Summary of Research

Prospects for a rapid *in vitro* regeneration system for propagation of the pesticidal tree *Melia volkensii*, Gurke.

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Abstract:
An *in vitro* system for regeneration of *Melia volkensii* Gurke shoots is described. *M. volkensii,* a drought-tolerant tree native to East Africa, has under-utilized potential as a source of botanical pesticides. Extracts of its fruits and seeds have larvicidal, growth retarding and anti-feedant effects on mosquito larvae and locusts. The species has been over-exploited for its timber. Production of adequate planting material for replenishment of declining stock is constrained by difficulties in propagation via seed and cuttings. The objective of this study was to develop an *in vitro* culture protocol for rapid and efficient shoot regeneration in two ecotypes of *M. volkensii*. Mature zygotic embryos were aseptically cultured on Gamborg *et al*'s B5 medium containing the plant growth regulator Thidiazuron. High frequency callus induction and regeneration were achieved. Callus subcultured to hormone-free ½ MS or B5 medium formed multiple somatic embryos which grew into micro shoots. ½ MS was superior to B5 medium for induction of somatic embryos. Thidiazuron concentration had no significant effect on callus induction but significant effects were observed on fresh mass of callus and somatic embryo induction (F_{test}, $p < 0.001$). Microshoots elongated well on B5 medium containing 0.1 mg/l Benzylaminopurine plus 5 or 10% (v/v) coconut water. Frequent multiple shoot induction, with 4 to 12 shoots per initial shoot, was also observed on the elongation medium. This protocol produced phenotypically normal shoots of height ≥ 5.0 cm in 3.5 months. Further work is in progress to attain rooting of the shoots. This study offers a simple protocol that could be optimized for exploitation in large scale *in vitro* cloning of *M. volkensii* mother trees with elite genotypes and phenotypes.
Key words: *In vitro*, regeneration, *Melia volkensii*, Thidiazuron.

Introduction

Melia volkensii Gurke (Meliaceae; mahogany family) is an important multipurpose tree native to the arid and semi arid lands of Kenya, Ethiopia, Somalia and Tanzania (Stewart and Blomley, 1994). It has received considerable attention in the search for botanical pesticides owing to the presence of limonoid triterpenes (Arnason *et al.,* 1987; Champaigne *et al.,* 1992). However, commercial exploitation of these botanical pecticides is yet to be realized. The wider adoption and commercialization of botanical pesticides has been limited by three drawbacks: low sustainability of source material, difficulties in standardization of botanical extracts and lack of regulatory approvals (Isman, 1997; 2006). For production of botanical pesticides on a commercial scale, the source plant biomass must be obtainable on an agricultural scale, which requires that the plant be either abundant in nature or amenable to cultivation (Isman, 2006). Natural variations in the chemistry of the active principles across geographical zones, genotypes and seasons also makes the standardization of the extracts difficult (Isman, 2006). These sustainability and standardization problems could be addressed through *in vitro* mass propagation. An efficient *in vitro* regeneration system could increase the availability of planting

material and the uniformity of extracts by allowing mass cloning of elite trees. Natural populations of *M. volkensii* have been over exploited for supply of valuable mahogany timber, wood fuel and termite resistant poles (Runo *et al.,* 2004). Currently, large-scale cultivation of *M. volkensii* is severely constrained by lack of adequate planting material due to difficulties in seed extraction, a complex mechanical seed dormancy and high post germination mortality (Indieka *et al.,* 2007). Vegetative propagation is also difficult due to poor rooting of cuttings (Stewart and Blomley, 1994). Tissue culture may offer the solution to these problems. The objective of the present study was to determine the TDZ-mediated *in vitro* culture responses of *M. volkensii* zygotic embryo explants from two agro-climatic zones of Eastern Kenya, as a prelude to a larger study on the effect of ecotype on regeneration in this species.

Literature Summary

Melia volkensii fruit and seed extracts contain several anti-insect limonoids including volkensin, meliavolkenin and salannin (Isman, 2006; Akhtar *et al.,* 2008). The fruit extract of *M. volkensii* is toxic to a broad range of insects (Mwangi and Rembold, 1988; Akhtar and Isman, 2004). Seed extracts have potent antifeedant and growth inhibitory properties against the army worm, locusts, larvae of cabbage looper and mosquito larvae. (Rajab *et al.,* 1988; Mwangi and Rembold, 1988; Kabaru and Mwangi, 2002; Akhtar and Isman, 2004; Akhtar *et al.,* 2008). There is a dearth of studies on *in vitro* culture of *M. volkensii.* To our knowledge, the only published works in this area are by Indieka *et al.,* (2007) and the authors of this paper (Mulanda *et al.,* 2012). Indieka *et al.,* (2007) obtained plant regeneration from cotyledon explants via direct somatic embryogenesis using Murashige and Skoog (MS) (1962) medium supplemented with combinations of the cytokinin 6-Benzylaminopurine (BAP) and the auxin 2,4-Dichloro- phenoxyacteic acid (2,4-D). However, the shoot regeneration and rooting frequency attained were suboptimal for large-scale production of planting material. Mulanda *et al.,* (2012) reported high frequency plant regeneration (up to 96.67%) from mature zygotic embryo explants on Gamborg *et al.*'s (1968) B5 medium supplemented with 0.05 to 4 mg/l of low-cost agrochemical Thidiazuron (Phenyl-1,2,3-thidiazol-5-ylurea; or simply TDZ), as the sole plant growth regulator. However, both studies relied on plant materials from a single agro-climatic zone in eastern Kenya hence the need for a study of the effect of ecotype.

