

## *In vitro* plant regeneration of *Zelkova schneideriana*, an endangered woody species in China, from leaf explants

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### SUMMARY

*Zelkova schneideriana* Hand.-Mazz. is a high-value plant used for hardwood timber production in China. Because of over-harvesting and limited numbers of seedlings for plantations, *Z. schneideriana* has become an endangered species in China. To grow this plant sustainably, we generated a protocol to reproduce this species using tissue culture. Leaf explants were cultured on Wood Plant Medium (WPM), Murashige and Skoog (MS), 1/2MS (main elements), or B5 media variously supplemented with 6-benzylaminopurine (BA), naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), or 2, 4-dichlorophenoxyacetic acid (2,4-D) to induce callus, adventitious shoots, and rooting. WPM was the most effective medium for callus induction and resulted in two types of calli from leaf explants. One was soft and straw-yellow coloured on WPM containing 0.45, 4.54, 9.08, or 13.62  $\mu\text{M}$  2,4-D or on WPM supplemented with 4.44  $\mu\text{M}$  BA in combination with 0.45 or 4.54  $\mu\text{M}$  2,4-D. The other callus type was friable and green on WPM supplemented with 0.44, 4.44, 8.88, or 13.32  $\mu\text{M}$  BA or on WPM supplemented with 4.44  $\mu\text{M}$  BA in combination with 0.54 or 5.37  $\mu\text{M}$  NAA. Only the latter callus type was observed to regenerate plantlets. WPM supplemented with 4.44  $\mu\text{M}$  BA plus 2.68  $\mu\text{M}$  NAA had a 64.5% shooting rate, with 6.75 shoots per callus. Rooting at 70.0%, with an average of 3.6 roots per shoot, was obtained on WPM supplemented with 2.46  $\mu\text{M}$  IBA in combination with 2.0  $\text{g l}^{-1}$  activated charcoal (AC). In total, 86% of regenerated *Z. schneideriana* plantlets survived after acclimation in a greenhouse at  $24^\circ \pm 2^\circ\text{C}$  under a 16 h photoperiod provided by cool-white fluorescent lights (PPFD =  $65.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), and 100% survived after transplanting to an experimental field plot.

Ten species of *Zelkova* exist worldwide, and three are native to China. They are *Z. schneideriana* Hand.-Mazz., *Z. serrata* (Thunb.) Makino, and *Z. sinica* Schneid. (Chen and Huang, 1999). Although most *Zelkova* species are growing for quality hardwood timber, *Z. schneideriana* is also one of the most important landscape species because of its large crown and high disease resistance (Cao *et al.*, 2005; Jin and He, 2005). *Z. schneideriana* is highly valued in China, Korea, and other East Asian countries (Lo *et al.*, 1995). There is now an increasing demand for *Z. schneideriana* trees in China. However, *Z. schneideriana* has become a rare and endangered species in China due to uncontrolled commercial logging and the lack of effective propagation methods (Fu and Jin, 1992).

Micropropagation of tree species offers a rapid means to produce clonal planting stock for re-forestation programmes, gardening, and the conservation of elite and rare germplasm (Fenning and Gershenzon, 2002). Regeneration has been accomplished in over 20 endangered woody plants (Wu *et al.*, 2006) such as

*Davidia involucrata* (Jin *et al.*, 2007), *Emmenopterys henryi* (Xiong *et al.*, 2008), and *Elaeagnus mollis* (Yan *et al.*, 2003). Although several previous studies have shown micropropagation and rooting of *Z. serrata* (Gao *et al.*, 1996) and *Z. sinica* (Jin *et al.*, 2006), a complete protocol for plant regeneration has not been established for *Z. schneideriana*, particularly for the induction of adventitious shoots from leaf explants.

The objective of this study was to develop a complete regeneration system for the rare and endangered plant, *Z. schneideriana* Hand.-Mazz., using *in vitro* leaf explants. Our studies should provide guidance for future genetic improvement of landscape species, contribute to the conservation of rare germplasm, and help to meet the increased demand for *Z. schneideriana* trees for gardening and timber production.

