CHAPTER 8

JACKET PLUM (*PAPPEA CAPENSIS*) PLANT REGENERATION THROUGH INDIRECT SOMATIC EMBRYOGENESIS

8.1 Abstract

The objective was to determine an effective protocol for mass multiplication of clonal jacket plum (*Pappea capensis*) planting materials through somatic embryogenesis. Leaf and cotyledon sections were cultured on Murashige and Skoog (MS) medium with different supplements for callus induction. Calli from leaf sections were transferred onto MS medium with different supplements for somatic embryo regeneration. The MS medium with 0.1 mg l^{-1} thidiazuron (TDZ) alone or with 0.1 mg l^{-1} indole-3-butyric acid (IBA), and also a combination of 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg l^{-1} benzylaminopurine (BAP) were effective for callus induction. Embryos from leaf calli (juvenile tissues) were observed under light conditions. Cotyledon explants failed to yield embryogenic calli. Three-quarter strength MS medium with 0.05 mg l^{-1} TDZ and 0.3 mg l^{-1} casein hydrolysate (CH) were effective in somatic embryo germination despite a strong rhizogenic capacity. Three-quarter strength MS medium supplemented with 0.2 mg l^{-1} BAP and 0.3 mg l^{-1} CH was effective in the germination of rhizogenic embryos into complete plantlets. MS medium with high TDZ (>1.0 mg l^{-1}) concentrations promoted secondary callus production. Somatic embryos were successfully germinated into plants (65%) and there was 60% survival rate after hardening off under mist.

8.2 Introduction

Jacket plum (*Pappea capensis* L.) seed germination is always erratic and seedling growth is extremely slow (Venter & Venter, 1996). Clonal propagation is important because planting materials are genetically proven to be superior and hence there is the perpetuation of desirable traits. However, there has been no scientific research done on jacket plum tree species to achieve clonal mass propagation. Somatic embryogenesis, defined as production of embryos from somatic cells (direct embryogenesis) or from callus (indirect embryogenesis), is a potential technique that enables mass propagation of some woody tree crops (Bajaj, 1986). Through somatic embryogenesis, cells develop into embryos and eventually regenerate into complete plants.

Somatic embryogenesis has been reported in more than 150 woody plant species (Dunstan, Tautorus & Thorpe, 1995) and this is a preferred technique for mass propagation of superior or clonal tree species. Somatic embryogenesis has several advantages over other forms of propagation and these include the rapid multiplication rate of plantlets and the uniformity of somatic plantlets (Raghavan, 1986). It enables production of millions of units of uniform planting materials within a short time. Moreover, a major advantage is the ability to regenerate both roots and shoots simultaneously. Consequently, this eliminates the rooting stage (Raghavan, 1986; Gupta, 1995) which is required when plantlets are regenerated through organogenesis. Rooting micro-cuttings regenerated through organogenesis is difficult, especially for many woody plants. Le Roux and van Staden (1991) reported *in vitro* rooting of between 2% and 30% for *Eucalyptus smithii* while Ahee and Duhoux (1994) reported less than 30% in *Faidherbia albida* micro-shoots. This *in vitro*

rooting problem can be averted through somatic embryogenesis. Furthermore, somatic embryos have functional root and shoot meristems, and hence regeneration of plantlets is always better (Raghavan, 1986). Therefore, an efficient and reproducible protocol for jacket plum plantlet regeneration through the somatic embryogenesis technique is needed. The objective of this trial was to determine an effective and reproducible protocol for mass multiplication of clonal jacket plum planting materials through somatic embryogenesis.

8.3 Materials and methods

8.3.1 Plant Material

P. capensis seeds and stem cuttings were collected from the National Botanical Gardens (refer to 6.3.1 for site description). A few stem cuttings were successfully rooted in a mist propagation chamber with 400 µmol m^{-2} s^{-1} PAR and 70 to 95% relative humidity (Chapter 7). Rooted stem cuttings were transferred to the glasshouse where they were preconditioned with Benlate (Benomyl, 0.1 g l^{-1}) for four weeks in order to free them from pathogens. Tender leaves (adult source) were excised from these stock plants and stirred in water with a few drops of Teepol (0.05%, 10 min). They were washed under running tap (30 min) and surface decontaminated in 1.75% NaOCl (8 min). They were rinsed in sterile water for four consecutive times under the airflow cabinet.

