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Topophysic effects differ between node and organogenic cultures of the eucalypt *Corymbia torelliana* × *C. citriodora*

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Abstract. The eucalypt *Corymbia torelliana* × *C. citriodora* is planted widely in India, Brazil and Australia although plantation establishment has been limited by inadequate seed supply and low amenability to propagation via cuttings. This study optimised node culture and organogenic culture methods for *in vitro* propagation of *Corymbia* hybrids by identifying explant position (topophysic) effects on rooting, shoot elongation and shoot proliferation. Strong, negative morphogenic gradients in shoot elongation and proliferation capacity were evident from the cotyledonary node to the fourth or fifth node of seedlings when their nodes were transferred to node culture (without benzyladenine). These topophysic effects were related to differences in rooting capacity of individual nodes. Root formation in node culture was associated with formation of long multi-nodal axillary shoots, and so higher rooting of shoots from the cotyledonary node or first true-leaf node was associated with higher shoot proliferation. However, all nodes were equally capable of shoot proliferation in organogenic culture (with 2.2 μM benzyladenine), where rooting and rapid stem elongation did not occur. Most shoots (61–100%) from both node culture and organogenic culture were converted to plantlets, with plantlet conversion and primary root number not differing significantly among explant node positions. The strong topophysic effect in node culture, combined with the lack of a topophysic effect in organogenic culture, provides for an optimised clonal propagation system based on segregation of nodes from the same seedling into separate node and organogenic culture pathways.

Keywords Adventitious roots · Adventitious shoots · *Eucalyptus* · Organogenesis · Plantlet · Topophysis

Abbreviations

BA 6-Benzyladenine

IBA Indole-3-butyric acid

IVS *In vitro* soil-less

MS Murashige and Skoog

NAA α-Naphthaleneacetic acid

Introduction

Explants from many organs are capable of producing adventitious shoots or roots, but explants from different organs usually vary greatly in their morphogenic capacity (George 1993). Morphogenic capacity can also vary depending on the position of explants within an organ. For example, shoot regeneration from hypocotyl or epicotyl segments often depends on their distance from the cotyledonary node (Costa et al. 2004; Chen et al. 2008; Germanà et al. 2008) and regeneration from excised leaves often depends on their distance from the shoot apex (Corredoira et al. 2008; Aggarwal et al. 2010; Feng et al. 2010). Positional effects on subsequent growth or development of explants or cuttings are termed 'topophysis' (George 1993; Mitchell et al. 2004). Topophysic effects can be large, and an understanding of topophysis for optimised selection of initial explants can significantly improve the efficiency of a micropropagation system (Cheng et al. 1992; George 1993; D'Souza and Sharon 2001; Lee-Espinosa et al. 2008).

We have recently developed node and organogenic culture methods for micropropagation of the previously difficult-to-propagate eucalypt, *Corymbia torelliana* × *C. citriodora* (Trueman and Richardson 2007; Hung and Trueman 2010). Although methods have been established for commercial-scale propagation of cuttings of some eucalypts such as *E. grandis* and *E. urophylla* (Assis et al. 2004; Saya et al. 2008), most plantation eucalypts including *Corymbia citriodora*, *C. torelliana*, *E. cloeziana*, *E. dunnii*, *E. globulus*, *E. marginata* and *E. nitens* are considered recalcitrant (Aimers-Halliday et al. 1999; Luckman and Menary 2002; Assis et al. 2004; Fogaça and Fett-Neto 2005; Woodward et al. 2006; Almeida et al. 2007; Smith and Henson 2007; Schwambach et al. 2008; Trueman and Richardson 2008). *C.*

torelliana × *C. citriodora* is grown in India, Brazil and Australia, where it combines rapid growth and disease resistance from *C. torelliana* with the excellent wood quality and stem form of *C. citriodora* (Bisht et al. 2002; Lee 2007; Arya et al. 2009; Lee et al. 2009). However, inadequate seed supply and low amenability to cuttings propagation have hampered plantation establishment of these hybrids (Lee 2007; Shepherd et al. 2007, 2008; Trueman and Richardson 2008; Lee et al. 2009). Growers in Australia have been planting *C. citriodora* seedlings on sites where they would prefer to grow *C. torelliana* × *C. citriodora*.

