CHAPTER 7

IN VITRO PROPAGATION OF JACKET PLUM (*PAPPEA CAPENSIS*) TREE SPECIES

7.1 Abstract

The objective of the trial was to determine an effective propagation protocol for jacket plum (*Pappea capensis*) tree species. Experiments on *in vitro* propagation and rooting of stem cuttings were carried out. Stem cuttings were dipped in half strength Murashige and Skoog medium (MS) or water before application of Seradix® No. 2 and 3, Dip 'n Grow or 0.1 g l^{-1} indole-3-butyric acid (IBA, liquid form). Juvenile shoot explants were cultured on MS medium with different supplements for shoot multiplication and root regeneration. Dipping stem cuttings in MS medium prior to application of rooting hormones improved bud break and survival of cuttings for more than two months, but there was poor rooting (11%). For micro-propagation, significant differences ($P \leq 0.05$) in number of micro-shoots regenerated and number of micro-shoots that formed roots. MS medium with 2.0 mg l^{-1} benzylaminopurine (BAP) was significantly superior to either 1.5 mg l^{-1} kinetin, 5.0 mg l^{-1} BAP, or 1.0 mg l^{-1} BAP and 5.0 mg l^{-1} gibberellic acid (GA_3) with respect to shoot multiplication. IBA (0.5 mg l^{-1}) was superior (42%) to other MS medium supplements. *In vitro* rooting was improved to 64% when 0.5 mg l^{-1} IBA medium supplement was repeated with one-month exposure to half strength MS medium before the second exposure to rooting treatment.

7.2 Introduction

Jacket plum (*Pappea capensis* L.) tree is a good fodder for livestock and the seed oil (74%) is fairly viscous (Swart, 1991; Venter & Venter, 1996), and hence can be exploited as a potential source of bio-diesel. Such diesel-fuels are renewable and contribute lower amounts of net greenhouse gasses to the atmosphere than does the fossil diesel (Bouaid *et al*., 2005; Ramadhas *et al*., 2005; Canoira *et al*., 2006). Recently, there is increasing interest in selecting superior germplasm for multiplication of jacket plum trees. Consequently, trials on the contributions of jacket plum trees to bio-diesel have been initiated at the University of Pretoria, South Africa. However, wide cultivation of these species or managing them in their natural habitats will largely depend on efficient propagation techniques that result in large quantities of good quality planting materials. The only known method of propagation is by seeds, but seedling growth is extremely slow (Palmer & Pitman, 1972; Anonymous, 1997).

Vegetative propagation is always preferred but rooting is a setback for many woody tree species. No scientific research appears to have been done on vegetative propagation of jacket plum tree species. The challenge for vegetative propagation of many tropical woody trees is root regeneration, unlike that in sexually propagated planting materials where seedlings easily develop normal and functional roots (Kwapata *et al*., 1999). Therefore, the objective of this trial was to determine an effective propagation protocol for *P. capensis* tree species.

7.3 Materials and methods

7.3.1. Stem cuttings

Semi-hardwood cuttings (epicormic shoots) were collected from mature trees at the National Botanical Institute (1360 m above sea level, 25° 44'S, 28° 16'E), northeast of Pretoria in South Africa (Botha *et al*., 2000) from October 2004 to February 2005. Epicormic stem cuttings were selected because they are not completely juvenile and it is expected that they fruit earlier than seed-derived plants (George, 1993). The experiment was a randomised complete block design with a 2 × 4 factorial arrangement and four replicates. There were eighty (80) stem cuttings per treatment combination and a total number of 640 stem cuttings were used for this experiment. Rooting of stem cuttings was assessed over a period of 16 weeks.

The stem cuttings (10-15 cm long) were dipped in half strength Murashige and Skoog (Murashige & Skoog, 1962) medium for 12 hours or planted without pre-treating in MS medium. Rooting hormones applied were (i) Seradix® No. 2 (0.3% indole-3-butyric acid, IBA in powder form) (Bayer, Pretoria, South Africa), (ii) Seradix® No. 3, (iii) Dip 'n Grow (10 g l^{-1} IBA and 5 g l^{-1} NAA in liquid form) or (iv) IBA (0.1 g l^{-1} in liquid form). These stem cuttings were planted in tray flats containing sterile fine quartz sand growing medium. The trays were placed on mist beds where there was eight second jet of mists every four minutes. The conditions in the mist propagation chamber were at 70 to 95% relative humidity, 23 to 26 °C temperatures and 400 µmoles m^{-2} sec^{-1} photosynthetically active radiation (PAR).

