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In vitro Storage Delays the Maturation of African Mahogany (*Khaya senegalensis*) Clones

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ABSTRACT

This study determined whether *in vitro* storage of *Khaya senegalensis* clones could delay their maturation and increase their capacity for adventitious rooting. Forestry trees display the desired traits of high adventitious rooting, rapid stem growth and long internodes when they are propagated from juvenile rather than mature explants. *In vitro* storage has the potential to maintain the juvenility of clones during the period of clonal field selection but there is little empirical evidence to show that *in vitro* storage delays clonal maturation. This study assessed the maturation of 20 clones that had been stored for 12 months using two different methods: (1) as *ex vitro* stock plants in the nursery; or (2) as shoots *in vitro* at 25°C. *K. senegalensis* proved amenable to vegetative propagation, with high rooting percentages (76±4%) and adventitious root numbers (5.2±0.4) from the cuttings of nursery-stored stock plants. However, storing shoots *in vitro* for 12 months provided higher rooting percentages (89±2%) and adventitious root numbers (6.3±0.5). These results demonstrate that *K. senegalensis* clones did undergo significant maturation in the nursery and that *in vitro* storage delayed their maturation. *In vitro* storage is, therefore, an effective method for clonal archiving that minimises investments in water, fertiliser, pesticide and space while providing planting stock with the juvenile-phase characteristics required for forestry plantations.

Key words: *Cedrela*, cuttings, meliaceae, propagation, *Swietenia*, tissue culture

INTRODUCTION

An advantage of plant tissue culture is the capacity to preserve species with minimal climatic risk and without the large investments in water, fertiliser, pesticide and space that are associated with nursery or broad-acre germplasm repositories (Watt *et al.*, 2001; Benson, 2008; Sharma *et al.*, 2013). *In vitro* storage has also been used to preserve plant germplasm while pre-commercial clones are tested under field conditions (Mason *et al.*, 2002; Aimers-Halliday and Burdon, 2003; Trueman, 2006). Many plantation trees, such as those used for timber and pulp production, display the desired traits of high adventitious rooting, rapid stem growth, long internodes and commitment to vegetative development when they are propagated from juvenile instead of mature explants (Greenwood, 1995; Mitchell *et al.*, 2004; Pijut *et al.*, 2011). The genetic gains made by a clonal

selection program can be eroded if selected clones have lost juvenility during the period of field testing and clonal archiving (Mitchell *et al.*, 2004; Rasmussen and Hunt, 2010). *In vitro* storage has the potential to slow the onset of maturation in pre-commercial clones (Aimers-Halliday and Burdon, 2003; Trueman, 2006) but there is little empirical evidence to demonstrate that minimal growth storage delays clonal maturation.

This study compared the adventitious rooting capacity of *ex vitro* stock plants from 20 clones that had been stored using two different methods: (1) as nursery stock plants; and (2) as shoots *in vitro*. The study species was the African mahogany, *Khaya senegalensis*, which is native to western and central Africa but which has been introduced widely across tropical Australia, China, Vietnam, Malaysia, Indonesia and Sri Lanka for high-value timber and amenity plantings (Arnold *et al.*, 2004). Low seed production in seed orchards and plantations has limited the availability of desired genotypes of *K. senegalensis* and so development of micropropagation and rooted cutting methods for germplasm storage and mass production are priorities of a genetic improvement program aimed at selecting high-quality timber-yielding clones (Nikles *et al.*, 2008; Hung and Trueman, 2011a, b, 2012).

MATERIALS AND METHODS

Plant material and treatments: Twenty *K. senegalensis* clones were produced by germinating seeds from a Burkina Faso provenance *in vitro* (Fig. 1a) and then proliferating their shoots in full-strength MS media (Fig. 1b), as described by Hung and Trueman (2011a). After approximately 5 months, shoots from each of the 20 clones were subdivided randomly and transferred to three treatments:

