CHAPTER 2

IN VITRO PROPAGATION OF MATURE *UAPACA KIRKIANA* Müell Arg. TREES

2. 1 Abstract

The objectives of study were to determine efficient decontamination and micro-propagation protocols for mature *Uapaca kirkiana* plant materials. The efficacy of sodium hypochlorite (NaOCl), calcium hypochlorite, $Ca(OCl_2)_2$ and mercuric chloride ($HgCl_2$) as surface sterilants was evaluated. Field collected shoots and lateral shoots from grafted trees, preconditioned with Benomyl (0.1 g l^{-1}), were used. Murashige and Skoog (MS) media supplemented with benzylaminopurine (BAP), thidiazuron (TDZ) or kinetin were evaluated for shoot multiplication. Rooting was evaluated on different concentrations of indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA). Pre-conditioning of stock plants was important and $HgCl_2$ was equally effective in decontaminating shoot (80%) and leaf explants (59%). New shoots (lateral shoots) responded positively to shoot multiplication on three quarter MS medium with a combination of 0.2 mg l^{-1} BAP, 0.04 mg l^{-1} NAA and 0.3 mg l^{-1} casein hydrolysate. High TDZ (>0.1 mg l^{-1}) concentrations increased callus formation, and hence suppressed shoot multiplication. Callused explants on TDZ could not survive when transferred onto MS medium with BAP. Rooting of micro-cuttings (36%) was achieved with MS medium supplemented with 2.5 mg l^{-1} IBA. Plantlets were hardened off, but failed to survive when potted. Micro-propagation of mature *U. kirkiana* was, therefore, shown to be feasible.

2.2 Introduction

U. kirkiana trees have a long juvenile phase (about 10-12 years) when sexually propagated. This frustrates potential fruit tree growers (Akinnifesi *et al*., 2004, 2006). By using mature plant materials for *in vitro* propagation enables multiplication of superior proven plants to achieve precocious fruiting. However, micro-propagation of mature woody trees is difficult because of poor regenerative ability, and hence low multiplication rate (Pierik, 1987). Furthermore, there is often a need to rejuvenate material in such cultures and consequences of such methods on subsequent tree performance need to be evaluated on this species.

Studies on micro-propagation of *U. kirkiana* trees have previously been carried out (Maliro, 1997; Chishimba *et al*., 2000; Nkanaunena, 2002), but successes were not achieved for the explants collected from mature stock plants. Nkanaunena (2002) reported no success in micro-grafting of scions from mature trees due to fungal contamination. In all the studies done to date, successful micro-propagation protocols of *U. kirkiana* trees were only achieved using seedlings as stock plants (Maliro, 1997; Chishimba *et al*., 2000; Nkanaunena, 2002). However, it is difficult to ascertain the gender and future production characteristics of *U. kirkiana* seedlings. This is important for *U. kirkiana* trees since they are dioecious and superior provenances have already been collected and characterised. Use of explants of proven genetic potential is preferred to seedlings or embryos (juvenile plant materials).

Micro-propagation of mature woody plants largely depends on successful rejuvenation and culture asepsis. Therefore, effective decontamination and rejuvenation protocols are

prerequisites for micro-propagation of mature stock plants. Lack of rejuvenation and contamination are the major obstacles to overcome *in vitro* propagation of mature *U. kirkiana* plants (Maliro, 1997; Nkanaunena, 2002). Sodium hypochlorite (NaOCl, 2%) was found to be ineffective in decontaminating *U. kirkiana* explants excised from mature stock plants and preconditioned in a non-mist propagation unit (Nkanaunena, 2002). Maliro (1997) reported high fungal contamination on *U. kirkiana* leaf explants and trials for mature plants failed at the initial stage due to fungal contamination. Culture asepsis and micro-propagation of *U. kirkiana* was only achieved from seedlings (juvenile plant material) (Maliro, 1997; Chishimba *et al*., 2000; Nkanaunena, 2002). In addition, this was achieved with long hours of washing explants and the use of concentrated sterilants. This indicates the presence of endogenous, endophytic or cryptic contamination which is often difficult to eliminate. According to Mwamba (1995), *U. kirkiana* trees live and thrive in the wild in association with symbiotic microbes. However, there has been no research study done to elucidate the effects of these organisms on *in vitro* contamination.

