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In vitro propagation of the African mahogany *Khaya senegalensis*

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Abstract A protocol was developed for shoot proliferation and plantlet formation of *Khaya senegalensis*, an important medicinal and timber plantation species introduced to Australia and southern Asia from western and central Africa. We assessed effects of the plant growth regulators, benzyladenine, kinetin, naphthalene acetic acid and gibberellic acid, on shoot proliferation and subsequent plantlet conversion. Shoot proliferation over four passages was higher in media containing benzyladenine than in media containing other growth regulators, and optimal proliferation from seed of three different sources was consistently obtained in medium containing 4.4 μM benzyladenine. Shoots from this medium were converted to plantlets at high frequencies (76–90%) after treatment with 19.6 μM indole-3-butyric acid, and almost all plantlets were successfully acclimatized to nursery conditions. These methods provide the means for establishing in vitro and ex vitro clone banks of juvenile *K. senegalensis* trees for field selection of desired genotypes and tropical plantation establishment.

Keywords *Khaya* · Mahogany · Meliaceae · Plantlet · Propagation · Rooting

Introduction

The genus *Khaya* comprises seven species, native to tropical Africa and Madagascar, within subfamily Swietenioideae of the mahogany family Meliaceae (Pennington and Styles 1975; Styles 1981). Meliaceae contains many commercially important tree species including *Azadirachta indica* (neem tree), *Cedrela odorata* (Spanish cedar), *Melia azedarach* (paradise tree), *Swietenia macrophylla* (big-leaf mahogany) and *Toona ciliata*

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(Australian red cedar) (Vila et al. 2002; Mroginski et al. 2003; Quraishi et al. 2004; Schottz et al. 2007; Cameron 2010). The four *Khaya* species that occur in western Africa, *K. anthotheca*, *K. grandifoliola*, *K. ivorensis* and *K. senegalensis*, are commonly known as the African mahoganies. These species are valued for timber, medicinal products and amenity plantings throughout their natural distributions in western, central and eastern Africa (Pennington and Styles 1975; Styles 1981; Nikles et al. 2008). *Khaya* provides a major source of revenue to many countries because of its hard, durable timber, which is widely sought for construction, furniture and carpentry (Ofori et al. 2007; Nikles et al. 2008; Opuni-Frimpong et al. 2008a). The highly desired bark, seeds, leaves and other parts of *Khaya* trees are also used traditionally for treatment of diseases including cancer, diarrhoea, dysentery and malaria (Zhang et al. 2007; Makut et al. 2008; Kubmarawa et al. 2009). The African mahoganies are classified as 'vulnerable' because of habitat loss and over-harvesting for timber and medicinal products (Ofori et al. 2007; Nikles et al. 2008).

One of the African mahoganies, *K. senegalensis*, has been introduced widely across tropical Australia, China, Vietnam, Malaysia, Indonesia and Sri Lanka for forestry plantations and amenity plantings (Arnold et al. 2004). The species has proven suitable for high-value timber plantations in northern Australia, where the trees are not exposed to the mahogany shoot borer, *Hypsipyla robusta* (Arnold et al. 2004; Nikles et al. 2008). A genetic improvement program, aimed at selecting high-quality timber-yielding clones and families, commenced in Australia in 2001. However, limited seed production in seed orchards and plantations has limited the availability of desired genotypes of *K. senegalensis*, and development of micropropagation methods for mass production and germplasm storage is a main priority of the program (Nikles et al. 2008).

There are very few reports on micropropagation of *Khaya* species. Initial shoot induction has been described from the single-node shoots of freshly-germinated *K. senegalensis* seeds (Danthu et al. 2003). These authors induced axillary shoots in full-strength Murashige and Skoog (MS) medium containing 2.2 or 8.9 μM benzyladenine (BA), although the maximum number of shoots obtained after the single passage of their study was only 2.5 per seedling. Axillary shoot proliferation has been described from nodal explants of *K. ivorensis* stock plants, with final shoot numbers being highest in full-strength MS medium containing 22.2 μM BA, 0.5 μM α-naphthalene acetic acid (NAA) and 2.9 μM gibberellic acid (GA_3) (Mathias 1988). Reports on micropropagation of other Meliaceae trees have also used MS media containing BA for initiating or proliferating shoots, with the BA frequently, though not always, supplemented with another growth regulator such as NAA, indole-3-butyric acid (IBA), GA_3 or kinetin (Daniel et al. 1999; Vila et al. 2002; Mroginski et al. 2003; Tacoronte et al. 2004; Schottz et al. 2007; Shahin-uz-zaman et al. 2008; Hajari et al. 2009; Husain and Anis 2009).

The aim of our research was to develop methods for micropropagation of *K. senegalensis*, including (1) shoot induction and shoot proliferation to establish a laboratory archive of juvenile clones, and (2) root formation to generate plantlets of the same clones. Very high proliferation rates are not necessary for establishment of the ex vitro clone banks, because *Khaya* species can be propagated from cuttings of juvenile stock plants (Nikles et al. 2008; Opuni-Frimpong et al. 2008b) and so sufficient rooted cuttings for initial field tests can be obtained from just a few stock plants of each clone. Therefore, we aimed to develop methods for axillary shoot proliferation (Schottz et al. 2007; Trueman and Richardson 2007; Hajari et al. 2009; Husain and Anis 2009) rather than adventitious shoot production, which might pose a greater risk of somaclonal variation (George 1993; Pijut et al. 2007). Specifically, we present the results of large factorial experiments assessing the

effects of BA, kinetin, NAA or GA_3, singly or in combination, on shoot induction, shoot proliferation and subsequent IBA-induced plantlet conversion.

