Environmental and Experimental Biology (2019) 17: 169–177 *Original Paper*
DOI: 10.22364/eeb.17.17

In vitro regeneration and propagation from fasciated stems of *Vitex rotundifolia*

Hanzhi Liang[1,2], Yuping Xiong[1,4], Beiyi Guo[1,4], Haifeng Yan[5], Shuguang Jian[1], Hai Ren[1], Xinhua Zhang[1], Yuan Li[1], Songjun Zeng[1], Kunlin Wu[1], Feng Zheng[1], Jaime A. Teixeira da Silva[3]*, Youhua Xiong[2]*, Guohua Ma[1]*

[1]Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, the Chinese Academy of Sciences, Guangzhou, 510650, China
[2]College of Horticulture and Landscape Architecture, Zhongkai University of Agriculture and Engineering, Guangzhou, 264000, China
[3]P.O. Box 7, Miki-cho Post Office, Miki-cho, Ikenobe 3011-2, Kagawa-ken, 761-0799, Japan
[4]University of Chinese Academy of Sciences, Beijing 100039, China
[5]Cash Crop Institute of Guangxi Academy of Agricultural Sciences, Nanning, 530007, China

*Co-corresponding authors, E-mail: jaimetex@yahoo.com; youhuachina@126.com; magh@scib.ac.cn

Abstract

An efficient plant proliferation and regeneration system via fasciated stems was established for the first time in *Vitex rotundifolia* L. Aseptic nodal segments were used as explants and cultured on Murashige and Skoog medium supplemented with different plant growth regulators alone and in different combinations to investigate the induction, proliferation, axillary shoot initiation and elongation from fasciated stems and subsequent regeneration. The obtained results show that the growth of fasciated stems can be regulated by cytokinins. A higher concentration of cytokinins induced both the formation of axillary shoots from normal stems and the development of fasciated stems. Anatomical analyses revealed that fasciated stems (5 to 25 mm wide) were much wider than normal *in planta* shoot stems (2 to 3 mm wide). The pith cells of fasciated stems developed laterally to 0.05 – 0.08 mm in width, while normal stems were usually 0.02 to 0.04 mm in width. Flow cytometry indicated no obvious changes in chromosomes among the four types of shoots. This is the first report on the development of axillary shoots from fasciated stems in *V. rotundifolia* tissue culture. Our protocol serves as a new form of clonal plant regeneration.

Key words: axillary shoots, fasciated stem, anatomical analysis, flow cytometer, rooting, *Vitex rotindifolia*.
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, 6-furfurylaminopurine; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; PGRs, plant growth regulators; TDZ, thidiazuron; ZEA, zeatin.

Introduction

Vitex rotundifolia L. (Verbenaceae) is a deciduous shrub found in tropical and temperate regions, including China, India, Japan and Korea, where it grows on beaches and coastal zones forming a sprawling groundcover and displays tolerance to salinity stress (Yeeh et al. 1996). It is a well-known medicinal herb, and its fruits, which are used as a component of traditional Chinese medicine Fructus Viticis, to treat colds, headaches, migraines, and neuralgia, exhibit a variety of pharmacological activities including anti-inflammatory, cytotoxic, antimicrobial, anti-nociceptive, antihyperprolactinemia as well as the induction of apoptosis in human colorectal cancer cells (Kawazoe et al. 2001; Ono et al. 2002; Sohn and Ko 2009; Lee et al. 2013; Song et al. 2017). In recent years, market demand has increased for new applications and the development of new products, many based on natural resources, and the lack of suitable resource conservation protocols is leading to their exhaustion. *V. rotundifolia* has been listed in the Catalogue of National Key Protected Wild Medicinal Material Species as a national class-III endangered and conserved plant species in China (Liang et al. 2012).

Vitex species such as *Vitex doniana* can be propagated by seeds (Abubakar et al. 2015). However, this method is slow and tedious since it relies on the seasonal collection of seeds from mother plants. Therefore, to strengthen the conservation, development and long-term utilization of this plant resource, it is necessary to establish an efficient *in vitro* micropropagation and plant regeneration protocol. A recent review of the micropropagation of *Vitex* species (Teixeira da Silva et al. 2016) indicates that only a single regeneration protocol exists for *V. rotundifolia* (Park et al. 2004). In that study, shoots were regenerated from nodal segments in the presence of a cytokinin, 6-benzyladenine (BA) on Nitsch basal medium (Nitsch, Nitsch 1969). Other than that study, to the best of our knowledge, there is no recorded regeneration protocol for this species.

Fasciation is the deformation of shoots that typically involves broadening of the shoot apical meristem, resulting in a fasciated stem and changes in leaf arrangement (Iliev, Kitin 2011). Fasciation has been found in over 100 species of vascular plants (Iliev, Kitin 2011). Exogenously applied cytokinins can induce fasciation in some plant species (Varga et al. 1988; Iliev 1996; Papafotiou et al. 2001). However, shoot fasciation has not yet been reported in V. rotundifolia. Plant regeneration from fasciated stems has not been well studied in any plant. In this study, an efficient protocol was established for the high-frequency regeneration of V. rotundifolia via two pathways, namely axillary shoot formation from normal and fasciated stems. We also investigated the effects of different types and concentrations of plant growth regulators (PGRs) on the induction of fasciated stems. Anatomical analysis and ploidy analysis by flow cytometry were also performed.

Materials and methods

Plant material

Cuttings of *V. rotundifolia* mother plants obtained from Wenchang County, Hainan province, China, were transplanted in South China Botanical Garden, Guangzhou, China, on July 28, 2016. One month later, semi-lignified softwood stems with shoots were selected as explants. Explants were surface sterilized with 0.1% (w/v) $HgCl_2$ for 8 min followed by repeated washes with sterilized distilled water, and 0.5 to 1.0 cm long stem segments with a single node each were excised aseptically and cultured on Murashige and Skoog (1962; MS) medium without any PGRs to develop new nodal shoots. Newly formed nodal shoots that developed after 30 days were used to excise nodal stem segments with two axillary shoots, which were used as explants for the next step of our protocol.