- Some shoots were converted immediately to plantlets by transferring them to half-strength MS medium containing 19.6 μM indole-3-butyric acid (IBA) for 1 week, hormone-free half-strength MS medium for 3 weeks and then to potting mix in 90-cm^3 propagation tubes, as described by Hung and Trueman (2011a). The plantlets were transferred to a translucent-white polyethylene propagation chamber for 4 weeks and then transplanted into 1.6-L pots (Fig. 1c, d) filled with a stock plant mix (Kilkenny *et al.*, 2012; Trueman *et al.*, 2013a, b) consisting of a 75/25 (v/v) mixture of shredded pine bark and perlite, with 3 kg of 8-9 month slow release Osmocote™ fertiliser (Scotts International, Heerlen, The Netherlands), 3 kg of lime (Unimin, Lilydale, Australia), 1 kg of gypsum (Queensland Organics, Narangba, Australia), 1 kg of Micromax™ granular micronutrients and 1 kg of Hydroflo™ soil wetting agent (both from Scotts Australia, Baulkham Hills, Australia) incorporated per m^3. These 'Nursery' plantlets were then stored and managed as stock plants under full sunlight for 12 months in Gympie (26°11'S, 152°40'E), Australia
- Other shoots were transferred to full-strength MS medium and stored for 12 months at 25°C with a 16 h photoperiod (~100 μmol $m^{-2} s^{-1}$ irradiance; Fig. 1e), with subculture to fresh medium every 2 months. Shoots were then converted to plantlets (as described above) and these 'Laboratory' plantlets were transferred to the polyethylene chamber, transplanted into 1.6 L pots with stock plant mix and then moved to full sunlight alongside the Nursery stock plants
- Other shoots were transferred to full-strength MS medium and stored for 12 months at 14°C ith a 16 h photoperiod (~10 μmol m^{-2} sec^{-1} irradiance), with subculture to fresh medium after 6 months. However, no shoots survived 12 months of this 'Cool storage' treatment