7.3.2 Pilot micro-propagation experiment

Jacket plum seeds were soaked in 98% sulphuric acid (3 min) to remove the seed coats (testas) and then rinsed in sterile water. The seeds were nicked and decontaminated in 1.75% sodium hypochlorite (7 min) before rinsing in sterile water for four consecutive times. The seeds were germinated on hormone-free, half strength MS medium supplemented with 3% sucrose, solidified with 0.3% (w/v) gellan gum (Gelrite®). The medium pH was adjusted to 5.6±2 with 1 N KOH or 1 N HCl before addition of Gelrite. Ten (10) ml of MS medium with supplements was dispensed into 25 × 125 mm test tubes and tubes were covered with caps before autoclaving at about 100 ^{o}C under 121 psi pressure (15 min).

Eleven different MS medium supplements were evaluated and the promising supplements were selected for further experimentation. Epicotyl shoots were excised from two-week old seedlings and explanted on MS medium supplemented with mg l^{-1} of either (i) 0.1 thidiazuron (TDZ), (ii) 0.1 TDZ and 0.1 indole-3-butyric acid (IBA), (iii) 2.0 benzylaminopurine (BAP), (iv) 1.0 BAP and 1.0 kinetin (Kin), (v) 0.1 BAP and 0.1 Kin, (vi) 0.2 BAP and 0.1 Kin, (vii) 1.5 Kin and 0.05 α-naphthaleneacetic acid (NAA), (viii) 3.0 BAP and 0.01 IBA, (ix) 5.0 BAP and 0.01 IBA, (x) 0.1 BAP and 0.05 NAA, or (xi) 3.0 BAP and 0.1 indole-3-acetic acid (IAA). The MS medium supplements were selected based on rate of bud break, growth of micro-shoots, presence of necrotic shoot tips and callusing. The MS medium, with 3% sucrose and pH 5.6±2 adjusted with 1 N KOH or 1 N HCl, were gelled with 0.3% Gelrite. The MS medium (10 ml aliquot) was dispensed into 25 × 125 mm test tubes and tubes were then covered with caps before autoclaving at about 100 ^{o}C under 121 psi pressure (15 min). Ten test tubes were used per treatment and then sealed with

parafilm strips. This experiment was carried out for a period of four weeks, but not repeated.

7.3.3 Shoot multiplication

This experiment was a completely randomised design with six treatments and three replicates. There were twenty test tubes per treatment and this experiment was carried out for a period of 12 weeks. Six MS medium supplements were selected. These supplements (mg l^{-1}) were: (i) 1.5 kinetin; (ii) 5.0 BAP; (iii) 0.1 BAP and 0.05 NAA; (iv) 2.0 BAP; (v) 1.0 BAP and 1.0 kinetin or (vi) 1.0 BAP and 5.0 gibberellic acid (GA_3). For the last medium treatment, 5.0 mg l^{-1} GA_3 replaced kinetin since a combination of 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} kinetin promoted a high amount of callus formation despite a good bud break. The MS medium with 3% sucrose, pH 5.6±2 and solidified with 0.3% Gelrite was then dispensed into 25 × 125 mm test tubes. Each test tube contained 10 ml aliquot of MS medium. Tubes were then covered with caps. They were autoclaved at about 100 ^{o}C under 121 psi pressure (15 min). Excised shoots with two nodes were cultured and ensuing micro-shoots were subcultured three times on the same MS medium. Test tubes were sealed with parafilm strips and incubated. Data collected included the number of micro-shoots produced per responding explant, rate of callusing and shoot tip necrosis.

