\pm$ 0.89 efg	0.50 $\pm$ 0.19 efg
$\frac{1}{2}$ DKW	IBA	100.0 $\pm$ 0 a	7.8 $\pm$ 1.30 a	3.48 $\pm$ 0.50 a
$\frac{1}{2}$ DKW	NAA	86.7 $\pm$ 0.1 abc	5.4 $\pm$ 1.14 bc	1.94 $\pm$ 0.42 c
$\frac{1}{2}$ DKW	IAA	26.7 $\pm$ 0.1 ef	3.2 $\pm$ 1.10 ef	0.82 $\pm$ 0.23 def
$\frac{1}{2}$ B ₅	IBA	46.7 $\pm$ 0.14 d	4.8 $\pm$ 0.84 bcd	2.24 $\pm$ 0.44 c
$\frac{1}{2}$ B ₅	NAA	33.3 $\pm$ 0.12 de	3.8 $\pm$ 0.30 def	0.88 $\pm$ 0.24 de
$\frac{1}{2}$ B ₅	IAA	13.3 $\pm$ 0.14 f	1.6 $\pm$ 0.89 g	0.32 $\pm$ 0.13 g

IAA = 3-indoleacetic acid; NAA = naphthaleneacetic acid; IBA = indole-3-butyric acid; DKW = Driver and Kuniyuki Walnut; MS = Murashige and Skoog; B₅ = Gamborg B₅ medium.

^zData were collected after 4-week culture of shoot explants.

^yIBA, NAA, and IAA concentrations were all 0.25 μM .

^xDifferent letters within a column represent significant difference at $P < 0.05$ by Fisher's protected least significant difference test.

resulted in 100% rooting of microcuttings (Table 5). The $\frac{1}{2}$ MS with 0.25 μM IBA or 0.25 μM NAA had 93.3% or 83.3% rooting, $\frac{1}{2}$ DKW with 0.25 μM NAA had 86.7% rooting. However, $\frac{1}{2}$ DKW with 0.25 μM IBA induced more root numbers and longer roots, 7.8 per shoot and 3.48 cm (Fig. 1C), respectively, than the other treatments. The optimum rooting medium thus was $\frac{1}{2}$ DKW medium supplemented with 0.25 μM IBA.

Plantlets, after transplanting into a substrate, grew vigorously (Fig. 1D) and had 99% survival rate when grown in a shaded greenhouse. Two months later, numerous roots had been produced, and plants were ready for transplanting to the field for commercial production.

Discussion

An in vitro shoot culture method for propagating *Weigela florida* ‘Tango’ was developed in this study. Axillary shoots were produced from single-node stems cultured on DKW medium without addition of growth regulators. The axillary shoots then were used as explants cultured on DKW medium supplemented with 8.88 μM BA with 0.27 μM NAA for shoot proliferation. The shoots could be used as explants for continuous multiplication or used as microcuttings for subsequent rooting in $\frac{1}{2}$ DKW medium containing 0.25 μM IBA. Plantlets were easily acclimatized in a shaded greenhouse after transplanting into a substrate. Two months after growing in the shaded greenhouse, plants were ready for planting into the field for commercial production.

This protocol is different from previously reported ones for micropropagating *Weigela* species. The previously reported methods used MS as basal medium, and multiplication rates varied from two to five axillary shoots per explant. The present study identified DWK as a better medium for in vitro shoot culture where an average of 6.67 axillary shoots per explant was produced. The effectiveness of DKW in shoot induction could be attributed the medium's mineral concentrations and compositions. DKW may have concentrations of mineral elements that are comparatively similar to those normally

needed for *W. florida* growth. Nas and Read (2004) proposed that the composition of a culture medium for a particular species should resemble the seed composition. Efficient multiplications were achieved by Bouman and Tiekstra (2005) using media with macronutrients resembling the elemental composition in adult leaves of *Gerbera* and *Cymbidium*. In the case of *Weigela*, a study found that manganese (Mn) and zinc (Zn) concentrations in leaves of *W. florida* were 28 and 27 mg·kg⁻¹, respectively (Chong et al., 2004). Concentrations of MnSO₄ in B₅, MS, and DKW were 10, 16.9, and 33.5 mg·L⁻¹, respectively. Zinc nitrate was 17 mg·L⁻¹ in DKW, but ZnSO₄ in MS was 8.6 mg·L⁻¹ and only 2 mg·L⁻¹ in B₅. Therefore, DKW has Mn and Zn concentrations more closely matching *Weigela*'s leaf analysis concentrations. Additionally, DKW has nickel (Ni), but Ni is absent in B₅ and MS media. Ni is an essential micronutrient for plants (Brown et al., 1987; Eskew et al., 1983; Hand and Reed, 2014; Ragsdale, 1998). The same DKW medium has been shown to improve in vitro shoot multiplication of *Cornus wilsoniana* (Li et al., 2015). It might be possible that Ni is important to the growth of *Weigela* and its absence in the other media might affect axillary shoot production.

The present study agrees with the previous reports (Ochatt, 1993; Wang et al., 2000; Weigle and Stephens, 1991; Yuan and Zhang, 2006) that BA and NAA were appropriate for *Weigela* micropropagation. For rooting of microcuttings, IBA resulted in highest rooting percentage, greater root numbers, and longer root lengths than the other auxins tested. Plants had a 99% survival rate in the shaded greenhouse. The higher survival rate could be due to the plant status during the acclimatization period where plants were well rooted. Among the 1,000 plants produced using this established method, off-type plants were not observed during the acclimatization period and growth in the shaded greenhouse. Since the propagation is based on preexisting meristems, not through a regeneration process; somaclonal variation is generally not expected (Chen and Henny, 2006). The established protocol has been used for propagation of ‘Tango’ for

more than 2 years in our laboratory. It produces repeatable results in weigela multiplication and rooting. We believe that the use of this method could lead to rapid commercial propagation of new hybrids and cultivars of *W. florida*.

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