### MATERIALS AND METHODS

*Plant material, sterilisation procedures, and preparation of explants*

Young fully-expanded leaves (approx. 1.0 cm  $\times$  1.5 cm) were collected from 12 adult *Z. schneideriana*

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trees (approx. 20-years-old) in an experimental field plot at the Central South Forestry University, Changsha, China. The leaves were surface-sterilised with 70% (v/v) ethanol for 1 min, rinsed three-times with sterile distilled water, followed by 6% (v/v) H<sub>2</sub>O<sub>2</sub> for 5 min, then rinsed three-times again with sterile distilled water. After disinfection, leaf explants with their midrib (in approx. 0.5 cm × 0.5 cm sections) with the leaf margins wounded by complete removal of the leaf edges, were placed firmly (abaxial-side up) in 100 mm × 15 mm Petri dishes containing 25 – 30 ml of various callus induction media: (i) WPM (Lloyd and McCown, 1981); MS (Murashige and Skoog, 1962); 1/2MS (half-strength MS); or B5 (Gamborg *et al.*, 1968). Each medium was supplemented with 4.44 μM 6-benzylaminopurine (BA) and 5.37 μM naphthaleneacetic acid (NAA). Explants were also placed on WPM supplemented with 0, 0.45, 4.54, 9.08, or 13.62 μM 2,4-dichlorophenoxyacetic acid (2, 4-D); on WPM supplemented with 0.44, 4.44, 8.88, or 13.32 μM BA; on WPM supplemented with 4.44 μM BA in combination with 0.54 or 5.37 μM NAA; or on WPM supplemented with 4.44 μM BA in combination with 0.45 or 4.54 μM 2, 4-D. Each experiment was replicated three-times, with 15 leaf explants per treatment per replicate.

All media were solidified using 6.0 g l<sup>-1</sup> agar (GS1200; Beijing Gentel Co., Beijing, P.R. China) supplemented with 30.0 g l<sup>-1</sup> sucrose, and the pH was adjusted to 5.7. The media were then autoclaved at 1.08 × 10<sup>5</sup> N m<sup>-2</sup> for 15 min at 121°C.

Cultures were incubated at 24° ± 2°C under a 16 h photoperiod provided by cool-white fluorescent lights (PPFD = 65.5 μmol m<sup>-2</sup> s<sup>-1</sup>). The calli formed were transferred to fresh medium (WPM containing 4.44 μM BA and 5.37 μM NAA) every 4 weeks.

#### *Adventitious shoot induction from calli*

Calli (7 – 10 mm<sup>2</sup> in area) which had been sub-cultured four-times, were excised and placed on adventitious shoot-induction medium. The number of adventitious shoots per explant was recorded after 4 weeks. Adventitious shoots were maintained on WPM supplemented with 4.44 μM BA and 2.67 μM NAA and transferred to the same fresh medium every 3 – 4 weeks. The experiment was replicated three-times, with 15 calli per treatment per replicate. Shoots were maintained for the root induction experiment.

#### *Rooting of adventitious shoots*

Shoots (2 – 3 cm in length) from calli were excised and placed on root-induction media. The root induction media used were: (i) WPM, 1/2WPM, MS, or 1/2MS each supplemented with 2.46 μM indole-3-butyric acid (IBA); or (ii) WPM supplemented with different concentrations of NAA, IBA, or 2.0 g l<sup>-1</sup> activated charcoal (AC). All of the root-induction media were supplemented with 20.0 g l<sup>-1</sup> sucrose and solidified with 7.0 g l<sup>-1</sup> agar (Beijing Gentel Co.). The experiment was replicated three-times, with 15 shoots per treatment per replicate.