Node and organogenic cultures of *C. torelliana* × *C. citriodora* are both derived from the first four or five nodes of *in vitro* seedlings (Trueman and Richardson 2007; Hung and Trueman 2010). For node culture, the excised nodes are transferred to cytokinin-free proliferation medium, where they form roots and long axillary shoots with multiple nodes. For organogenic culture, the excised nodes are instead transferred to media containing benzyladenine (BA), in which they do not form roots or long shoots, but proliferation appears to occur through a combination of axillary shoot production, basal stem callogenesis and shoot regeneration. The relative contributions of axillary and adventitious shoots to *C. torelliana* × *C. citriodora* shoot proliferation have not been determined definitively; however, extensive callus formation is a prerequisite for high rates of proliferation. Node culture is considered ideal when high rates of proliferation are not required and it is important to reduce the risk of genetic irregularities arising from a callus phase (George 1993; Pijut et al. 2007). Organogenic culture has greater value when very high proliferation rates are required (George 1993; Nehra et al. 2005).

We have suggested previously that a dual node and organogenic culture approach for each clone might be useful for field testing, germplasm storage and

plantation establishment of *C. torelliana* × *C. citriodora* (Trueman and Richardson 2007; Hung and Trueman 2010). Node culture could be used to raise sufficient shoots and plantlets of each clone for initial establishment of nursery stock plants, field testing and clonal storage, while organogenic culture of desired clones is used for laboratory storage and *en masse* production of plantation trees. The efficiency of a dual culture approach for *C. torelliana* × *C. citriodora* clones could be greatly enhanced if morphogenic gradients in proliferation capacity (i.e. topophysis) exist along the seedling stem and if the direction of these gradients differs between node and organogenic cultures. Nodal explants of the same clone could then be segregated into node and organogenic culture media, at the onset of shoot proliferation, for optimal shoot and plantlet production in a dual culture system.

This study investigated whether proliferation capacity differs among the first five nodes of *C. torelliana* × *C. citriodora* seedlings in node culture and organogenic culture, and whether morphogenic gradients differ between the two culture methods. We examined the proliferation of individual nodal explants in optimal media from our recent study (Hung and Trueman 2010), and then assessed their plantlet formation in an *in vitro* soil-less (IVS) system developed by Newell et al. (2003, 2005).

Materials and methods

Seed germination and shoot induction

Full-sibling seeds of two *C. torelliana* × *C. citriodora* subsp. *variegata* families were obtained from the Hardwood Tree Improvement Group, Agri-Science Queensland. The families, 1CT2-013 × 1CV2-109 (‘family 13’) and 1CT2-019 × 1CV2-114

('family 19'), were produced by controlled pollination of individual trees. Seeds were surface sterilised as described previously (Hung and Trueman 2010) and plated (ten seeds per 90-mm Petri dish) onto germination medium consisting of half-strength Murashige and Skoog (MS) basal salts (PhytoTechnology Laboratories, Shawnee Mission, Kansas) and 20 g L^{-1} sucrose, with pH adjusted to 5.8 prior to addition of 8 g L^{-1} agar (Bacto Laboratories, Liverpool, Australia) and autoclaving (121°C, 20 min). Seeds were germinated at 25°C with a 16-h photoperiod (approx. 50 μmol m^{-2} s^{-1} irradiance using fluorescent tubes) for 2 weeks.

After germination, all shoots longer than 5-mm were excised just above the root collar and transferred randomly to 375-mL glass jars containing 50 mL of one of four MS media: (1) half-strength MS medium without naphthaleneacetic acid (NAA); (2) half-strength MS medium with 0.05 μM NAA; (3) full-strength MS medium without NAA; or (4) full-strength MS medium with 0.05 μM NAA. Media for shoot induction (first passage) and proliferation (second to fourth passages; see section below) contained 30 g L^{-1} sucrose, solidified with 8 g L^{-1} agar, and with pH adjusted to 5.8 prior to autoclaving (121°C, 20 min). Jars were positioned randomly in the growth room and maintained at 25°C with a 16-h photoperiod (approx. 100 μmol m^{-2} s^{-1} irradiance).

All shoots formed roots and elongated to produce multiple nodes during the next four weeks (Fig. 1a), at which stage each node was dissected for transfer (Fig. 1b). The shoot apex with several young leaves was counted, excised and transferred as a single node. Most shoots of family 13 had four nodes, and most shoots of family 19 had five nodes. Shoots with fewer or more nodes in each family were not included in the analysis (i.e. the cotyledonary node was node 1 for both families, and the apical node was node 4 in family 13 and node 5 in family 19).

