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Full Length Research Paper

Effective preconditioning methods for *in vitro* propagation of *Uapaca kirkiana* Müell Arg. tree species

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The objective of the study was to determine efficient preconditioning methods for *in vitro* multiplication of *Uapaca kirkiana* plant materials from mature stock plants. The efficacy of sodium hypochlorite (NaOCl), calcium hypochlorite {$Ca(OCl_2)_2$} or mercuric chloride ($HgCl_2$) as surface sterilant was evaluated in decontaminating explants excised from grafted and field-collected *U. kirkiana* trees. Different Murashige and Skoog (MS) medium supplements were evaluated for shoot multiplication and root regeneration. Results indicated that preconditioning grafted *U. kirkiana* trees before excising explants and decontaminating explants in 0.1% w/v $HgCl_2$ were effective methods in establishing aseptic cultures (80%). Lateral shoots (new shoots) responded positively to shoot multiplication on ¾ strength MS medium supplemented with a combination of 0.1 mg/L benzylaminopurine, 0.04 mg/L naphthaleneacetic acid and 0.3 mg/L casein hydrolysate. High concentrations of thidiazuron (>0.1 mg/L) suppressed bud break. Rooting (36%) was achieved with ½ MS medium supplemented with 2.5 mg/L indole-3-butyric acid. Plantlets were successfully hardened off. *In vitro* multiplication of mature *U. kirkiana* plant materials was achieved using lateral shoots excised from grafted *U. kirkiana* trees after preconditioning with fungicides.

Key words: Decontaminants, fungi, euphorbiaceae, phenols, rejuvenation.

INTRODUCTION

Uapaca kirkiana Müell Arg. (wild loquat) is a priority tree crop selected for domestication in many countries of southern Africa (Akinnifesi et al., 2006). The tree bears edible fruits which are important sources of income and food to many rural community dwellers. Furthermore, these fruits serve as food reserves during seasonal food shortage (Maghembe and Seyani, 1991; Saka et al., 2002). Recent findings have shown that indigenous fruit trees of the Miombo ecosystem are capable of reducing vulnerability to poverty for more than 30% during a seasonal food shortage period (Mithöfer et al., 2006). However, lack of improved planting materials which result in precocious fruiting ability, superior fruit load and fruit size have been major bottlenecks to spontaneous cultiation by smallholder farmers in southern Africa (Akinnifesi et al., 2004).

U. kirkiana fruit trees have a long juvenile phase (10 - 12 years) when sexually propagated and this frustrates many potential *U. kirkiana* tree growers (Akinnifesi et al., 2004, 2006).Therefore, reliable propagation techniques that use mature plant materials are required to achieve precocious fruiting. Vegetative propagation methods such as air layering, budding, stem cuttings and grafting have been evaluated at SADC-ICRAF Makoka Agricultural Research Station in Malawi. Budding and rooting stem cuttings have yielded poor results while air layering is promising, but the root development after tree establishment was problematic (Mhango et al., 2000). Grafting has been the most promising method with 80% graft-take

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(Akinnifesi et al., 2004; 2006). However, growth irregularities, at the scion/stock union suspected to be due to graft incompatibility, have been observed in many grafted *U. kirkiana* trees in the nursery and the field (Mng'omba et al., 2007). Furthermore, there is a steady decline in tree survival from 100% at 6 months after tree establishment (MAE) to 67% at 13 MAE and the early growth in *U. kirkiana* was not significantly affected by fertilization, manure and irrigation (Akinnifesi et al., 2007).

In vitro propagation methods using mature plant materials enable multiplication of proven genetic potential plants and for precocious fruiting. However, micro-propagation of many mature woody plants is always difficult because of poor regenerative ability, and hence low multiplication rate (Pierik, 1987). Moreover, culture contamination is another major constraint to micro-propagation techniques (Enjalric et al., 1998). Many contaminants are visible at primary initiation except cryptic contaminants. Axenic cultures are preferred, and hence contaminated cultures are often discarded as they cause death of explants by exuding toxins or overgrowing the explants (Cassells, 1991; George, 1993). However, many adult plants live and thrive with symbiotic association, but such symbiotic microbes become contaminants in the culture media. According to Herman (1990), endogenous or endophytic microbes are often difficult to decontaminate although some are beneficial for the growth of explants.