To date no scientific study has been done on alternative methods to achieve culture asepsis of mature *U. kirkiana* plants. Different chemotherapy and stock plant preconditioning methods have also not been evaluated. The objectives of this study were to develop efficient decontamination and micro-propagation protocols for *U. kirkiana* explants excised from mature stock plants.

2.3. Materials and methods

2.3.1 Plant material

U. kirkiana shoots were collected from mature and healthy trees at Chongoni natural forest (Dedza district) in Malawi in January and June 2004. Also, grafted *U. kirkiana* trees (one-year old after grafting) were also collected from Makoka Research Station in Malawi in January 2005. All of these grafted trees were washed to be free of soil, wrapped in moist newspaper, placed in a cooler box and transported to the University of Pretoria (South Africa) within three days.

2.3.2 Site description

Chongoni forest is at 1632 m above sea level, latitude 14^{o} 19' S and longitude 34^{o} 16' E (Ngulube *et al*. 1997). Makoka lies at 1029 m above sea level, latitude 15^{o} 30'S and longitude 35^{o} 15' E. The total annual rainfall ranges from 560 to 1600 mm, with a ten year-mean of 930 mm. The rainfall is unimodal with most of the rains falling between November and April. Temperature varies between 16 ^{o}C and 32 ^{o}C (Akinnifesi *et al*., 2004). The trees were taken to the University of Pretoria Experimental Farm. This site lies 25^{o} 45' S; 28^{o} 16' E (at an altitude of 1372 m above sea level). The grafted trees were kept under mist for two days before potting into a nursery soil mixture (small stones, pine bark and ash in a 1:1:2 proportion, pH = 6.8, CEC = 0.66). They were kept under mist for a week before transferring to the glasshouse for preconditioning. Benomyl (Benlate, 0.1 g l^{-1}), a systemic fungicide, was applied once a week and the trees were pruned to induce lateral branch development. The trees were acclimatized for four weeks before the trials commenced. Old shoots collected after pruning the grafted trees were used for *in vitro* culture experiments. Watering of grafted trees was done in the morning and three times per week.

2.3.3 Efficacy of sodium hypochlorite and calcium hypochlorite on field collected explants

Field collected *U. kirkiana* shoots were washed in Benomyl (0.14 g l^{-1}) with a few drops of Teepol (0.05%, 30 min). The shoots were then dipped in 50% ethanol (20 sec) and washed under running tap water (1 h). They were further decontaminated, in a laminar flow cabinet, using three different treatments, namely either (i) 3.5% NaOCl (15 min), (ii) 40 mg l^{-1} $Ca(OCl_2)_2$ (15 min) or (iii) 3.5% NaOCl (5 min) with subsequent 1.4% NaOCl (15 min). Disinfectants were decanted and explants were then rinsed in sterile water for four consecutive times. Shoots were trimmed (0.5 - 1 cm long) and cultured on Murashige and Skoog (Murashige & Skoog, 1962) basal media without plant growth regulators. The experiment was laid out in a completely randomised design with three treatments and twenty explants per treatment. The experiments were replicated three times. In case of contamination, the explants were re-decontaminated in 3.5% NaOCl (15 min), 40 mg l^{-1} $Ca(OCl_2)_2$ (15 min) or mercuric chloride (0.1% w/v $HgCl_2$, 8 min).

2.3.4 Efficacy of mercuric chloride on shoot explants from grafted trees

Old shoots and new lateral shoots collected from grafted *U. kirkiana* trees (scions) and shoots from forest trees were washed in Benlate (0.14 g l^{-1}) with a few drops of Teepol. They were then washed under running tap water (20 min) and decontaminated with mercuric chloride (0.1% w/v $HgCl_2$, 8 min). They were further rinsed in sterile water for six consecutive times, trimmed (0.5 - 1 cm) and then explanted onto MS medium without plant growth regulators.

A completely randomised design was used with three treatments (three types of explants). There were twenty shoots per treatment and three replicates.

2.3.5 Effect of medium supplements on contamination of leaf explants

Leaves excised from preconditioned grafted *U. kirkiana* trees (scions) were washed in Teepol (15 min) and surface sterilised in 0.1% $HgCl_2$ (8 min). They were then rinsed in sterile water for five consecutive times. Leaf sections (approximately 1 cm^2) were explanted on MS media supplemented with either (i) 1.0 mg l^{-1} indole-3-butyric acid (IBA) and 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA), (ii) 0.2 mg l^{-1} thidiazuron (TDZ) and 0.5 mg l^{-1} NAA, (iii) 0.5 mg l^{-1} benzylaminopurine (BAP) and 1.0 mg l^{-1} NAA or (iv) 0.1 mg l^{-1} TDZ and 4.0 mg l^{-1} NAA. The experiment was laid out in a complete randomised block design with four treatments (plant growth regulators). There were ten leaf explants per treatment and three replicates.