Shoot proliferation via node or organogenic culture

The four or five explant nodes from each seedling (i.e. one clone) were placed together in a 375-mL glass jar containing 50 mL of fresh medium. Explants from shoots in half-strength MS induction media (1 and 2, above) were transferred to their same medium (1 or 2) for node culture (Fig. 1c). Explants from shoots in full-strength MS media (3 and 4, above) were transferred to their same medium but now containing 2.2 μM BA for organogenic culture (Figs 1d–f). These half-strength and full-strength MS media are the most suitable for node culture and organogenic culture, respectively (Hung and Trueman 2010). The original position of each node on the seedling shoot was recorded on the outside of the jar, and these explant node positions were tracked for all proliferated shoots in subsequent passages. The numbers of replicate clones per medium were 5–10 and 6–8 for families 13 and 19, respectively. New nodes and clusters of regenerating callus were excised and transferred to fresh medium after 4 weeks, 10 weeks and 15 weeks. Proportion of shoots with roots, shoot length, and total number of transferred shoots were calculated for each of the original explant nodes after every passage.

Plantlet conversion

A random subsample of five shoots (where available) was taken from each clone, and the shoots were transferred to glass jars containing 50 mL of half-strength MS medium with 20 g L^{-1} sucrose, 8 g L^{-1} agar and 19.6 μM indole-3-butyric acid (IBA). Shoots were incubated for 7 d in darkness at 25°C, and then transferred to a sterile

well-watered vermiculite / perlite (50/50, v/v) medium based on the *in vitro* soil-less (IVS) method of Newell et al. (2003, 2005) as described by Hung and Trueman (2010). The shoots were maintained at 25°C with a 16-h photoperiod (approx. 100 μmol m^{-2} s^{-1} irradiance) for 4 weeks, and then gently extracted to determine the percentage of shoots that had formed roots (Fig. 1g) for each of the nodal explants. All plantlets were transferred to a polyethylene nursery chamber (Fig. 1h).

Statistical analyses

Proportions of shoots with roots at 4 weeks were compared using chi-squared tests, with significance test values adjusted using sequential Dunn-Šidák corrections, because rooting values for each node at this stage were either 0 or 1 (i.e. binomially distributed). Proportions of shoots with roots at 10 and 15 weeks, and shoot lengths and shoot numbers at 4, 10 and 15 weeks, were analysed by 2-way ANOVA (node × NAA level) for each family because significant interactions were detected by 3-way ANOVA (family × node × NAA level) but not by 2-way ANOVA. Proportions were arcsine square root transformed, and shoot lengths and shoot numbers were square root or log transformed, when variance was heterogeneous. Duncan's multiple range tests were performed when significant differences among nodes were detected by ANOVA. Results for the two NAA levels (nil and 0.05 μM) were pooled because there were no significant node × NAA interactions in 2-way ANOVA, and because NAA had no significant effect on shoot proliferation in either family. Means are reported with standard errors, and treatment differences or interactions were regarded as significant at $P < 0.05$.

Results and discussion

Rooting, shoot elongation and shoot proliferation differed greatly among the first four or five nodes of *C. torelliana* × *C. citriodora* seedlings when they were propagated in node culture. Shoots derived from the first (cotyledonary) node had higher rooting and shoot length than shoots from nodes 3 and 4 on all occasions for family 13 (Figs 2a, 2c) and higher rooting or shoot length than shoots from nodes 3, 4 or 5 on some occasions for family 19 (Figs 2b, 2d). In each case, the higher rooting or shoot length was translated into higher proliferation and, for both families, the final number of shoots produced from the cotyledonary node was higher than from other nodes (Figs 2e, 2f).

These effects were not the result of a simple difference between the cotyledonary node and the true leaf nodes, as a gradient existed from the cotyledonary to the second node and from the second node to subsequent nodes. This gradient was clearer for the family (13) with higher amenability to node culture. In this family, rooting, length and proliferation of shoots from node 2 did not differ from those of the cotyledonary node at 4 or 10 weeks, and final rooting, shoot length and shoot number from node 2 were intermediate between those of the cotyledonary node and nodes 3 and 4. However, there was a greater distinction in proliferation capacity between the cotyledonary node and the true leaf nodes for the family (19) with lower amenability to node culture. Here, rooting and length of shoots derived from node 2 did not differ significantly from those of the cotyledonary node at 4 and 10 weeks, but shoot numbers on every occasion differed significantly between all true leaf nodes and the cotyledonary node. A notable feature of family 19 was the very poor proliferation

capacity of the true leaf nodes (nodes 2–5), which typically provided just one or two shoots in node culture (Fig. 2f).