Micro-propagation of mature woody plants largely depends on successful rejuvenation. Maliro (1997) and Nkanaunena (2002) reported that contamination and poor rejuvenation were the major obstacles to *in vitro* propagation of mature *U. kirkiana* plants. Success in culture asepsis and propagation of *U. kirkiana* plants was achieved using seedlings (Chishimba et al., 2000; Maliro, 1997; Nkanaunena, 2002). However, it is difficult to ascertain the gender and future production characteristics of *U. kirkiana* seedlings since *U. kirkiana* trees are dioecious (Mng'omba, 2007). Furthermore, unsuccessful micro-propagation protocols using adult *U. kirkiana* plant materials, due high fungal contamination at the initial stages, have been reported. These reports suggest the presence of endogenous, endophytic or cryptic contamination in *U. kirkiana* explants and such type of contaminants are difficult to eliminate. To date, there has been no scientific research done to achieve *in vitro* asepsis of mature *U. kirkiana* plants, and hence no micro-propagation breakthrough has been achieved. However, different chemotherapy and stock plant preconditioning methods have not been evaluated. The objectives of this study were to develop efficient decontamination and micro-propagation protocols for adult *U. kirkiana* plant materials.

MATERIALS AND METHODS

Plant material

U. kirkiana shoots were collected from mature trees (female) at Chongoni forest in Malawi. Grafted *U. kirkiana* trees (one year old after grafting) were also collected from SADC-ICRAF Makoka Agricultural Research Station in Malawi in 2005. All of these grafted trees were washed to be free of soils, wrapped in moist newspaper and placed in a cooler box. They were taken to the University of Pretoria Experimental Farm (South Africa) within three days.

Site description

Chongoni forest in Dedza district lies 1632 m above sea level, latitude 14° 19' S and longitude 34° 16' E (Ngulube et al., 1997). Makoka research station lies 1029 m above sea level, latitude 15° 30' S and longitude 35° 15' E. It has an annual rainfall between 560 and 1600 mm. Temperature varies between 16 and 32°C. The rainfall for both sites is unimodal and falls between November and April (Akinnifesi et al., 2004). The University of Pretoria Experimental Farm lies 1372 m above sea level, 25° 45' S; 28° 16' E.

The grafted *U. kirkiana* trees were kept under mists for two days before they were potted. The growth medium used was a nursery soil mixture (small stones, pine bark and ash, pH = 6.8, CEC = 0.66). The trees were kept under mists for one week before transferring them to the glasshouse for preconditioning. Benomyl (Benlate, 0.1 g/L), a systemic fungicide, was applied once a week and the trees were acclimatized for four weeks before commencement of the trials. The grafted trees were pruned to induce lateral shoot development and all the old shoots collected after pruning the trees were used as explants. Watering of grafted *U. kirkiana* trees was done to the pots in the morning and three times per week.

Efficacy of sodium and calcium hypochlorite on shoot explants

Field-collected *U. kirkiana* shoots were washed in Benomyl (0.14 g/L) with a few drops of Teepol (0.05%, 30 min). They were dipped in 50% ethanol (20 s) and washed under running tap water (1 h). The shoots were further decontaminated under a laminar flow cabinet in (i) 3.5% NaOCl (15 min), (ii) 40 mg/L $Ca(OCl_2)_2$ (15 min) or (iii) 3.5% NaOCl (5 min) and subsequently 1.4% NaOCl (15 min). Disinfectants were decanted and explants rinsed in sterile water for four consecutive times. Shoots were trimmed (0.5 - 1 cm long) and explanted on Murashige and Skoog (Murashige and Skoog, 1962) media without plant growth regulators.