Media and culture conditions

The basal nutrient media used in all shoot induction experiments consisted of MS salts and vitamins (Murashige, Skoog 1962), except for the root induction media, which consisted of half-strength MS (½ macro-elements). All media contained 3.0% sucrose and were solidified with 0.7% agar (Sigma-Aldrich, St. Louis, MI, US). Media was dispensed into jars (10 cm in height, 6 cm in diameter) after pH was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min. Among the PGRs tested, thidiazuron (TDZ), zeatin [ZEA, or *trans*-6-(4-hydroxy-3-methylbut-2-enylamino purine)] and indole-3-acetic acid (IAA) were filter sterilized through a 0.22 µm filter (Millipore Co., Bedford, MA, USA) and added to the media after it had cooled to 40 – 50 °C. However, BA, 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), 6-furfurylaminopurine (KIN) and α-naphthaleneacetic acid (NAA) were added directly to media prior to autoclaving at 121 °C for 15 min. All culture jars were placed in a culture room at 25 ± 2 °C under a 12-h photoperiod with a photosynthetic photon flux density of 50 $\mu mol^{-2}\ s^{-1}$ supplied by cool white fluorescent lamps (2 × 40 W tubes, Philips).

Induction of axillary shoots from normal stems

Nodal segments with two axillary buds were inoculated on MS media supplemented with 2.5 to 12.5 µM of IAA, NAA, KIN, ZEA, and BA. MS medium without any PGRs served as the control (Table 1). Axillary shoot response, number of shoots per explant and mean shoot height were investigated after culture for 30 days.

Induction of fasciated stems and adventitious shoots

In order to study the effects of different concentrations of PGRs (2,4-D, TDZ, KIN, ZEA, BA) and their combinations on the induction of fasciated stems and axillary shoots from their surface, nodal segments with two axillary shoots were inoculated on MS media supplemented with different concentrations of PGRs to induce fasciated stems (Table 2). The response of fasciated stems and the number of axillary shoots that formed per fasciated stem were determined after culture for 30 days.

Fasciated stem recovery and axillary shoot elongation and proliferation

Clusters of axillary shoots derived from fasciated stems were cut into 10 axillary shoots per cluster, which had equal height and were inoculated on MS media supplemented with 2.5 to 12.5 µM KIN, NAA, or BA alone, or BA in combination with 0.5 µM NAA. PGR-free MS medium was used as the control (Table 3). Shoots that elongated more than 0.5 cm were regarded as elongated shoots. The response of elongated shoots and the mean height of axillary shoots were determined after culture for 30 days.

Anatomical analysis

Wild-type shoots, axillary shoots from normal stems, fasciated stems and axillary shoots from fasciated stems were harvested and sectioned transversely by freehand sections. The sections were stained with toluidine blue O solution (TBO, Sigma-Aldrich). Cross sections were examined with a light biological microscope (BDS200; OPTEC, Chongqing, China). The size of pith cells was measured by the in-built grid.

Flow cytometry

The wild-type shoots, adventitious shoots from normal stems, fasciated stems and axillary shoots from fasciated stems were used as test materials, and the wild-type shoots of *Lycopersicon esculentum* (Ayele et al. 1996) were used as an external standard. About 100 mg from each sample was chopped with a new sharp razor blade in a culture Petri dish with 2 mL HEPES dissociation buffer containing 10 mM $MgSO_4$, 50 mM KCl, 5 mM HEPES, 6.5 mM dithiothreitol, 0.25% (v/v) Triton X-100 (pH 8.0), to break cell walls and cell membranes and free the nuclear suspension. After filtration through a 33 µm nylon mesh, samples

Table 1. Effects of different PGRs on induction of adventitious shoots and their proliferation from normal stems of *Vitex rotundifolia* after 30 days of culture. Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$). Adventitious shoot response = (number of explants from adventitious shoots after inoculation / number of explants) ×100%

PGRs (μM)	Adventitious shoot response (%)	Number of shoots per explant	Mean shoot length (cm)
Control	0 f	1.3 ± 0.8 f	3.2 ± 0.2 d
IAA 0.5	0 f	3.3 ± 0.5 ef	3.4 ± 0.1 cd
IAA 12.5	0 f	5.3 ± 0.5 ef	3.8 ± 0.2 bcd
NAA 0.5	0 f	4.2 ± 0.7 ef	3.4 ± 0.1 cd
NAA 12.5	0 f	3.5 ± 0.5 ef	3.5 ± 0.1 cd
KIN 0.5	6.89 ± 1.74 f	5.1 ± 0.4 ef	3.8 ± 0.2 bcd
KIN 12.5	21.1 ± 2.9 e	5.1 ± 0.4 ef	4.9 ± 0.1 a
ZEA 0.5	5.6 ± 2.9 f	6.2 ± 0.3 de	3.6 ± 0.3 cd
ZEA 12.5	20.0 ± 1.9 e	10.0 ± 0.8 cd	5.2 ± 0.2 a
BA 0.5	56.7 ± 1.9 c	14.9 ± 0.0 b	3.4 ± 0.2 cd
BA 2.5	75.6 ± 2.9 a	21.4 ± 3.7 a	3.8 ± 0.2 bc
BA 5.0	77.8 ± 2.9 a	21.9 ± 1.1 a	4.2 ± 0.2 b
BA 12.5	81.1 ± 2.9 a	21.6 ± 0.6 a	3.7 ± 0.2 bcd
TDZ 0.5	67.8 ± 2.9 b	16.4 ± 0.8 b	2.3 ± 0.1 e
TDZ 2.5	33.3 ± 1.9 d	14.0 ± 2.9 b	1.5 ± 0.1 f
TDZ 5.0	22.2 ± 2.9 e	14.0 ± 1.8 bc	1.2 ± 0.1 f
TDZ 12.5	18.9 ± 2.9 e	14.5 ± 2.0 b	1.1 ± 0.1 f

were treated with 10 μl (1523 μM) of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), which served as a DNA fluorochrome. After incubation of the mixture on ice for 15 min in the dark, the fluorescence of the nuclear suspension was measured using a Partec CyFlow Space flow cytometer (PARTEC GmbH, Münster, Germany). The flow rate varied between 20 and 50 events per second and the fluorescence of 5000 to 10000 nuclei was measured per sample. Histograms were evaluated using FloMax software (version 2.81, Quantum Analysis GmbH, Münster, Germany). The coefficient of variation (CV) and maximum peak height were recorded. Analyses were performed twice for each sample.