These gradients in proliferation capacity are very similar to those observed in node cultures of other plants. Shoot proliferation is highest from basal nodes, lower from middle nodes, and lowest from apical nodes of *in vitro* seedlings of *Quercus robur* (Volkaert et al. 1990) and *Corymbia citriodora* (Koriesh et al. 2003). In the case of *C. citriodora* (Koriesh et al. 2003), shoot proliferation was monitored for a single passage (compared with three passages in the present study) but shoot numbers did decline from 4.6 to 2.9 to 2.3 from basal to intermediate to apical nodes, respectively, very similar to the values obtained in the first proliferation passage for *C. torelliana* × *C. citriodora* (Figs 2e, 2f). Proliferation is also highest when nodes are dissected from the basal, rather than apical, end of less-juvenile shoots of *Vitis rotundifolia* (Sudarsono and Goldy 1991), *Maytenus ilicifolia* (Pereira et al. 1995), *Rosa* spp. (Ma et al. 1996) and *Nothofagus obliqua* (Martínez Pastur et al. 1998).

In contrast, a morphogenic gradient was not evident for shoot length or shoot proliferation when the first four or five nodes of *C. torelliana* × *C. citriodora* seedlings were propagated in organogenic culture. Shoots did not form roots in organogenic culture, and significant effects of node position on shoot length were inconsistent across families and only evident at 4 weeks (Figs 3a, 3b). Shoot numbers were slightly higher from node 3 than from other nodes for family 13 at 4 weeks, but node position had no effect on shoot numbers in organogenic culture at 10 or 15 weeks (Figs 3c, 3d). These results differ from many other species, where proliferation capacity in organogenic culture is highest from explants dissected from the apical end of shoots. The percentage of explants forming adventitious shoots and the number of shoots per regenerating explant decline progressively from 82.5% to 20.0% and 4.5 to

1.8, respectively, from the most apical to the fourth-most apical node of *Alstroemeria* shoots (Lin et al. 1998). Pre-existing meristematic structures are not evident in the *Alstroemeria* leaf axils, but adventitious shoot production is induced by a treatment of thidiazuron and IBA followed by BA, which promotes the development of meristemoids from nodal epidermal cells (Lin et al. 1998). Shoot production declines from 18.5 to 11.0 shoots per explant between the four most-apical nodes and the next four nodes of *Vanilla planifolia* shoots (Lee-Espinosa et al. 2008). These authors described the BA-induced *V. planifolia* shoots as 'adventitious' although their cellular origin, like those of *C. torelliana* × *C. citriodora*, has not been determined histologically. In practice, it can be difficult to distinguish between shoots arising from existing meristems (axillary shoot proliferation), from differentiated cells of a freshly transferred piece of plant tissue (direct or permissive organogenesis) or from dedifferentiated callus cells (indirect organogenesis) when meristems and other tissues are surrounded by callus (George 1993).

The topophysic effects on shoot proliferation evident in node cultures of *C. torelliana* × *C. citriodora* resulted from differences in rooting capacity and shoot length of individual nodes. The first four or five nodes were equally capable of shoot proliferation in organogenic culture, where rooting and rapid stem elongation do not occur but proliferation occurs through an apparent combination of axillary shoot proliferation, basal stem callogenesis and shoot regeneration (Figs 1d–f). However, there was a strong, negative morphogenic gradient in rooting capacity from the first to the fourth node in node culture. A causal relationship between rooting and shoot length cannot be demonstrated, although neither shoot length of the rooted shoots nor shoot length of the unrooted shoots varied significantly among nodes at 10 or 15 weeks (data not presented). For example, among the nodes at 15 weeks, mean shoot

lengths of rooted shoots varied between 52.5 ± 3.8 and 55.8 ± 6.1 mm (family 13) and between 55.7 ± 4.9 and 60.0 ± 13.0 mm (family 19), whereas mean shoot lengths of unrooted shoots varied between 13.7 ± 0.9 and 16.1 ± 1.0 mm (family 13) and between 12.2 ± 0.8 and 13.3 ± 1.5 mm (family 19). In any case, root formation was associated with rapid formation of long, multi-nodal axillary shoots (Fig. 1c), and so higher rooting from the cotyledonary node (and second node for family 13) was associated with higher shoot proliferation. These topophysic effects in node culture reflect those described in vegetative propagation of *Eucalyptus grandis* seedlings, where rooting of cuttings declines progressively from >95% at the cotyledonary node to <5% at the eleventh node (Paton et al. 1970).