7.3.4 Root regeneration

Micro-cuttings produced from axillary shoots with two leaves were excised and cultured on half strength MS medium supplemented with IAA, IBA, NAA or their different combinations. The experiment was a randomised complete block design with ten rooting treatments (mg l^{-1}), namely: (i) 0.1 IBA; (ii) 0.5 IBA and 0.5 NAA; (iii) 1.0 IBA; (iv) 1.0

IBA and 0.5 IAA; (v) 0.5 IBA; (vi) 0.1 NAA; (vii) 0.5 IBA and 0.5 IAA; (viii) 0.5 IAA; (ix) 0.5 NAA or (x) 1.5 IBA. The half strength MS medium also contained 3% sucrose and gelled with 0.3% Gelrite. The MS medium pH was adjusted to 5.6±2 with 1 N KOH or 1 N HCl before Gelrite was added. The culture medium was dispensed into 25 × 125 mm test tubes. Test tubes containing 10 ml aliquot of MS medium were covered with caps. They were autoclaved at about 100 ^{o}C under 121 psi pressure (15 min) and then sealed with parafilm strips after culture initiation. Micro-cuttings were maintained on the same MS medium for four weeks.

Plantlets were transferred onto half strength MS medium without hormones soon after root induction. Micro-cuttings that failed to root were transferred onto half strength MS medium without hormones after four weeks of exposure to the rooting hormones. Data collected included the number of rooted explants and the number of roots per responding explant. Plantlets were hardened off in a mist bed with 70 to 95% relative humidity, 23 to 26 ^{o}C and 400 µmoles m^{-2} sec^{-1} PAR.

7.3.5 Repeated exposure of micro-cuttings to IBA

All micro-cuttings that failed to root were maintained on half strength MS medium without hormones for four weeks. Terminal shoots (3-4 cm long) were excised from these micro-cuttings and cultured on half strength MS medium supplemented with 0.5 mg l^{-1} IBA, since this was the most effective rooting treatment established from the previous experiment. The medium pH was adjusted to 5.6±2 with 1 N KOH or 1 N HCl. The culture medium contained 3% sucrose and was solidified with 0.3% Gelrite. All test tubes (25 × 125 mm) containing 10 ml aliquot of MS medium was covered with caps. The tubes were autoclaved

at about 100 ^{o}C under 121 psi pressure (15 min) and the sealed with parafilm strips after cultures were explanted. There were 15 test tubes used to assess the rooting capacity of these micro-cuttings and this was repeated three times. All the cultures were on the same MS medium for a period of four weeks (section 2.3.8 describes incubation conditions). Plantlets were hardened off in a mist bed with 70 to 95% relative humidity, 23 to 26 ^{o}C temperature and 400 µmoles m^{-2} sec^{-1} PAR.

7.3.6 Statistical analysis

Data transformation for shoot multiplication experiments was done using arcsine (Steel & Torrie, 1980). The data were subjected to analysis of variance (ANOVA) using GenStat (Rothamsted Experimental Station).

7.4 Results and discussion

7.4.1 Stem cuttings

There was early leaf loss in all the stem cuttings followed by bud break and the formation of new leaves. Stem cuttings pre-treated in MS medium remained green and continued to produce shoots for 12 weeks. Many stem cuttings not pre-treated in MS medium wilted within 8 weeks and died. Although stem cuttings pre-treated in MS medium continued to produce new leaves, rooting was very poor (11%). This occurred despite the fact that the cuttings were from epicormic shoots. There were no significant differences ($P \leq 0.05$) between the rooting treatment combinations. Dipping cuttings in MS medium only extended the leaf regeneration period and bud break, but not rooting. This study confirmed

that there are rooting difficulties in jacket plum trees and that with hardwood and mature stem cuttings, rooting will be harder to achieve.

7.4.2 Pilot micro-propagation experiment

Inclusion of auxins, especially IBA, increased the rate of callusing at the bases of explants. MS medium supplemented with TDZ and IBA highly favoured callus production (Figure 7.1) at the bases and shoot tips of explants and hence this medium was not selected. A combination of 5.0 mg l^{-1} BAP and 0.01 mg l^{-1} IBA resulted in good bud break despite high amounts of callus formation (Figure 7.1). This medium formulation was selected but IBA was excluded. An MS medium supplemented with 2.0 mg l^{-1} BAP gave the highest bud break, but low amount of callus formation (Figure 7.1). An MS medium supplemented with 0.1 mg l^{-1} BAP and 0.05 mg l^{-1} NAA resulted in high bud break and rapid growth of micro-shoots despite the high amounts of callus formation at the bases of explants (Figures 7.1 and 7.2A). This MS medium formulation was also selected.