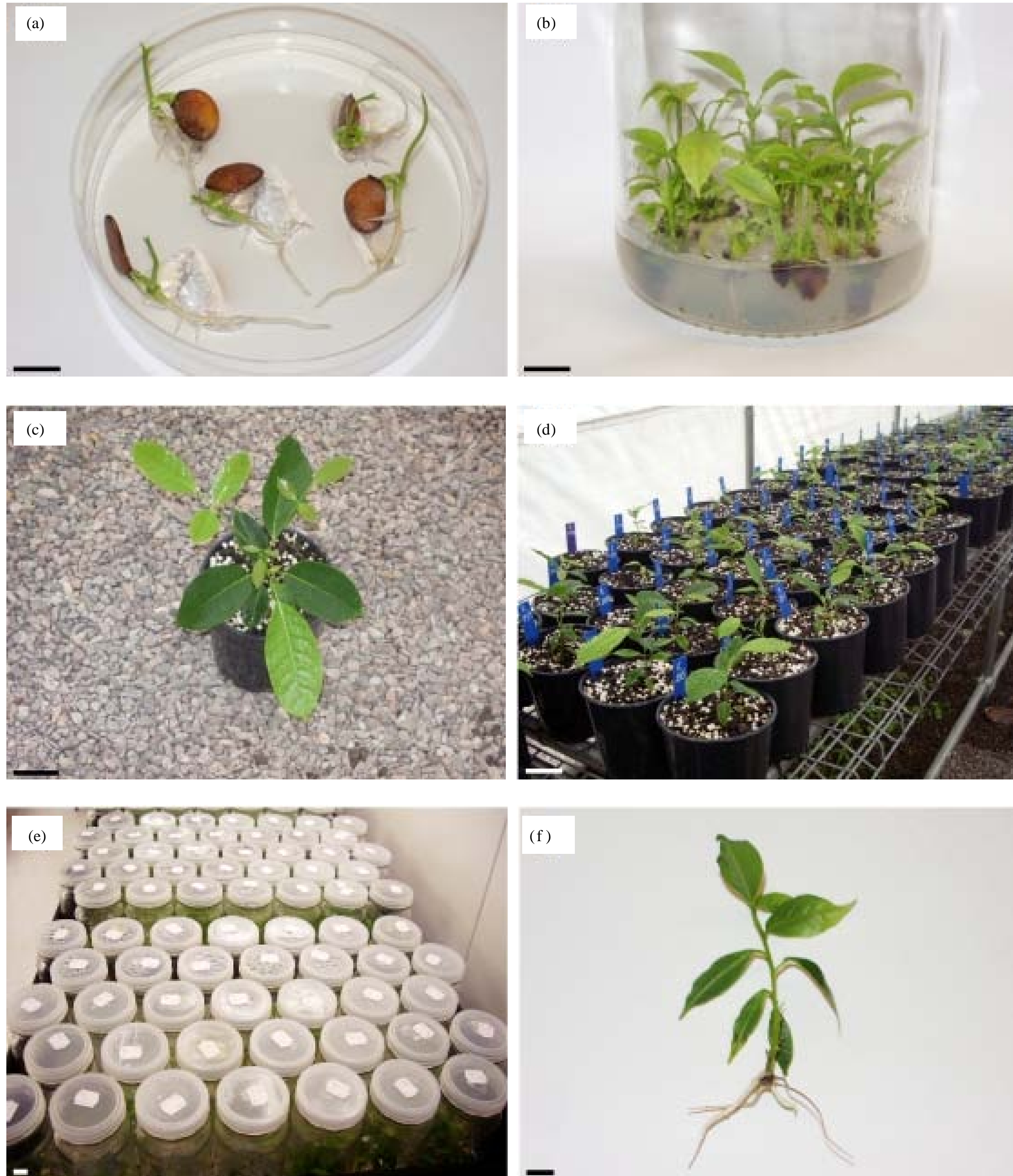

Fig. 1(a-f): Propagation, storage and rooted cutting production of *Khaya senegalensis* clones. (a) Seed germination *in vitro*, (b) Shoot proliferation *in vitro*, (c) Plantlet growing in a 1.6 L pot, (d) Stock plants in the polyethylene chamber, (e) Laboratory storage of shoots at 25°C and (f) Rooted cutting, Scale bars: (a), (b), (e), (f) 1 cm (c), (d) 3 cm

Stock plant management: The 'Nursery' and 'Laboratory' plants were managed as stock plants for a further 9 months by regular pruning to ~30-cm height and ~20-cm diameter. Cuttings were then collected to produce fresh stock plants from each clone and each treatment. This ensured that subsequent treatment differences would be due to the maturational state, rather than the size, of the stock plants. The cuttings, approx. 4-cm length, were dipped 0.5 cm into powder containing 3 g k^{-1} IBA (Opuni-Frimpong *et al*., 2008) and placed 1 cm deep in a 90 cm^3 tube containing a cutting mix (Kilkenny *et al*., 2012; Trueman *et al*., 2013a, b) consisting of a 75/25 (v/v) mixture of perlite and shredded pine bark with 3 kg of 8-9 month slow-release Osmocote™ fertiliser and 1 kg of gypsum incorporated per m^3. Propagation tubes were placed in an adjacent glasshouse, with mist irrigation provided for 10 sec every 15 min from 0600-1800 h and 10 sec every 20 min from 1800-0600 h. Rooted cuttings (Fig. 1f) were transplanted to 1.6-L pots with stock plant mix, moved to full sunlight and managed as stock plants for another 12 months (Fig. 2).

Adventitious rooting capacity: Cuttings were harvested from each stock plant on two occasions at 2 months apart, treated with 3 g k^{-1} IBA, placed into cutting mix and moved to the glasshouse under mist irrigation (as above). At least five cuttings were set for each treatment×clone combination on each occasion. All cuttings were gently removed at 8 weeks after insertion and the number of adventitious roots on each cutting was recorded. Rooted cuttings were rinsed carefully to remove the cutting mix and the roots were dissected from the shoot. The roots and shoot were placed in separate paper bags, dried at 55°C and weighed.

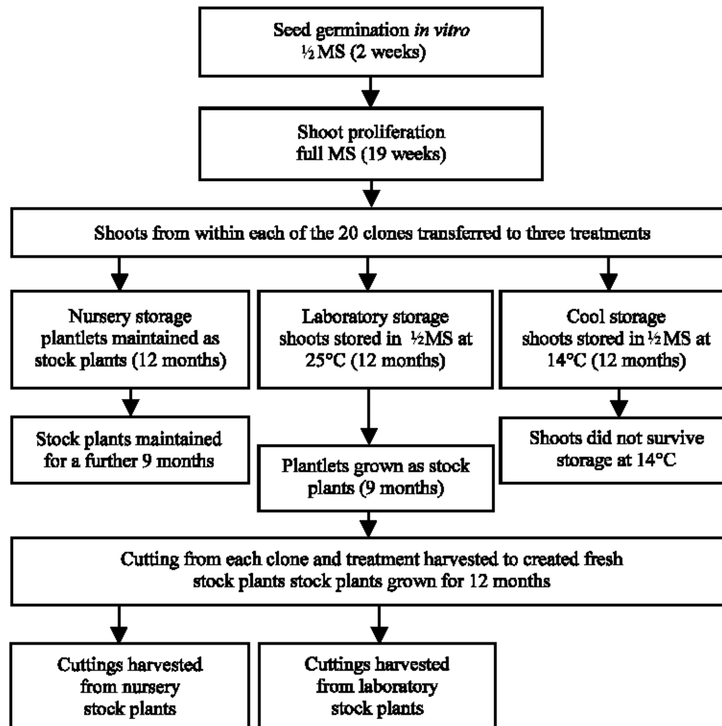

Fig. 2: Summary of the propagation and storage treatments for shoots and stock plants of *Khaya senegalensis*