The experiment was laid out in a completely randomised design with three treatments and replicated three times. There were ten test tubes (25 × 125 mm) per treatment. In case of contamination, the explants were re-decontaminated.

Efficacy of mercuric chloride on shoot explants

The lateral (new shoots excised from the scions) and old shoots excised from grafted *U. kirkiana* trees and field-collected shoots were washed in Benlate (0.14 g/L) with a few drops of Teepol (30 min). They were then washed under running tap water (20 min) and further decontaminated in 0.1% w/v $HgCl_2$ (8 min) solution. They were rinsed in sterile water for six consecutive times, trimmed (0.5 - 1 cm) and then explanted onto Murashige and Skoog media (MS) without plant growth regulators.

A completely randomised design was used with twenty test tubes (25 × 125 mm) per treatment and three replicates. Three sources of explants formed the treatments (lateral and old shoots from grafted trees and field-collected shoots).

Effect of medium supplements on contamination of leaf explants

U. kirkiana leaves were excised from grafted trees and washed in Benlate (0.14 g/L) with a few drops of Teepol (15 min). They were

surface decontaminated in 0.1% w/v $HgCl_2$ (8 min) and rinsed in sterile water for five consecutive times. Leaf sections (1 cm^2) were explanted on MS medium supplemented with either (i) 1.0 mg/L indole-3-butyric acid (IBA) and 0.1 mg/L α-naphthaleneacetic acid (NAA), (ii) 0.2 mg/L thidiazuron (TDZ) and 0.5 mg/L NAA, (iii) 0.5 mg/L benzylaminopurine (BAP) and 1.0 mg/L NAA or (iv) 0.1 mg/L TDZ and 4.0 mg/L NAA.

This 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.

Shoot multiplication

Aseptic shoot explants from the decontamination experiments were collected and explanted on three quarter (¾) strength MS medium supplemented (mg/L) 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, 0.02 NAA and 0.3 CH, (vi) 0.5 BAP and 0.02 NAA, (vii) 1.0 BAP, 0.04 NAA and 0.3 CH or (viii) 0.2 Kinetin and 0.02 NAA.

The experiment was laid out as a complete randomised block design with ten explants per treatment and three replicates.

Root regeneration

Micro-shoots obtained from lateral shoot explants were exposed to different ½ strength MS medium supplements for rooting. The ½ MS medium supplements (mg/L) used were either (i) 0.5 IBA, (ii) 1.0 IBA, (iii) 2.5 IBA, (iv) 1.0 NAA or (v) 0.5 NAA with 0.5 IBA.

The experiment was laid out as a complete randomised block design with five treatments and three replicates. Plantlets were then hardened off under mists with 70 - 90% relative humidity and 400 µmol m^{-2} s^{-1} PAR. The plantlets were potted and then placed in the glasshouse.

Culture conditions

All the MS media used in this trial 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®). All the test tubes (25 × 125 mm) were autoclaved at 100°C under 121 psi pressure for 15 min and sealed with parafilm strips before culture incubation. All cultures were then incubated under 12 h of photoperiod and 60 µmol m^{-2} s^{-1} PAR supplied by two cool white fluorescent tubes. Temperatures in the incubation room were maintained at 23 ± 2°C.

Statistical analysis

Data collected were subjected to analysis of variance (ANOVA) using GenStat Discovery edition 2 (Rothamsted Experimental Station).

RESULTS AND DISCUSSION

Efficacy of sodium and calcium hypochlorite on shoot explants

There were no aseptic shoot cultures obtained regardless of the type and concentration of disinfectants used. Cultures were contaminated and overgrown by unidentified fungi. The fungal hyphae first appeared on the top part of explants and progressed to the explant–medium contact. The part of explants inside the media and the media itself were free from visible contamination. Colonization of fungi progressed with time and all explants were covered in fungal mycelia after a week. However, the part of explants inside the MS media remained free from visible contaminants. The present results indicate the presence of endogenous, cryptic or endophytic fungi in *U. kirkiana* explants.