P. capensis seeds were scarified in 98% sulphuric acid (3 min) and rinsed in sterile water for four consecutive times. They were germinated on hormone-free, half strength Murashige and Skoog (Murashige & Skoog, 1962) medium containing 3% sucrose and pH was adjusted to 5.6±2 with either 1 N KOH or HCl and gelled with 0.3% Gelrite®. The MS

medium was autoclaved at about 100 ^{o}C and 121 psi (15 min) before culture initiation. Sections of cotyledons (seed leaves) and the first three leaves excised from aseptic seedlings were cultured on MS medium with different supplements for callus induction.

8.3.2 Callus induction

The experiment was laid out in a split-plot design with three sources of stock plants as the main plot (juvenile and adult leaf tissues, and cotyledons) and plant growth regulators as sub-plots. Leaf (1 cm^2) and cotyledon sections were cut and placed on 25 ml aliquot of MS medium with 4% sucrose, pH 5.6±2, different medium supplements and gelled with 2.5 g l^{-1} Gelrite®. The culture media were dispensed in 9-cm diameter Petri dishes. There were eight explants per Petri dish and the medium supplement combinations and concentrations (mg l^{-1}) used were either (i) 0.1 TDZ and 0.1 IBA, (ii) 0.1 TDZ, (iii) 1.0 BAP and 1.0 Kin, (iv) 0.1 BAP and 0.05 NAA, (v) 0.2 BAP and 1.0 (2,4-D), or (vi) 0.2 BAP and 1.0 NAA. (Refer to section 2.3.8 for incubation conditions). Data collected included percentage callus induction based on visual scores.

8.3.3 Induction of somatic embryos

The experiment was a randomised block design laid out in 10 × 2 factorial arrangement and replicated three times. Calli induced from leaf explants were transferred onto three quarter strength MS media which were dispensed into 9-cm diameter Petri dishes for somatic embryo induction, but calli from cotyledons were discarded. The three-quarter strength MS medium contained 4% sucrose, pH was adjusted to 5.6±2 with either I N KOH or HCl and then the medium was solidified with 2.5 g l^{-1} Gelrite®. Three-quarter strength MS medium supplements (mg l^{-1}) used were either (i) 0.3 casein hydrolysate (CH), (ii) 0.5 (2,4-D) and

1.5 NAA, (iii) 5.0 gibberellic acid (GA_3), (iv) 0.05 TDZ and 0.3 CH, (v) 2.0 BAP and 0.5 IAA, (vi) 1.0 BAP and 0.1 NAA, (vii) 1.0 TDZ and 0.3 CH, (viii) 2.0 kinetin and 0.5 IAA, (ix) 2.5 TDZ and 0.5 NAA, or (x) 2.0 TDZ.

Petri dishes were covered and sealed with parafilm strips before incubation. One set of treatments was exposed to light and another to dark conditions (refer to section 2.3.8 for incubation conditions). There were five Petri dishes per treatment combination. Petri dishes were later placed at a slanting position (approximately at an angle of 30 - 45^{o}).

8.3.4 Germination of somatic embryos

This experiment was a randomised block design with five Petri dishes per treatment and with three replicates. Calli at globular stage were transferred onto fresh medium (see section 8.3.3) every three to four weeks for embryo development. For plantlet formation, embryos at the cotyledonary stage were cultured on different MS medium with 3% sucrose, pH adjusted to 5.6±2 and solidified with 2.5 g l^{-1} Gelrite®. The medium supplements (mg l^{-1}) were either (i) hormone-free MS, (ii) half (½) strength MS, (iii) MS with 0.3 CH, (iv) three quarter strength MS with 0.2 BAP and 0.3 CH, (v) ½ strength MS with 0.3 CH, (vi) three quarter strength (¾) MS with 2.0 BAP and 0.3 CH, (vii) ¾ MS with 0.05 TDZ and 0.3 CH, (viii) ¾ MS with 1.0 TDZ and 0.3 CH, or (ix) ½ MS with 5.0 GA_3. In case of embryos showing strong rhizogenic capacity, they were transferred onto MS medium with 2.0 mg l^{-1} BAP and 0.3 mg l^{-1} CH dispensed in 150 × 10 mm test tubes. Data collected included number of plantlets regenerated on different MS media.