#### *Statistical analysis*

A randomised complete block design (RCB) trial with each treatment, and three replications with 15 samples per treatment per replication was used. Data for all dependent variables (i.e., callus induction rate, shoot initiation rate,

shoot count, root count, and root induction rate) were analysed by one-way ANOVA to test for statistical significance. Tukey's standardised test at *P* < 0.05 was applied for means separations. All analyses were conducted using the MINITAB computer package (Downy and Wearden, 1983).

## RESULTS AND DISCUSSION

### *Effects of different basal media on callus induction*

Almost all treatments induced some calli. Callus began to form on the wounded margins of leaf explants as early as 4 – 10 d after the explants had been placed on WPM, MS, 1/2MS, or B5 media supplemented with 4.44 μM BA and 5.37 μM NAA. The type of basal medium had a significant effect on callus induction in *Z. schneideriana* (Table I). After 4 weeks in culture, 30.0 – 95.7% of leaf explants were covered with calli, but no shoot regeneration was observed. WPM was found to be the optimum medium for callus induction, followed by MS medium. The highest induction rate (95.7%) occurred when leaf explants were cultured on WPM plus 4.44 μM BA. WPM was therefore best for *Z. schneideriana* leaf tissue culture, which was consistent with the conclusion of Gao *et al.* (1996) using *Z. serrata*.

### *Effects of growth regulators on callus induction*

Leaf explants were placed on WPM supplemented with different growth regulators to induced callus. Calli were observed at the ends of the midvein or around the wounded leaf edges. Callus induction was significantly affected by the particular combination of plant growth regulators (Table II). Leaf explants became larger, but no calli appeared on WPM without added phytohormones, whereas all phytohormone-containing media induced two types of calli. The first type of callus was soft and watery (Figure 1A) and was formed on WPM medium containing 0.45, 4.54, 9.08, or 13.62 μM 2,4-D, or on WPM supplemented with 4.44 μM BA in combination with 0.45 or 4.54 μM 2,4-D. The highest rate of callus induction was 97.8%. But, this type of callus frequently turned brown during later sub-culture. The second type of callus was fast-growing, friable, and yellow-green or green (Figure 1B). It was formed on WPM supplemented with 0.44, 4.44, 8.88, or 13.32 μM BA; or on WPM supplemented with 4.44 μM BA in combination with 0.54 or 5.37 μM NAA. The optimum medium for this type of callus induction was WPM supplemented with 4.44 μM BA and 5.37 μM NAA, and the induction rate was 95.6%. Only the second type of callus had any morphogenic competence for regeneration during sub-culture. These calli were further sub-cultured for shoot and root regeneration studies.

TABLE I  
Effects of different basal media, supplemented with 4.44 μM BA and 5.37 μM NAA, on callus induction from leaf explants of *Z. schneideriana*

Culture medium	Time of first callus appearance (d)	Callus texture	Callus induction rate
WPM	4.4 a <sup>†</sup>	Friable	95.7 a
MS	5.5 b	Hard	71.7 b
1/2MS	8.6 c	Hard	36.7 c
B5	10.0 c	Hard	30.0 c

<sup>†</sup>All values are means (n = 15), and those followed by a different lower-case letter within a column are significantly different at *P* < 0.05 according to Tukey's test. The data were collected after culturing leaf explants on each callus induction medium for 4 weeks.