Table 2. Effects of different PGRs and their combination on induction of fasciated shoots of *Vitex rotundifolia*. Values represent means ± SD. Different letters within a column indicate significant differences according to the Duncan's multiple range test ($P < 0.05$). Fasciated stem response = (number of fasciated stems / number of explants) ×100%.

PGRs (μM)	Fasciated stem response (%)	Number of adventitious shoots per fasciated stem
Control	0.0 f	0.0 h
2,4-D 5.0	0.0 f	0.0 h
TDZ 5.0	0.0 f	0.0 h
KIN 2.5	0.0 f	0.0 h
KIN 5.0	7.8 ± 1.1 e	7.3 ± 0.7 g
KIN 12.5	21.1 ± 2.9 cd	28.9 ± 1.7 e
KIN 25.0	23.3 ± 1.9 cd	31.4 ± 1.5 e
ZEA 2.5	0.0 f	0.0 h
ZEA 5.0	8.9 ± 1.1 e	17.0 ± 0.8 f
ZEA 12.5	20.0 ± 1.9 d	28.6 ± 0.7 e
ZEA 25.0	22.2 ± 2.9 cd	37.8 ± 0.8 d
BA 2.5	26.7 ± 1.9 c	31.2 ± 0.7 e
BA 5.0	46.7 ± 1.9 b	68.1 ± 3.4 b
BA 12.5	60.0 ± 1.9 a	78.0 ± 2.2 a
BA 25.0	57.8 ± 4.8 a	74.9 ± 4.71 a
BA 5.0 + NAA 0.5	43.3 ± 1.9 b	60.7 ± 3.9 c
BA 5.0 + NAA 0.5 + TDZ 0.5	56.56 ± 1.1 a	73.6 ± 2.7 ab

Table 3. Effects of plant growth regulators (PGRs) on elongation of axillary shoots from fasciated stems of *Vitex rotundifolia*. Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$)

PGRs (μM)	Shoot elongation (% response)	Mean shoot length (cm)
Control	11.7 ± 1.0 g	0.3 ± 0.0 e
KIN 2.5	17.2 ± 1.1 def	0.4 ± 0.0 de
KIN 7.5	16.7 ± 0 efg	0.4 ± 0.1 de
KIN 12.5	16.1 ± 1.1 fg	0.4 ± 0.0 de
BA 2.5	15.0 ± 1.9 fg	0.4 ± 0.0 de
BA 7.5	16.1 ± 1.5 fg	0.3 ± 0.1 e
BA 12.5	15.6 ± 0.6 fg	0.4 ± 0 de
NAA 2.5	33.9 ± 2.0 b	1.6 ± 0.1 b
NAA 7.5	58.3 ± 1.9 a	3.0 ± 0.1 a
NAA 12.5	58.9 ± 2.0 a	3.0 ± 0.2 a
BA 2.5 + NAA 2.5	25.6 ± 2.8 c	0.7 ± 0.1 c
BA 7.5 + NAA 2.5	22.2 ± 2.4 cd	0.6 ± 0.1 cd
BA 12.5 + NAA 2.5	21.7 ± 1.0 cde	0.6 ± 0.0 cd

Root formation

When elongating axillary shoots reached 3 to 4 cm in height with four leaves, they were excised from axillary shoot clusters and transferred to half-strength MS media supplemented with 2.5 to 15.0 μM NAA or 2.5 to 25 μM IBA alone, or a combination of 5.0 to 10.0 μM IBA and 5.0 to 15.0 μM NAA, for root formation. After culture for a total of 30 days on these media, the rooting response, number of roots per shoot and mean root length were determined (Table 4).

Acclimatization

DNase In order to acclimatize the resulting plantlets in a greenhouse, jars were gradually opened over 3 to 7 days. 100 plantlets with 4 to 6 leaves were selected respectively from different rooting media containing IBA or NAA, alone or in combination. Agar was gently washed off roots with tap water. Plantlets derived from three rooting media were pooled and transplanted to square plastic bags (10 × 10 × 12 cm) with a substrate of yellow soil and peat (3:1, v/v). After 30 days, survival percentage was calculated and surviving plants were transferred to a field in May 2017.

Statistical analyses

All experiments were repeated three times within a two-week interval. Each experiment contained six explants per jar and five jars per treatment. The data were reported as mean ± SD (standard deviation). Percentage values

Table 4. Effects of plant growth regulators (PGRs) in rooting medium (half-strength MS) on the rooting of *Vitex rotundifolia* after 30 days of culture. Values represent means ± SD. Different letters within a column indicate significant differences according to Duncan's multiple range test ($P < 0.05$). Rooting response (%) = (number of plantlets with roots / number of explants) ×100%

PGRs (μM)	Rooting response (%)	Number of roots per shoot	Mean root length (cm)
Control	0.0 f	0.0 g	0.0 d
NAA 2.5	0.0 f	0.0 g	0.0 d
NAA 5.0	8.3 ± 4.2 e	48.7 ± 3.2 e	1.76 ± 0.9 c
NAA 10.0	3.8 ± 2.1 ef	63.7 ± 2.3 d	2.0 ± 1.0 bc
NAA 15.0	4.8 ± 0.6 ef	67.7 ± 1.5 cd	1.9 ± 0.3 bc
IBA 2.5	16.3 ± 2.4 d	17.2 ± 2.1 f	5.4 ± 0.2 a
IBA 5.0	40.1 ± 2.6 c	17.4 ± 1.1 f	5.7 ± 0.8 a
IBA 10.0	63.4 ± 1.0 b	24.0 ± 0.7 f	5.0 ± 0.2 a
IBA 15.0	65.8 ± 1.2 ab	22.1 ± 1.4 f	5.3 ± 0.1a
IBA 25.0	58.1 ± 2.3 ab	20.6 ± 1.7 f	5.3 ± 0.6 a
IBA 5.0 + NAA 5.0	45.2 ± 1.9 c	71.8 ± 3.3 cd	3.4 ± 0.4 b
IBA 5.0 + NAA 10.0	44.6 ± 1.8 c	82.3 ± 1.7 ab	3.0 ± 0.2 bc
IBA 5.0 + NAA 15.0	46.4 ± 2.7 c	82.8 ± 4.4 ab	3.4 ± 0.1 b
IBA 15.0 + NAA 5.0	66.6 ± 3.2 ab	75.3 ± 8.8 bc	3.4 ± 0.3 b
IBA 15.0 + NAA 10.0	67.4 ± 1.7 ab	86.4 ± 3.8 a	2.9 ± 0.5 bc
IBA 15.0 + NAA 15.0	70.5 ± 3.2 a	86.3 ± 3.5 a	3.1 ± 0.1 bc

