# Micropropagation of *Stewartia pseudocamellia*[1]

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## Abstract

Single-node explants were excised from shoots of actively growing, 2-year-old seedlings of *Stewartia pseudocamellia* Maxim. (Japanese stewartia) on three dates associated with specific stock plant growth stages. Following surface sterilization, explants were placed on agar-solidified Woody Plant Medium (WPM) containing either no growth regulators or N-(3-methyl-2-butenyl)-1H-purin-6-amine (2iP) at 5.0 or 10.0 ppm (24.6 or 48.2 μM) or 0.025 or 0.05 ppm (0.11 or 0.23 μM) N-phenyl-N-1,2,3-thiadiazol-5-ylurea (TDZ). The most frequent budbreak was noted for explants placed on media containing 2iP at either concentration. Explants cultured at the softwood stage had less contamination and greater budbreak than explants taken from more mature stem tissue. In another study, the three distal, axillary nodes of each shoot were excised at 4-day intervals for 28 days beginning 52 days after stock plants were potted following cold storage at 7C (44F). Explants were surface sterilized and placed on WPM supplemented with 10 ppm (49.2 μM) 2iP either alone or in combination with 3 ppm (8.6 μM) gibberellic acid ($GA_3$). Neither $GA_3$ nor node position influenced budbreak frequency or shoot elongation. Days after potting (stock plant growth stage) influenced frequency of budbreak and shoot elongation with the optimal period for explant collection being 56 to 72 days after stock plants were potted. Elongated shoots (one microcutting per explant) were produced on both media. Microcuttings ≥10 mm (0.4 in) were rooted using ex vitro procedures and acclimatized to greenhouse conditions.

**Index words:** Japanese stewartia, Theaceae, tissue culture, in vitro propagation, landscape species.

## Significance to the Nursery Industry

*Stewartia pseudocamellia* Maxim. (Japanese stewartia) is a small tree which is highly prized as a landscape plant because of its showy flowers, exfoliating bark, and attractive fall color. Unfortunately, the species is not widely utilized, due in part to propagation difficulties.

Results of this study indicated that *S. pseudocamellia* can be propagated successfully by micropropagation. Single node shoot explants were surface sterilized and established on Woody Plant Medium (6). Axillary shoot elongation (one per explant) was influenced by the physiological condition (growth stage) of the stock plants. Seventy-nine percent rooting of microcuttings was achieved using *ex vitro* procedures and plantlets were acclimatized to greenhouse conditions.

## Introduction

*Stewartia pseudocamellia* (Theaceae), Japanese stewartia, is a small to medium sized tree (1). The species is native to Japan and Korea where it typically grows along streambanks (5). In cultivation, it reaches a height of 6–9 m (20–30 ft) and develops a pyramidal to oval growth habit (4). *Stewartia pseudocamellia* is valued as a landscape plant because of several desirable traits. Showy white flowers with white filaments and yellow to orange anthers occur in June and July, a time when flowering of many woody ornamentals has passed. Exfoliating bark is spectacular, exposing an underlying layer which is cinnamon in color. Older specimens develop smooth mottled bark with beautiful, irregular patches which are tan and reddish brown (5). Dark green, elliptic-obovate leaves are 5–9 cm (2–3.5 in) long and have an attractive fall color range of yellow to reddish purple. *Stewartia pseudocamellia* is relatively pest free and grows well in USDA Zones 5–8 (2).

Use of *S. pseudocamellia* as a landscape specimen has been limited because propagation of the species has been difficult (2). Sexual (seed) propagation is slow and unreliable. Seeds of *S. pseudocamellia* are reported to be doubly dormant and in nature require 2 years to germinate. Even when successful, germination is often sporadic. In addition, availability of viable seed is a problem because viability typically decreases following collection (4). Vegetative propagation of *S. pseudocamellia* by stem cuttings has been more successful with cuttings rooting in high percentages. However, rooted cuttings are difficult to overwinter and high mortality is often observed the spring following rooting (3).

