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***In vitro* biotechnology of *Melia volkensii*, a high potential forestry tree from eastern Africa**

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Introduction

Melia volkensii belongs to the Meliaceae family. It is endemic to the semi-arid and arid areas of Kenya, Somalia and Tanzania. Its natural range spreads from dry bush land to wooded grassland, situated between 400 and 1600 metres above sea level. Remarkably, it combines drought resistance with fast growth. The main purpose of developing new plantations is for production of termite-resistant mahogany-like timber to be harvested after 15-20 years. Furthermore, its leaves and seeds contain medicinal and insecticidal compounds which can supply an additional income to the farmers. The species has been overexploited and both habitat fragmentation and loss of wild elite trees have been reported (Runo et al. 2004). Even though its local importance, *M. volkensii* is a challenging tree to propagate. The fragile seeds shelter in a woody endocarp which is difficult to open without damaging the fragile seeds. The seeds germinate poorly (Stewart and Blomley 1994) and despite all efforts, seedling production is not able to satisfy demand. Therefore, during the last decade, plant tissue culture tools have been explored for propagation, somatic embryogenesis and adventitious shoot and root induction.

***In vitro* culture initiation**

Seedlings

After cracking the endocarp, fresh seeds with an intact seed coat are rinsed in 70% ethanol, sterilized for 15 min in a 10% NaOCl solution with 0.005% detergent (teepol), and subsequently rinsed three times in sterile distilled water. Usually it's enough to remove the seed coat at the micropylar end but often longitudinal incisions are made through the seed coat, as a scarification treatment. The seeds start to germinate within one week on MS medium (Murashige and Skoog 1962) supplemented with 20 g l⁻¹ sucrose at pH 5.4 (**Fig. 1**). Each cotyledon has an axillary meristem that produces extra shoots when the main shoot axis is damaged. Without cotyledons, the development of the seedling is very poor. About two weeks after germination, the seedling can be subdivided into nodes to start micropropagation.





Figure 1. *M. volkensii* seedlings, 14 days after sowing *in vitro*.

Plus trees

Shoots from plus trees are grafted on seedlings and grown under hygienic conditions or forced from branches (**Fig. 2**). When good growth is achieved, the shoots are prepared for *in vitro* culture initiation by removing the leaves while remaining a stub of the petiole. Then the shoots are rinsed in 70% ethanol, sterilized for 5 min in a 0.5% HgCl₂ solution and then 15 min in a 10% NaOCl solution (both with 0.005% Tween 20) and subsequently rinsed three times in sterile distilled water.



Figure 2. New shoots are forced from branches of elite trees.

Micropropagation

Although *Melia volkensii* can be propagated on MS medium supplemented with BA, more shoots of higher quality are produced with the topolin derivatives MemTR and mTR (Olchemim.cz) (unpublished results). Excessive curling of the leaves can be avoided by using containers with filter lids. A better general quality of the shoots is obtained in this way. Well-closed polypropylene Microbox ® (Saco2, Belgium) with or without integrated filter of different pore size (L, XXL or XXL+) where compared in this regard. The medium was MS supplemented with 20 g l⁻¹ sucrose and 10 µM mTR. The largest leaf surface area was obtained when a filter was present with no effect of the filter pore size (**Fig. 3**). Without aeration of the headspace, the plant growth is slow and plants developed curly leaves as well as white calluses on the stem.