Description of Research

Seeds were obtained from mature fruits collected from wild trees in two agro-climatic regions of eastern Kenya, Mavuria provenance in Mbeere District and Nguutani provenance in Mwingi West District. Zygotic embryos were surface sterilized for 15 minutes in 10% Jik® commercial bleach with 2 drops of Teepol® detergent added as a surfactant and rinsed with sterile water. Callus was induced on Gamborg *et al*'s (1968) B5 salts supplemented with Murashige and Skoog (1962) MS vitamins and organics, 20 g/l sucrose and 12 g/l Oxoid® agar. TDZ was added at concentrations of 0, 0.05, 0.125, 0.25, 0.5, 1, 2 and 4 mg/l and pH adjusted to 5.80 ± 0.2. Media was dispensed into culture bottles and autoclaved for 20 minutes at 1.06 kg cm^{-2} steam pressure (121^{o} C). Five zygotic embryos were placed in each bottle, with three replicate bottles for each concentration of TDZ and ecotype, in a complete randomized design. The experiment was repeated three times. Callused zygotic embryos were subcultured to two types of hormone-free medium, ½ MS and B5, for induction of somatic embryos and microshoots, then to ½ MS plus 0.1 mg/l BAP ± 5 or 10% (v/v) coconut water for shoot elongation. Incubation temperatures were

29.8 ± 0.8 and 25.5 ± 0.1^{o}C max/min respectively (mean ± S.E). Other conditions were fluorescent light ~ 60 µmol photons m^{-2} s^{-1} and 16 hours light: 8 hours dark photoperiod. Images were taken using a Keyence® (Z35) VHX Digital Scanning Microscope and a Sony digital camera (Model DSC-W390). Data were subjected to one-way Anova using SPSS version 17.0.

Research Results and Application

A high frequency of callus induction was observed within 4 to 10 days in all TDZ concentrations and on hormone-free medium. TDZ concentration had no significant effect on percent callus induction in both ecotypes. Endogenous plant growth regulator (PGR) levels in the zygotic embryos may be sufficient for callus induction. TDZ concentration had significant effect on fresh mass of callus in both ecotypes (Table 1). Tukey's HSD located the significance at 0.05 mg/l TDZ. It appears very low TDZ concentration is favorable for callogenesis as reported in other plant species (Guo *et al.,* 2011). Callus masses were significantly larger in Mbeere than Mwingi ecotype but more explant-level variability in callus fresh mass was seen in Mbeere ecotype than Mwingi (Table 1). This explant-level variability may be attributed to the asynchronous flowering and fruit setting in *M. volkensii* which causes fruits on the same branch to be at different stages of development (Orwa *et al.,* 2009). Callus had to be transferred to hormone-free B5 or ½ MS medium to form somatic embryos. TDZ was crucial for formation of somatic embryos as these only formed in calluses from media with TDZ. This agrees with findings in a number of plant species (Guo *et al.,* 2011). TDZ modulates both auxin and cytokinin effects in plant tissues, removing the need for application of combinations of PGRs (Huetteman and Preece, 1993). It appears that presence of TDZ in the medium confers embryogenic competence, but its depletion or withdrawal is essential for actual development of the embryos. The somatic embryos were induced as green globular structures (Figures 1a; b), which conforms to the findings of Ammirato (1987) in other species. The globular embryos developed leaf primordia and formed well defined microshoots (Figure 1c). ½ MS + 0 PGR may be superior to B5 + 0 PGR in percentage induction and vigor of somatic embryos. These results also suggest the existence of ecotypic differences in regeneration ability. Microshoots elongated on ½ MS medium with either 0.1 mg/l BAP ± 5 or 10 % coconut water (Figure 1d). Media having coconut water allowed multiple shoot development in the range of 4 to 12 shoots. Similar results were obtained by Indieka *et al.,* 2007 on MS medium with 0.5 mg/l BAP and 0.2 mg/l IAA. This study shows the amenability of *M. volkensii* to *in vitro* propagation from mature zygotic embryos. This protocol gave multiple phenotypically normal shoots of ≥ 5cm height in 105 days (3.5 months). It is a rapid and fairly simple protocol that could be optimized for application in clonal forestry for commercial supply of timber and botanical pesticides, and in other tissue culture related technologies such as *in vitro* conservation and genetic modification of the species. The only remaining obstacle is the apparent difficulty in rooting, for which further work is in progress.

Table 1. Summary of the effects of TDZ concentration on % callus induction and fresh mass.