Contaminated *U. kirkiana* explants were removed from the MS media after three weeks. Observations indicated that they were still green (alive) though not actively growing. However, it was difficult to declare these fungi '*vitropathic*', but proliferation of these fungi on top of explants could be attributed to the weakening of membrane or cell wall accelerated by disinfectant. Discharge of *U. kirkiana* sap (nutrients) from the cells could have stimulated an outgrowth of endogenous fungi. According to Darworth and Callan (1996), endogenous or endophytic fungi become pathogenic to the host plants only when the plants are stressed. In this experiment, the stress could be attributed to the weakened cell walls or other unfavourable *in vitro* conditions such as limitation in oxygen or light conditions. Helander et al. (1996) reported that mutualism depends on the prevailing plant condition. According to Mwamba (1995), *U. kirkiana* trees live and thrive with symbiotic mycorrhizae, but such mutualistic association might be broken once the host plant is stressed.

Maliro (1997) and Nkanaunena (2002) obtained no aseptic cultures from mature *U. kirkiana* explants when decontaminated in 2% NaOCl. This confirms that *U. kirkiana* trees live and survive in association with endogenous or cryptic microbes which become *in vitro* contaminants. The present results indicate low efficacy of NaOCl and $Ca(OCl_2)_2$ sterilants 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. Concentrated disinfectants and long exposure time might injure explants. In this trial, there was death of the old shoot explants when re-decontaminated in 0.1% w/v $HgCl_2$. This indicates that $HgCl_2$ was too strong for the weakened explants. In addition, there was resurgence of contaminants when explants were re-decontaminated either in NaOCl or $Ca(OCl_2)_2$.

Efficacy of mercuric chloride on shoot explants

Culture asepsis was achieved with 0.1% w/v $HgCl_2$ from preconditioned stock plants (80%). The results show that preconditioning stock plants was effective in establishing aseptic cultures. $HgCl_2$ was equally effective in decontaminating explants. Moreover, lengthy washing of explants in running tap water was reduced (20 min). However, $HgCl_2$ was less effective on field-collected explants. Therefore, preconditioning grafted *U. kirkiana* trees was necessary to achieve culture asepsis.

Table 1. *Uapaca kirkiana* leaf culture asepsis (%) on Murashige and Skoog (MS) medium supplemented with different combinations of indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), thidiazuron (TDZ) or benzylaminopurine (BAP) (Mean with the same letters indicates no significant difference at P≤0.05).

MS supplements (mg L)	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

Effect of medium supplements on contamination of leaf explants

No significant difference (P ≤ 0.05) was detected amongst treatment means with respect to culture contamination (Table 1). This indicates that plant growth regulators used in this trial did not promote or influence culture contamination. Maliro (1997) reported a high rate of *in vitro* contamination for *U. kirkiana* leaf explants. This suggests that almost every part of *U. kirkiana* tree might be colonised by these endogenous or cryptic fungi. In this trial, $HgCl_2$ was effective in decontaminating *U. kirkiana* leaf explants.

Observations made from this trial showed phenol accumulation in the MS media, especially from the old shoot explants. Phenol accumulation was also observed from leaf explants. However, it was difficult to record differences in browning intensity in the MS media due to this phenol accumulation. This is because of frequent transferring of aseptic explants onto fresh media for shoot multiplication. Phenol accumulation was absent when lateral shoot explants were cultured. This indicates that the lateral shoot explants were more rejuvenated than the old shoot explants since excessive phenol exudates are the major characteristics of mature plant tissues when explanted onto the culture media.

