Plant regeneration through somatic embryogenesis of jacket plum (*Pappea capensis*)

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Abstract The study was undertaken to identify an effective culture growing system for mass multiplication of jacket plum (*Pappea capensis*) through somatic embryogenesis. Calli derived from leaf sections were transferred onto Murashige and Skoog (MS) medium with different supplements. The most effective medium for callus induction was MS supplemented with 0.1 mg litre⁻¹ thidiazuron (TDZ) alone or with 0.1 mg litre⁻¹ indole-3-butyric acid (IBA) or a combination of 1.0 mg litre⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg litre⁻¹ benzylaminopurine (BAP). Light exposure promoted embryo induction. Three-quarter strength MS medium supplemented with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ casein hydrolysate (CH) was effective for embryo germination and the most effective culture medium for plantlet conversion was three-quarter strength MS supplemented with 0.2 mg litre⁻¹ BAP and 0.3 mg litre⁻¹ CH. There was 60% survival of plants under a mist propagation chamber. The study shows that it is possible to improve *P. capensis* somatic embryogenesis through manipulation of some culture medium constituents and incubation conditions.

Keywords embryogenic callus; germination; plantlet conversion; somatic embryo

INTRODUCTION

Jacket plum (*Pappea capensis* L.) belongs to the Litchi family (Sapindaceae) (Fivaz & Robbertse 1993; Venter & Venter 1996). The tree grows up to 3.9 m tall and can be deciduous or evergreen depending upon the prevailing environmental conditions (Anon.1997; van Wyk & Gericke 2000). According to Fivaz & Robbertse (1993), this monoecious tree is widely distributed throughout southern Africa, but absent in the western Kalahari and northern Namibia. It is fairly adapted to a wide range of ecological areas (van Wyk & Gericke 2000).

*Pappea capensis* produces fleshy fruits which can be processed into vinegar, jelly, and jam (Palmer & Pitman 1972). The seeds are rich in edible, non-drying, and fairly viscous oil, c. 74% (Swart 1991). The oil is used for making soap and metal lubrication (Venter & Venter 1996; van Wyk & Gericke 2000). This oil can also be exploited as an alternative source of bio-diesel fuels and this can provide an alternative source of income generation to rural communities in South Africa (Mng’omba et al. 2007). The tree is known to be drought-tolerant and grows on marginal lands (Venter & Venter 1996; van Wyk & Gericke 2000).

Research studies were inititated at the University of Pretoria to exploit the potential of *P. capensis* as an alternative source of bio-diesel fuel. However, there has been no germplasm collection, characterisation, and improvement. Moreover, the trees are still growing in the wild. There is a need to develop a reliable propagation method to adequately select and multiply the desirable ideotypes for tree improvement and cultivation. The available reports indicate that *P. capensis* is propagated by seeds, but seedling growth has been extremely slow (Palmer & Pitman 1972).
Poor rooting ability of epicormic stem cuttings has been reported despite pre-treatments (Mng’omba et al. 2007). Furthermore, the cuttings took more than 12 weeks to root. In addition, micro-propagation via organogenesis has been accomplished, but shoot tip necrosis and in vitro rooting problem limit mass multiplication of *P. capensis* (Mng’omba et al. 2007).

In vitro propagation technique through somatic embryogenesis offers an opportunity for tree improvement (through genetic transformation) and mass multiplication. According to Kayim & Koc (2006), protoplast fusion technologies rely on embryogenic callus culture and such technologies are often used for genetic improvement. Furthermore, embryogenic cultures or somatic embryos are amenable to in vitro cryo-preservation and this could provide a long-term storage solution for tree seeds, especially for trees that bear recalcitrant seeds. For instance, it has been reported that *Pinus glauca* cultures maintained stability after 3–4 years of cryo-preservation (Merkle & Dean 2000). However, the major challenge is that many woody tree crops are difficult to propagate through somatic embryogenesis (Merkle & Dean 2008). To our knowledge, there has been no study on somatic embryogenesis of *P. capensis* despite being identified as a potential source of bio-diesel fuels. The objective of the study was to identify efficient culture growing conditions for mass multiplication of *P. capensis* through somatic embryogenesis.