8.3.5 Culture conditions

Culture conditions were as described previously (Refer to section 2.3.8).

8.3.6 Statistical analysis

Data were subjected to analysis of variance (ANOVA) using GenStat (Rothamsted Experimental Station). Standard errors of mean values were calculated.

8.4 Results and discussion

8.4.1 Callus induction

Table 8.1 shows percentage callus induction of cotyledon sections and leaves from mature and juvenile explants on different MS medium supplements after four weeks. There were highly significant differences ($P \leq 0.01$) in callus induction with respect to the source of explants, medium supplements and the interactions between sources of explants and medium supplements. A significant amount of callus was obtained from leaf sections excised from juvenile stock plants for all MS medium supplements evaluated. The callus was mainly induced along the cut surfaces of the leaves. There was a poor response from cotyledon sections for all of the media evaluated. Callus could not easily be induced from the cotyledons. MS medium with 0.2 mg l^{-1} BAP and 1.0 mg l^{-1} 2, 4-D were slightly effective in callus induction for cotyledon explants after eight weeks. However, calli from the cotyledons were very loose, friable and not suitable for embryogenesis. These were discarded from further investigation.

MS medium supplemented with either 0.1 mg l^{-1} TDZ alone, 0.1 mg l^{-1} TDZ and 0.1 mg l^{-1} IBA or a combination of 0.2 mg l^{-1} BAP and 1.0 mg l^{-1} 2,4-D were superior in callus induction. MS medium supplemented with 0.1 mg l^{-1} TDZ was selected for further proliferation of callus to achieve adequate amounts for the somatic embryogenesis experiment.

8.4.2 Induction of somatic embryos

Leaf explants from juvenile stock plants resulted in rapid formation of green (chlorophyllous), compact and nodular calli (Figure 8.1A-B) when cultured on three quarter strength MS medium supplemented with 0.05 mg l^{-1} TDZ and 0.3 mg l^{-1} CH (Table 8.2) under light exposure. After four weeks, globular shaped embryos (Figure 8.2A) were observed from the compact and nodular calli. Transferring the calli on the same medium supplements led to regeneration of cotyledonary embryos after a further three to four weeks (Figure 8.2B-C).

The calli exposed to darkness were white or yellow and there was poor growth. Consequently, there was rapid degeneration and necrosis of calli. Raghavan (1986) reported that light conditions promote somatic embryogenesis. However, other authors have reported that dark conditions are stimulatory to embryogenesis, especially for embryo maturation. In this trial, dark conditions were inhibitory to somatic embryo induction for *P. capensis*. Therefore, somatic embryogenesis experiments continued under 12 h of light exposure, since light promoted development of embryogenic calli.

There was prolific growth of somatic embryos for Petri dishes placed on slanting positions (30 - 40^{o} angle). This is attributed to the absence of a film of liquid MS medium accumulating around the embryos. The film of liquid MS medium was inhibitory to embryo proliferation due to inadequate oxygen needed for the respiration of the embryos. At a slanting position, the liquid from the semi-solid MS medium accumulated at the lower part of the Petri dishes and the volume increased with time.

The data in Table 8.2 show that a high concentration of TDZ decreased the number of embryos being regenerated. TDZ has been reported to induce somatic embryos in many recalcitrant tree species such as white ash, *Flaximus americana* and neem, *Azadirachta indica* (Singh *et al*., 2003). In this trial, low TDZ concentrations were stimulatory to embryogenesis on calli excised from juvenile stock plants. Higher TDZ concentrations (>1.0 mg l^{-1}) resulted in production of secondary calli, and hence suppressed further somatic embryo growth and development. Huetteman & Preece (1993) reported that high concentrations of TDZ stimulated callus proliferation, but inhibited shoot production in walnut trees. This trial shows similar findings. Singh *et al*. (2003) reported that exposing pigeon pea (*Cajanus cajan*) somatic embryos to high concentrations of TDZ improved somatic embryogenesis. This suggests that the response to high concentrations of TDZ might depend on plant species. In this trial, a few somatic embryos were observed from leaves from mature stock plants, but the regeneration capacity was low (<3%). For a better embryogenesis response, the juvenile plant materials should be used.