Shoot multiplication

Data in Figure 1 show mean number of micro-shoots obtained on different MS medium supplements. There were significant differences (P ≤ 0.05) amongst treatment means with respect to mean number of shoots obtained and percentage callus formation. Three quarter strength MS medium supplemented with a combination of 0.1 mg/L BAP, 0.04 mg/L NAA and 0.3 mg/L CH was effective in shoot multiplication. However, high BAP concentrations (≥1.0 mg/L) decreased the number of micro-shoots obtained. Chishimba et al. (2000) reported that high cytokinin concentrations inhibited shoot multiplication of *U. kirkiana* seedlings.

In this trial, there were stunted *U. kirkiana* micro-shoots

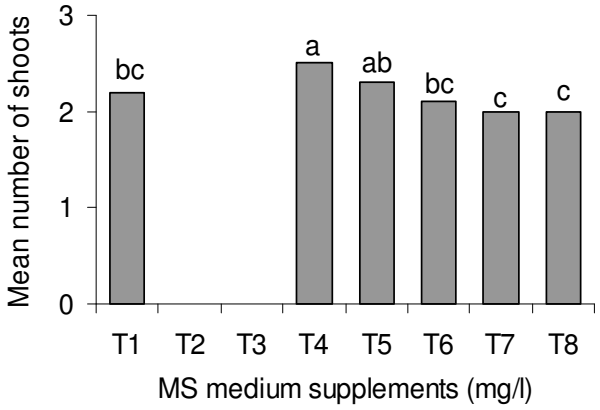

Figure 1. Mean number of *Uapaca kirkiana* micro-shoots obtained on Murashige and Skoog (MS) medium supplemented (mg/L) with either (T1) 0.05 thidiazuron (TDZ) and 0.3 casein hydrolystae (CH), (T2) 0.1 TDZ and 0.01 indole-3-butyric acid (IBA), (T3) 0.2 TDZ and 0.3 CH, (T4) 0.1 benzylaminopurine (BAP), 0.04 α-naphthaleneacetic acid (NAA) and 0.3 CH, (T5) 0.2 BAP, 0.02 NAA and 0.3 CH, (T6) 0.5 BAP and 0.02 NAA, (T7) 1.0 BAP, 0.04 NAA and 0.3 CH or (T8) 0.2 kinetin and 0.04 NAA) (Means with the same letters are not significantly different at P ≤ 0.05).

obtained on MS media supplemented with low concentrations of TDZ. Comparing the data in Figures 1 and 2 show that, prolific callus formation negatively affected the bud break. There were no micro-shoots regenerated on explants exposed to ¾ MS medium supplemented with high concentrations of TDZ. The old shoot explants excised from grafted *U. kirkiana* trees did not respond positively to different MS medium supplements except forming callus on ¾ MS medium supplemented with TDZ.

Figure 2 shows significant differences (P ≤ 0.05) with respect to callus formation when *U. kirkiana* explants were exposed to different MS medium supplements. There was no callus formation on ¾ MS medium supplemented with BAP (Figure 3A). High concentrations of TDZ (0.1 - 0.2 mg/L) resulted in a significant amount of callus formation (Figure 3B). However, transferring such callused explants onto different MS media with BAP did