2.3.6 Shoot multiplication

All aseptic explants from previous experiments (grafted trees) were cultured on ¾ MS media supplemented (mg l^{-1}) with either (i) 0.05 TDZ and 0.3 casein hydrolysate (CH), (ii) 0.1 TDZ and 0.01 IBA, (iii) 0.2 TDZ and 0.3 CH, (iv) 0.1 BAP, 0.04 NAA and 0.3 CH, (v) 0.2 BAP, 0.04 NAA and 0.3 CH, (vi) 0.5 BAP and 0.04 NAA, (vii) 1.0 BAP, 0.04 NAA and 0.3 CH or (viii) 0.2 kinetin and 0.04 NAA. The experiment was a complete randomised block design with ten explants per treatment and three replicates.

2.3.7 Root regeneration

Rooting trial involved only micro-shoots regenerated from lateral shoot explants and half strength MS media were supplemented (mg l^{-1}) with either (i) 0.5 IBA, (ii) 1.0 IBA, (iii) 2.5 IBA, (iv) 1.0 NAA or (v) 0.5 NAA and 0.5 IBA. In case of callused shoot explants,

especially those on MS medium supplemented with high concentrations of TDZ, they were immediately transferred onto MS medium supplemented with BAP.

2.3.8 Culture conditions

All the MS media used contained 3% sucrose and pH was adjusted to 5.6±2 with 1 N KOH or 1 N HCl and then solidified with 0.3% (w/v) gellan gum (Gelrite®). The MS medium (10 ml aliquot) was dispensed into 25 × 125 mm test tubes and then covered with caps before autoclaving at about 100 ^{o}C under 121 psi pressure (15 min). Test tubes were sealed with parafilm strips after culture initiation and then incubated under a 12 h photoperiod and 60 µmol m^{-2} sec^{-1} PAR using two cool white fluorescent tubes per shelf. Temperatures were maintained at 23±2 ^{o}C. All plantlets produced were hardened off in a mist bed with 70-95% relative humidity and 400 µmol m^{-2} sec^{-1} PAR. Within the mist enclosure, there was eight second jet of mist at four minute interval.

2.3.9 Statistical analysis

Data were analysed using GenStat 4.24 DE (Rothamsted Experimental Station) following angular transformation (Steel & Torrie, 1980).

2.4 Results and discussion

2.4.1 Efficacy of sodium hypochlorite and calcium hypochlorite on field collected explants

There were no aseptic shoot cultures obtained from field grown stock plants regardless of type and concentration of disinfectants used. Cultures were heavily contaminated and

overgrown by unidentified fungi. The fungal hyphae were seen first growing from the top part of explants and progressed to the explant-medium contact. Colonization of microbes progressed with time and all explants were heavily covered in fungal mycelia after a week. The results indicate the presence of endogenous, cryptic or endophytic fungal in *U. kirkiana* tree species.

Explants were removed from the MS medium after three weeks and they were still green (alive) though not actively growing. It was difficult to declare the fungi '*vitropathic'* but their proliferation on top of explants could be attributed to the weakening of membrane or cell wall accelerated by disinfectant and the presence of oxygen. Discharge of plant nutrients from plant cells could have stimulated an outgrowth of endogenous fungi. Darworth and Callan (1996) reported that endogenous or endophytic fungi become pathogenic to the host plants only when the plants are stressed. In this trial, the stress could be due to low nutrient uptake, weakened cell walls or low light conditions. Helander, Neuvonen & Ranta (1996) reported that mutualism depends on the prevailing plant condition, but such mutualistic association may be broken once plants are stressed. *U. kirkiana* trees live and thrive with symbiotic mycorrhizae (Mwamba, 1995).