8.4.3 Germination of somatic embryos

Three quarter strength MS medium with 0.05 mg l^{-1} TDZ and 0.3 mg l^{-1} CH was effective (65%) in germination of *P. capensis* somatic embryos despite a strong rhizogenic capacity (i.e. embryos with good roots but poor shoot development). These rhizogenic embryos (Figure 8.3A-B) were later germinated on three quarter strength MS medium supplemented with 0.2 mg l^{-1} BAP and 0.3 mg l^{-1} CH. Successful embryo separation led to formation of single *P. capensis* plantlets (Figure 8.4A-B), but multiple and fused plantlets were obtained when clusters of embryos were left to grow together. Early separation of somatic embryos was important in order to avoid fusion of somatic embryos.

Generally, plantlets regenerated on MS medium supplemented with TDZ at low concentration showed some moderate of callus formation at the root zone (Figure 8.4B) and high concentrations resulted in profuse secondary callusing. Therefore, lower concentrations of cytokinin, especially TDZ (0.05 mg l^{-1}) improved somatic embryogenesis in *P. capensis*. Continuous exposure of *P. capensis* embryos to low concentrations of TDZ was necessary for somatic embryogenesis. This is in agreement with findings reported in pigeon pea study by Singh *et al*. (2003).

In this trial, casein hydrolysate seemed to promote greening (chlorophyllous) and proliferation of somatic embryos. According to Singh & Chand (2003) and Robichaud *et al*. (2004) amino acids are known to increase the number and size of somatic embryos and improve the conversion of embryos into plantlets. Casein hydrolysate (200 mg l^{-1}) promoted somatic embryo maturation in *Dianthus* species (Pareek & Kothari, 2003) and the

results of this study suggest that casein hydrolysate (CH) also promotes embryo development in *P. capensis*.

8.5 Conclusion

MS medium supplemented with 0.1 mg l^{-1} TDZ was effective in callus induction on leaf sections excised from juvenile stock plants of *P. capensis*. Three quarter strength MS medium supplemented with 0.05 mg l^{-1} TDZ and 0.3 mg l^{-1} CH promoted somatic embryogenesis under 12 h of light exposure and when Petri dishes were placed on a slanting position (30^{o} - 40^{o}). Three quarter strength MS medium supplemented with 0.2 mg l^{-1} BAP and 0.3 mg l^{-1} CH promoted germination of rhizogenic embryos into complete plantlets. With this protocol, mass multiplication of *P. capensis* planting material through somatic embryogenesis was achieved. Therefore, an effective and reproducible protocol for mass multiplication of *P. capensis* planting material has been determined.

Tables

Table 8.1 Percentage callus induction from leaf and cotyledon sections of jacket plum (*Pappea capensis*) tree species after weeks on Murashige and Skoog (MS) medium supplemented with (mg l^{-1}) (T1) 0.1 thidiazuron and 0.1 indole-3-butyric; (T2) 0.1 thidiazuron; (T3) 1.0 benzylaminopurine (BAP) and 1.0 kinetin; (T4) 0.1 BAP and 0.05 α-naphthaleneacetic acid (NAA); (T5) 0.2 BAP and 1.0 dichlorophenoxyacetic acid (2,4-D); or 0.2 BAP and 1.0 NAA.