Statistical analyses: The percentage of cuttings forming roots, the average number of adventitious roots per rooted cutting and the average root and shoot masses of rooted cuttings, were calculated for each treatment×clone combination on each occasion. Data were analysed by paired t-tests, with the pairs comprising the two treatments within each of the 20 clones on each occasion. Means are reported with standard errors and treatment differences were regarded as significant at $p<0.05$.

RESULTS AND DISCUSSION

K. senegalensis proved highly amenable to vegetative propagation, with rooting percentages from routine nursery stock plants (76±4%; Fig. 3a) being above the 70% level preferred by commercial nurseries (Trueman, 2006; Hunt *et al.*, 2011). These rooting percentages were very

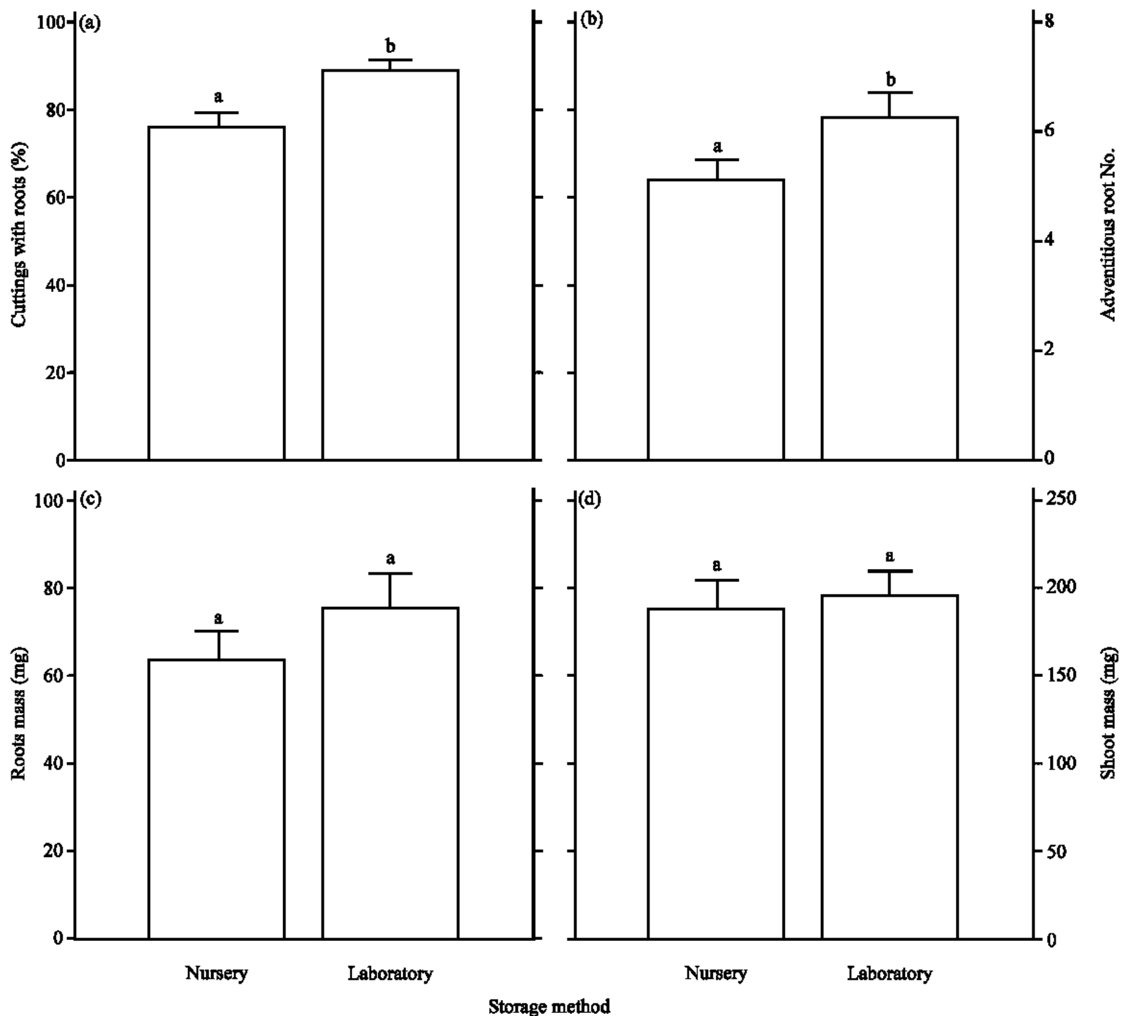

Fig. 3(a-d): Juvenility indicators for *Khaya senegalensis*. (a) Percentage of cuttings that formed adventitious roots, (b) Number of adventitious roots per rooted cutting, (c) Root mass per rooted cutting and (d) Shoot mass per rooted cutting, for *ex vitro* clones that were stored simultaneously for 12 months as nursery stock plants (Nursery) and as shoots *in vitro* (Laboratory). Means with different letters are significantly different paired t-tests; $p<0.05$; n = 35-38

similar to those from 12-month-old stock plants of two other African mahoganies, *K. anthotheca* (80±3%) and *K. ivorensis* (75±3%) (Opuni-Frimpong *et al.*, 2008). Nevertheless, the alternative method of storing *K. senegalensis* shoots *in vitro* for 12 months provided a higher rooting percentage (89±2%; Fig. 3a), very similar to the rooting percentage (90±5%) obtained with juvenile shoots from the same provenance *in vitro* (Hung and Trueman, 2011a). This demonstrates that *K. senegalensis* clones underwent maturation when they were stored as nursery stock plants and it supports previous findings from *K. anthotheca* that rooting percentages decline (to 66±3%) as the stock plant age increases from 12 to 36 months (Opuni-Frimpong *et al.*, 2008).

The juvenility of *in vitro*-stored shoots of *K. senegalensis* was also evident as an increased number of adventitious roots per rooted cutting (from 5.2±0.4-6.3±0.5 roots; Fig. 3b). Root mass (Fig. 3c), shoot mass (Fig. 3d) and total cutting mass (not presented) were not affected significantly by the clonal storage method. The capacity to increase root number by maintaining clones *in vitro* could be important for plantation establishment because the stability of *ex vitro* derived trees can be compromised by a low number of adventitious roots (Mokotedi *et al.