Maliro (1997) and Nkanaunena (2002) obtained no aseptic cultures from mature *U. kirkiana* explants when 2% NaOCl was used. This confirms that *U. kirkiana* trees live and survive in association with endogenous or cryptic microbes. The results also show low efficacy of NaOCl and $Ca(OCl_2)_2$ at the concentrations and exposure time used. Chishimba *et al.* (2000) used 30% NaOCl to decontaminate *U. kirkiana* seedlings but there was no report on the number of aseptic or dead cultures. High concentrations of disinfectants and

long exposure time may injure explants. In this trial, there was death of old shoot explants when re-decontaminated in 0.1% $HgCl_2$, an indication that $HgCl_2$ was too strong for the already weakened explants. There was also resurgence of contaminants when explants were re-decontaminated either in NaOCl or $Ca(OCl_2)_2$. This shows that these two sterilants are not effective in decontaminating *U. kirkiana* explants.

2.4.2 Efficacy of mercuric chloride on explants from young preconditioned grafted trees

About 80% culture asepsis was achieved with 0.1% $HgCl_2$ for explants excised from young preconditioned *U. kirkiana* trees. The results show that preconditioning stock plants was effective to achieve high *in vitro* culture asepsis. $HgCl_2$ was equally effective in decontaminating explants. Use of $HgCl_2$ reduced lengthy washing of explants under running tap water (20 min) compared to other sterilants evaluated in this study. However, $HgCl_2$ was less effective on explants which were directly collected from the field. Therefore, preconditioning grafted *U. kirkiana* trees played an important role to achieve culture asepsis and this is important for plants that harbour cryptic or endogenous microbes.

2.4.3 Effect of medium supplements on decontamination of leaf explants

No significant difference ($P \leq 0.05$) was detected amongst treatments (plant growth regulators) with respect to contamination (Table 2.1). This indicates that different plant growth regulators used in this experiment did not promote or influence *in vitro* contamination of leaf explants. This also indicates that decontaminating *U. kirkiana* leaf explants, excised from grafted trees, in 0.1% $HgCl_2$ solution was effective in controlling *in vitro* contamination. Maliro (1997) reported a high rate of *in vitro* contamination of *U.*

kirkiana leaf explants. This suggests that almost every part of *U. kirkiana* plants is associated with endogenous or cryptic fungi which are difficult to decontaminate.

2.4.4 Effect of explant age on *in vitro* phenol production

Observations made from this trial showed that phenol accumulation in the MS medium was mainly from the old shoots (scions) collected from the field or grafted trees. There was also production of phenols into the MS medium from the mature fully-expanded leaves from grafted trees. However, it was difficult to record differences in browning intensity or phenol content in the MS medium. This is because of frequent transferring of aseptic explants onto fresh medium for shoot multiplication experiments. Phenol production was visibly absent from new lateral shoot explants, and hence these were preferred to the old shoots. This indicates that lateral shoots were probably rejuvenated unlike the old shoots (scions) since excessive exudates (phenols) are major characteristics of mature tissues. According to Ochatt, Davey & Power (1990), disinfectants that precipitate protein are preferred to those that oxidise. This is especially important for woody explants that are associated with high production of phenols into the growth media. They further reported that $HgCl_2$ is preferred to NaOCl since the latter increases accumulation of phenols due to oxidation.

2.4.5 Shoot multiplication

The number of shoots produced per responding explant and the amount of callus formation on different medium supplements varied widely amongst treatments (Table 2.2). Three quarter strength MS medium supplemented with a combination of 0.1 mg l^{-1} BAP, 0.04 mg l^{-1} NAA and 0.3 mg l^{-1} casein hydrolysate (CH) was effective in shoot multiplication (2.5

shoots per responding explant). However, increasing BAP concentration to 1.0 mg l^{-1} resulted in a decrease in the number of shoots produced. Chishimba *et al*. (2000) also reported that high cytokinin concentrations inhibited shoot multiplication of *U. kirkiana* explants using juvenile plant materials (seedlings) and that low concentrations of BAP were effective in shoot multiplication.

In this trial, growth of micro-shoots was slow (Figure 2.1A) but high TDZ concentrations (0.1-0.2 mg l^{-1}) resulted in an excessive amount of callus formation (Figure 2.1B). Stunted micro-shoots were also observed on MS medium with TDZ and prolific callusing negatively affected bud-break (number of shoots produced). The old shoot explants excised from grafted trees did not respond positively to different MS medium supplements except a high amount of callusing on MS medium supplemented with TDZ (Figure 2.1C).