Materials and methods

Experiment 1: shoot induction, proliferation, and plantlet conversion

Khaya senegalensis seeds from a natural provenance in Burkina Faso were obtained from the Hardwood Tree Improvement Group, Agri-Science Queensland. Seeds were refrigerated at 4°C until use. Seven hundred and eighty seeds were used for this experiment. Batches of five seeds were washed in 70% (v/v) ethanol for 1 min in 70-ml vials containing one drop of Tween 20, rinsed in sterile distilled water for 1 min, and then transferred to new vials containing sodium hypochlorite (1, 5 or 10%; w/v) and one drop of Tween 20. The vials were swirled for 5, 10, 20 or 40 min on an orbital shaker at 110 rpm. After three subsequent rinses in sterile distilled water, seeds were plated (five seeds per 90-mm Petri dish) onto germination medium consisting of half-strength MS basal salts and 20 g l^{-1} sucrose, solidified with 8 g l^{-1} agar, with pH adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Seeds were germinated at 25°C under a 16-h photoperiod ($\sim$50 μmol m^{-2} s^{-1} by cool white fluorescent tubes). Sodium hypochlorite concentration and duration had no significant effect on seed contamination or the percentage of seeds producing suitable shoots for transfer to shoot induction medium (2-way ANOVA; $P > 0.05$). Therefore, all upright, uncontaminated shoots were transferred randomly after 2 weeks to shoot induction media (Fig. 1).

Shoots were dissected at the point of emergence from the seed coat, and transferred to 375-ml glass jars containing 50 ml of one of the ten shoot induction treatments: 0, 1.1, 2.2, 4.4 or 8.9 μM BA combined with 0 or 0.3 μM NAA (Sigma, St. Louis, MO). All shoot induction and shoot proliferation media (see section below) consisted of full-strength MS medium containing 30 g l^{-1} sucrose, solidified with 8 g l^{-1} agar, with pH adjusted to 5.8 prior to autoclaving at 121°C for 20 min, and with jars maintained at 25°C under a 16-h photoperiod ($\sim$100 μmol m^{-2} s^{-1}). The number of replicate shoots per induction treatment ranged from 65 to 75. Length of the main shoot, and numbers of nodes and macroscopic lateral shoots, were recorded after 4 weeks. Each node, and any lateral shoot >10-mm in length, was dissected for transfer, and the number of excised shoots transferred to proliferation medium was recorded.

All excised shoots from each seedling (i.e. one clone) were then transferred to a 375-ml jar containing 50 ml of one of the eight shoot proliferation treatments: (1) control (no hormones), (2) 0.3 μM NAA, (3) 1.5 μM GA_3, (4) 4.7 μM kinetin, (5) 4.4 μM BA, (6) 4.4 μM BA + 0.3 μM NAA, (7) 4.4 μM BA + 1.5 μM GA_3, or (8) 4.7 μM kinetin + 1.5 μM GA_3. The 65–75 clones from within each of the ten shoot induction treatments were randomly transferred across the eight proliferation treatments, and so the number of replicate clones for each of the 80 induction × proliferation treatment combinations was 8–10. Each node, and any lateral shoot >10-mm in length, was dissected for transfer to fresh medium after 5 weeks. The same process was repeated after 10 weeks. After 15 weeks, shoots were dissected for root formation by only removing basal callus or existing roots. The number of shoots transferred was recorded after each of the three passages in proliferation medium.

After 15 weeks in proliferation medium, shoots were transferred to 375-ml glass jars containing half-strength MS medium supplemented with 19.6 μM IBA, and the jars were