*, 2010) and because tree survival and growth can be positively related to the number of adventitious roots (Haines *et al.*, 1992; Goldfarb *et al.*, 1998; Foster *et al.*, 2000). However, these studies showed benefits as the number of adventitious roots increased from 1 to 5, whereas most rooted cuttings of *K. senegalensis* possessed at least 5 roots. The adventitious root numbers on cuttings compared favourably with plantlets produced from juvenile shoots of the same provenance *in vitro*, which generally produced 3-4 roots (Hung and Trueman, 2011a).

This study has demonstrated that juvenility of *K. senegalensis* clones, expressed as a greater capacity to form adventitious roots, can be maintained by storing shoots *in vitro* rather than as *ex vitro* nursery plants. These results highlight one of the potential benefits of plant tissue culture; specifically, the ability to store pre-commercial varieties in their juvenile phase and delay their maturation (Mason *et al.*, 2002; Aimers-Halliday and Burdon, 2003; Trueman, 2006). Clonal forestry programs have typically used nursery or broad-acre repositories of pruned stock plants (i.e., 'hedges') to maintain archives of clones during the field selection period or to rejuvenate selected clones after field selection (Aimers-Halliday and Burdon, 2003; Mitchell and Jones, 2006; Trueman, 2006; Saya *et al.*, 2008; Brondani *et al.*, 2012). However, *in vitro* storage, which requires less investment in water, fertiliser, pesticide and space, appears more effective for clonal forestry programs that require planting stock to possess the juvenile-phase characteristics of high adventitious rooting, rapid stem growth, long internodes and commitment to vegetative ratherthan reproductive growth (Greenwood, 1995; Mitchell *et al.*, 2004; Majada *et al.*, 2012).

CONCLUSION

The African mahogany, *K. senegalensis*, was highly amenable to vegetative propagation but stock plants did undergo significant maturation in the nursery. Maturation could be delayed by storing shoots *in vitro*. Rooting percentages were raised from 76±4 to 89±2% by storing shoots of each clone in the laboratory for 12 months rather than maintaining stock plants of each clone in the nursery for the same period.

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