Most *C. torelliana* × *C. citriodora* shoots were converted to plantlets in IVS medium, both after node culture and organogenic culture (Fig. 4), and almost 100% of the plantlets were successfully transferred to the nursery. The rooting percentages (61–100%) were comparable with those of other Myrtaceae species in the IVS media of Newell et al. (2003, 2005). Importantly, although shoot proliferation gradients differed between node and organogenic cultures, plantlet conversion and the number of primary roots per plantlet did not differ significantly among node positions. This allows for the segregation of individual seedling nodes into a dual node and organogenic culture system for shoot proliferation and subsequent plantlet formation.

We envisage that the cotyledonary node could be transferred to node culture to establish a small number of plantlets of each clone as a nursery clonal archive (Fig. 1h) and source of cuttings for initial field testing. The true leaf nodes of each seedling could, meanwhile, be transferred to organogenic culture for laboratory storage or rapid proliferation (Figs 1d–f). Selection of *C. torelliana* × *C. citriodora* clones for disease resistance, frost tolerance, height and diameter takes 3–6 years (Lee 2007; Lee

et al. 2009) and so a clonal forestry program would, ideally, incorporate a laboratory method to store clones, possibly using minimal growth conditions or cryopreservation (Watt et al. 2000; Trueman 2006; Bunn et al. 2007; Sommerville et al. 2008; Padayachee et al. 2009).

In conclusion, establishment of *C. torelliana* × *C. citriodora* plantations has been hampered by limited hybrid seed supply and low amenability to propagation as rooted cuttings (Lee 2007; Shepherd et al. 2007, 2008; Trueman and Richardson 2008; Lee et al. 2009). We have shown that *C. torelliana* × *C. citriodora* can be propagated *in vitro* with very high rates of shoot proliferation and plantlet conversion, and that plant production can be optimised by appropriate explant selection based on an understanding of the different topophysic effects in node and organogenic culture.

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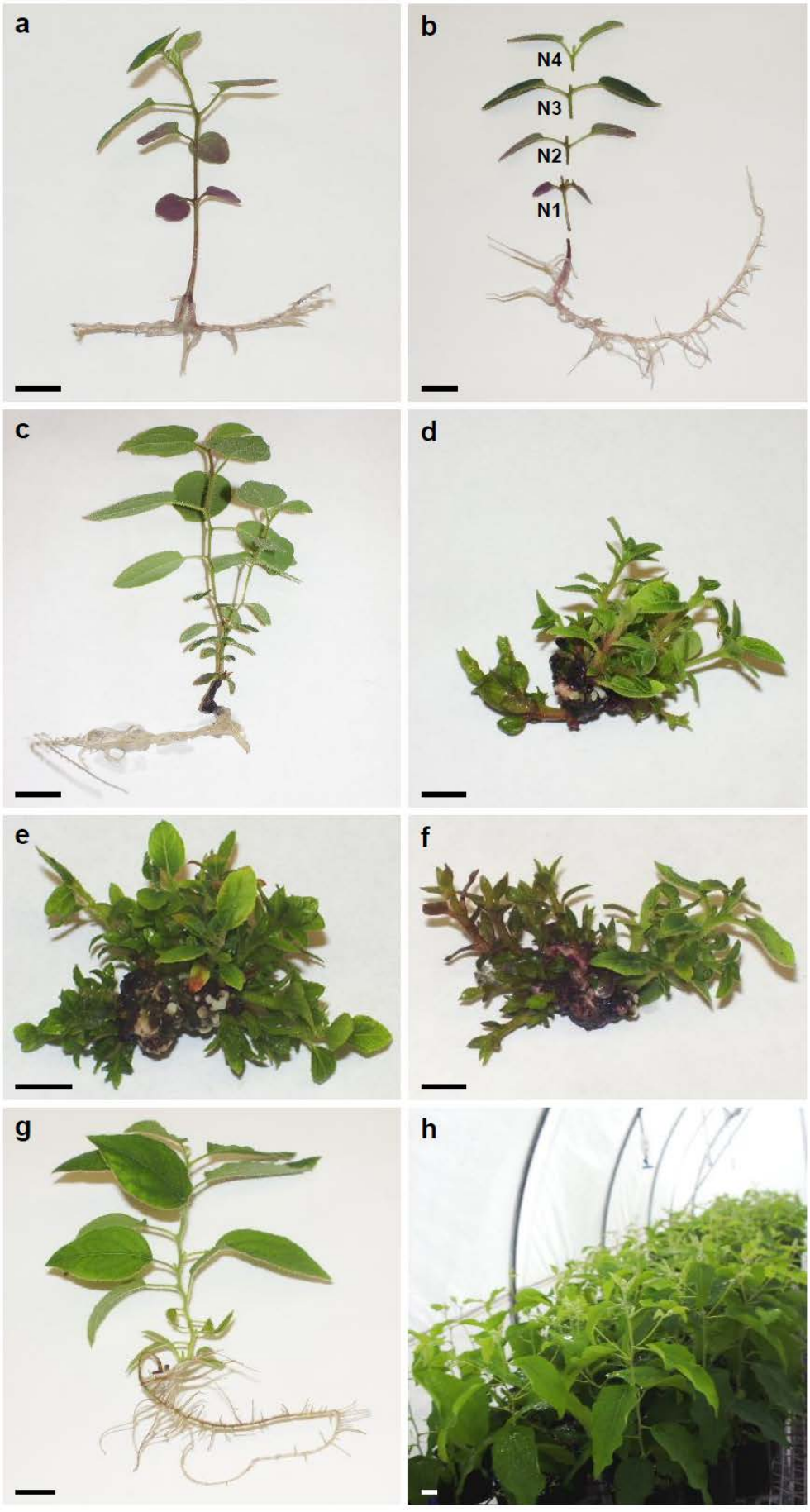
Figure captions