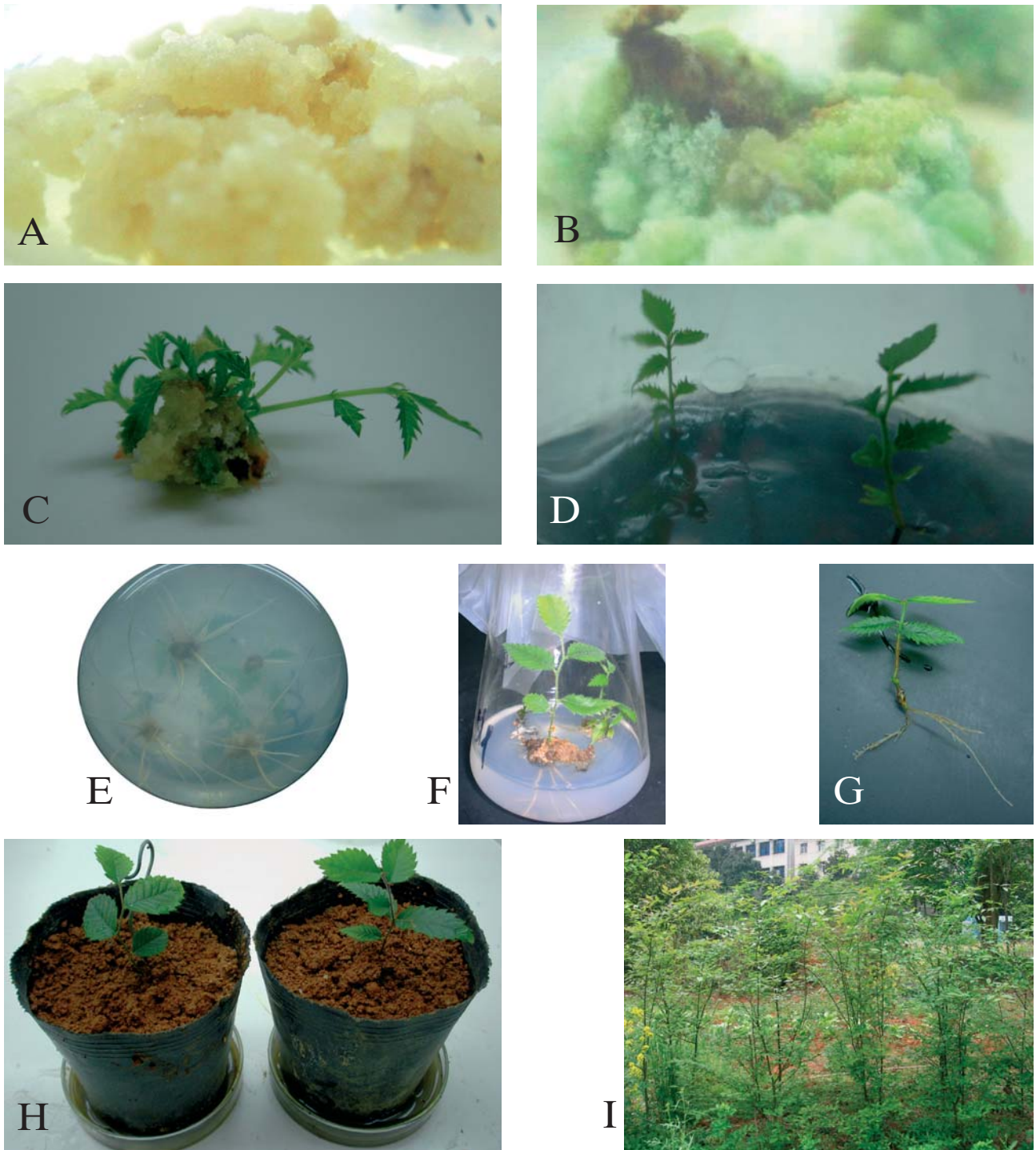


FIG. 1

Regeneration of *Zelkova schneideriana* from leaf explants on WPM supplemented with various growth regulators. Panel A, straw-yellow callus on WPM plus 4.44  $\mu\text{M}$  BA + 0.45  $\mu\text{M}$  2,4-D; Panel B, green callus on WPM plus 4.44  $\mu\text{M}$  BA + 5.37  $\mu\text{M}$  NAA; Panel C, shoot organogenesis on WPM plus 4.44  $\mu\text{M}$  BA in combination with 2.69  $\mu\text{M}$  NAA; Panel D, rooting on WPM + 2.46  $\mu\text{M}$  IBA + 2.0  $\text{g l}^{-1}$  AC; Panel E, roots on WPM + 2.46  $\mu\text{M}$  IBA; Panel F, roots on WPM + 2.69  $\mu\text{M}$  NAA; Panel G, plantlet; Panel H, regenerated plants grown in a greenhouse; and Panel I, regenerated (100 – 150 cm-high) plants grown in the field (approx. 2-years old).