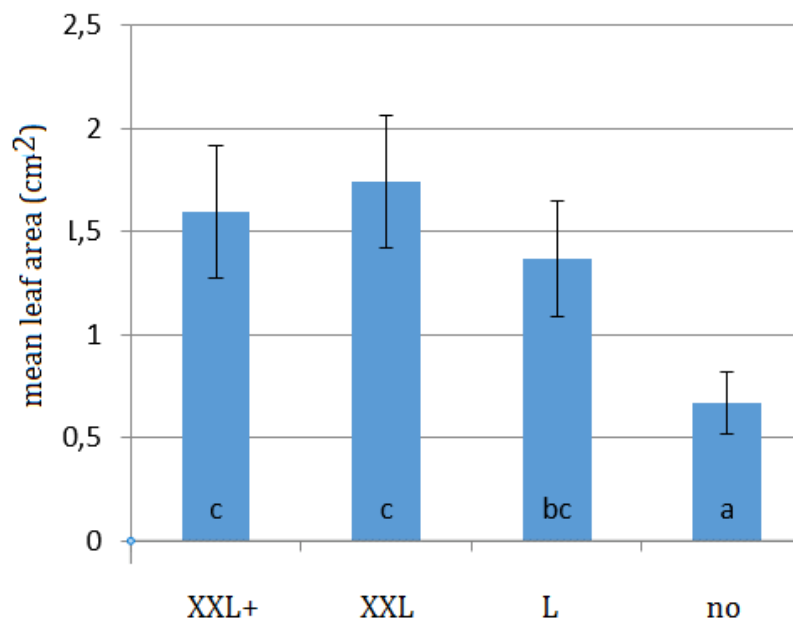


Figure 3. Effect of integrated filter on mean leaf area (cm²). Filter pore size is decreasing from ‘XXL+’ to ‘XXL’ and ‘L’. Bars: standard deviation. Significant differences between means (Tuckey’s tests, 95%) are indicated by different letters.

Callus induction

Melia is known to easily develop calluses *in vitro*. Mulanda (2016) succeeded in inducing callus on carpels, leaves, petioles/rachis, mature zygotic embryos, root segments and seedling epicotyls on several media including i) MS medium supplemented with 0 – 8 mg l⁻¹ BAP combined with 0.05 - 0.4 mg l⁻¹ of either 2,4-D or NAA, or ii) MS medium supplemented with 0 – 1 mg/l TDZ with or without 0.2 mg l⁻¹ 2,4-D, and B5 medium supplemented with 2 mg l⁻¹ kinetin.

Somatic embryogenesis

Indieka et al. (2007) regenerated somatic embryos on cotyledons derived from fresh, immature seeds. After 4 weeks *in vitro* culture on MS medium with 0.5 mg l⁻¹ BA and 0.2 mg l⁻¹ 2,4-D, up to 60% of the cotyledonary explants initiated embryogenic cultures. In this work, we confirmed these results. Direct somatic embryogenesis after 5 weeks induction is illustrated in **Fig. 4**. The somatic embryos germinate easily on hormone free MS medium (**Fig. 5**).

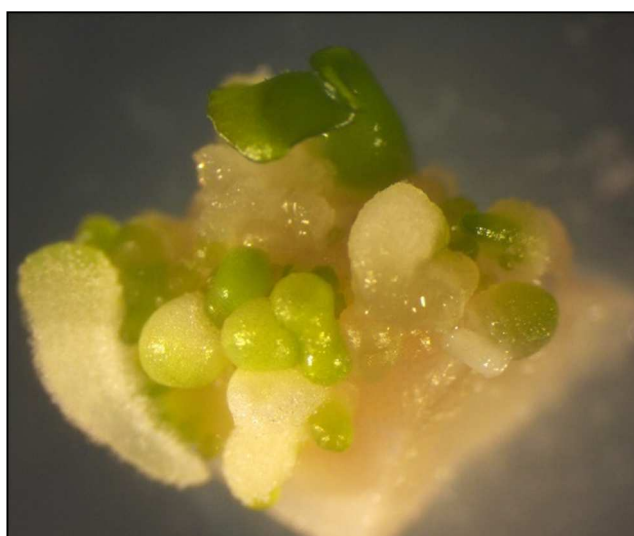


Figure 4. Somatic embryos developing on an isolated cotyledon from *M. volkensii* seedling.



Figure 5. Germinated somatic embryo of *M. volkensii* developing on MS medium without plant growth regulator.

Somatic embryogenesis was also induced by Mulanda et al. (2012) on zygotic embryos excised from mature seeds using the B5 medium (Gamborg et al. 1968) supplemented with MS vitamins, 20 g l⁻¹ sucrose and 0.05 mg l⁻¹ – 4 mg l⁻¹ TDZ. After 21 days, callus masses were subcultured to hormone-free B5 medium. Multiple somatic embryos are initiated within 14 days and further developed into shoots. Shoot elongation was achieved when transferred to ½ MS medium supplemented with 0.1 mg l⁻¹ BA and 10% coconut water.

Since the performance of seedlings is unknown, efforts should be made to obtain somatic embryos derived from explants of selected, elite trees.

Adventitious shoot regeneration from leaf explants of elite trees

Werbrouck (2017) successfully induced adventitious shoots on leaf explants with 10 µM INCYDE-C1, INCYDE-F, TDZ, 2iP or CPPU. TDZ and 2iP were however not very effective compared to INCYDE-C1, INCYDE-F and CPPU. The well-known synthetic phenyl urea cytokinins CPPU and TDZ are inhibitors of cytokinin oxidases/dehydrogenases (CKX) (Chatfield and Armstrong 1986; Laloue and Fox 1989). But INCYDE-C1 or -F and CKX are also involved in the catabolism of endogenous cytokinins such as Z and 2iP by oxidative removal of their side chain (Zatloukal et al. 2008). Remarkably, the INCYDEs could awaken dormant meristems in the secondary axils of the *M. volkensii* composite leaves (**Fig. 6**).