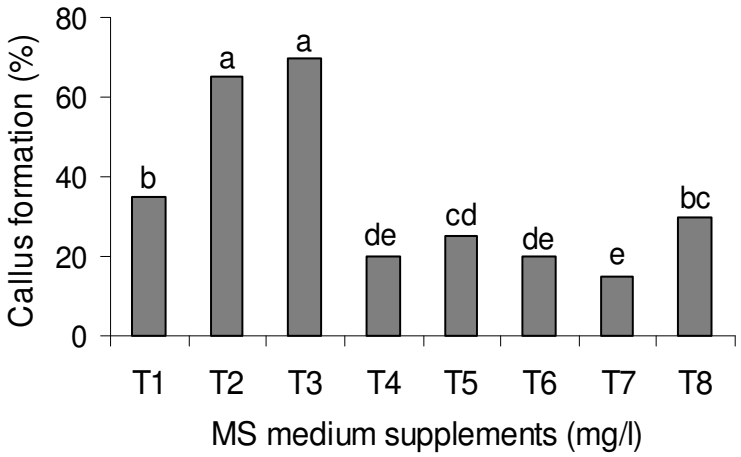

Figure 2. Callus formation (%) of *Uapaca kirkiana* micro-shoots on Murashige and Skoog (MS) medium supplemented (mg/l) with either (T1) 0.05 thidiazuron (TDZ) and 0.3 casein hydrolystae (CH), (T2) 0.1 TDZ and 0.01 indole-3-butyric acid (IBA), (T3) 0.2 TDZ and 0.3 CH, (T4) 0.1 benzylaminopurine (BAP), 0.04 α-naphthaleneacetic acid (NAA) and 0.3 CH, (T5) 0.2 BAP, 0.02 NAA and 0.3 CH, (T6) 0.5 BAP and 0.02 NAA, (T7) 1.0 BAP, 0.04 NAA and 0.3 CH or (T8) 0.2 kinetin and 0.04 NAA) (Means with the same letters are not significantly different at $P \leq 0.05$).

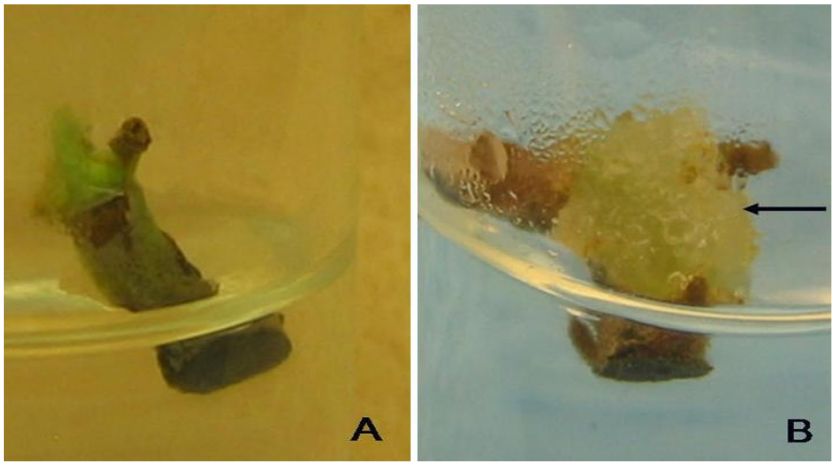

Figure 3. *Uapaca kirkiana* explants on Murashige and Skoog medium supplemented with (A) 0.1 mg/L benzylaminopurine, 0.04 mg/L α-naphthaleneacetic acid and 0.3 mg/L casein hydrolysate (B) callus formation on 0.2 mg/L thidiazuron three weeks after culture initiation.

not promote further growth of the explants. The explants remained alive for a while and eventually died. This could be attributed to a dose effect in that BAP could not promote growth of callused explants after being exposed to a more effective plant growth regulator (TDZ).

Rooting of micro-shoots

There was 36% rooting of *U. kirkiana* micro-cuttings only on ½ MS medium supplemented with 2.5 mg/L IBA. This rooting percentage is the first report on *in vitro* rooting explants excised from mature of *U. kirkiana* stock plants. More-over, many woody tree explants do not easily regenerate roots and this is attributed to a rejuvenation problem (Maliro, 1997). According to Franclet et al. (1987), juvenility in scions is short-lived. Therefore, repeated pruning is important to achieve a certain degree of juvenility for the explants. George (1993) reported that explants excised from deeply pruned trees resulted in low browning of the MS media and increased rooting ability. This further indicates that pruning plays a major role in