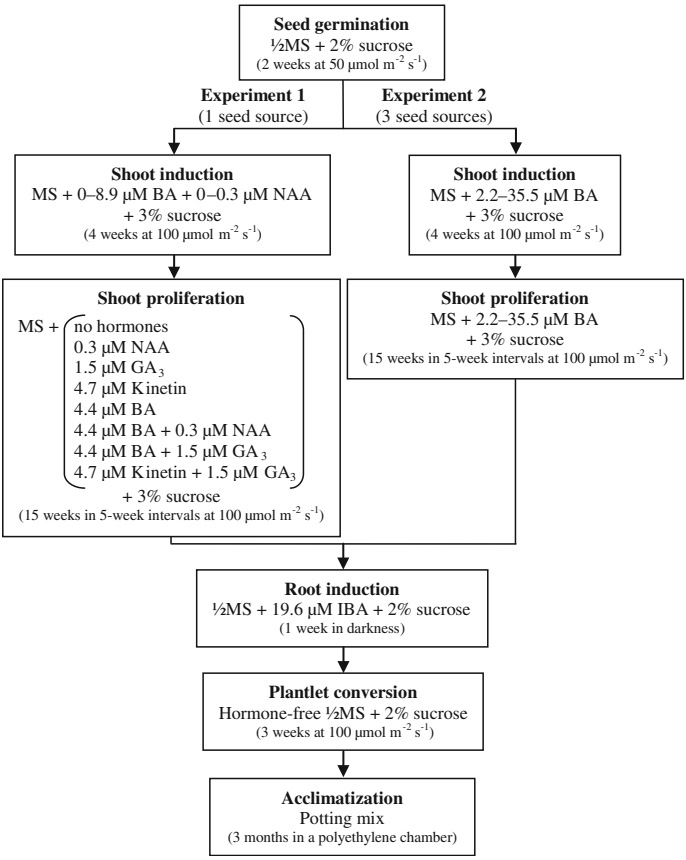

Fig. 1 Summary of the in vitro propagation experiments for *Khaya senegalensis*

placed in darkness at 22°C for 1 week. Shoots were then transferred to hormone-free half-strength MS medium and maintained at 25°C under a 16-h photoperiod ($\sim$100 µmol m^{-2} s^{-1}) for a further 3 weeks. All shoots were then gently extracted to determine the proportion of shoots from each clone that had formed roots and the number of adventitious roots per rooted shoot. Plantlets were rinsed of agar and transplanted into a potting mix containing composted pine bark, perlite, lime and slow-release Osmocote^{TM} fertiliser (Scotts International, Heerlen, The Netherlands) in 90-cm^3 propagation tubes (Trueman and Richardson 2008). Plantlets were grown in a polyethylene propagation chamber for 4 weeks, with 1-min misting provided at 10-min intervals for the first 2 weeks to maintain high relative humidity.

Experiment 2: optimised shoot induction and proliferation

This experiment assessed the optimal BA concentration for shoot induction and proliferation, using *K. senegalensis* seeds from three different sources: (1) seeds from a natural provenance in Burkina Faso, obtained from the Hardwood Tree Improvement Group, Agri-Science Queensland; (2) open-pollinated seeds of a selected Uganda-provenance clone (number 104) in a plantation trial in the Northern Territory, Australia, obtained from the

Northern Territory Department of Resources; and (3) a mixed seedlot (number 20851) from a plantation in Weipa, Queensland, Australia, obtained from the CSIRO Australian Tree Seed Centre. The Weipa plantation had been established from open-pollinated seeds of street trees in Darwin, Australia, which had mostly been planted using seeds from Senegal provenances. The three seed sources are referred to as 'Burkina Faso', 'Northern Territory' and 'Queensland', respectively, because the African provenances that contributed to the pedigrees of the open-pollinated Northern Territory and Queensland seeds are not completely known. All seeds were refrigerated at 4°C until use.

The seeds were surface-sterilised with 70% ethanol for 1 min and 5% NaOCl for 10 min, and germinated using the methods described for experiment 1 (above). After 2 weeks, 100 upright shoots per seed source were dissected at the point of emergence from the seed coat and transferred randomly to one of five media for shoot induction and shoot proliferation: full-strength MS medium with 2.2, 4.4, 8.9, 17.8 or 35.5 μM BA (Fig. 1). Shoots were induced on this medium for 4 weeks, and then dissected and transferred to fresh medium of the same composition for three 5-week passages of shoot proliferation. The vessels, media volumes, culture conditions, dissection methods, and number and duration of passages were as described for experiment 1. The number of shoots transferred after each proliferation passage was recorded for each clone.

After 15 weeks of shoot proliferation, a subsample of five shoots per clone from the treatments with 2.2, 4.4, 8.9 or 17.8 μM BA was transferred to root induction medium, using the methods described for experiment 1. Insufficient shoots were available from the other treatment (35.5 μM BA) to attempt plantlet conversion. Shoots were gently extracted after 4 weeks to determine the proportion of shoots from each clone that had formed roots and the number of adventitious roots per rooted shoot. Plantlets were transplanted to potting mix and grown in a polyethylene propagation chamber, as described for experiment 1.

Statistical analyses

Data for shoot length, and numbers of nodes, lateral buds and transferred shoots after 4 weeks in shoot induction medium, were analysed by 1-way ANOVA (10 induction media). The number of shoots transferred after each passage in proliferation medium, the proportion of shoots forming roots, and root number per rooted shoot were analysed by 2-way ANOVA (10 induction media × 8 proliferation media) in experiment 1 because there were no significant interactions between these two factors, and by 1-way ANOVA (5 media) in experiment 2. Proportions were arcsine square root transformed, and lengths or numbers were square root or log transformed, when variance was heterogeneous. Duncan's multiple range tests were performed when significant differences among treatments were detected by ANOVA. Means are reported with standard errors, and treatment differences or interactions were regarded as significant at $P < 0.05$.

Results and discussion

Experiment 1

Seedlings of *K. senegalensis* grew vigorously and formed a well-developed root system and a shoot with length > 20 mm within 2 weeks of plating (Fig. 2a). After germination, a medium containing cytokinin was the most effective for shoot induction. Addition of BA to induction medium did not affect the length of shoots or, in most cases, the number of nodes