Since propagation of *S. pseudocamellia* by conventional methods is relatively inefficient, we decided to investigate the feasibility of propagating *S. pseudocamellia* by micropropagation (tissue culture). By developing a successful protocol for micropropagation, *S. pseudocamellia* may become more readily available to the landscape industry. Such a protocol might also be applicable to other desirable species of *Stewartia* including two which are native to the southeast United States, *S. ovata* Cav. (mountain stewartia) and *S. malacodendron* L. (silky camellia). Previous reports indicated that *Camellia japonica* L. (Japanese camellia), also in the Theaceae, has been propagated successfully via micropropagation using nodal segments as explants (8). Thus, protocols from these investigations were used as guidelines during the initial stages of this research.

## Materials and Methods

*Stock plant growth stage and growth regulators (experiment 1).* Bare root, 2-year old seedlings of *S. pseudocamellia* were removed from a coldroom on February 28, 1993, following storage at 7C (44F) for 90 days. They were then potted in 11.4 liter (3 gal) plastic containers using a pine bark medium supplemented with 1.9 and 0.9 kg/m$^3$ (2.0 and 1.5 lb/yd$^3$) dolomitic lime and Micromax (Grace/Sierra, Milpitas, CA), respectively. Each container was top dressed with 19 g (0.6 oz) of an 18N–2.6P–9.9K slow-release fertilizer

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(Osmocote 18–6–12, Grace/Sierra), and the plants maintained in a greenhouse at days/nights of 24 ± 4/16 ± 4C (75 ± 7/61 ± 7F) with natural photoperiod and irradiance (light intensity).

Budbreak and subsequent growth occurred rapidly following potting. However, explant collection did not begin until terminal shoots approximately 18.5 cm (7.3 in) in length and consisting of six to eight nodes were available. Explants were collected April 6, May 6, and June 6, 1993, dates associated with specific stages of active growth. Shoots collected April 6, 1993 [37 days after potting (DAP)] were soft and very succulent and the stems were light green in color. When the stems were bent, they would snap and separate into two distinct pieces. Shoots collected May 6, 1993 (67 DAP), were in the softwood stage, having more mature stems that had developed a burgundy cast. They were firm and pliable and when pressure was applied, did not break or snap. Shoots collected June 6, 1993 (97 DAP), were past the softwood stage and the proximal portions had become lignified.

Following collection, shoots were placed in running tap water for 10 min. The leaf blades were then removed, shoots were decapitated (terminal buds removed), and the shoots divided into single node segments. The nodal sections were then gently agitated in 70% ethanol for 2 min, rinsed with tap water, and washed with soapy water [1% Ivory liquid (Proctor & Gamble, Cincinnati, OH)] for 5 min. They were then submersed for 10 min with gentle agitation in 0.53% sodium hypochlorite, containing six drops per liter Tween-20 [polyoxyethylene sorbitan monolaurate (Sigma Chemical, Co., St. Louis, MO)]. After being rinsed 3 times in sterile distilled water, 2 mm of stem tissue was excised from each end of an explant so that a single node explant remained which was 1.5 cm (0.6 in) in length.

Nodal explants were placed horizontally into 15 × 60 mm (0.6 × 2.4 in) plastic petri dishes (one per dish) containing 10 ml Woody Plant Medium [WPM (6)] solidified with 1% TC agar (JRH Biosciences, Lenexa, KS), containing no growth regulators or N-(3-methyl-2-butenyl)-1H-purin-6-amine (2iP) at 5.0 or 10.0 ppm (24.6 or 48.2 μM) or N-phenyl-N-1,2,3-thiadiazol-5-ylurea (TDZ) at 0.025 or 0.05 ppm (0.11 or 0.23 μM). The pH of the medium was adjusted to 5.6 with 1 N KOH prior to autoclaving for 15 min at 121C (250F).