**MATERIALS AND METHODS**

**Plant material**

*Pappea capensis* seeds and epicormic shoots were collected in August 2005 from Pretoria National Botanical Gardens (1360 m a.s.l., latitude 25°44′S, longitude 28°16′E, an annual rainfall of 750 mm) (Botha et al. 2000). Fresh seeds were scarified in 98% sulfuric acid (3 min) and rinsed in sterile water four consecutive times. They were germinated on hormone-free and half-strength Murashige and Skoog (Murashige & Skoog 1962) MS medium containing 3% sucrose. The cotyledons and the first three leaves were excised from aseptic *P. capensis* seedlings for callus induction.

A few *P. capensis* epicormic stem cuttings were rooted under a mist propagation chamber (21–26°C temperature and 70–95% relative humidity (RH)). The rooted cuttings were transferred to the glasshouse where they were preconditioned with Benlate (Benomyl, 1.0 g litre⁻¹) for 4 weeks. Tender and fully expanded leaves were collected and washed under running tap water (30 min). They were surface decontaminated in 1.75% sodium hypochlorite (8 min) and rinsed in sterile water four consecutive times. Leaf sections were cultured on MS medium with different supplements for callus induction.

**Media preparation and culture condition**

The pH of the culture medium was adjusted to 5.6 ± 2 with KOH or HCl and solidified with 2.5 g litre⁻¹ Gelrite®. Culture medium was sterilised at 121°C and 104 kPa (15 min). The Petri dishes were covered and then sealed with parafilm strips after culture initiation. They were then incubated under a 12 h photoperiod and 60 µmol m⁻² s⁻¹ photosynthetically active radiation (PAR) cool white fluorescent light. Temperatures were maintained at 23 ± 2°C.

**Callus induction**

Leaf sections (1 cm²) and cotyledons were cultured on 25 ml MS medium with 4% sucrose and different supplements. The MS medium was dispensed in 9-cm diameter Petri dishes. The experiment was laid out in a split-plot design with three sources of stock plants as the main-plots (seedlings, stem cuttings, and cotyledons) and plant growth regulators (MS supplements) as subplots. The MS medium supplements (mg litre⁻¹) used were: (1) 0.1 thidiazuron (TDZ) and 0.1 indole-3-butyric acid (IBA); (2) 0.1 TDZ; (3) 1.0 benzylaminopurine (BAP) and 1.0 kinetin (Kin); (4) 0.1 BAP and 0.05 α-naphthaleneacetic acid (NAA); (5) 0.2 BAP and 1.0 of 2,4 dichlorophenoxycetic acid (2,4-D); or (6) 0.2 BAP and 1.0 NAA. There were eight explants per Petri dish and five Petri dishes per treatment. The experiment was replicated three times.

**Somatic embryo induction**

Calli derived from leaf explants were transferred onto three-quarter strength MS medium with 4% sucrose for embryo induction. The supplements (mg litre⁻¹) used were: (1) 0.3 casein hydrolysate (CH); (2) 0.5 (2,4-D) and 1.5 NAA; (3) 5.0 gibberellic acid (GA₃); (4) 0.05 TDZ and 0.3 CH; (5) 2.0 BAP and 0.5 indole-3-acetic acid (IAA); (6) 1.0 BAP and 0.1 NAA; (7) 1.0 TDZ and 0.3 CH; (8) 2.0 kinetin and 0.5 IAA; (9) 2.5 TDZ and 0.5 NAA; or (10) 2.0 TDZ. There were five Petri dishes per treatment. One set of treatment was exposed to light, while another set was exposed to dark conditions. After 3–4 weeks, calli in the Petri dishes were slightly submerged in the culture medium and there was no positive response. Therefore, all the Petri dishes were then placed at a
slanting position (approximately at 30–40° angle). The experimental design was a randomised block laid out in a factorial arrangement and with three replicates.