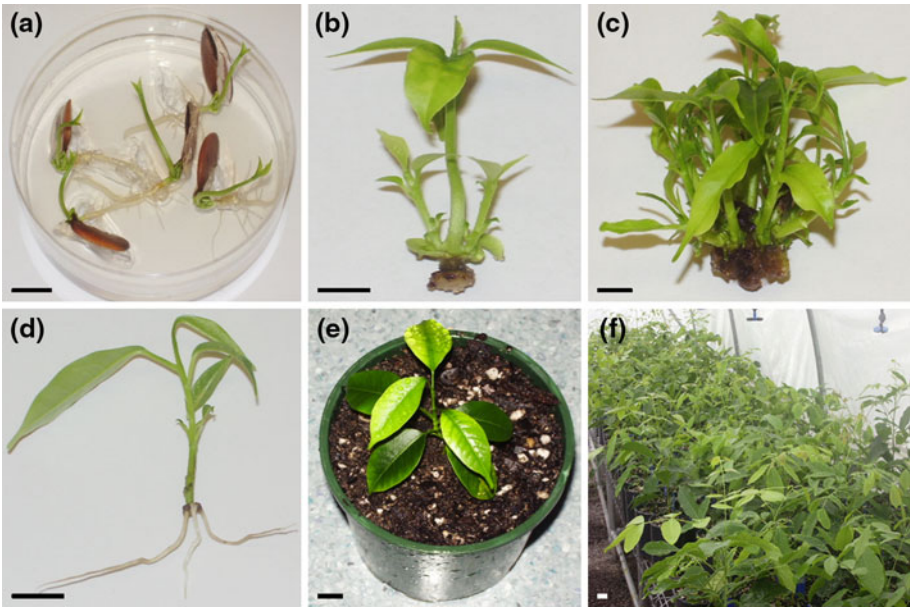

Fig. 2 Micropropagation of *Khaya senegalensis* from seeds: **a** germination on ½MS medium; **b** shoots induced on MS medium supplemented with 8.9 μM BA and 0.3 μM NAA; **c** shoots proliferating on MS medium with 4.4 μM BA; **d** BA-derived shoot forming roots on ½MS medium after treatment with 19.6 μM IBA; **e** 1-month acclimatisation of a plantlet; and **f** 3-month acclimatisation of plantlets. *Bar* 1 cm

per shoot (Table 1). However, all concentrations of BA promoted development of macroscopic lateral buds (Table 1; Fig. 2b). The highest numbers of shoots transferred to the second passage (2.3 ± 0.1–2.6 ± 0.2 per explant) were obtained from media supplemented with 8.9 μM BA alone, or 4.4 or 8.9 μM BA in combination with 0.3 μM NAA, whereas the lowest shoot number (1.2 ± 0.1 per explant) was obtained in the medium containing 1.1 μM BA without NAA (Table 1). These results are similar to those of Danthu et al. (2003), who obtained highest induction (2.4 ± 0.5 shoots) on full-strength MS medium containing 8.9 μM BA and 0.3 μM IBA but also on medium containing 2.2 μM BA and 0.3 μM IBA (2.3 ± 0.4 shoots). The number of shoots induced using *K. senegalensis* seedling shoots is low compared with the numbers obtained from shoot tips and nodal segments of *Cedrela odorata* (Maruyama et al. 1989), *Melia azedarach* (Ahmad et al. 1990; Thakur et al. 1998; Husain and Anis 2009) and *Azadirachta indica* (Arora et al. 2010). Therefore, the induced shoots required subsequent proliferation before each clone could yield sufficient material for a combined clone bank of in vitro shoots and ex vitro plantlets.

Carry-over effects of shoot induction media on shoot numbers were observed after the second and third passages but not after the fourth passage, i.e. after 5 and 10, but not after 15, weeks in proliferation medium (data not presented), and there were no significant interactions between induction and proliferation media. Consequently, mean shoot numbers for each of the eight proliferation media were pooled across ten induction media (Fig. 3). Shoot proliferation was consistently highest in media containing BA but lacking GA_3, and final shoot numbers were lowest in media containing GA_3. This gibberellin, in combination with BA, had previously been shown to improve shoot proliferation in

Table 1 Shoot length, and numbers of nodes, lateral shoots and transferred shoots following a 4-week exposure of *Khaya senegalensis* seedling shoots to one of ten shoot induction media

BA (μM)	NAA (μM)	Shoot length (mm)	Number of nodes	Number of lateral buds	Number of shoots transferred
0	0	56.6 ± 1.6ab	2.0 ± 0.1a	0.2 ± 0.1 g	1.7 ± 0.1def
0	0.3	53.6 ± 1.5ab	2.0 ± 0.1a	0.2 ± 0.1 g	1.8 ± 0.1de
1.1	0	48.5 ± 1.4b	1.5 ± 0.1c	1.3 ± 0.1ef	1.2 ± 0.1 g
1.1	0.3	49.5 ± 1.6b	1.9 ± 0.1ab	0.8 ± 0.1f	1.5 ± 0.1efg
2.2	0	48.6 ± 1.4b	1.8 ± 0.1b	1.6 ± 0.1 cd	1.4 ± 0.1 fg
2.2	0.3	57.7 ± 6.6ab	2.0 ± 0.1a	1.1 ± 0.1ef	1.7 ± 0.1ef
4.4	0	48.3 ± 1.3b	1.9 ± 0.1ab	2.4 ± 0.2b	2.0 ± 0.1 cd
4.4	0.3	63.6 ± 8.1a	2.0 ± 0.1a	1.9 ± 0.1c	2.4 ± 0.2ab
8.9	0	50.0 ± 1.3b	2.0 ± 0.1a	3.5 ± 0.2a	2.3 ± 0.1bc
8.9	0.3	47.9 ± 1.2b	1.9 ± 0.1ab	3.4 ± 0.3a	2.6 ± 0.2a