7.4.3 Shoot multiplication

Data in Table 7.1 shows mean number and condition of micro-shoots produced on different MS media for a period of 12 weeks. There were significant differences ($P \leq 0.05$) amongst treatments. MS medium with 2.0 mg l^{-1} BAP was superior (4.1 shoots per explant) to other treatments. Matu *et al*. (2006) found that BAP was superior to kinetin in shoot multiplication in *Maytenus senegalensis* trees. Our results agree with their findings. Shoot tip necrosis was observed when micro-shoots were kept on this medium for four weeks or longer before subculturing. This could be attributed to prolific micro-shoot growth and

build-up toxicity with time in the culture tubes. Once there was shoot tip death, new micro-shoots were regenerated (Figure 7.2B).

In vitro shoot tip necrosis has been reported in *Pistacia vera* explants on MS medium and this is attributed to slow calcium absorption (Barghchi and Alderson, 1985). Bhalla and Mulwa (2003) also reported a high number of necrotic shoot tips (80%) in macadamia shoots. In this trial, adding 0.3 mg l^{-1} casein hydrolysate slightly reduced shoot tip necrosis and frequent subculturing (short passages) was effective in reducing this problem.

A combination of cytokinin and a low auxin concentration, or the use of another cytokinin improved growth of micro-shoots. This agrees with the findings of Huetteman and Preece (1993). MS medium with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} kinetin, and 0.1 mg l^{-1} BAP and 0.05 mg l^{-1} NAA resulted in a high rate of shoot multiplication. Jain *et al*. (1990) also found cytokinin and auxin combination to be effective for *Morus* species but Islam *et al*. (1993) found the combination to be less effective in *M. laevigata*. Figure 7.3A shows three micro-shoots produced on MS medium supplemented with 2.0 mg l^{-1} BAP which was an optimal formulation. Bhalla and Mulwa (2003) reported the highest percentage bud break and shoot multiplication on MS medium containing 2.0 mg l^{-1} BAP. Subculturing ortet explants on MS medium supplemented with 2.0 mg l^{-1} BAP after excising micro-shoots promoted growth of a prolific single micro-shoot (Figure 7.3B). This prolific growth makes it possible to get multiple micro-cuttings within two weeks.

7.4.4 Root regeneration

Roots were observed after 10-15 days. Figure 7.4 shows rooting percentage of jacket plum micro-cuttings cultured on different medium supplements. There were significant differences amongst rooting treatments ($P \leq 0.05$). The MS medium supplemented with 0.5 mg l^{-1} IBA was superior in rooting (42%) jacket plum micro-cuttings. *In vitro* rooting of micro-shoots is affected by several factors (Rugini *et al*., 1993; Kwapata *et al*., 1999). Le Roux and van Staden (1991) reported a wide range in rooting percentage within *Eucalyptus* species. In their trial, a range of 0 to 67% rooting was obtained for *E. macarthurii*, 2 to 30% for *E. smithii* and 21 to 100% for *E. saligna*. This indicates that woody perennial tree species show variations in *in vitro* rooting capacity. Figure 7.6 shows significant differences ($P \leq 0.05$) amongst treatments with respect to mean number of roots. A significant number of roots (3) per plantlet was obtained on MS medium supplemented with 0.5 mg l^{-1} IBA (Figure 7.5). These data show that IBA was superior to NAA and IAA in root regeneration. Matu *et al*. (2006) also found that IBA was better than NAA and IAA in root regeneration.

A combination of IBA and NAA induced callusing at the bases of explants (Figure 7.6A). Johnston and Armstrong (2003) reported callus development at the bases, petioles and leaves of Christmas bush (*Ceratopetalum gummiferum* Sm.) explants on MS medium supplemented with IBA or NAA. Despite the large amount of callus, they reported a high rooting percentage (80-93%) and this suggests that callus development does not hinder rooting. However, *in vitro* rooting experiments for woody tree species have shown different responses (Williams, Taji & Bolton, 2003). In this study, observations showed that MS medium supplemented with a combination of 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} IAA resulted in

slender or weak roots. Some of the thread-like roots curled away from the rooting medium. It was further observed that micro-cuttings with a few or no leaves had good root regeneration (Figure 7.6B-C) and they were able to regenerate new shoots and leaves upon transferring to half strength MS medium. Huetteman and Preece (1993) attributed rooting difficulties to 'carry over' effects from the shoot multiplication medium. Scott, Carter & Street (1961) and Maliro (1997) attributed a delay in root induction, and fewer and weaker roots occurring in many woody tree species to inadequate light quality exposure.