Significant differences were observed for explants on three quarter strength MS medium supplemented with either BAP or TDZ (Table 2.2). There was no callusing of explants on ¾ MS medium supplemented with BAP as shown in Figure 2.1A, but the amount of callus formation was significantly high on three quarter strength MS medium supplemented with 0.2 mg l^{-1} TDZ and 0.3 mg l^{-1} CH (Figure 2.1B-C). It was observed that higher concentrations of TDZ stimulated profuse amount of callusing but transferring such callused explants onto three quarter strength MS medium with BAP did not promote further growth of shoot explants or calli. Such explants remained alive for some weeks but eventually died. This could be attributed to a high TDZ dose effect in that BAP could not promote growth of callused explants after being exposed to TDZ. Therefore, a low TDZ

concentration (0.05 mg l^{-1}) was better for shoot multiplication although *U. kirkiana* explants are amenable to callusing.

The present trial was compounded by inadequate supply of new lateral shoots as the grafted trees in the glasshouse died after the fourth collection of lateral shoots. This could be attributed to either the effect of severe pruning or to a lack of mycorrhizae. The trees could be sensitive to wounding due to frequent lateral shoot collection and the initial pruning. It is also speculated that Benlate might have eliminated any remnant symbiotic microbes from the trees. The most likely cause could be due to poor acclimatisation of *U. kirkiana* grafted trees to the new glasshouse habitat together with the use of soils deficient in mycorrhizae. According to Mwamba (1995), survival of *U. kirkiana* trees is associated with mycorrhizae and possibly other unknown endophytes. The trees were transported without the soils where mycorrhizal inocula are often present. The death of even the rootstocks suggests that soil conditions might have played a vital role, and hence this rules out graft incompatibility as a possible cause of poor stock plant survival. Generally, this confirms that maintenance of symbiotic microbes (mycorrhizae) is critical for the survival of *U. kirkiana* trees. According to Högberg (1982), *U. kirkiana* trees grow and survive due to the presence of fungal mycorrhizae.

In the present trial, there was a positive response from new lateral shoots excised from grafted trees compared to the shoots taken from older trees. This indicates that *U. kirkiana* is amenable to *in vitro* propagation if manipulated properly. The use of new shoots and preconditioned stock plants overcame high contamination rates and allowed rejuvenation. Therefore, with adequate preconditioned stock plants (grafted trees), micro-propagation of

mature *U. kirkiana* trees is feasible using the lateral shoot explants. According to Auge (1995) growth of explants can be seasonal due to changes in hormone balance in some plants at a typical seasonal stage. Moreover, the balance between the endogenous growth regulators and those in the media (exogenous) can affect the ultimate growth response of explants. Evaluation of other types, combinations and concentrations of plant growth regulators would also improve growth response of *U. kirkiana* lateral shoot explants as would an evaluation of the time of the year that explants were collected.

2.4.6 Rooting of micro-shoots

A few *U. kirkiana* micro-shoots were cultured onto the rooting half strength MS medium with four medium supplements being evaluated. The results show that rooting of *U. kirkiana* micro-cuttings was difficult although a few were successfully rooted (36%) on half strength MS medium supplemented with 2.5 mg l^{-1} IBA. Although this rooting percentage is low, it is the first report on *in vitro* rooting of *U. kirkiana* explants excised from mature stock plants. This low rooting is attributed to a rejuvenation problem. According to Franclet *et al*. (1987), juvenility in scions is short-lived and repeated pruning or grafting is useful for rejuvenation. Furthermore, through repeated pruning a certain degree of juvenility can be achieved. In this trial, death of stock plants hindered further investigation on rooting ability of *U. kirkiana* micro-shoots from pruned trees.

It was observed from this trial that lateral shoot explants did not cause any visible browning (phenol accumulation) of the different MS media. George (1993) reported that explants excised from heavily pruned trees resulted in low browning of the MS medium and increased rooting ability. Use of *U. kirkiana* young lateral shoot explants yielded positive

results unlike the old shoot explants in terms of growth response and low or no phenol production into the culture medium.

Figure 2.2 shows rooted *U. kirkiana* micro-cuttings cultured on half strength MS medium supplemented with 2.5 mg l^{-1} IBA. There was base callusing of plantlets and this is attributed to the high concentration of IBA that was used. However, a significant amount of callus formation was also observed in many explants during shoot multiplication and root regeneration stages. Chishimba *et al.* (2000) reported that there was low number of roots on *in vitro* propagated *U. kirkiana* seedlings. In this trial, the number of roots per plantlet was not more than two and this may indicate that rejuvenation was a problem. There could be a seasonality effect on rooting of *U. kirkiana* explants since some explants have rhizogenic capacity only during a particular period. The use of rooting hormones (auxins) may extend the rooting period to some extent (Auge, 1995), but these hormones cannot induce rooting in the unresponsive period. Woody plants have a poor regenerative ability and are often difficult to rejuvenate. Consequently, they have a low multiplication rate. They also exude toxic substances (phenols) that hinder *in vitro* growth of explants (Pierik, 1987).