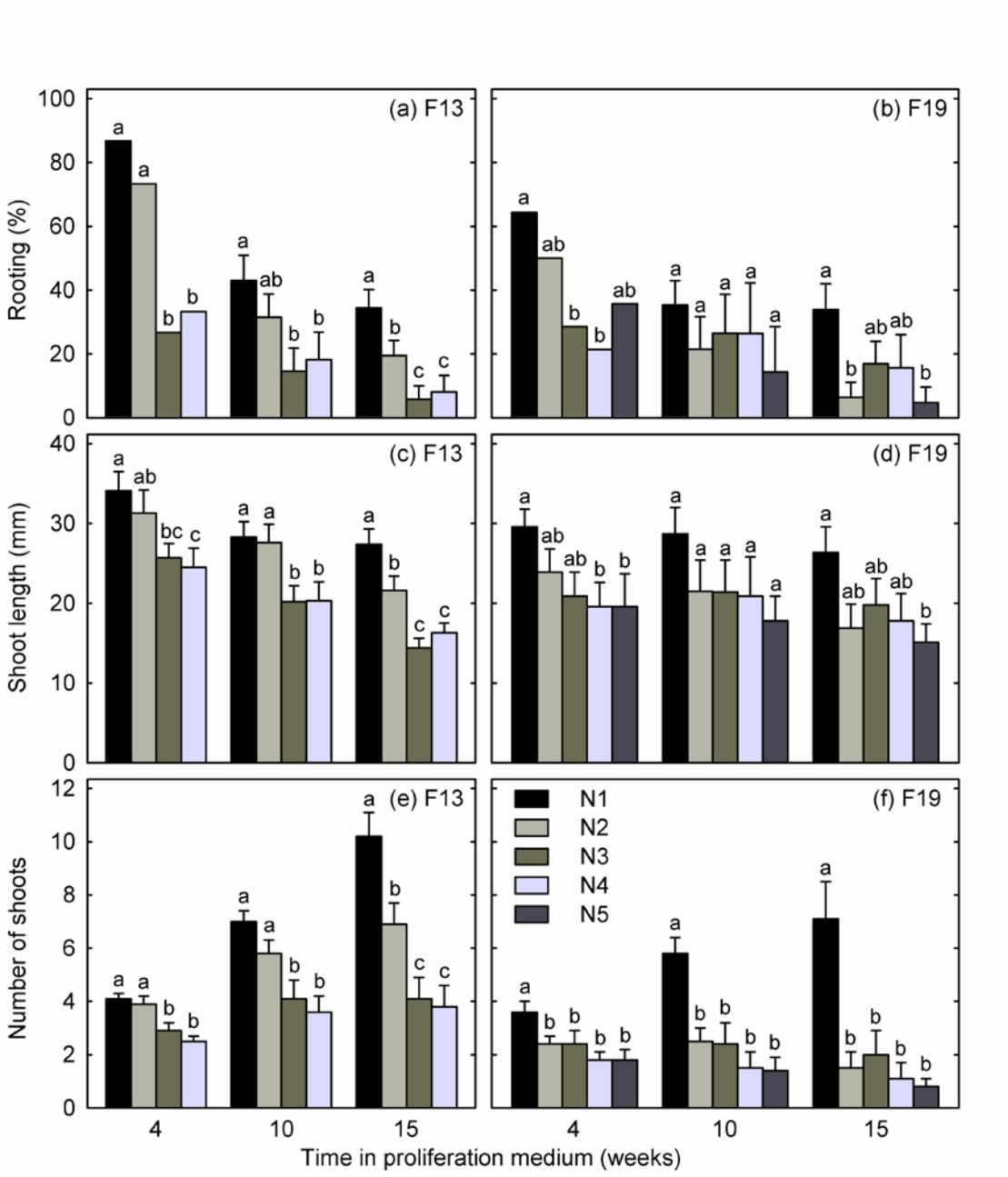
Fig. 1. Node culture and organogenic culture of *C. torelliana* × *C. citriodora*. (**a**) Seedling stem with four macroscopic nodes after 4 weeks in induction medium. (**b**) Seedling stem dissected into four nodes (N1 – N4) for transfer to proliferation medium. (**c**) Shoot that has produced roots and multi-nodal axillary shoots in node culture. (**d–f**) Explants that have formed axillary shoots, basal callus and adventitious shoots in organogenic culture. (**g**) Plantlet extracted from *in vitro* soil-less medium. (**h**) Plantlets transferred to the nursery. *Bar* = 1 cm.