Source of explants	MS medium supplements (mg l^{-1})						
	T1	T2	T3	T4	T5	T6	Mean
Leaves (mature)	60	51	40	33	60	30	46b
Leaves (juvenile)	78	78	50	42	83	63	66a
Cotyledons	40	42	18	12	53	13	39c
Mean	59b	57b	36c	29d	67a	36c	

CV (%) = 9.4

LSD (0.01) Source of explants (S) = 6.7**

Medium treatment (T) = 5.8**

S × T = 9.9^{NS}

** highly significant differences at P≤0.01

NS = no significant differences at P≤0.01

Numbers with the same letters are not significantly different at P≤0.01

Table 8.2 *Pappea capensis* somatic embryos induced on three quarter strength Murashige and Skoog (MS) medium with different supplements from juvenile and mature tissues after 12 weeks. Data were recorded with standard error

Three quarter MS medium supplements (mg l^{-1})	Embryos regenerated (%)		Conditions of embryos
	juvenile	mature	
0.3 CH	0.0	0.0	brown/compact
0.5 (2,4-D) + 1.5 NAA	6.7±3.3d	0.0	brown/loose
5.0 GA_3	0.0	0.0	brown/compact
1.0 BAP + 0.1 NAA	0.0	0.0	brown/compact
2.0 BAP + 0.5 IAA	0.0	0.0	white/friable
0.05 TDZ + 0.3 CH	60.0±5.8 a	5.0±1.5 a	green/nodular
1.0 TDZ + 0.3 CH	36.7±3.3 b	1.0±0.5 b	green/compact
2.0 Kin + 0.5 IAA	0.0	0.0	white/compact
2.5 TDZ + 0.5 NAA	20.0±5.8 c	0.0	green/loose
2.0 TDZ	26.7±3.3bc	0.0	green/friable

Means followed by same letters within a column are not significantly different at P≤0.05

Table 8.3 Effect of Murashige and Skoog (MS) medium with different supplements on *Pappea capensis* somatic embryos after 10 weeks

MS with supplements ($mg\ l^{-1}$)	Embryo germination (%)	Condition of embryos
MS	0[c]	browning of embryos
½ MS	0[c]	browning of embryos
MS + 0.3 CH	0[c]	greening of embryos
¾ MS + 0.2 BAP + 0.3 CH	45[b]	root and shoot regeneration
½ MS + 0.3 CH	0[c]	greening of embryos
¾ MS + 2.0 BAP + 0.3 CH	0[c]	browning of embryos
¾ MS + 0.05 TDZ + 0.3 CH	65[a]	rhizogenesis
¾ MS + 1.0 TDZ + 0.3 CH	0[c]	rhizogenesis
½ MS + 5 GA_3	0[c]	greening of embryos

Means followed by same letters within a column are not significantly different at $P \leq 0.05$

Figures

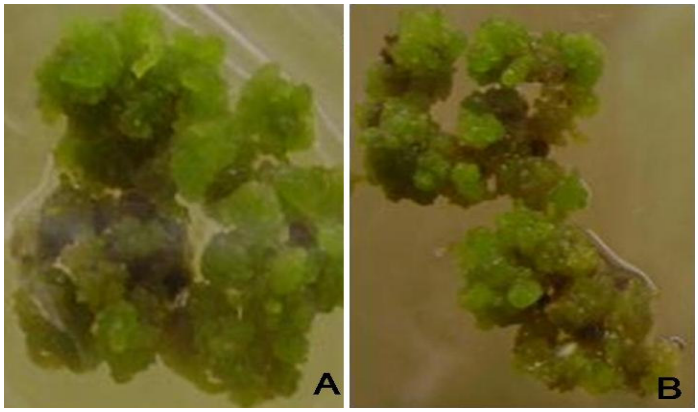

Figure 8.1 Jacket plum (*Pappea capensis*) calli induced on three quarter strength Murashige and Skoog medium with 0.05 mg l^{-1} thidiazuron and 0.3 mg l^{-1} casein hydrolysate: (A) chlorophyllous and embryogenic calli after three weeks; (B) calli after four weeks