From this trial, survival of *U. kirkiana* plantlets was poor and this could have been due to the absence of symbiotic mycorrhizae. Mwamba (1995) reported a high survival of *U. kirkiana* seedlings due to presence of fungal mycorrhizae. The fungal mycelia increased the volume of soil from which *U. kirkiana* seedlings were able to extract plant nutrients and water. In the present trial, only a few *U. kirkiana* (three) plantlets survived up to six months (Figure 2.3), and it is likely that the presence of symbiotic mycorrhizae could have enhanced *U. kirkiana* plantlet growth and survival.

2.5 Conclusion

Preconditioning grafted *U. kirkiana* trees and decontaminating explants in 0.1% w/v mercuric chloride (8 min) were effective methods to achieve high *in vitro* culture asepsis. *In vitro* propagation of mature *U. kirkiana* tree species is feasible with lateral shoots excised from preconditioned grafted trees. Three quarter strength MS medium supplemented with 0.1 or 0.2 mg l^{-1} BAP, 0.04 mg l^{-1} NAA and 0.3 mg l^{-1} CH was effective for shoot multiplication and half strength MS medium supplemented with 2.5 mg l^{-1} IBA was effective in root regeneration. However, root regeneration needs further investigation as only a few micro-shoots were evaluated. From the present study, *in vitro* propagation of mature *U. kirkiana* trees is feasible and the present micro-propagation protocol can yield better results, especially if carried out in the natural habitat of *U. kirkiana* trees so that the stock plants (grafted trees) are not stressed due to absence of mycorrhizae. A detailed scientific study on the seasonality of rejuvenation period in *U. kirkiana* plants needs investigation. Suitable period can be exploited to increase multiplication of *U. kirkiana* plantlets without facing difficulties in shoot multiplication and root regeneration.

Tables

Table 2.1 *Uapaca kirkiana* leaf culture percentage asepsis explanted on Murashige and Skoog (MS) medium supplemented with benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA), thidiazuron (TDZ) and indole-3-butyric acid (IBA). There were no significant differences ($P \leq 0.05$) amongst treatments.

MS supplements (mg l^{-1})	Leaf culture asepsis (%)
1.0 IBA + 0.1 NAA	87.5 ± 6.3 [a]
0.2 TDZ + 0.5 NAA	93.1 ± 0.7 [a]
0.5 BAP + 1.0 NAA	92.4 ± 0.7 [a]
0.1 TDZ + 4.0 NAA	91.7 ± 0.0 [a]
CV (%)	5.7
LSD ($_{0.05}$)	10.3

Table 2.2 *Uapaca kirkiana* shoot multiplication on three quarter strength Murashige and Skoog (MS) medium supplemented with thidiazuron (TDZ), benzylaminopurine (BAP), kinetin (Kin), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) or casein hydrolysate (CH). Means are calculated with standard errors

MS supplements (mg l^{-1})	Number of shoots	Callus formation (%)
0.05 TDZ + 0.3 CH	2.2 ± 0.06^{bc}	35.0 ± 5.0^{b}
0.1 TDZ + 0.01 IBA	0.0^{d}	65.0 ± 5.0^{a}
0.2 TDZ + 0.3 CH	0.0^{d}	70.0 ± 5.0^{a}
0.1 BAP + 0.04 NAA + 0.3 CH	2.5 ± 0.06^{a}	20.0 ± 5.0^{de}
0.2 BAP + 0.02 NAA + 0.3 CH	2.3 ± 0.18^{ab}	25.0 ± 5.0^{cd}
0.5 BAP + 0.02 NAA	2.1 ± 0.03^{bc}	20.0 ± 5.0^{de}
1.0 BAP + 0.04 NAA + 0.3 CH	2.0 ± 0.03^{c}	15.0 ± 2.0^{e}
0.2 Kin + 0.04 NAA	2.0 ± 0.12^{c}	30.0 ± 5.0^{bc}
CV (%)	9.1	14.2