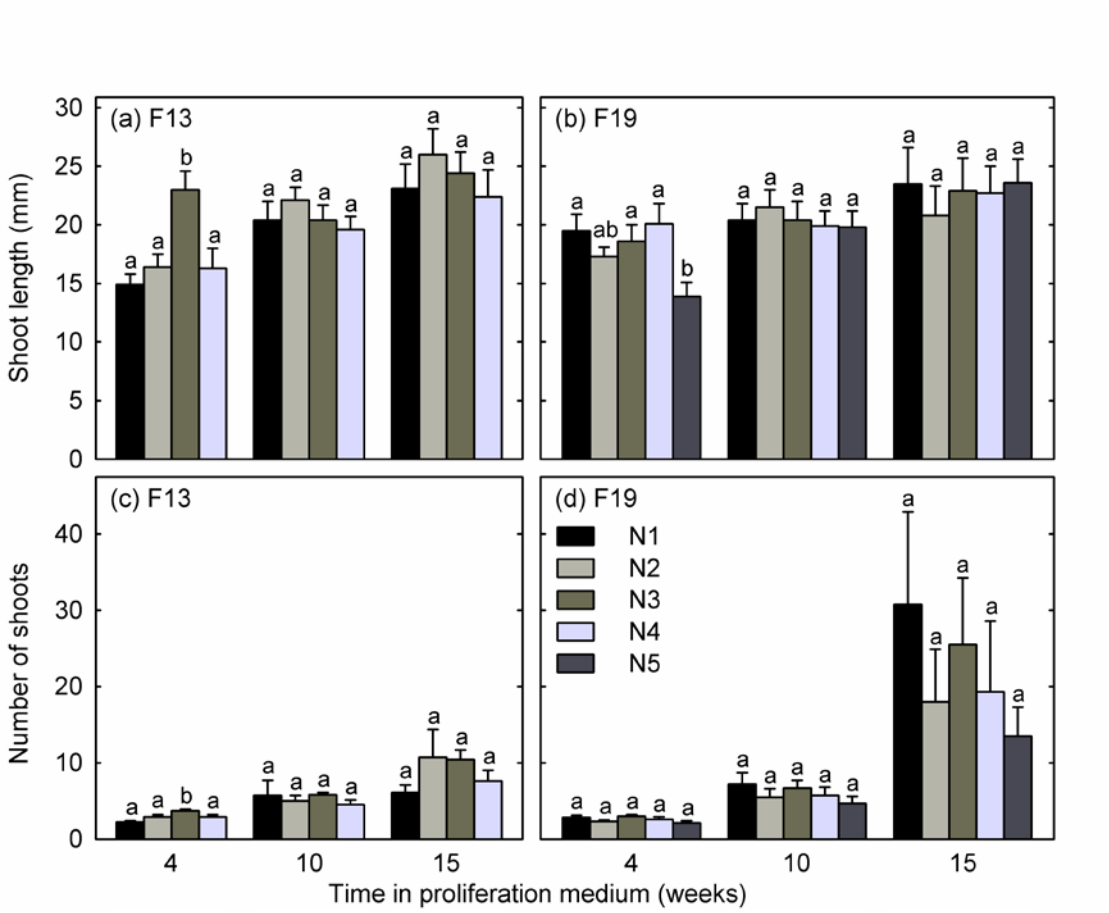
Fig. 2. Effect of seedling node position on (**a**, **b**) rooting, (**c**, **d**) shoot length and (**e**, **f**) shoot production of two *C. torelliana* × *C. citriodora* families (F13, F19) after 4, 10 and 15 weeks of proliferation in node culture. 'N1' is the cotyledonary node; 'N4' or 'N5' is the apical node for families 13 and 19, respectively. Means are provided with s.e. (n = 6–17). Different letters indicate significant differences among four or five nodes (ANOVA and Duncan's multiple range test, $P < 0.05$).