Means (± s.e.) with different letters are significantly different (ANOVA and Duncan's multiple range test; $P < 0.05$; $n = 65$–75 clones)

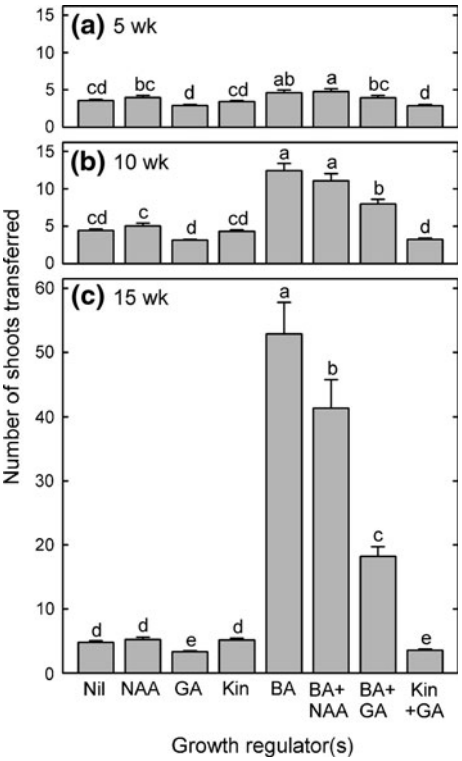

Fig. 3 Effect of growth regulators on number of shoots transferred per *Khaya senegalensis* clone after **a** 5 weeks, **b** 10 weeks, and **c** 15 weeks in shoot proliferation medium. The growth regulators were 0.3 μM NAA (NAA), 1.5 μM GA_3 (GA), 4.7 μM kinetin (Kin) or 4.4 μM BA (BA) singly or in combination. Means (+s.e.) with different letters at each time point are significantly different (2-way ANOVA and Duncan's multiple range test; $P < 0.05$; $n = 81$–90 clones)

cultures of *Khaya ivorensis* (Mathias 1988), *Azadirachta indica* (Chaturvedi et al. 2004), *Naregamia alata* (Daniel et al. 1999), *Melia azedarach* (Vila et al. 2002) and *Cedrela odorata* (Peña-Ramírez et al. 2010). The use of 4.4 μM BA in proliferation medium promoted *K. senegalensis* shoot production by 1.3-, 2.8- and 11.0-fold over nil-hormone

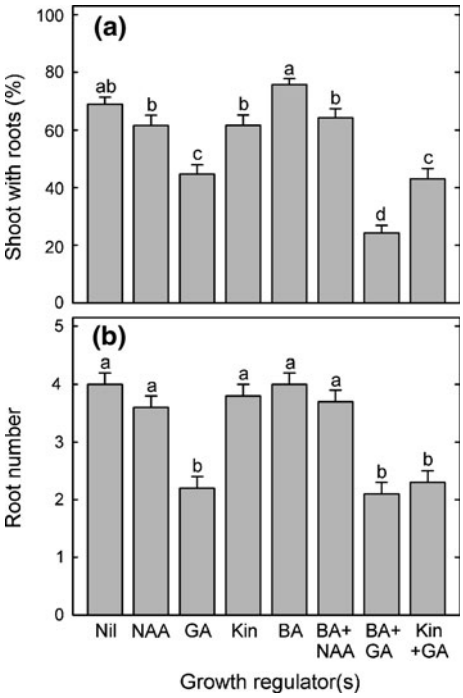

Fig. 4 Effect of growth regulators in shoot proliferation medium on **a** percentage of shoots with roots and **b** number of adventitious roots per rooted shoot for *Khaya senegalensis* clones after a 1-week treatment of shoots with 19.6 μM IBA followed by 3-week culture on ½MS medium. The growth regulators were 0.3 μM NAA (NAA), 1.5 μM GA_3 (GA), 4.7 μM kinetin (Kin) or 4.4 μM BA (BA) singly or in combination. Means (+s.e.) with different letters are significantly different (2-way ANOVA and Duncan's multiple range test; $P < 0.05$; $n = 80$–88 clones)

medium across the three successive proliferation passages, with a cumulative increase in shoot number from 4.6 ± 0.3 to 12.4 ± 1.0 to 52.9 ± 4.9 per clone (Fig. 3a–c).

NAA or kinetin failed to increase shoot proliferation over any of their respective controls (Fig. 3). Indeed, addition of 0.3 μM NAA to proliferation medium containing 4.4 μM BA decreased final shoot production by 22% (Fig. 3c). These results contrast with the positive effects of combined BA and NAA treatments in *Azadirachta excelsa* (Liew and Teo 1998) and *Azadirachta indica* (Khatun et al. 1998), but they accord with the greater effectiveness of BA compared with kinetin or zeatin for shoot proliferation of *Melia azedarach* (Ahmad et al. 1990; Husain and Anis 2009) and *Cedrela odorata* (Peña-Ramírez et al. 2010). Almost all *K. senegalensis* shoots from all treatments were suitable for transfer to fresh medium after each passage. Callus was frequently formed at the base of shoots in the presence of BA (Fig. 2c) or kinetin, but basal callus was removed after each passage to promote nutrient and growth regulator uptake for axillary shoot proliferation or, after the fourth passage, root formation.