Means with the same letters in a column are not significantly different at $P \leq 0.05$

Figures

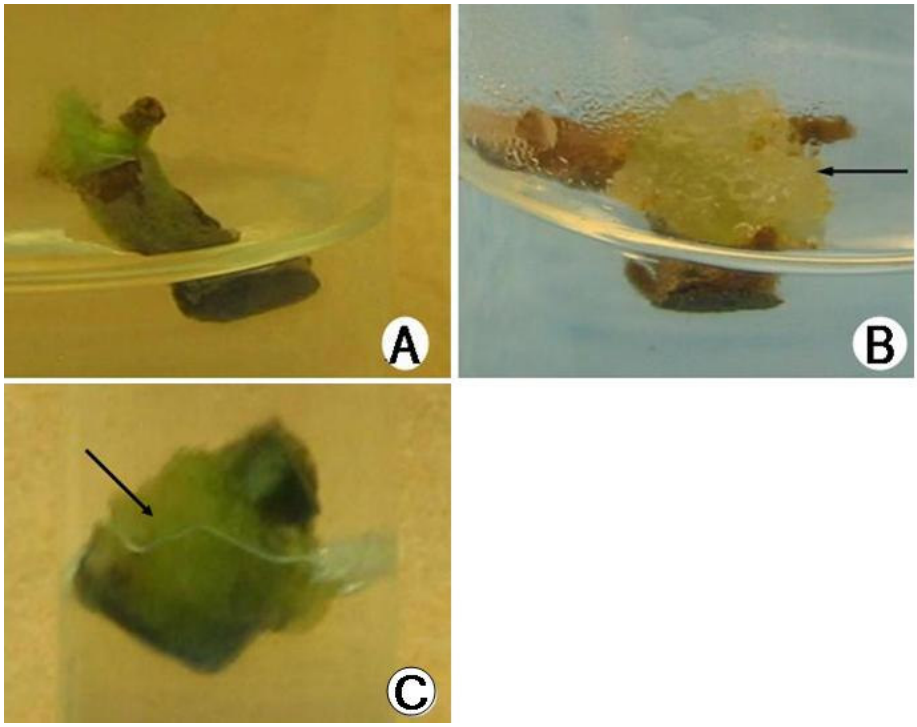

Figure 2.1 *Uapaca kirkiana* explants on three quarter strength Murashige and Skoog medium supplemented with (A) 0.1 mg l^{-1} benzylaminopurine (BAP), 0.04 mg l^{-1} α-naphthaleneacetic acid (NAA) and 0.3 mg l^{-1} casein hydrolysate (CH); (B) callusing on 0.2 mg l^{-1} thidiazuron (TDZ) and 0.3 mg l^{-1} CH; (C) old shoot explant not responding to three quarter MS medium supplemented with a combination of 0.05 mg l^{-1} TDZ and 0.3 mg l^{-1} CH after three weeks

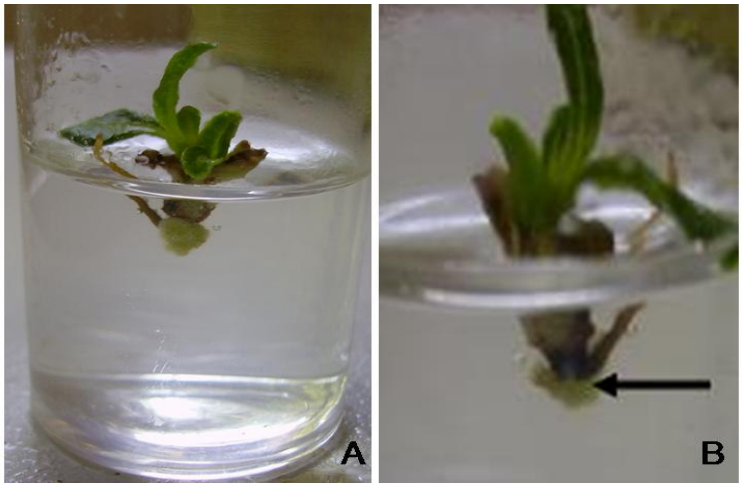

Figure 2.2 *Uapaca kirkiana* root regenerated on half strength Murashige and Skoog medium supplemented with 2.5 mg l^{-1} indole-3-butyric acid (IBA). Arrow shows base callusing of a plantlet

Figure 2.3 Six months old *Uapaca kirkiana* plant growing in a pot