Dishes were sealed with Parafilm M (American National Can, Greenwich, CT) and cultures were maintained at 25 ± 1C (77 ± 2F) under a 16-hr photoperiod provided by a combination of two GE model F48PG17CW cool-white, fluorescent power groove lamps (GE Lighting, Cleveland, OH) and two 75-watt soft-white incandescent bulbs suspended 76 cm (2.5 ft) above the plates. The lamps and bulbs provided a photosynthetic photon flux [PPF (400–700 nm)] of 45 to 57 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (3.8 to 4.2 klx), plus photomorphogenic radiation [PR (700–850 nm)] of 6.2 to 9.8 $W \cdot m^{-2}$ as measured at the shelf surface on which the dishes were placed. These and all other light measurements were recorded with a LI COR LI-185A quantum/radiometer/photometer (LI-COR, Lincoln, NE).

For each trial, dishes were arranged in a completely randomized design. There were five treatments, each replicated 20 times. After 3 weeks, data were recorded for percent contamination and percent budbreak (explants which developed one or more leaves) and subjected to two-way analysis of variance (ANOVA) procedures (9).

*Node position, growth regulators, and stock plant growth stage (experiment 2).* Additional stock plants, similar to those used for experiment 1 were removed from the coldroom on February 6, 1995, following storage at 7C (44F) for 90 days. They were then potted and grown under similar cultural conditions as described for experiment 1.

Terminal shoots, similar in length to those collected for experiment 1, were surface sterilized following the aforementioned procedures. In a previous trial, budbreak, and subsequent axillary shoot elongation, occurred with explants collected from the middle portion of a shoot. Therefore, shoots were decapitated and the three distal axillary nodes of each shoot were used as explants. They were kept separate to determine the influence of nodal position on frequency of budbreak and shoot elongation. Individual explants were placed in Magenta GA-7 culture vessels (Magenta Corp., Chicago, IL), each containing 50 ml WPM (6) supplemented with 10 ppm (49.2 μM) 2iP either alone or in combination with 3 ppm (8.6 μM) $GA_3$. In a preliminary study, greater shoot elongation was observed for explants placed on WPM (6) containing 10 ppm 2iP in combination with 3 ppm $GA_3$.

The experiment was replicated every 4 days from March 31, 1995, to April 26, 1995. Replication over time represented a succession of growth stages similar to those described in experiment 1. Culture vessels were arranged in a randomized complete block design with a factorial arrangement of treatments. For each trial, there were four blocks with two growth regulators (10 ppm 2iP with and without 3 ppm $GA_3$) × three nodal positions per block. Explants were maintained under conditions similar to those described for the previous experiment.

After 4 weeks, explants of noncontaminated cultures were transferred to identical culture vessels containing media of the same composition. After an additional 4 weeks, data were recorded for frequency of budbreak and shoot elongation (shoots consisting of one or more nodes). Data were subjected to three-way analysis of variance (ANOVA) procedures (9).

Regardless of growth regulators, all microcuttings ≥10 mm (0.4 in) were excised from the original explants and the bases were treated with Hormodin 1 [1000 ppm (0.1%) indole-3-butyric acid (IBA) in talc] (MSD AGVET, Division of MERCK & Co., Inc., Rahway, NJ). Shoots were then inserted into 78-cell, plastic plug trays [vol per cell = 50 $cm^3$ (3.1 $in^3$)] containing a steam pasteurized medium of peat:perlite (1:1 by vol). The trays were placed on a raised greenhouse bench under intermittent mist, which operated daily 6 sec every 3.3 min during daylight hours. Microcuttings were maintained at days/nights of 24 ± 4/16 ± 4C (75 ± 7/61 ± 7F) under natural photoperiods and irradiance, which was reduced by approximately 40% with a greenhouse shading

**Table 1. Two-way analysis of variance for percent contamination and percent budbreak of nodal explants of *Stewartia pseudocamellia* as influenced by days after potting [DAP (stock plant growth stage)] and growth regulators (GR) (experiment 1).**

| | F ratio | |
|---|---|---|
| **Source of variation** | **Percent contamination** | **Percent budbreak** |
| DAP | 13.00** | 5.74* |
| GR | $0.31^{NS}$ | 18.91** |
| DAP × GR | $0.65^{NS}$ | $2.23^{NS}$ |

NS, *, **, Nonsignificant or significant at the 5% or 1% level, respectively.