were arcsine transformed prior to analysis. Means were statistically analyzed by one-way analysis of variance (ANOVA) and treatment means were considered to be significantly different from controls after analysis with Duncan's multiple rage test at $P < 0.05$ using SPSS v. 19.0 (IBM, New York, NY, USA).

Results

Effect of PGRs on induction of axillary shoots from normal nodal segments

Significant differences were observed in axillary shoot formation among the media supplemented with different PGRs or PGR combinations (Table 1). Nodal explants failed to form axillary shoots on PGR-free medium or on media supplemented with auxins (IAA or NAA), forming only individual shoots. In contrast, axillary shoot clusters formed from nodal segments that had been cultured on media supplemented with cytokinins (KIN, ZEA, BA and TDZ) after culture for 30 days (Fig. 1A). Each cytokinin displayed a different efficacy of axillary shoot formation from nodal explants. Compared to other cytokinins, BA was the most effective PGR for axillary shoot induction and proliferation. Maximum axillary shoot response (81.1%) and highest mean number of axillary shoots per explant (21.9) were observed on medium supplemented with 5.0 to 12.5 μM BA. KIN or ZEA showed an almost similar morphogenetic response and both induced fewer axillary shoots, but they induced longer shoots, and longest shoots reached 5.2 cm in medium containing 12.5 μM ZEA. Compact callus as well as axillary shoots were induced after nodal explants were cultured on medium supplemented with 0.5 to 12.5 μM TDZ (Fig. 1B).

Effects of PGRs on induction of fasciated stems and subsequent axillary shoots

Some fasciated stems (5 to 20 mm wide) (Fig. 1C – E) developed on media supplemented with higher concentrations of select cytokinins. A low concentration (0.5 μM) of KIN or ZEA could not induce any fasciated stems, or only a few at higher concentrations (≥ 5.0 μM; Table 2). The maximum fasciated stem response and number of axillary shoots per fasciated stem were observed on media supplemented with 12.5 to 25.0 μM BA. Higher concentrations (≥ 25.0 μM) of BA had a negative effect on axillary shoot growth and shoots were stunted with slightly compact leaves. The combination of BA and NAA resulted in significant differences in the fasciated stem response compared with BA alone, but adding 0.5 μM TDZ increased the fasciated stem response and the number of axillary shoots per fasciated stem, which was higher than stems cultured on medium supplemented with BA and NAA. The optimized PGR to induce fasciated stems and axillary shoots was 12.5 μM BA or a combination of 0.5 μM BA, 0.5 μM NAA and 0.5 μM TDZ (Table 2).

Effect of PGRs on recovery of fasciated stems and adventitious shoot elongation

Fasciated stem recovery and axillary shoot elongation from these stems was favorable when NAA was used compared with KIN, BA or no PGRs (Table 3). As the concentration of NAA increased, shoot elongation and mean shoot length of axillary shoots also increased. The maximum frequency (58.9%) of shoot elongation and mean shoot length (3.0 cm) of axillary shoots were observed on MS medium containing 7.5 to 12.5 μM NAA. Not only the frequency of shoot elongation, but also the mean shoot height, when cultured on medium containing BA and NAA, were higher than in the treatment of only BA. On medium supplemented with BA or a higher concentration of KIN, some axillary shoots developed secondary fasciated stems.

Anatomical analysis

Fasciated shoots were characterized by lateral growth and the formation of fasciated stems. Cortical layers were radially additionally elongated in wild-type shoots (Fig. 2A black arrows), but not in other types of shoots. The cortical layers of fasciated shoots were plicated and their cell volume of pith was considerably larger than other types of shoots. The diameter of the stems of fasciated shoots ranged from 5 to 25 mm, which was larger than normal shoots (2 to 3 mm), such as wild-type shoots, axillary shoots from normal stems and axillary shoots from fasciated stems. The development of vascular tissue was observed in all shoots, as observed by cross-sections (Fig. 2). However, pith cells were larger and developed laterally by 0.05 to 0.08 mm in fasciated stems (Fig. 2C), while almost orbicular pith cells formed in normal stems, including wild-type shoots, axillary shoots from normal stems and axillary shoots from fasciated stems (Fig. 2A, 2B, 2D). No significant differences in anatomical features were observed among axillary shoots from fasciated stems and those derived from normal stems. Other cells showed no obvious changes.

Flow cytometry

Ploidy analysis by flow cytometry showed that all samples had distinct peaks with coefficients of variation ranging from 4.16 to 10.06 (Fig. 3) while the external standard, *L. esculentum*, had a typical double peak (Fig. 3A). The relative fluorescence intensity of each of the four types of shoot nuclei was 57.77, 56.92, 57.92, and 61.23, respectively (Fig. 3B – E), showing uniform fluorescence. This indicates that the ploidy level (or chromosome number) did not change, or differ much, among the four shoot types.