#### *Adventitious shoot induction from callus*

The second type of callus (friable, green) was transferred to shoot-induction medium. Bud primordia began to develop within 2 – 3 weeks, when the callus turned brown. The bud primordia turned green, and shoots started to differentiate and elongate (Figure 1C). Adventitious shoots (1 – 2 cm in length) were obtained after 4 weeks. The numbers of shoots per callus are recorded in Table III.

Table III showed that 0.0 – 64.5% of calli induced adventitious shoots on all induction media. The shoots grew well on WPM supplemented with 4.44  $\mu\text{M}$  BA, or on WPM supplemented with 2.68  $\mu\text{M}$  NAA in combination with 2.22, 4.44, or 8.88  $\mu\text{M}$  BA. No shoots, but callus was observed on WPM supplemented with 10.74  $\mu\text{M}$  NAA in combination with 4.44  $\mu\text{M}$  BA, or on WPM supplemented with 4.93  $\mu\text{M}$  IBA in combination with 2.22, 4.44, or 8.88  $\mu\text{M}$  BA. Shoots grew slowly on all

TABLE II  
Effects of different phytohormones on callus induction from leaf explants of *Z. schneideriana*

Phytohormone ( $\mu\text{M}$ )			Callus induction rate	Callus texture	Callus colour
2,4-D	BA	NAA			
0	0	0	0.00	–	–
0.45	0	0	97.78 a <sup>†</sup>	Soft	Straw yellow
4.54	0	0	82.32 b	Soft	Straw yellow
9.08	0	0	71.10 b	Soft, watery	Tawny
13.62	0	0	84.76 b	Softer, watery	Straw yellow
0	0.44	0	48.90 c	Friable	Green
0	4.44	0	83.85 b	Friable	Green
0	8.88	0	73.30b	Hard	Green
0	13.32	0	40.00 c	Very hard	Green
0	4.44	0.54	53.33c	Friable	Yellow-green
0	4.44	5.37	95.51 a	Friable	Yellow-green
0.45	4.44	0	88.94 b	Soft	Straw yellow
4.54	4.44	0	95.54 a	Soft, watery	Straw yellow

<sup>†</sup>All values are means (n = 15), and those followed by a different lower-case letter within a column are significantly different at  $P < 0.05$  according to Tukey's test.

TABLE III  
Effect of different combinations of phytohormones on shoot regeneration from callus cultures of *Z. schneideriana*

Growth regulator or combination ( $\mu\text{M}$ )	Shoot initiation rate (%)	Avg. no. of shoots per callus	Observations
<b>BA</b>			
2.22	15.57 d <sup>†</sup>	1.14 c	Shoots grew slowly
4.44	44.42 b	3.38 b	Shoot grew well and most of them reach approx. 1 cm high
8.88	28.90 c	1.50 c	Shoots grew slowly
<b>BA:NAA</b>			
2.22:2.68	37.77 b	2.35 b	Shoots grew well
4.44:2.68	64.50 a	6.75 a	Shoots grew well and most of them reach approx. 1–2 cm high
8.88:2.68	52.27 b	3.13 b	Shoot grew well and most of them reach approx. 1 cm high
2.22:5.37	13.33 d	1.66 c	Shoots grew slowly and were $\leq 1$ cm high; vitrification happened to some shoots
4.44:5.37	11.10 d	1.20 c	Ditto
8.88:5.37	15.53 d	1.50 c	Ditto
4.44:10.74	0	0	No shoots formed
<b>BA:IBA</b>			
2.22: 2.46	22.07 c	1.10 c	Shoots grew slowly, the callus grew fast and frequently turned brown, some calli produced roots
4.44: 2.46	14.50 d	2.71 b	Ditto
8.88: 2.46	12.72 d	1.16 c	Ditto
2.22: 4.93	0	0	No shoots, the callus grew fast and frequently turned brown, some calli produced roots
4.44: 4.93	0	0	
8.88: 4.93	0	0	