**Embryo maintenance and maturation**

Calli at globular stage were transferred every 3–4 weeks onto culture medium with 4% sucrose and different supplements for embryo development, maintenance, and maturation. The culture medium used were: (1) ¾ MS with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH; (2) ½ MS; (3) ¾ MS with 0.5 mg litre⁻¹ (2,4-D) and 1.5 mg litre⁻¹ NAA; (4) MS; (5) ¾ MS with 1.0 mg litre⁻¹ BAP and 0.1 mg litre⁻¹ NAA; (6) ¾ MS with 1.0 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH; or (7) ¾ MS with 2.0 mg litre⁻¹ kinetin and 0.5 mg litre⁻¹ IAA.

**Germination of somatic embryos**

Somatic embryos at cotyledonary stage were separated into single embryos and transferred onto the culture medium containing 3% sucrose. The culture medium and supplements used for the embryo germination were: (1) MS; (2) ½ MS; (3) MS with 0.3 mg litre⁻¹ CH; (4) ¾ MS with 0.2 mg litre⁻¹ BAP and 0.3 mg litre⁻¹ CH; (5) ½ MS with 0.3 mg litre⁻¹ CH; (6) ¾ MS with 2.0 mg litre⁻¹ BAP and 0.3 mg litre⁻¹ CH; (7) ¾ MS with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH; (8) ¾ MS with 1.0 TDZ and 0.3 mg litre⁻¹ CH; or (9) ½ MS with 5.0 mg litre⁻¹ GA₃.

Individual embryos were transferred into 150 × 25 mm test tubes. The experiment was laid out in a randomised block design and repeated three times. There were 10 test tubes per treatment. Plantlets were hardened off on a mist bed in a propagation chamber with 70–95% RH, 21–26°C temperature and 400 µmol m⁻² s⁻¹ PAR.

**Statistical analysis**

The data collected were subjected to analysis of variance (ANOVA) using GenStat 4.24 DE 2005 (Rothamsted Experimental Station). Means were calculated with standard errors or deviations.

**RESULTS**

**Callus induction**

There were significant differences ($P \leq 0.05$) in callus formation among the sources of explants, culture medium supplements, and the interaction between sources of explants and culture medium supplements (Table 1). A significant amount of callus was formed on explants derived from leaves (excised from the seedlings and stem cuttings) irrespective of the culture media evaluated. The callus was mainly induced along the cut surfaces of the leaves. There was a poor response from cotyledon explants on all the culture media evaluated. The callus was mainly induced along the cut surfaces of the leaves. There was a poor response from cotyledon explants on all the culture media evaluated. Calli derived from the cotyledon explants were of poor quality (very loose and mushy), and hence unsuitable for somatic embryogenesis. Furthermore, it took more than 4 weeks to obtain callus from the cotyledon explants. Therefore, this source of explants was discarded from further investigation.

**Table 1**

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<th>Callus formation (%) from three sources of explants</th>
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<td><strong>MS medium supplement (mg litre⁻¹)</strong></td>
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<tr>
<td>0.1 TDZ + 0.1 IBA</td>
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<tr>
<td>0.1 TDZ</td>
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<td>1.0 BAP + 1.0 Kin</td>
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<td>0.1 BAP + 0.05 NAA</td>
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<td>0.2 BAP + 1.0 2,4D</td>
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<td>0.2 BAP + 1.0 NAA</td>
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LSD (5%) = 9.4
Source of explants (S) = 4.1 $P \leq 0.05$
Medium treatment (T) = 4.3 $P \leq 0.01$
S × T = 7.4 $P \leq 0.05$
The MS medium with 0.1 mg litre\(^{-1}\) TDZ alone or in combination with 0.1 mg litre\(^{-1}\) IBA or a combination of 0.2 mg litre\(^{-1}\) BAP and 1.0 mg litre\(^{-1}\) 2,4-D resulted in high production of callus across the three different sources of explants (Table 1). The MS medium supplemented with 1.0 mg litre\(^{-1}\) BAP and 0.05 mg litre\(^{-1}\) NAA produced significantly low amount of callus. Therefore, the MS medium supplemented with 0.1 mg litre\(^{-1}\) TDZ was used for further proliferation and maintenance of calli.