Table 2. **Effect of growth regulators and days after potting (stock plant growth stage) on percent contamination and percent budbreak of nodal explants of *S. pseudocamellia* (experiment 1).**

| | Date cultured | | | |
|---|---|---|---|---|
| | May 6, 1995[z] | | June 6, 1995[y] | |
| Growth regulator | Contamination (%)[x] | Budbreak (%)[x] | Contamination (%)[x] | Budbreak (%)[x] |
| Nontreated | 15.0 ± 8.2 | 65 ± 10.9 | 50 ± 11.5 | 40 ± 11.2 |
| 5 ppm 2iP | 15.0 ± 8.2 | 85 ± 8.2 | 45 ± 11.4 | 55 ± 11.2 |
| 10 ppm 2iP | 12.5 ± 7.6 | 90 ± 6.9 | 40 ± 11.2 | 55 ± 11.4 |
| 0.025 ppm TDZ | 20.0 ± 9.2 | 10 ± 6.9 | 25 ± 9.9 | 15 ± 8.2 |
| 0.05 ppm TDZ | 15.0 ± 8.2 | 5 ± 5.0 | 35 ± 10.9 | 15 ± 8.2 |

[z]Softwood growth stage.

[y]Transitional growth stage between softwood and semi-hardwood.

[x]Mean ± 1 SE. Each value is based on 20 observations.

compound. To control fungi, cuttings were sprayed initially and weekly thereafter alternating 3a,4,7,7a-tetrahydro-2[(trichloromethyl) thiol]-1H-isoindole-1,3(2H)-dione (captan) and tetrachloroisophthalonitrile (daconil) at 2.4 g/liter (0.32 oz/gal) and 2.5 ml/liter, (0.32 oz/gal), respectively.

Four weeks under intermittent mist were followed by acclimatization to greenhouse conditions. After 2 weeks under greenhouse conditions, data were recorded for the percentages of cuttings that rooted. A plantlet was considered rooted when roots emerged from drain holes in the bottom of the plug tray.

## Results and Discussion

*Stock plant growth stage and growth regulators (experiment 1).* Days after potting [DAP (stock plant growth stage)] significantly affected survival and contamination ($P = 0.0005$) of the cultures (Table 1). Explants collected April 6, 1993 (37 DAP), turned brown within minutes when subjected to the previously described method of sterilization and did not survive. Those collected May 6, 1993 (67 DAP), were not injured by the sterilization procedures and contamination was ≤20% (Table 2). When shoots were collected June 6, 1993 (97 DAP), sterilization became more difficult, with contamination ranging from 25% to 50%, indicating that a crucial growth stage existed for successful sterilization.

Frequency of budbreak was influenced by growth stage ($P = 0.0186$) and growth regulators ($P = 0.0001$) (Table 1). Budbreak was highest (90%) for explants cultured May 6, 1993 (67 DAP), on WPM containing 10 ppm 2iP (Table 2). A reduction in budbreak was observed for explants cultured on media containing TDZ and resulting growth appeared stunted and less vigorous than those cultured on media containing 2iP.

Following callus removal and subsequent transfer to media of the same composition, explants showed tissue browning, necrosis, and high mortality. In addition, media discoloration was noted, which may have been a response to wounding caused when callus was removed from the original explant. These findings were similar to those of Samartin et al. (8) when working with *Camellia japonica*. Improvements were noted when callus was not removed during transfer. Therefore, in trials which followed, callus removal was discontinued.