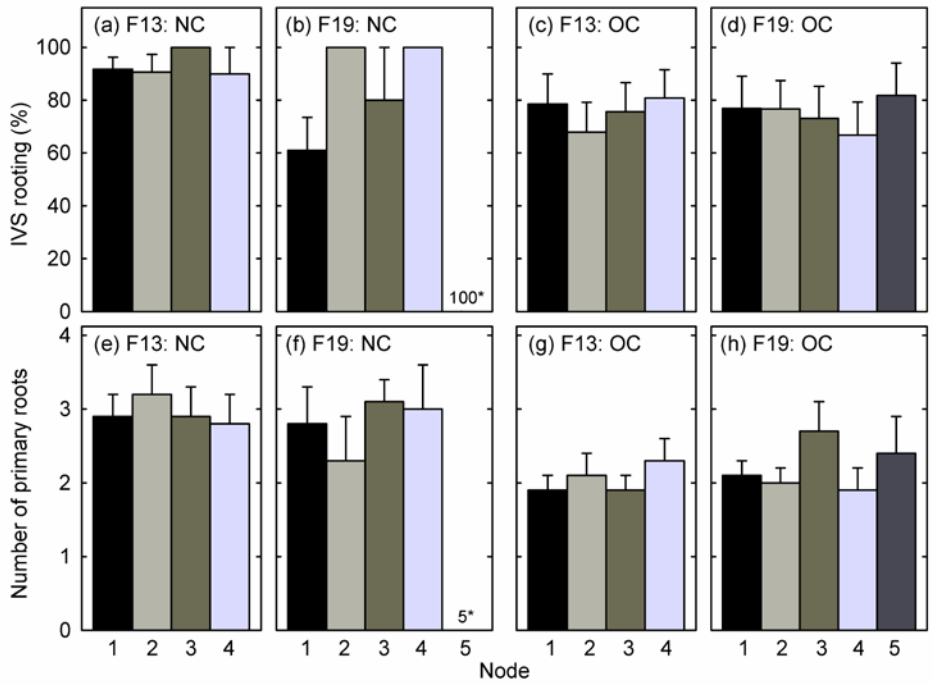
Fig. 3. Effect of seedling node position on (**a**, **b**) shoot length and (**c**, **d**) shoot production of two *C. torelliana* × *C. citriodora* families (F13, F19) after 4, 10 and 15 weeks of proliferation in organogenic culture. 'N1' is the cotyledonary node; 'N4' or 'N5' is the apical node for families 13 and 19, respectively. Means are provided with s.e. (n = 13–15). Different letters indicate significant differences among four or five nodes (ANOVA and Duncan's multiple range test, $P < 0.05$).

Fig. 4. Effect of seedling node position on (**a**, **b**, **c**, **d**) percentage of shoots with roots and (**e**, **f**, **g**, **h**) number of primary roots per plantlet for two *C. torelliana* × *C. citriodora* families (F13, F19) raised in (**a**, **b**, **e**, **f**) node culture (NC) or (**c**, **d**, **g**, **h**) organogenic culture (OC) and then transferred to *in vitro* soil-less (IVS) mixture. Node 1 is the cotyledonary node; Node 4 or Node 5 is the apical node for families 13 and 19, respectively. 100* and 5* represent results from a single shoot. Four or five means (+ s.e.) did not differ significantly (ANOVA, $P > 0.05$, n = 3–16).


a
b
N4
N3
N2
N1
c
d
e
f
g
h


(a) F13
(b) F19
(c) F13
(d) F19
(e) F13
(f) F19
Rooting (%)
Shoot length (mm)
Number of shoots
Time in proliferation medium (weeks)
N1
N2
N3
N4
N5


(a) F13
(b) F19
(c) F13
(d) F19
Shoot length (mm)
Number of shoots
N1
N2
N3
N4
N5
Time in proliferation medium (weeks)
4
10
15


(a) F13: NC
(b) F19: NC
(c) F13: OC
(d) F19: OC
IVS rooting (%)
100*
(e) F13: NC
(f) F19: NC
(g) F13: OC
(h) F19: OC
Number of primary roots
5*
Node