### Somatic embryo induction

Calli derived from seedlings (leaves) resulted in green, compact, and nodular callus formation on three-quarter strength MS medium supplemented with 0.05 mg litre\(^{-1}\) TDZ and 0.3 mg litre\(^{-1}\) CH under the light condition (Table 2). The calli under the dark condition turned either white or yellow, but there was no profuse growth. Consequently, there was degeneration and necrosis of the calli after 3 weeks. Since the dark condition was inhibitory to
Pappea capensis somatic embryogenesis, the experiment continued under 12 h of light exposure.

Pappea capensis somatic embryo induction was promoted when the Petri dishes were placed at a slanted position (30–40° angle) 4 weeks after callus culture incubation. A few stages of somatic embryogenesis derived from seedlings are shown in Fig. 1. The calli became compact, nodular, and green (chlorophyllous) on three-quarter strength MS medium supplemented with 0.05 mg litre⁻¹ TdZ and 0.3 mg litre⁻¹ casein hydrolysate (CH). After 4 weeks, globular-shaped embryos (Fig. 1A) were regenerated from the compact and nodular calli. Transferring such calli onto the same culture medium led to the cotyledonary embryo regeneration after 3–4 weeks (Fig. 1B–C).

The highest number (60 ± 5.8%) of somatic embryos (globular or torpedo stage) regenerated was obtained from leaves excised from the seedlings. This was when the calli were cultured on three-quarter strength MS medium supplemented with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH (Table 2). The data show that high TDZ concentration (≥1 mg litre⁻¹) decreased the number of somatic embryos
regenerated. A few somatic embryos (<5%) were obtained from calli derived from leaves of stem cuttings. The three-quarter strength MS medium supplemented with either CH alone, GA₃, BAP with NAA, or IAA or kinetin with IAA was ineffective in somatic embryo induction.

*Pappea capensis* somatic embryo maturation was promoted by continuous exposure to three-quarter strength MS medium supplemented with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH. However, there was rapid degeneration of embryos on many culture medium treatments evaluated. Therefore, three-quarter strength MS medium supplemented with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH was superior for *P. capensis* embryo development, maintenance, and maturation. There was no indefinite production of somatic embryos and the calli that did not initially produce embryos turned yellow and eventually degenerated. Furthermore, no secondary embryo production was observed from the primary embryos.

**Germination of somatic embryos**

The highest germination of somatic embryos (65%) was achieved on three-quarter strength MS medium supplemented with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH and there was precocious root development (Fig. 2). Complete plantlet (shoot and root development) formation was obtained on three-quarter strength MS medium supplemented with 0.2 mg litre⁻¹ BAP and 0.3 mg litre⁻¹ CH despite lower percentage germination (45%) of embryos. The culture medium without plant growth regulators and also without or with only CH led to browning and eventually the death of the embryos. No embryo germination was obtained on the other culture media tested. Somatic embryos with precocious rooting developed shoots later after transferring them onto three-quarter strength MS medium with 0.2 mg litre⁻¹ BAP and 0.3 mg litre⁻¹ CH.

*Pappea capensis* somatic embryos that germinated on all the culture media supplemented with BAP showed no secondary callus formation (Fig. 3A), whereas somatic embryos that germinated on all culture media supplemented with TDZ showed some callus formation, especially at the root zones (Fig. 3B).

**DISCUSSION**

To our knowledge, this is the first report for somatic embryogenesis of jacket plum. In this study, the highest rate of somatic embryogenesis was achieved using leaf explants derived from seedlings, cultured on three-quarter strength MS medium supplemented with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH and then exposed to light condition. Therefore, the key factors that significantly influenced embryogenesis and plant regeneration of jacket plum include plant growth regulators, culture medium strength, plant tissue type, and light condition and slanting position of the Petri dishes.

The study shows that the culture medium supplemented with low TDZ concentration was stimulatory to *P. capensis* somatic embryogenesis. According to Singh et al. (2003), TDZ has been effective in inducing somatic embryogenesis in recalcitrant trees such as white ash (*Fraxinus americana*) and neem (*Azadirachta indica*). In this study, the use of high TDZ concentration (>1.0 mg litre⁻¹) resulted in the formation of secondary callus on plantlets. Huetteman & Preece (1993) reported that high TDZ concentration stimulated callus proliferation, but inhibited shoot production in walnut trees. In this study, there was precocious root development, but not for shoot as illustrated in Fig. 2. This could be attributed to TDZ dosage effect or possibly to the internal hormonal imbalance. Singh et al. (2003) reported successful embryo germination on lower TDZ concentration (one 10th) after somatic embryo induction on culture medium supplemented with 10–20 µM TDZ. This study shows a similar trend although Singh et al. (2003) did not mention the quality of somatic embryos produced with respect to secondary callus formation. *P. capensis* somatic embryo maturation could be promoted by continuous exposure to low TDZ concentration.