In this trial, shoot tip necrosis was absent in all the jacket plum plantlets, even those with weak and few roots. However, shoot tip necrosis was pronounced during *in vitro* shoot multiplication. Therefore, this means poor absorption of plant nutrient by micro-shoots plays a major role in the development of shoot tip necrosis. There could be seasonality effect on rooting of micro-cuttings, a common phenomenon in many woody tree species. Poor rooting has been reported in many tropical woody trees (Kwapata *et al*., 1999) and that there is often less than 30% of explants that regenerate normal and functional roots (Ahee and Duhoux, 1994). This is often attributed to inadequate light quality and intensity (Torrey, 1952; Maliro, 1997). However, there are several factors that affect *in vitro* rooting of micro-shoots (Rugini *et al*., 1993). In our study, root induction was done under 12 hours of light and darkness using cool white fluorescence tubes. Amin & Jaiswal (1993) improved rooting of jackfruit explants from 40% after the fourth subculture to 80% after the tenth subculture.

7.4.5 Repeated exposure of micro-cuttings to IBA

In this study, rooting of jacket plum micro-cuttings was improved from 42% to 64% when maintained on half strength MS medium for 4 weeks before exposing them to 0.5 mg l^{-1} IBA for the second time (Figure 7.7). It is suspected that 'carry over' effect was broken by exposing micro-cuttings to hormone free MS medium for four weeks or longer. Amin and Jaiswal (1993) improved rooting of jackfruit (*Artocarpus heterophyllus*) explants from 40% after the fourth subculture to 80% after the tenth subculture. In this trial, rooting was improved by exposing micro-cuttings to MS medium supplemented with 0.5 mg l^{-1} IBA for the second time.

7.4.6 Acclimatization

Acclimatization of the regenerated plantlets was done to assess survival. Plantlets were removed from the MS medium and the roots were washed with distilled water to remove the MS medium. Plantlets were hardened off in a mist propagation bed and 70% survival rate of plantlets was achieved after they were hardened off.

7.5 Conclusion

Jacket plum stem cuttings (epicormic shoots) are difficult to root despite a good to produce shoots. MS medium treatment with 2.0 mg l^{-1} BAP was optimal for rapid multiplication of micro-shoots and ortet explants. This makes rapid and mass production of jacket plum plantlets possible. Repeated exposure of micro-cuttings to 0.5 mg l^{-1} IBA improved rooting to 64%. It would be interesting to investigate the effect of repeated exposure of jacket plum stem cuttings to rooting hormones, especially those in liquid, on rooting capacity.

Table

Table 7.1 Mean number and condition of jacket plum (*Pappea capensis*) micro-shoots produced on Murashige & Skoog (MS) medium supplemented with benzylaminopurine (BAP), kinetin (Kin), α-naphthaleneacetic acid (NAA) and gibberellic acid (GA_3)

MS medium Supplements (mg l^{-1})	Mean number of shoots per explant	Condition of micro-cuttings
1.5 Kin	2.9 ± 0.2^{bc}	no shoot tip necrosis
5.0 BAP	2.3 ± 0.1^{c}	no shoot tip necrosis
0.1 BAP + 0.05 NAA	3.4 ± 0.4^{ab}	base callusing
2.0 BAP	4.1 ± 0.7^{a}	shoot tip necrosis
1.0 BAP + 1.0 Kin	2.5 ± 0.1^{bc}	little shoot tip necrosis
1.0 BAP + 5.0 GA_3	2.0 ± 0^{c}	no shoot tip necrosis
CV (%)	21.7	
$LSD_{(0.05)}$	1.05	

Numbers with the same letters within a column are not significantly different ($P \leq 0.05$)