*Node position, growth regulators, and stock plant growth stage (experiment 2).* The ANOVA showed that node position had no effect on budbreak or shoot elongation and an interaction was observed for growth regulator × nodal position (Table 1). However, after further examination of the data, the interaction did not appear to have any biological significance. The days at which explants were collected from the stock plants significantly influenced percent budbreak ($P = 0.0001$) and shoot elongation ($P = 0.0001$) (Table 3).

Microcuttings averaging 32 mm (1.2 in) in length and consisting of 3–4 nodes, developed only on explants collected 56 to 72 DAP (Table 4). Growth regulators did not influence microcutting length. For explants placed on WPM (6) containing 10 ppm 2iP alone, shoot elongation was highest (67%) with those collected 60 DAP. When explants were placed on WPM supplemented with 10 ppm 2iP in combination with 3 ppm $GA_3$, 75% shoot elongation was achieved with those collected either 60 or 64 DAP. Stock plant condition at this stage was similar to the softwood growth stage described in experiment 1. Once the stock plants had passed the softwood stage and the new growth had become lignified, the occurrence of shoot elongation decreased rapidly. After stock plants had been containerized more than 72 days, elongation was negligible. These results are not surprising since a report by Read (7) indicated that, for many woody species, successful propagation via tissue culture is dependent on healthy stock plants which are actively growing.

After 4 weeks under intermittent mist, and a subsequent acclimatization period, 79% of the microshoots had rooted. Plantlets were maintained under greenhouse conditions similar to those described for stock plants. After 4 weeks, the

Table 3. **Three-way analysis of variance for frequency of budbreak and axillary shoot elongation of nodal explants of *Stewartia pseudocamellia* as influenced by days after potting [DAP (stock plant growth stage)], growth regulators (GR), and node position (NP) (experiment 2).**

| | F value | |
|---|---|---|
| Source of variation | Frequency of budbreak | Frequency of axillary shoot elongation |
| DAP | 30.82** | 18.23** |
| GR | 5.21* | $0.41^{NS}$ |
| NP | $2.38^{NS}$ | $1.41^{NS}$ |
| DAP × GR | 2.42* | $0.51^{NS}$ |
| DAP × NP | $0.91^{NS}$ | $0.87^{NS}$ |
| GR × NP | $0.58^{NS}$ | 4.24* |
| DAP × GR × NP | $1.32^{NS}$ | $0.91^{NS}$ |

NS, *, **, Nonsignificant or significant at the 5% or 1% level, respectively.

**Table 4. Frequency of budbreak and axillary bud elongation (one per explant) of nodal explants of *S. pseudocamellia* taken at 4 day intervals beginning 52 days after potting of stock plants (DAP) following storage at 7C (44F) for 90 days. Explants were cultured on Woody Plant Medium containing 10 ppm 2iP or 10 ppm 2iP + 3 ppm $GA_3$ (experiment 2).**

| | Treatment | | | |
|---|---|---|---|---|
| | 10 ppm 2iP | | 10 ppm 2iP + 3 ppm $GA_3$ | |
| DAP[z] | Budbreak (%)[y] | Elongation (%)[y] | Budbreak (%)[y] | Elongation (%)[y] |
| 52 | 25.0 ± 13.0 | 0.0 ± 0.0 | 50.0 ± 15.1 | 0.0 ± 0.0 |
| 56 | 100.0 ± 0.0 | 50.0 ± 15.1 | 100.0 ± 0.0 | 33.3 ± 14.2 |
| 60 | 100.0 ± 0.0 | 66.7 ± 14.2 | 91.7 ± 8.3 | 75.0 ± 13.0 |
| 64 | 100.0 ± 0.0 | 58.3 ± 14.9 | 100.0 ± 0.0 | 75.0 ± 13.0 |
| 68 | 100.0 ± 0.0 | 25.0 ± 13.0 | 91.7 ± 8.3 | 33.3 ± 14.2 |
| 72 | 100.0 ± 0.0 | 41.7 ± 14.9 | 100.0 ± 0.0 | 50.0 ± 15.1 |
| 76 | 8.3 ± 8.3 | 0.0 ± 0.0 | 41.7 ± 14.9 | 0.0 ± 0.0 |
| 80 | 6.7 ± 14.2 | 0.0 ± 0.0 | 33.3 ± 14.7 | 0.0 ± 0.0 |

[z]Replication on eight dates representing various stock plant growth stages. At 52 days after potting, shoots were very soft and succulent. From 56 to 72 days, shoots were in a softwood condition and at 76 to 80 days, shoots were in a semi-hardwood (lignified) condition.