Continuous exposure of somatic embryos to culture medium supplemented with TDZ and CH could be factors responsible for the development and maturation of the embryos. According to Singh et al. (2003), continuous exposure to TDZ was necessary for a successful somatic embryogenesis in pigeon pea. *P. capensis* plantlets derived from continuous exposure of embryos to TDZ showed secondary callus formation, but this was absent on culture medium supplemented with BAP during shoot development. This is attributed to the effect of dosage as callusing of explants is a common phenomenon for the culture medium supplemented with TDZ.

Amino acids also play a vital role in somatic embryogenesis and CH is an important source of amino acids necessary for plant growth and development. Pareek & Kothari (2003) reported that CH promoted embryo maturation of *Dianthus*...
ornamental species. According to Robichaud et al. (2004), the significance of amino acids in embryo induction has been elucidated in many plants. Amino acids increased the number and size of somatic embryos regenerated and also improved the conversion of embryos to plantlets (Singh & Chand 2003; Robichaud et al. 2004). In this study, CH could have played a vital role in somatic embryogenesis although optimal concentrations need to be investigated by testing different CH concentrations. This is to improve the present findings and our understanding on the effects of CH on P. capensis somatic embryogenesis.

There are many factors which play a vital role in somatic embryogenesis and these include organic acids and vitamins (Pullman et al. 2006). Since the plantlet conversion was low (45%) in this study, other culture medium supplements should be evaluated to improve the current findings. The strength of the culture media could also play a major role in somatic embryogenesis. Singh & Chand (2003) reported a successful embryo germination of Dalbergia species on plain MS medium, whereas Rai et al. (2007) reported that half-strength MS medium was superior for embryo germination in guava. The response of embryos to different culture medium formulations could depend on the plant species. Therefore, culture medium strength could significantly influence somatic embryogenesis. In this study, three-quarter strength MS medium was generally superior for P. capensis somatic embryogenesis.

This study has shown that dark conditions were inhibitory for the induction of P. capensis somatic embryogenesis. According to Raghavan (1986), light conditions promote somatic embryogenesis. Furthermore, Nato et al. (2000) reported that light could be synergistic to the activities of plant growth regulators since many plant cell activities are modulated by light and plant growth regulators.

In this study, P. capensis somatic embryo induction was promoted when the Petri dishes were placed at a slanted position (30–40° angle). This indicates that placing the Petri dishes at an angle prevented accumulation of a film of liquid MS medium around the somatic embryos. Therefore, this suggests that accumulation of liquid MS medium around the embryos could be inhibitory to embryo proliferation, possibly as a result of insufficient oxygen supply for respiration of the embryos. Furthermore, there was condensation of liquid inside the Petri dish and this could also cause anoxia. Accumulation of the liquid in the culture medium, especially at the lower part of the slanting Petri dishes increased with time.

In conclusion, many factors could be involved in controlling the somatic embryogenesis process in P. capensis. Such factors include plant growth regulators, light exposure, culture medium composition and strength, and their interactions. In this study, MS medium supplemented with 0.1 mg litre⁻¹ TDZ was effective for callus induction, whereas three-quarter strength MS medium with 0.05 mg litre⁻¹ TDZ and 0.3 mg litre⁻¹ CH promoted embryo induction and maturation under 12 h of light exposure. Three-quarter strength MS medium supplemented with 0.2 mg litre⁻¹ BAP and 0.3 mg litre⁻¹ CH promoted conversion of embryos into plantlets with little or no secondary callus formation. However, further studies on other factors such as the effects of cold storage, desiccation, and sucrose concentration are warranted to improve the percentage germination of P. capensis somatic embryos.

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