Root formation

Single shoots transferred to rooting medium supplemented with a low concentration (2.5 μM) of NAA or free of PGRs did not show any rooting response after culture for 30 days (Table 4). Half-strength MS medium supplemented with IBA or high concentrations of NAA (≥ 5.0 μM), or

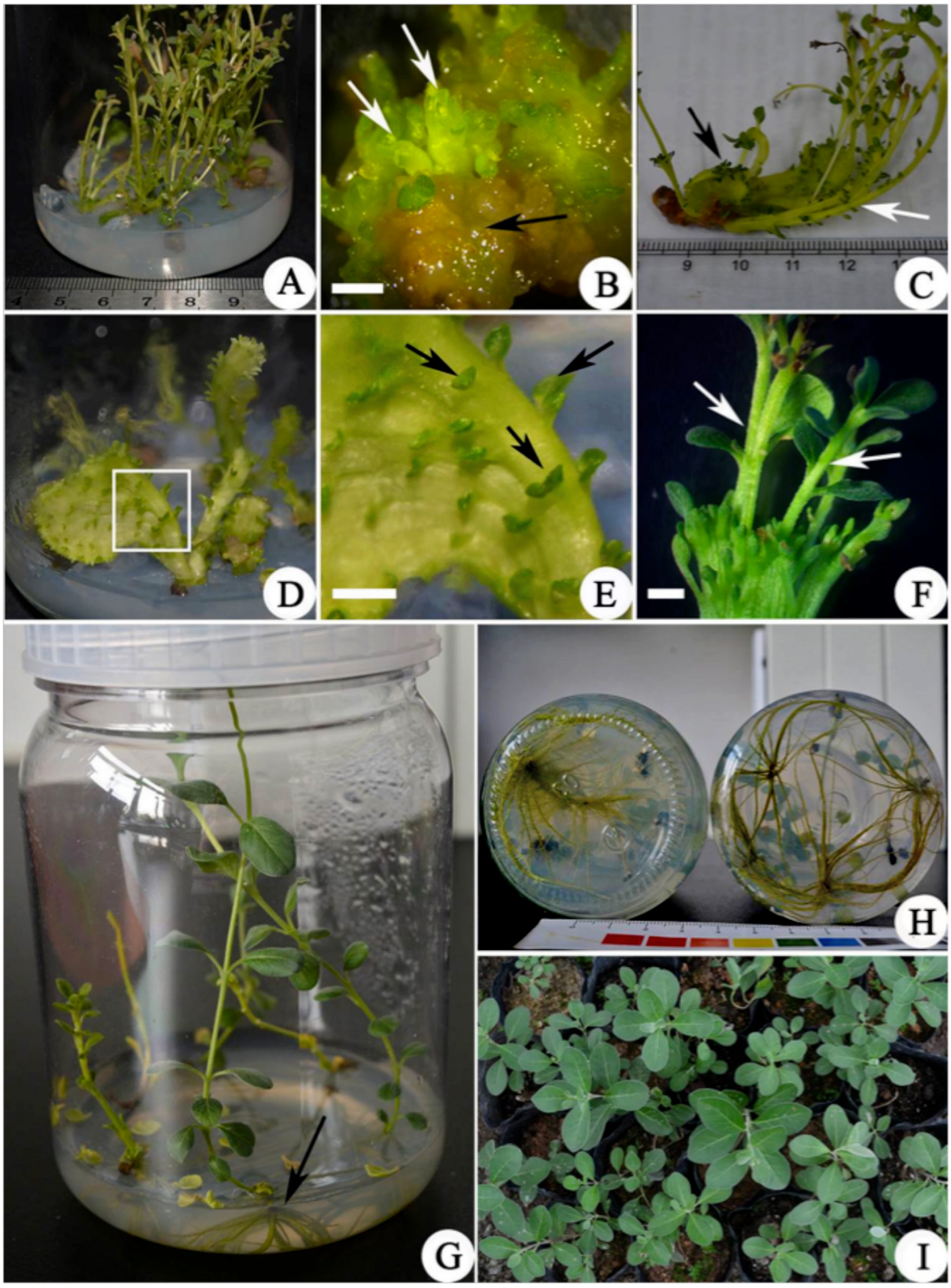

Fig. 1. Induction of fasciated stems and plant regeneration of *Vitex rotundifolia*. Axillary shoot development from normal stems on MS medium containing 0.5 μM BA after culture for 30 days (A). Adventitious shoots (white arrows) and compact callus (black arrows), induced on the base of stems, was observed on MS medium containing 2.5 μM TDZ after culture for 30 days (B). Adventitious shoot formation from fasciated stems (black arrows) and normal stems (white arrows) on MS medium containing 2.5 μM BA after culture for 30 days (C). (D) Fasciated stems developed from nodal explants on MS medium containing a high concentration (25.0 μM) of BA, and many adventitious shoots formed on the surface after culture for 30 days (D). Enlargement of a part of Fig. D (white frame) (E). Fasciated stem recovery and adventitious shoot elongation (white arrows) on medium containing 5.0 μM NAA after culture for 30 days (F). Root formation (black arrows) on half-strength MS medium supplemented with 15.0 μM IBA after culture for 30 days (G). Root formation with a poor rooting response and development of a lateral root system (left) on half-strength MS media containing 15.0 μM NAA, and root formation with a high rooting response and poor lateral roots (right) on half-strength MS media containing 15.0 μM IBA after culture for 30 days (H). Acclimatized plantlets in square plastic bags (10 × 10 × 12 cm) with a mixture of yellow soil and peat (3:1, v/v) for 30 days after transplanting from *in vitro* conditions (I). Bars (B, E, F) = 0.5 cm.