Conversion to plantlets was highest for shoots obtained from the optimal proliferation medium; i.e. containing 4.4 μM BA alone (Figs. 3c, 4a). This medium provided significantly higher rooting (75.7 ± 2.1%) than all of the other media containing growth regulators. Rooting was lowest (24.3 ± 2.6–44.7 ± 3.2%) for shoots obtained from the three proliferation media containing GA_3, and these media also provided the lowest numbers of adventitious roots per plantlet (Fig. 4b). Adventitious root numbers among the other media (3.6 ± 0.2–4.0 ± 0.2) did not differ significantly. There was a single carry-over effect of the shoot induction treatment on the percentage of shoots forming roots: 66.5 ± 3.7% of shoots derived from the induction treatment containing 2.2 μM BA and 0.3 μM NAA produced roots, which was significantly higher than rooting of shoots from the six induction treatments containing 0, 1.1 or 8.9 μM BA with or without 0.3 μM NAA

(51.0 ± 4.2–55.5 ± 4.3%; data not presented). Roots were formed at the base of shoots, with little or no callus production (Fig. 2d). Almost 100% of plantlets survived and were acclimatised successfully upon transfer to the propagation chamber (Fig. 2e, f).

Experiment 2

The first experiment demonstrated the effectiveness of medium containing a single hormone (BA, tested only at 4.4 μM) for proliferation of *K. senegalensis* shoots obtained from Burkina Faso seeds. The second experiment determined the optimal BA concentration for *K. senegalensis* micropropagation by assessing a wider range of concentrations (2.2–35.5 μM BA) for shoots from three different seed sources (Burkina Faso, Northern Territory, and Queensland). Shoot induction media containing 4.4 μM BA consistently provided longer shoots, with more nodes, than media containing 17.8 or 35.5 μM BA (Table 2). This medium promoted lateral shoot formation on Burkina Faso and Northern Territory seedling shoots, and it consistently provided one of the highest mean shoot numbers for transfer to the second passage. This mean was significantly higher than those of all other media for Burkina Faso seedlings, and significantly higher than those of the media containing 2.2, 17.8 or 35.5 μM BA for Northern Territory seedlings (Table 2). The only previous report of *K. senegalensis* shoot induction tested BA at 0, 2.2, 8.9 and 22.2 μM in combination with 0.3 μM IBA, and found highest induction in the media containing 2.2 or 8.9 μM BA (Danthu et al. 2003).

Table 2 Shoot length, and numbers of nodes, lateral shoots and transferred shoots following a 4-week exposure of *Khaya senegalensis* shoots from three different seed sources to one of five shoot induction media

BA (μM)	Shoot length (mm)	Number of nodes	Number of lateral buds	Number of shoots transferred
Burkina Faso				
2.2	57.3 ± 4.4a	2.0 ± 0.2a	2.0 ± 0.1a	3.1 ± 0.3a
4.4	55.0 ± 2.2ab	1.5 ± 0.1b	3.6 ± 0.4b	4.3 ± 0.4b
8.9	48.0 ± 2.7bc	1.5 ± 0.1b	2.8 ± 0.3b	3.0 ± 0.3a
17.8	40.8 ± 2.4c	1.3 ± 0.1b	3.0 ± 0.2b	3.1 ± 0.2a
35.5	33.8 ± 2.4d	1.2 ± 0.1b	3.1 ± 0.3b	2.8 ± 0.1a
Northern Territory				
2.2	50.3 ± 3.1ab	1.5 ± 0.1a	1.9 ± 0.2a	2.3 ± 0.2a
4.4	52.5 ± 2.3a	1.5 ± 0.1a	2.4 ± 0.2b	3.0 ± 0.3b
8.9	51.8 ± 2.6a	1.5 ± 0.1a	2.5 ± 0.2b	3.0 ± 0.2b
17.8	43.3 ± 2.0bc	1.1 ± 0.1b	2.3 ± 0.1b	2.4 ± 0.1a
35.5	38.8 ± 2.7c	1.1 ± 0.1b	2.1 ± 0.1ab	2.1 ± 0.1a
Queensland				
2.2	37.3 ± 2.4abc	1.2 ± 0.1a	1.9 ± 0.2a	2.2 ± 0.1a
4.4	42.7 ± 2.5a	1.7 ± 0.2b	2.3 ± 0.3a	2.6 ± 0.3a
8.9	38.4 ± 1.9ab	1.5 ± 0.1ab	2.1 ± 0.1a	2.2 ± 0.1a
17.8	34.0 ± 2.8bc	1.3 ± 0.1a	2.1 ± 0.1a	2.2 ± 0.1a
35.5	30.5 ± 2.4c	1.2 ± 0.1a	1.9 ± 0.2a	2.1 ± 0.1a

Means (± s.e.) with different letters within a seed source are significantly different (ANOVA and Duncan's multiple range test; $P < 0.05$; $n = 20$ clones)