	% callus induction		Fresh mass per callus/mg	
Component	Mbeere	Mwingi	Mbeere	Mwingi
Range of means	95.56 - 100	88.88 - 100	107.89 - 301.98	45.17 – 167.33
Optimal TDZ (mg/l)	N/S†	N/S†	0.05	0.05
F-value	1.317	2.158	4.725	5.560
d.f	(7, 64)	(7, 54)	(7, 64)	(7, 54)
F test Significance	*p >0.05*	*p >0.05*	*p < 0.001*	*p < 0.001*

R^2 value for regression upon TDZ concentration	0.768	0.157	0.252	0.252

† = Optimal *concentration was Not specific (N/S) as 100% callus induction was attained in most of the treatments.*

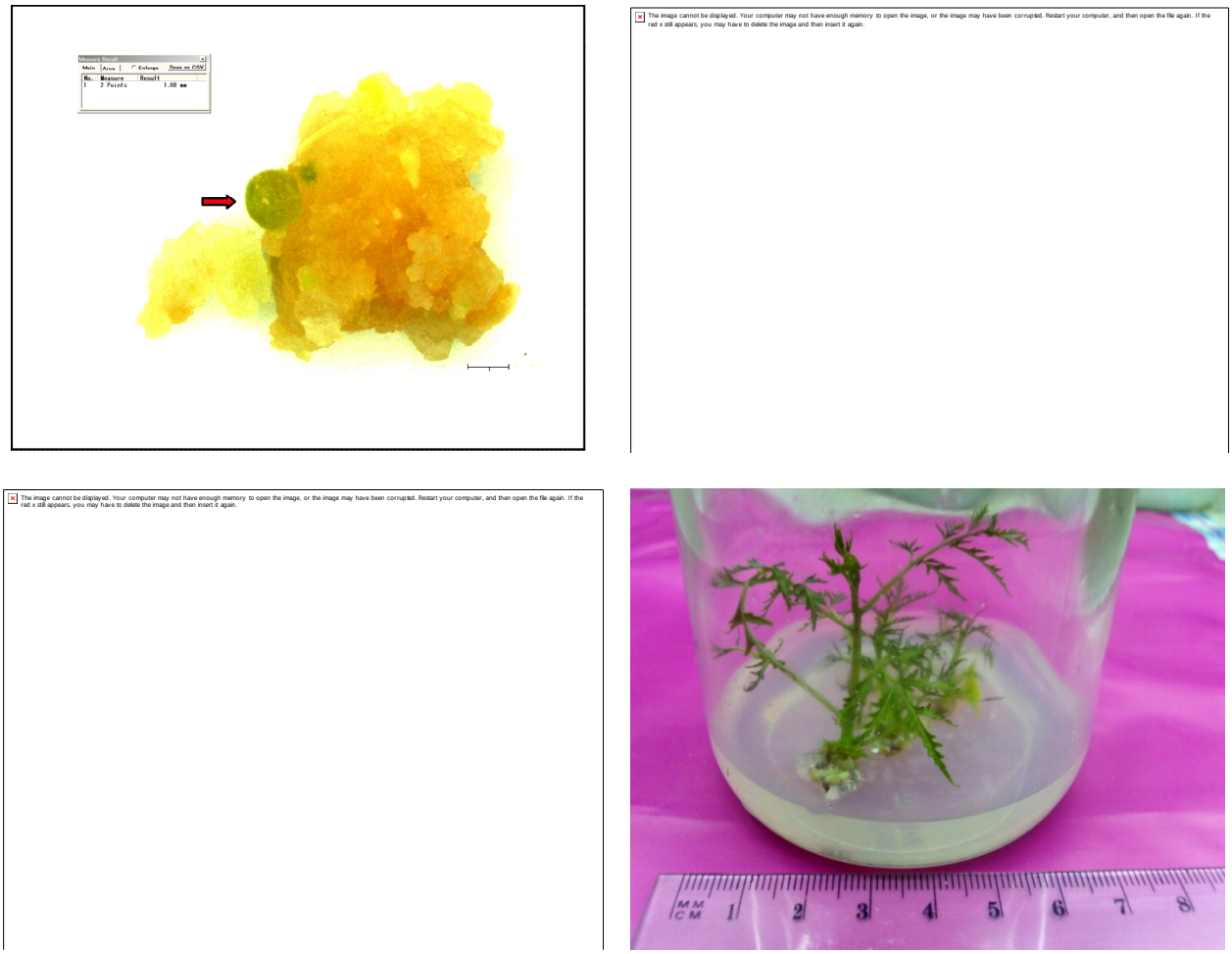

Figure 1. **A**. *Scanning photomicrograph; globular stage somatic embryo (arrow) forming from callus 14 days after transfer to B5 + 0 PGR medium; cumulative age = 35 days. Thin scale = 1mm.* ***B.*** *Scanning photomicrograph; Initial stages of shoot morphogenesis (arrows) after 15 days on B5+0 PGR medium; cumulative age = 36; Thin scale = 1mm.* ***C & D.*** *Multiple microshoots after 34 days on ½ MS + 0 PGR medium; cumulative age = 55 days* ***(C)*** *& Shoot elongation after 37 days on ½ MS + 0.1 mg/l BAP + 10% coconut water, cumulative age = 92 days* ***(D)***.

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