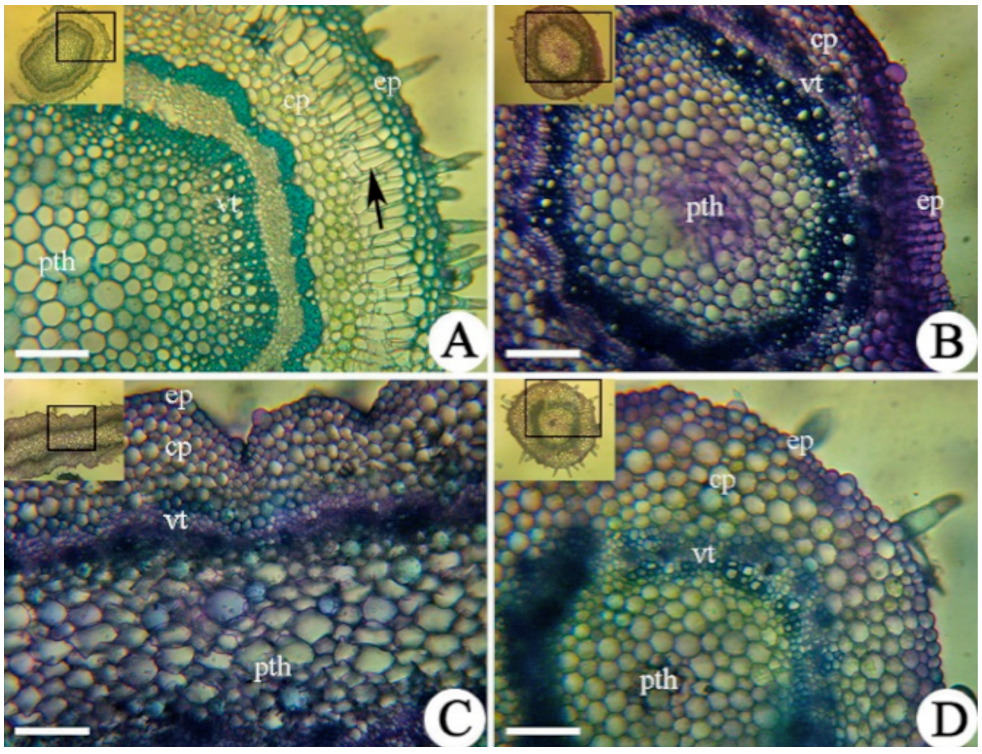

Fig. 2. Anatomical analysis of transverse stem section by inverted microscopy of different types of *in vitro* shoots of *Vitex rotundifolia*. A, wild-type shoots; B, shoot from normal stems; C, fasciated stem; D, shoot from fasciated stems; ep, epidermis; cp, cambial phloem; vt. vasular tissues; pth, pith. Bars = 0.1 mm.

their combination, stimulated root formation within 10 to 15 days. IBA was more effective than NAA in inducing rooting and in elongating roots. In contrast, NAA had a stronger effect on the number of roots per shoot, forming an extensive lateral root system. The combination of IBA and NAA formed more roots, and more lateral roots, than when each auxin was used singly. The maximum frequency (70.5%) of root formation, highest number of roots per shoot (86.3), and longest roots (3.1 cm) was achieved on half-strength MS medium supplemented with 15.0 µM IBA and 15.0 µM NAA.

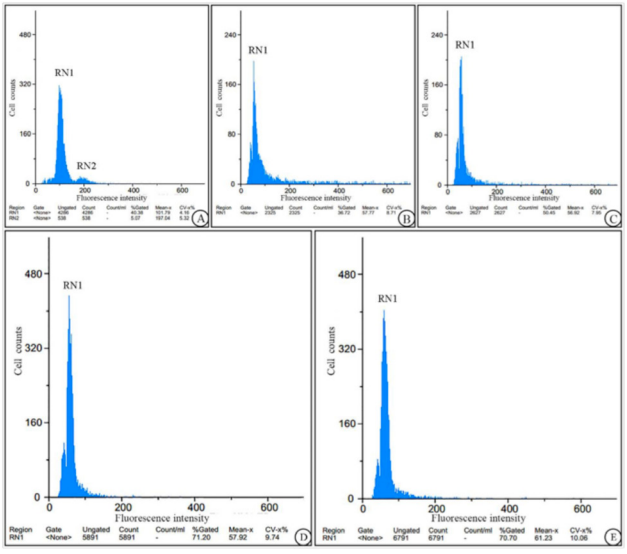

Fig. 3. Histograms of flow cytometry showing the relative fluorescence intensity of DAPI-stained nuclei in *Vitex rotundifolia*. A, external standard, *Lycopersicon esculentum*. B, wild-type shoots. C, adventitious shoots from normal stems. D, fasciated stems. E, adventitious shoots from fasciated stems.

Acclimatization

When agar was gently washed off rooted *in vitro* plantlets in tap water, roots were easily removed, accounting for the low number of roots per plantlets. The percentage of surviving transplanted plantlets cultured on half-strength MS medium containing IBA alone was lower than plantlets derived from half-strength MS medium containing NAA alone or a combination of IBA and NAA. All plantlets cultured on rooting medium were transferred to a yellow soil and peat (3/1, v/v) substrate. Plantlets from the NAA-only, IBA-only and NAA + IBA combination showed 82.0, 66.7 and 86.0% survival, respectively after 30 days (Fig. 1I).

Discussion

Shoot propagation and regeneration has been reported in several *Vitex* species (Hiregoudar et al. 2006; Balaraju et al. 2008; Teixeira da Silva et al. 2016). For example, the use of BA alone, or in combination with NAA, is a popular means to propagate *Vitex negundo* from nodal segments (Sharma et al. 2006; Usha et al. 2007; Ahmad et al. 2008). Using *in*

vitro leaf segments of *V. negundo* as explants, Jawahar et al. (2008) noted that many adventitious shoots formed from the surface of leaves on MS medium supplemented with 1.71 μM IAA and 1.33 μM BA. In *Vitex trifolia*, axillary shoots formed from *in vitro* nodes on MS medium after exposure to 5.0 μM TDZ, or a combination of 5.0 μM BA and 0.5 μM NAA (Ahmed and Anis 2012; Ahmad et al. 2013a). In our experiments, BA, KIN, ZEA and TDZ could induce adventitious shoots from normal nodal explants. BA, when applied at the same concentration, was better than KIN or ZEA for shoot proliferation, whereas KIN or ZEA elongated shoots more than BA. A similar phenomenon was reported in *V. negundo* (Ahmad, Anis 2011; Ahmad et al. 2013b) and *V. trifolia* (Ahmad et al. 2013c).