Subsequent shoot proliferation was also highest in the medium containing 4.4 μM BA (Fig. 5), although the final number of Northern Territory shoots produced was not significantly higher than from the media containing 2.2 or 8.9 μM BA (Fig. 5h). The final numbers of shoots produced in this medium were 106.7 ± 21.6, 75.0 ± 15.1 and 73.8 ± 15.9 from the Burkina Faso, Northern Territory and Queensland seed sources, respectively. Proliferation from all seed sources across all three passages was lowest in medium containing 35.5 μM BA, and final shoot numbers in the media containing 17.8 or 35.5 μM BA were consistently lower than in the media containing 2.2, 4.4 or 8.9 μM BA. Similar results were found in *Melia azedarach*, in which nodal segments produced fewer axillary buds with greater basal callusing at BA concentrations above 8.9 μM (Ahmad et al. 1990; Shahzad and Siddiqui 2001). In fact, for *K. senegalensis*, the highest BA

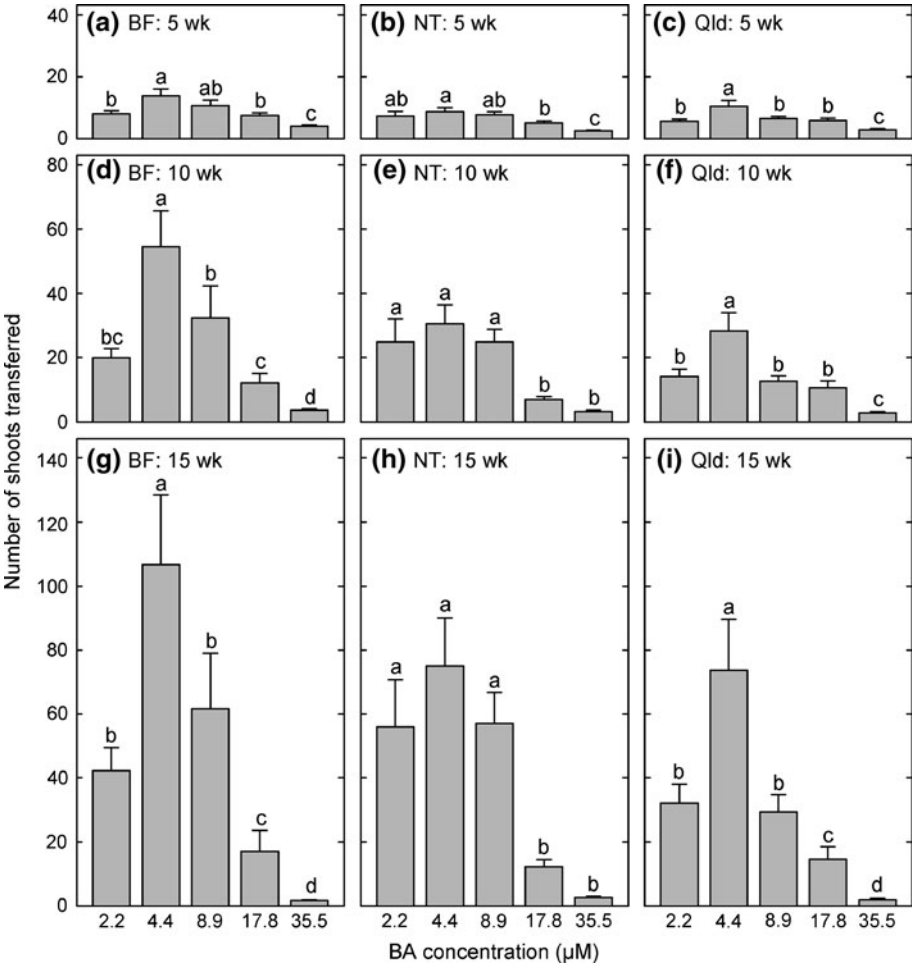

Fig. 5 Effect of BA concentration on number of shoots transferred per *Khaya senegalensis* clone after **a**, **b**, **c** 5 weeks, **d**, **e**, **f** 10 weeks, and **g**, **h**, **i** 15 weeks in shoot proliferation medium. Clones were derived from seeds obtained from **a**, **d**, **g** Burkina Faso (BF), **b**, **e**, **h** Northern Territory (NT) or **c**, **f**, **i** Queensland (Qld). Means (+s.e.) with different letters within each time point and seed source are significantly different (ANOVA and Duncan's multiple range test; $P < 0.05$; $n = 19$–20 clones)

Table 3 Percentages of clones with surviving shoots, and coefficients of multiplication during shoot induction (SI) and three passages of shoot proliferation (SP_1–SP_3), for *Khaya senegalensis* shoots in MS medium with 4.4 μM BA

Experiment	Seed source	No. of clones	Survival (%)	Coefficient of multiplication			
				SI	SP_1	SP_2	SP_3
1	Burkina Faso	9	100	2.2 ± 0.4	2.4 ± 0.3	2.6 ± 0.2	3.8 ± 0.4
2	Burkina Faso	20	100	4.3 ± 0.4	3.0 ± 0.3	3.5 ± 0.3	2.0 ± 0.1
2	Northern Territory	20	100	3.0 ± 0.3	2.7 ± 0.3	3.2 ± 0.2	2.4 ± 0.1
2	Queensland	20	100	2.6 ± 0.3	3.9 ± 0.3	2.6 ± 0.2	2.5 ± 0.2

concentration, 35.5 μM, completely inhibited shoot proliferation, with the final numbers of shoots produced in this medium (1.8 ± 0.2, 2.6 ± 0.4 and 2.0 ± 0.4; Fig. 5g–i, respectively) having not increased significantly (*t*-tests, $P > 0.05$, $n = 19$–20 clones) from the numbers available after shoot induction (Table 2). Survival of clones in medium containing 4.4 μM BA was 100%, and coefficients of multiplication (Sánchez and Vieitez 1991) for each of the 4- or 5-week passages ranged from 2.0 ± 0.1 to 4.3 ± 0.4 (Table 3). The final coefficients of multiplication (i.e. SP_3 in Table 3) would have been higher if all shoots had been dissected for further proliferation rather than a subsample being kept intact for subsequent plantlet conversion.