Figures

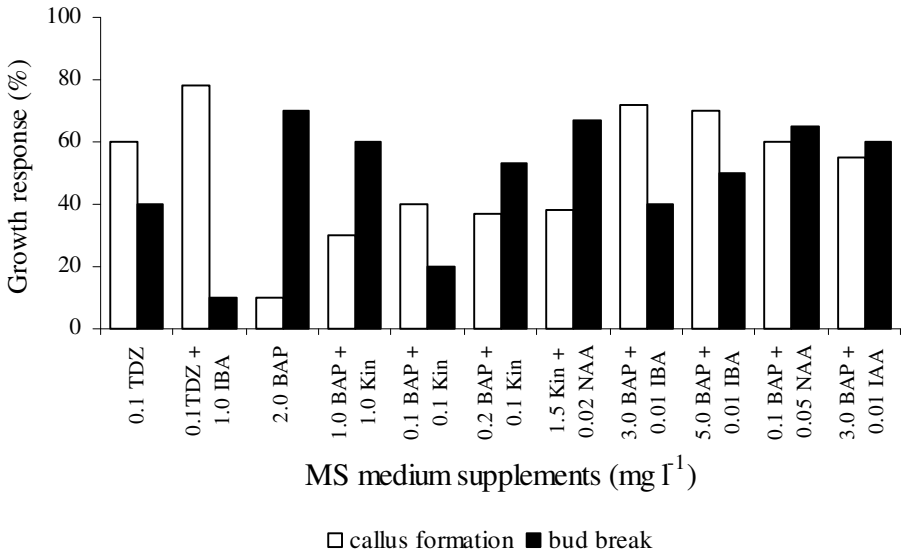

Figure 7.1 Callus formation and bud break (%) of jacket plum (*Pappea capensis*) micro-shoots explanted on Murashige and Skoog medium supplemented with different concentrations (mg l^{-1}) and combinations of benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), Kinetin (Kin), α-naphthaleneacetic acid (NAA) or thidiazuron (TDZ) four weeks after explanting

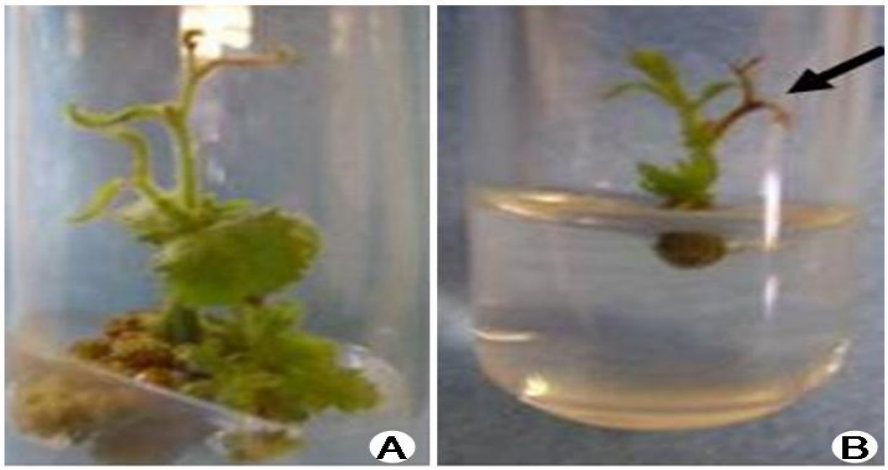

Figure 7.2 Jacket plum (*Pappea capensis*) micro-shoots on Murashige and Skoog medium (A) a high amount of callus formation at the bases of micro-shoots on 0.1 mg l^{-1} benzylaminopurine and 0.05 mg l^{-1} α-naphthaleneacetic acid; (B) micro-shoot with a necrotic shoot tip (arrow shows shoot tip death) and new micro-shoots being produced

Figure 7.3 Jacket plum (*Pappea capensis*) shoot multiplication on Murashige and Skoog medium supplemented with 2.0 mg l^{-1} benzylaminopurine (BAP) after two weeks: (A) three healthy micro-cuttings; (B) single shoot produced after subculturing the ortet explant