TDZ, a substituted phenylurea compound that exhibits both auxin and cytokinin-like functions (Guo et al. 2011), improved shoot proliferation to about 25 shoots per node in *V. negundo* (Ahmad and Anis 2007). However, prolonged exposure to TDZ for more than four weeks led to morphologically distorted, hyperhydric and fasciated shoots that negatively impacted the growth and multiplication of induced shoots of *V. trifolia* (Ahmed, Anis 2012). There was a poor axillary shoot induction response of *V. negundo* on MS medium with 2.27 μM TDZ (Sahoo, Chand 1998). In our experiment, TDZ induced the formation of both adventitious shoots and yellowish-brown compact callus (Fig. 1C) from normal nodes. Therefore, the use of TDZ is not a perfected choice for species in which other PGRs can effectively induce regeneration and should be avoided in *Vitex*, except for woody species (Ahmad et al. 2015).

Fasciation in Orbea gigantean was correlated with the accumulation of KIN and IAA, but there was no consistent phytohormone pattern in *Euphorbia coerulescens, Orbea cylindrica* and *Senecio stapeliiformis* (Omar et al. 2014), indicating that different plant species have different mechanisms to develop fasciation. Normal and fasciated shoots in *Prunus avium* were generated on MS media supplemented with 0.5 to 1.25 mg L^{-1} BA and the cross-sectional stem area of fasciated shoots was greater than that of normal shoots, while cytokinesis, morphogenetic activity, and the formation of axillary shoots increased in the cortex and pith during fasciated stem development (Kitin et al. 2005).

In the genus *Vitex*, fasciation has only been reported in *V. trifolia*, and shoots were distorted, hyperhydric and fasciated, and buds did not elongate after exposure to media with with TDZ for more than seven days (Ahmed, Anis 2012). TDZ has a number of negative effects on *in vitro* culture (Dewir et al. 2018). Our experiments are the first report on the development of fasciated stems from *V. rotundifolia* nodes in response to a high concentration of cytokinins. Anatomical analysis shown that pith cells developed laterally in fasciated stems and were larger than other types of shoots. Fasciated stems could be reverted to normal stems by elongating axillary shoots after reducing the concentration of cytokinins or by supplementing NAA. Flow cytometry showed no ploidy or chromosomal variation among the four types of shoots. Fasciated shoots had a large number of buds and initiated new shoots at their apices while normal shoots had a single dominant terminal bud. Therefore, plant regeneration via fasciated stems has an advantage of high proliferation ability, genetic uniformity and short proliferative cycle and could be used to mass produce *V. rotundifolia*.

Auxins such as IBA are widely used to induce root formation *in vitro* in *Vitex* species (Teixeira da Silva et al. 2016). In our study, IBA and NAA applied singly or in combination enhanced root formation, the maximum number (67.7) of roots with developed lateral roots forming on half-strength MS medium supplemented with a high concentration of NAA (≥ 7.5 μM). IBA was optimal for root formation (65.75%) but lateral root development was poor (Table 4). A similar result was observed for *V. negundo* (Narayanasamy 2000), but not for *V. trifolia* (Ahmed, Anis 2014). IBA combined with NAA had a high rooting response (70.5%) and formed a large number (86.4) of roots per shoot (Table 4). IBA and NAA functioned synergistically in root formation.

Conclusions

This study is the first protocol for *in vitro* axillary shoot initiation, proliferation and plant regeneration for *V. rotundifolia* from node explants via two distinct pathways, namely from normal stems and from fasciated stems. Given the ability of this protocol to produce a large number of genetically homogeneous shoots, it can be used for future biotechnological applications and serve as a practical and useful technique to efficiently propagate this medicinal plant.

Acknowledgements

HL conducted the majority of the experiments. YX, BG, HY, SJ, HR, XZ, LY, SZ, KW and FZ offered advice and assisted with several aspects of the experiments. JATdS offered critical assessment of the experiment, scientific advice, including with data analyses, and assisted in writing and revising the manuscript. GM and YX devised the experimental design and supervised the team. All authors saw and approved the final version for submission. The authors declare no conflicts of interest. This work was financially supported by the National Key Research and Development Program of China (2016YFC1403000/2016YFC1403002), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13020500) and the National Science and Technology Support Program (2015BAL04B04).

References

Abubakar S., Saba J.J., Umar I.D. 2015. Germination trial, proximate and elemental analysis of *Vitex doniana* (Linn)

fruits. *Int. J. Biol. Sci.* 2: 27–38.

Ahmad N., Anis M. 2011. An efficient *in vitro* process for recurrent production of cloned plants of *Vitex negundo* L. *Eur. J. For. Res.* 130: 135–144.

Ahmad N., Anis M. 2007. Rapid clonal multiplication of a woody tree, *Vitex negundo* L. through axillary shoots proliferation. *Agrofor. Syst.* 71: 195–200.

Ahmad N., Javed S.B., Khan M.I., Anis M. 2013. Rapid plant regeneration and analysis of genetic fidelity in micropropagated plants of *Vitex trifolia*: an important medicinal plant. *Acta Physiol. Plant.* 35: 2493–2500.

Ahmad N., Khan M.I., Ahmed S., Javed S.B., Faisal M., Anis M. 2013b. Change in total phenolic content and antibacterial activity in regenerants of *Vitex negundo* L. *Acta Physiol. Plant.* 35: 791–800.

Ahmad N., Wali S.A., Anis M. 2008. *In vitro* production of true-to-type plants of *Vitex negundo* L. from nodal explants. *J. Hortic. Sci. Biotechnol.* 83: 313–317.

Ahmad N., Shahid A., Javed S.B., Khan M.I., Anis M. 2015. Micropropagation of *Vitex* spp. through *in vitro* manipulation: current status and future prospectives. *J. Appl. Res. Med. Arom. Plants* 2: 114–123.

Ahmed M.R., Anis M. 2014. *In vitro* regeneration and the antioxidant enzymatic system on acclimatization of micropropagated *Vitex trifolia* L. *Agrofor. Syst.* 88: 437–447.

Ahmed M.R., Anis M. 2012. Role of TDZ in the quick regeneration of multiple shoots from nodal explant of *Vitex trifolia* L. - an important medicinal plant. *Appl. Biochem. Biotechnol.* 168: 957–966.

Ayele M., Doleže J., Duren M.V., Brunner H., Zapata-Arias F.J. 1996. Flow cytometric analysis of nuclear genome of the Ethiopian cereal tef [*Eragrostis tef* (Zucc.) Trotter]. *Genetica* 98: 211–215.