Most shoots from the medium containing 4.4 μM BA formed roots, with plantlet conversion frequencies of 90.0 ± 4.9% (Fig. 6a), 82.0 ± 4.8% (Fig. 6b) and 85.0 ± 5.2% (Fig. 6c). These means did not differ significantly from those of the media containing 2.2 μM BA for Burkina Faso shoots (Fig. 6a), any other BA concentration for Northern Territory shoots (Fig. 6b), and 2.2 or 8.9 μM BA for Queensland shoots (Fig. 6c). Plantlets from the medium containing 4.4 μM BA had more adventitious roots (3.5 ± 0.2, 2.6 ± 0.2 and 2.2 ± 0.1; Fig. 6d–f, respectively) than plantlets from one or both of the media containing 8.9 or 17.8 μM BA, depending on the seed source. Plantlets from all seed sources exhibited almost 100% survival after acclimatisation in the nursery, and their morphology was very similar to those produced in experiment 1 (Fig. 2e, f). The high rooting percentages achieved in this study, following application of 19.6 μM IBA for 7 d, are comparable with those obtained for the same species (87% and 96%) by applying 260 μM IBA for 1 or 7 d, respectively (Danthu et al. 2003). The current IBA concentration and exposure period are used for successful rooting (57–100%) of eucalypt hybrids (Hung and Trueman 2010, 2011), and rooting in the current study was higher than that obtained previously for other Meliaceae species including *Khaya ivorensis* (Mathias 1988), *Swietenia macrophylla* (Maruyama 2006) and *Toona ciliata* (Mroginski et al. 2003).

Conclusion

This study has developed highly effective methods for (1) shoot induction and shoot proliferation to establish a laboratory archive of juvenile clones, and (2) root formation to generate plantlets of the same clones. These methods provide a means for simultaneous in vitro and ex vitro clone banking of *K. senegalensis*, thus allowing simultaneous laboratory preservation, plantlet conversion, cuttings production and field testing of clones for high-value tropical timber plantations.

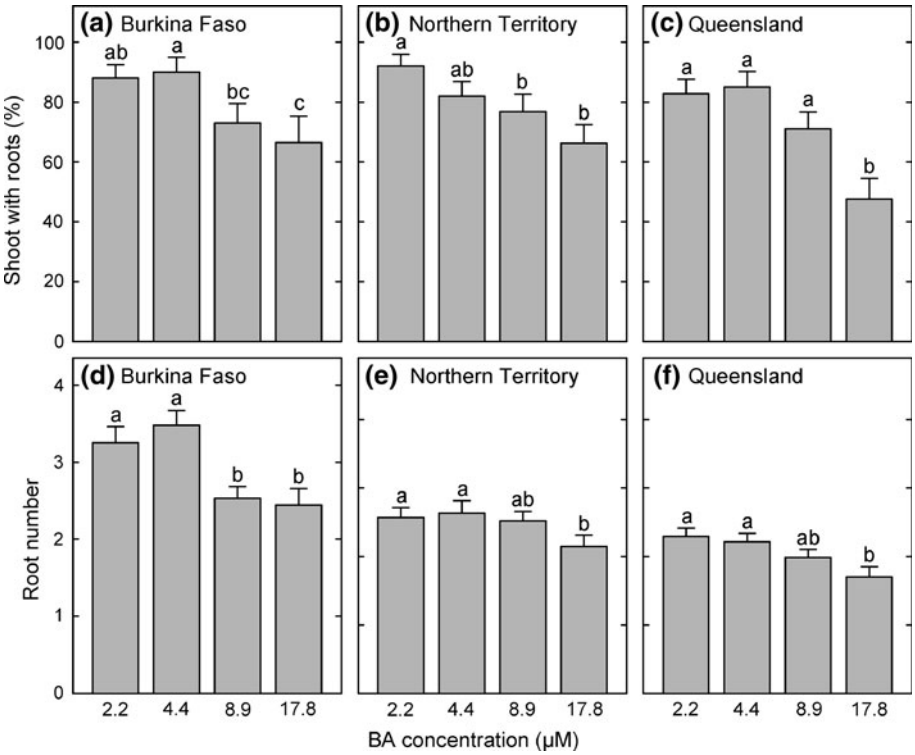

Fig. 6 Effect of BA concentration in the shoot proliferation medium on **a**, **b**, **c** percentage of shoots with roots and **d**, **e**, **f** number of adventitious roots per rooted shoot for *Khaya senegalensis* clones after a 1-week treatment of shoots with 19.6 μM IBA followed by 3-week culture on ½MS medium. Clones were derived from seeds obtained from **a**, **d** Burkina Faso, **b**, **e** Northern Territory or **c**, **f** Queensland. Means (+s.e.) with different letters within a seed source are significantly different (ANOVA and Duncan's multiple range test; $P < 0.05$; $n = 14$–20 clones)

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