Figure 6. Adventitious shoots developing on leaf explants from elite trees of *M. volkensis*.

In vitro rooting

Rooting of *in vitro* shoots is not self-evident. When 0.1-1 mg l⁻¹ IBA was added to ½ MS medium, the stem bases reacted by producing abundant callus but no roots (**Fig. 7**). On a hormone free ½ MS medium, only 10% of the shoots produced adventitious roots. But at least they grow out of the stem base, with minimal callus formation (**Fig. 8**).

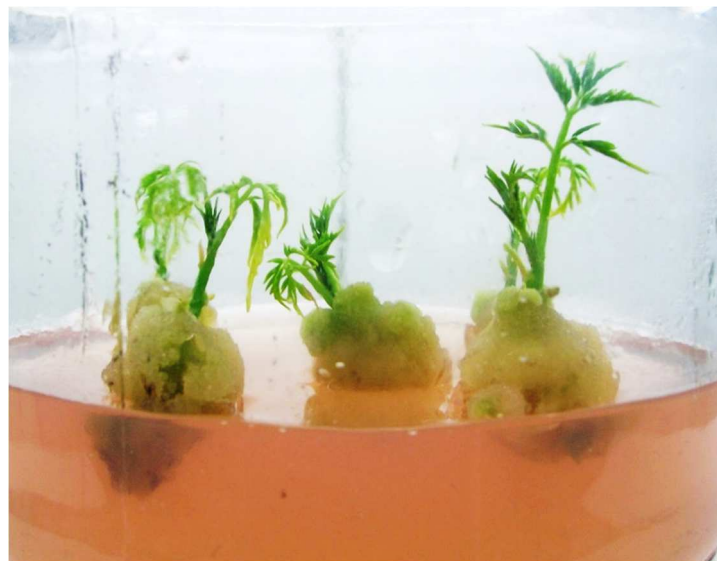


Figure 7. Excessive callus formation on MS medium with 2 mg/l IBA.

Moreover, the leaves turned yellowish and easily dropped. Pulse treatments with high IBA concentrations were also tested. When the *in vitro* shoots were placed in a semi solid $\frac{1}{2}$ MS medium with 200 mg l^{-1} IBA for 4 h, and afterwards were transferred to the same hormone free medium, 56% of the shoots rooted (unpublished results). Often the roots grow very fast, and apparently at the expense of the shoot. Shoots are very brittle and separated from the shoot vascular system by a large callus mass (**Fig. 9**). Such a root system is not functional during acclimatization.



Figure 8. Normal roots on hormone free medium.



Figure 9. Abnormal root and shoot after pulse treatment with IBA.

Acclimatization

Since *in vitro* roots show a shallower adventitious root system than seedlings (Mulatya et al. 2002), it can be expected that roots of *in vitro*-derived plantlets should be pruned in such a way that orthotropic root growth is stimulated. Three different main roots are shown in **Fig. 10**: a hook-shaped root, split root and tap root. Only the straight tap root promises a good tree anchorage. All types show the typical thickened morphology, illustrating the storage function of the root.

After root development, acclimatization was undertaken at KEFRI in a growth chamber set at 30°C . The rooted shoots were transferred into sterile sand covered with a polythene tube and were then kept in growth chambers for 30 days (**Fig. 11**). The plantlets were then transferred to coconut peat in a greenhouse and allowed to grow for about 30 more days to obtain fully acclimatized plants (**Fig. 12**). Overall, about 25% of the plantlets converted into plants of sufficient quality for field plantation.



Figure 10. Different morphology of the main root of *in vitro* plants: hook-shaped root (left), split root (center) and tap root (right).



Figure 11. Plantlets acclimatizing in sterile sand



Fig. 12 Fully acclimatized plants at KEFRI

Conclusion

Because of its precious termite-resistant timber and fast growth under harsh semi-arid conditions, there is a high demand for *Melia volkensii* plants. Current research is focusing on translating the gathered knowledge to commercial micropropagation of mature elite trees and to develop a robust rooting and acclimatization protocol. This will open up opportunities for planting massive multiclonal forests that could somewhat change the view of the savannahs.



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