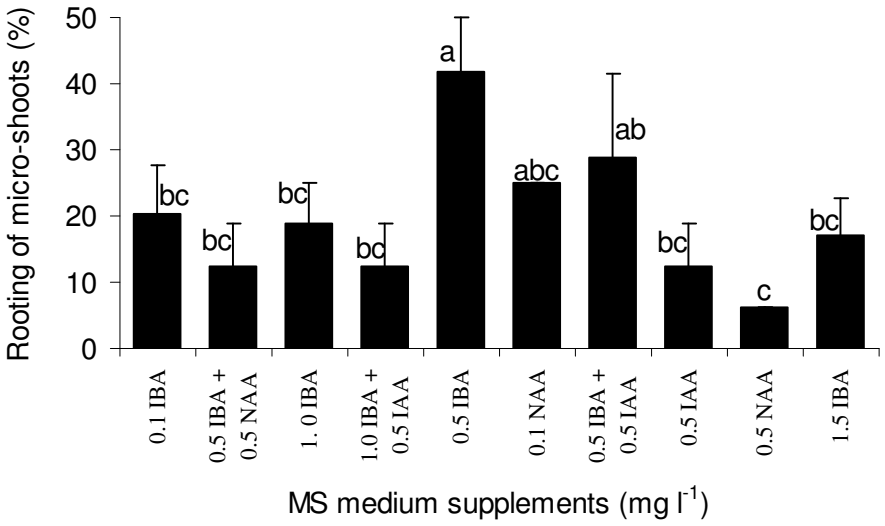

Figure 7.4 Rooting percentage of jacket plum (*Pappea capensis*) on half strength Murashige and Skoog medium supplemented with different concentrations (mg l^{-1}) and combination of indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) and indole-acetic acid (IAA) three weeks after explanting

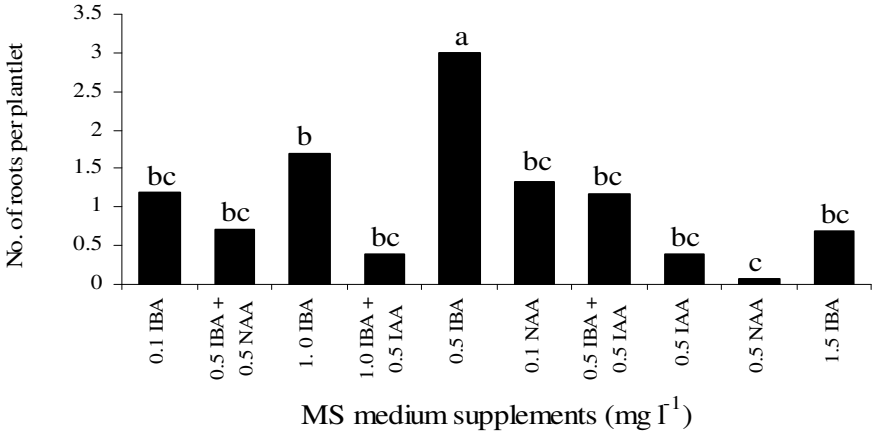

Figure 7.5 Mean number of roots regenerated on jacket plum (*Pappea capensis*) micro-cuttings rooted on different concentrations (mg l^{-1}) and combinations of indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) and indole-acetic acid (IAA)

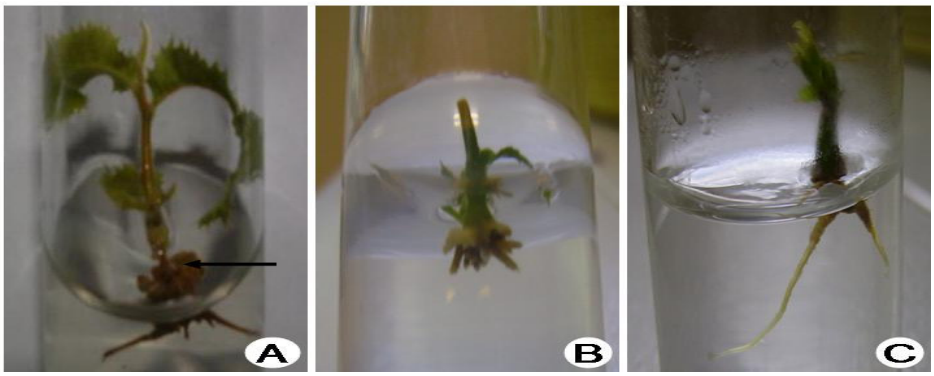

Figure 7.6 Rooted jacket plum (*Pappea capensis*) micro-cuttings on half strength Murashige and Skoog medium supplemented with either (A) 0.5 mg l^{-1} indole-3-butyric acid (IBA) and 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA) after three weeks or (B) 0.5 mg l^{-1} IBA or (C) 0.5 mg l^{-1} IBA and 50 mg l^{-1} thiamine HCl (arrow shows high amount of callusing at the base of plantlet)

Figure 7.7 Jacket plum (*Pappea capensis*) plantlet rooted on half strength Murashige and Skoog (MS) medium supplemented with 0.5 mg l^{-1} indole-3-butyric acid when subcultured on the same medium (three weeks old)