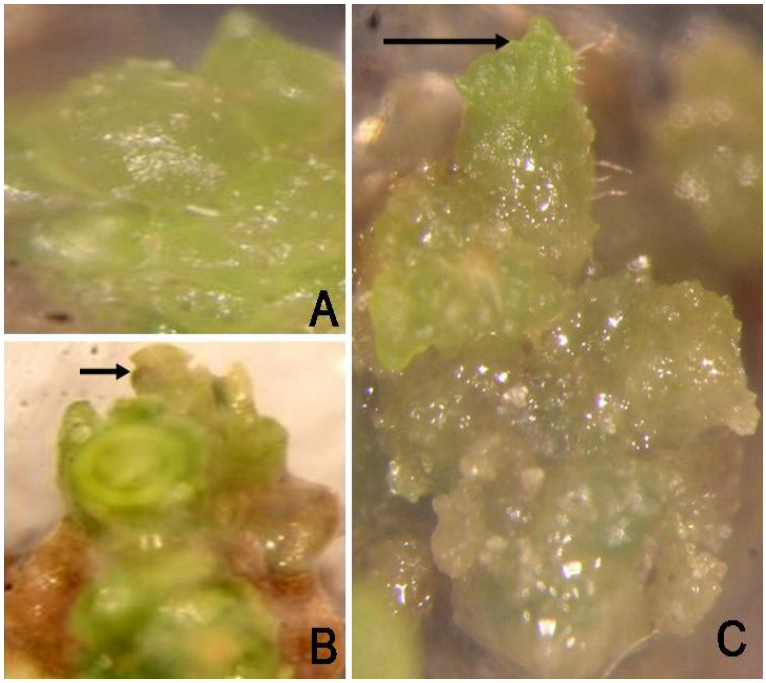

Figure 8.2 Jacket plum (*Pappea capensis*) somatic embryos on three quarter strength Murashige and Skoog medium with 0.05 mg l^{-1} thidiazuron and 0.3 mg l^{-1} casein hydrolysate: (A) early globular stage after 4 weeks; (B) open cotyledonary somatic embryo; (C) closed-up polycotyledonary somatic embryo (a long arrow shows a closed-up embryo tip while a short arrow shows an open tip)

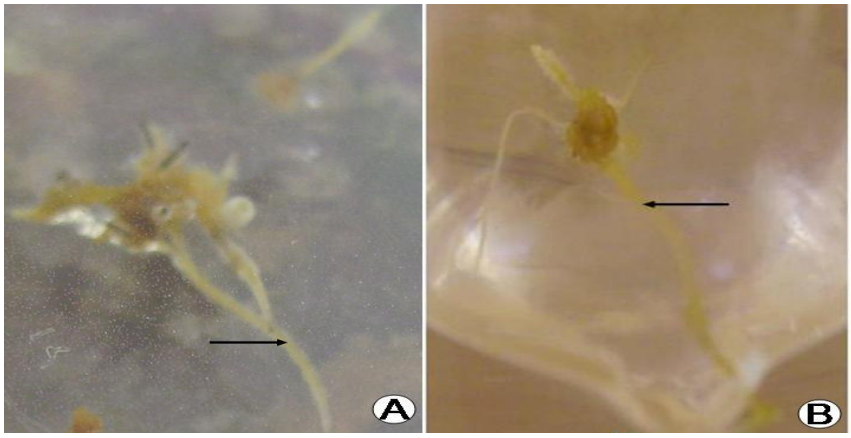

Figure 8.3 Jacket plum (*Pappea capensis*) embryos regenerated through indirect embryogenesis (A) cluster of somatic embryos on Murashige and Skoog medium with 0.05 mg l^{-1} thidiazuron and 0.3 mg l^{-1} casein hydrolysate (CH); (B) embryo germination on 0.2 mg l^{-1} benzylaminopurine and 0.3 mg l^{-1} CH five days after being separated from a cluster of embryos. Arrows indicate roots

Figure 8.4 Jacket plum (*Pappea capensis*) plantlets regenerated through embryogenesis after eight weeks on three quarter strength Murashige and Skoog medium supplemented with either (A) 0.2 mg l^{-1} benzylaminopurine (BAP) and 0.3 mg l^{-1} casein hydrolysate (CH) or (B) 0.05 mg l^{-1} thidiazuron and 0.3 mg l^{-1} CH and later on 0.2 mg l^{-1} BAP and 0.3 mg l^{-1} CH