Balaraju K., Agastian P., Preetamraj J.P., Arokiyaraj S., Ignaciamuthu S. 2008. Micropropagation of *Vitex agnuscastus* (Verbenaceae) - a valuable medicinal plant. *In Vitro Cell. Dev. Biol. Plant* 44: 436–441.

Dewir Y.H., Nurman S., Naidoo Y., Teixeira da Silva J.A. 2018. Thidiazuron-induced abnormalities in plant tissue cultures. *Plant Cell Rep.* 37: 1451–1470.

Guo B., Abbasi B.H., Zeb A., Xu L.L., Wei Y.H. 2011. Thidiazuron: A multi-dimensional plant growth regulator. *Afr. J. Biotechnol.* 10: 8984–9000.

Hiregoudar L.V., Bhat H.N.M.G., Nayeem A., Hema B.P., Hahn R.J. 2006. Rapid clonal propagation of *Vitex trifolia*. *Biol. Plant.* 50: 291–294.

Iliev I. 1996. *In vitro* propagation of *Betula pendula* Roth. 'joungii'. In: Iliev I, Zhelev P, Aleksandrov P (eds) *Propagation of Ornamental Plants.* Ministry of the Education, Science and Technology Publishing House, Sofia, pp. 44–54.

Iliev I., Kitin P. 2011. Origin, morphology, and anatomy of fasciation in plants cultured *in vivo* and *in vitro*. *Plant Growth Regul.* 63: 115–129.

Jawahar M., Ravipaul S., Jeyaseelan M. 2008. *In vitro* regeneration of *Vitex negundo* L. a multipurpose woody aromatic medicinal shrub. *Plant Tiss. Cult. Biotech.* 18: 37–42.

Kawazoe K., Yutani A., Tamemoto K., Yuasa S., Shibata H., Higuti T., Takaishi Y. 2001. Phenylnaphthalene compounds from the subterranean part of *Vitex rotundifolia* and their antibacterial activity against methicillin-resistant *Staphylococcus aureus*. *J. Nat. Prod.* 64: 588.

Kitin P., Iliev I., Scaltsoyiannes A., Nellas H., Rubos A., Funadas R. 2005. A comparative histological study between normal and fasciated shoots of *Prunus avium*, generated *in vitro*. *Plant Cell Tiss. Org. Cult.* 82: 141–150.

Lee C., Lee J.W., Jin Q., Lee H.J., Lee S.J. 2013. Anti-inflammatory constituents from the fruits of *Vitex rotundifolia*. *Bioorg. Med. Chem. Lett.* 23: 6010–6014.

Liang F., Zhou X.Z., Cao L. 2012. Investigation report on *Vitex trifolia* L. var. *simplicifolia* Cham, medicinal plant resources in China. *Med. Plant* 3: 16–19.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497.

Narayanasamy J. 2000. Mass propagation of *Vitex negundo in vitro*. *Plant Biotechnol.* 2: 151–155.

Nitsch J.P., Nitsch C. 1969. Haploid plants from pollen grains. *Science* 163: 85–87.

Omar A.F., Dewir Y.H., Elmahrouk M.E. 2014. Molecular identification of phytoplasmas in fasciated cacti and succulent species and associated hormonal perturbation. *J. Plant. Interact.* 9: 632–639.

Ono M., Yanaka T., Yamamoto M., Ito Y., Nohara T. 2002. New diterpenes and norditerpenes from the fruits of *Vitex rotundifolia*. *J. Nat. Prod.* 65: 537–541.

Park H.J., Min B.M., Cha H.C. 2004. Mass production of sand dune plant, *Vitex rotunditolia* via micropropagation. *J. Plant. Biotechnol.* 6: 165–169.

Papafotiou M., Balotis G., Louka P., Chronopoulos J. 2001. *In vitro* plant regeneration of *Mammillaria elongata* normal and cristate forms. *Plant Cell Tiss. Org. Cult.* 65: 163–167.

Sahoo Y., Chand P.K. 1998. Micropropagation of *Vitex negundo* L. a woody aromatic medicinal shrub, through high-frequency axillary shoot proliferation. *Plant Cell Rep.* 18: 301–307.

Sharma M.M., Jalootharia D.J., Khanna P., Batra A. 2006. An efficient *in vitro* mass propagation of a medicinally potent plant species *Vitex negundo* L. via nodal segments. *Phytomorphology* 56: 35–39.

Sohn S.H., Ko E.J., Oh B.G., Kim S.H., Kim Y.S., Shin M.K., Hong M.C., Bae H.S. 2009. Inhibition effects of *Vitex rotundifolia* on inflammatory gene expression in A549 human epithelial cells. *Ann. Allergy Asthma Immunol.* 103: 152–159.

Song H.M., Park G.H., Jin S.K., Jeong H.J. 2017. *Vitex rotundifolia* fruit extract induces apoptosis through the downregulation of ATF3-mediated bcl-2 expression in human colorectal cancer cells. *Am. J. Chin. Med.* 45: 901–915.

Teixeira da Silva J.A., Kher M.M., Nataraj M. 2016. Biotechnological advances in *Vitex* species, and future perspectives. *J. Genetic Eng. Biotechnol.* 14: 335–348.

Usha P.K., Benjamin S., Mohanan K.V., Raghu A.V. 2007. An efficient micropropagation system for *Vitex negundo* L. an important woody aromatic medicinal plant, through shoot tip culture. *Res. J. Bot.* 2: 102–107.

Varga A., Thoma H., Bruinsma J. 1988. Effects of auxins and cytokinins on epigenetic instability of callus-propagated *Kalanchoe blossfeldiana* Poelln. *Plant Cell Tiss. Org. Cult.* 15: 223–231.

Yeeh Y., Kang S.S., Chung H.G., Chung M.S., Chung M.G. 1996. Genetic and clonal diversity in Korean populations of *Vitex rotundifolia* (Verbenaceae). *J. Plant Res.* 109: 161–168.

Received 14 August 2019; received in revised form 9 October 2019; accepted 10 November 2019