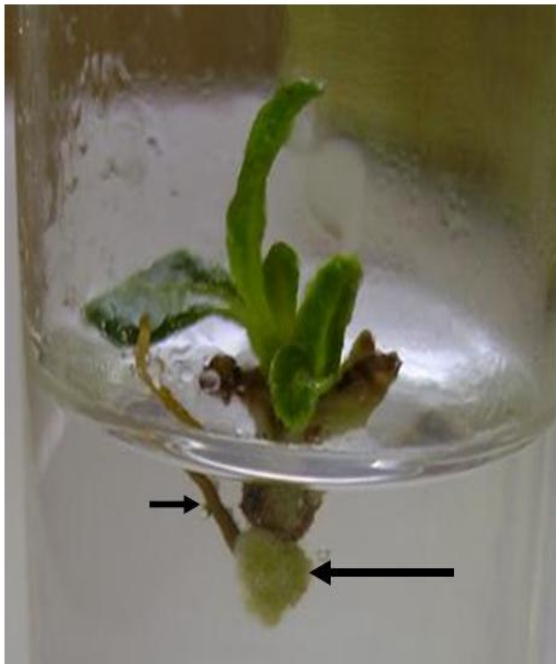

Figure 4. *Uapaca kirkiana* root regeneration on Murashige and Skoog medium supplemented with 2.5 mg/L indole-3-butyric acid (A small arrow shows a root and a thick arrow shows callusing at the base of a plantlet).

rejuvenation of stock plants.

Figure 4 shows a rooted *U. kirkiana* plantlet after explanted onto ½ strength MS medium supplemented with 2.5 mg/L IBA. However, there was callus formation at the bases of plantlets. This could be attributed to a high concentration of IBA and the nature of plants used in this trial. This is because callus formation was also observed in many *U. kirkiana* explants at different shoot multiplication and root regeneration stages. This indicates that formation of a great amount of callus could depend on plant species.

Chishimba et al. (2000) reported a low number of roots for *in vitro* propagated *U. kirkiana* seedlings. In this trial, the number of roots per plantlet was not more than two. This could be attributed to seasonality effect on rooting of woody explants. According to Augé (1995), some explants have rhizogenic capacity only during a particular period and the use of rooting hormones may extend the rooting period slightly. Furthermore, rooting hormones cannot induce rooting in the unresponsive period.

From this trial, survival of *U. kirkiana* plantlets after potting was poor and this could be attributed to the absence of symbiotic mycorrhizae. Mwamba (1995) reported a high survival rate of *U. kirkiana* seedlings when fungal mycorrhizae were present. This was attributed to the fact that fungal mycelia increase the volume of soil from which *U. kirkiana* seedlings were able to extract plant nutrients and water (Mwamba, 1995). In this trial, *U. kirkiana* plantlets survived up to six months. This observation confirms the importance of symbiotic mycorrhizae to *U. kirkiana* plantlet growth and survival even after a successful hardening off. Moreover, the grafted *U. kirkiana* stock plants placed in the glasshouse died. This could be attributed to poor acclimatisation to the new habitat, lack of mycorrhizae and other stresses. The trees were collected without the soils where mycorrhizal inocula are often present. Moreover, Benlate, systematic fungicide, might have eliminated any remnant symbiotic microbes from these *U. kirkiana* stock plants. According to Mwamba (1995) and Högberg (1982) survival of *U. kirkiana* trees is associated with mycorrhizae and possibly other unknown endophytes.

Conclusion

Preconditioning grafted *U. kirkiana* stock plants and decontaminating explants in 0.1% w/v mercuric chloride were the effective methods to achieve *in vitro* culture asepsis. Young lateral shoots from *U. kirkiana* scions were responsive to different ¾ MS medium supplements. Three quarter MS medium supplemented with a combination of 0.1 mg/L BAP, 0.04 mg/L NAA and 0.3 mg/L CH was effective in shoot multiplication and ½ MS medium supplemented with 2.5 mg/L IBA was effective in root regeneration. This trial had shown that mature *U. kirkiana* plants are amenable to *in vitro* propagation and the present protocol enables multiplication of mature *U. kirkiana* planting materials.

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