<sup>†</sup>Mean values followed by a different lower-case letter within a column are significantly different at  $P < 0.05$  according to Tukey's test.

other media. Callus cultured on WPM containing 2.46 or 4.93  $\mu\text{M}$  IBA produced roots, but no shoots, and these calli turned brown. In general, WPM supplemented with BA and NAA was better at inducing shoots than WPM supplemented with only BA, or WPM supplemented with BA and IBA. The optimum growth regulator combination for shoot induction in *Z. schneideriana* callus was therefore 4.44  $\mu\text{M}$  BA plus 2.68  $\mu\text{M}$  NAA. The shoot induction rate could reach 64.5%. The maximum percentage of calli showing shoot initiation and the maximum number of shoots per callus (6.75) were obtained on this medium.

We found several limitations, such as low shoot proliferation and vitrification, were pronounced in *Z. schneideriana* tissue culture. Sinha *et al.* (2000) reported that media additives such as polyvinylpyrrolidone (PVP), coconut water (CW), casein hydrolysate (CH), and glutamine were helpful for shoot organogenesis and could reduce these limitations. A similar phenomenon was observed in *Z. schneideriana*. Plantlets grew more strongly when the shoot sub-culture medium was supplemented with CH (data not shown).

#### Rooting of adventitious shoots

Roots were observed as early as 1 week after placing the adventitious shoots on root induction medium, and

most shoots produced roots by week-3. Overall, WPM as a basal medium had a better rooting response (55.3%) than 1/2WPM (40.0%), MS (26.8%), or 1/2MS (29.7%; Figure 2). AC enhanced the rooting rate from 55.3% to 70.0% when added to WPM supplemented with 2.46  $\mu\text{M}$

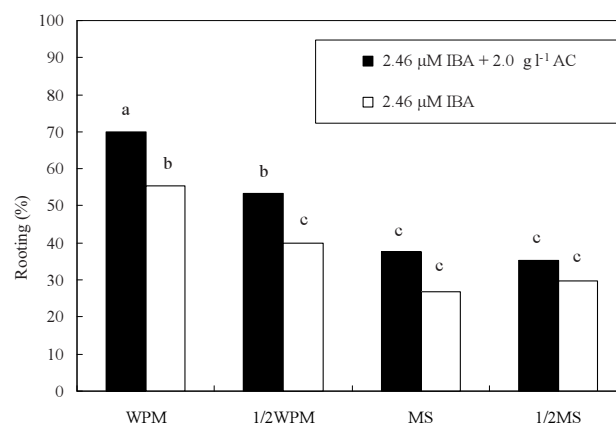


FIG. 2  
The effect of 2.0  $\text{g l}^{-1}$  activated charcoal (AC) and different basal media on the percentage rooting rate in *Z. schneideriana*. All treatments were supplemented with 2.46  $\mu\text{M}$  IBA. Mean column values with a different lower-case letter were significantly different at  $P < 0.05$  according to Tukey's test (n = 45).