[y]Mean ± 1 SE. Each mean is based on four replications, each with three subsamples.

plantlets were fertilized with 100 ppm N from a 15N–19.6P–4.2K water soluble fertilizer [Rhododendron Special (Grace/Sierra)]. Within 1 week, subsequent shoot growth was observed (Fig. 1).

The protocol described herein was developed over a 2-year period and incorporates the results of many preliminary trials. Although *S. pseudocamellia* was propagated successfully by micropropagation, large plantlet numbers were not achieved since explants produced only one microcutting per nodal segment. Attempts were made to initiate axillary shoot proliferation by placing decapitated microshoots horizontally on WPM (6) containing various growth regulators. However, these trials met with little success. Before the protocol will be applicable in a commercial situation, axillary shoot proliferation must be increased.

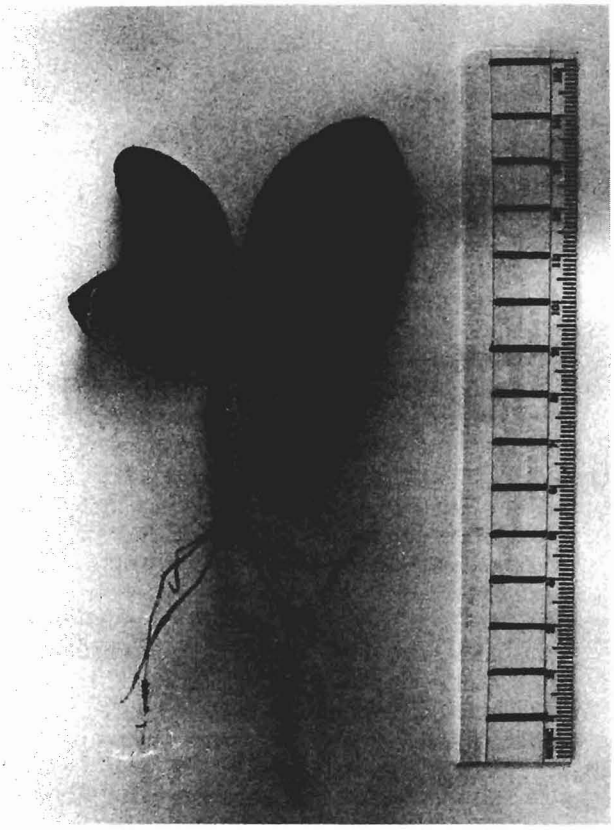

**Fig. 1. Rooted microcutting of *S. pseudocamellia* following acclimatization to greenhouse conditions. Scale divisions are in cm (0.4 in).**

Additional factors which need to be addressed include the availability of explants and stock plant management. Explant availability was limited because active growth of stock plants was short term (stock plants produced one flush of growth following budbreak). The procedures might be improved by removing bare root plants from cold storage at different times, making explants available over an extended period. Future studies might also focus on stock plant management (mineral nutrition, irradiance, photoperiod, temperature, etc.) as these factors affect subsequent *in vitro* performance of the explants.

*In vitro* rooting was not necessary since microshoots were rooted easily under intermittent mist following treatment with Hormodin 1. Whether or not Hormodin 1 was necessary for rooting is unknown since no additional IBA formulations or controls were utilized. Nevertheless, the fact that microshoots of the species root easily using *ex vitro* procedures should prove to be valuable in any further investigations.

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