TABLE IV  
 Rooting rate and percentage survival of regenerated *Z. schneideriana* plantlets in potting medium after 4 weeks in a greenhouse

NAA ( $\mu\text{M}$ )	IBA ( $\mu\text{M}$ )	Root induction rate (%)	Root number per shoot <sup>‡</sup>	Survival in greenhouse (%) <sup>§</sup>
0	0	0	0	–
0.54	0	23.33 d <sup>†</sup>	1.61 c	32.36
2.69	0	29.35 d	1.35 c	20.0
5.37	0	28.43 d	1.53 c	28.45
2.69 + 2.0 g l <sup>-1</sup> AC	0	33.33 d	1.69 c	45.50
0	0.49	33.0 d	1.47 c	69.32
0	2.46	55.33 b	1.93 b	72.0
0	4.93	45.50 c	2.15 b	67.23
0	2.46 + 2.0 g l <sup>-1</sup> AC	70.0 a	3.60 a	86.0

<sup>†</sup>Mean values followed by a different lower-case letter within a column are significantly different at  $P < 0.05$  according to Tukey's test.

<sup>‡</sup>Data represent the means of three replications per treatment.

<sup>§</sup>Percentage survival rate of regenerated plantlets transferred to potting medium and grown for 4 weeks under controlled greenhouse conditions.

IBA and 2.0 g l<sup>-1</sup> AC (Figure 2), and inhibited callus formation (Figure 1D).

Auxin was necessary for rooting in *Z. schneideriana*. Shoots on root induction medium without added auxin remained green and the leaves enlarged, but no rooting occurred (Table IV). Roots developed directly from adventitious shoots, without visible callus, when placed on WPM with IBA (Figure 1E), and roots developed mainly through callus formed at the basal end of shoots when placed on WPM with NAA (Figure 1F). Higher rooting percentages were observed in treatments with 0.49, 2.46, or 4.93  $\mu\text{M}$  IBA (30.0%, 55.3%, or 45.5%, respectively), compared to treatments with 0.54, 2.69, or 5.37  $\mu\text{M}$  NAA (23.3%, 29.4%, or 28.4%, respectively). Therefore, IBA induced a higher percentage of rooting than NAA. Root induction percentages for IBA at 0.49, 2.46, or 4.93  $\mu\text{M}$  were significantly different from one another. The numbers of roots per shoot on 0.49  $\mu\text{M}$  IBA were also markedly different to those on 2.46 or 4.93  $\mu\text{M}$  IBA (Table IV).

Overall, the highest rooting percentage (70%) was obtained when shoots were placed on WPM supplemented with 2.46  $\mu\text{M}$  IBA plus 2.0 g l<sup>-1</sup> AC (Figure 2; Table IV). The highest number of roots per shoot (3.60) was also obtained on WPM with 2.46  $\mu\text{M}$  IBA plus 2.0 g l<sup>-1</sup> AC (Table IV). The maximum number of roots developed per shoot was six (Figure 1G).

Difficulty in rooting has long remained a major challenge in the micropropagation of woody species *in vitro* (Harada and Murai, 1996). Our results confirmed that IBA was effective at promoting rooting during tissue culture of woody plant species, as shown by Lu *et al.*

(2001). We found that auxin in the rooting medium was a pre-requisite for root initiation, and that IBA was better than NAA. It has also been reported that regenerated woody plantlets may wilt and die during root induction (Gu *et al.*, 1999). A similar phenomenon was observed in *Z. schneideriana*. We found that this problem could be effectively alleviated by adding AC to the medium. This finding should have a significant impact on future micropropagation studies on woody plants *in vitro*.

#### Acclimatisation of rooted plantlets

*In vitro* plantlets grew actively during the acclimatisation process and no stress symptoms were observed after they were transplanted to larger pots and grown in a greenhouse at 24° ± 2°C, with a 16 h photoperiod at 85  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Figure 1H). After 3 months, plantlets ranged from 12 – 25 cm in height. In total, 86.0% of potted *Z. schneideriana* plantlets survived acclimatisation and were kept in a greenhouse for 5 months before transplanting to an experimental field plot. After 2 years, *Z. schneideriana* plantlets ranged from 100 – 150 cm in height and had a 100% survival rate in the experimental field plot (Figure 1I).

A reliable regeneration protocol has been established for *Z. schneideriana*. This work provides a foundation for further efforts to generate genetically improved *Z. schneideriana* and related woody species.

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