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Development of clonal propagation protocols for *Uapaca kirkiana* and *Pappea capensis*, two southern African trees with economic potential

By

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Submitted in partial fulfilment of the requirements for the Doctor of Philosophy degree (Horticulture)

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Declaration

I hereby declare that the thesis I am submitting for the Doctor of Philosophy degree (Horticulture) at the University of Pretoria is my own work and has not been submitted for a degree at any other institution.

………………………….…………….. ………………………………

Simon Alfred Mng'omba Date

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List of Abbreviations

BAP	benzylaminopurine
CH	casein hydrolysate
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
DW	dry weight
GA_3	gibberellic acid
Kin	kinetin
MS	Murashige and Skoog
NAA	naphthaleneacetic acid
PAR	photosynthetically active radiation
RP-HPLC	reverse phase high performance liquid chromatograph
psi	pounds per square inch
TDZ	thidiazuron
t_R	retention time
UV	ultra violet
2,4-D	dichlorophenoxyacetic acid

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Development of clonal propagation protocols for *Uapaca kirkiana* and *Pappea capensis*, two southern African trees with economic potential

By

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Abstract

Experiments were carried out with the objectives of developing propagation protocols for *Uapaca kirkiana* and *Pappea capensis* tree species of southern Africa, and evaluating the graft compatibility within *U. kirkiana* tree clones, provenances and species. Reverse phase high performance liquid chromatography (RP-HPLC), Folin-Ciocalteau reagent, fluorescence microscopy and callus fusion methodologies were used to diagnose graft compatibility. Results indicated that *U. kirkiana* culture asepsis was achieved with 0.1% w/v mercuric chloride ($HgCl_2$) and using pre-conditioned grafted trees. Sodium hypochlorite (NaOCl) improved *P. capensis* seed asepsis and germination, and discarding

floating seeds improved germination. Murashige and Skoog (MS) medium with 2.0 mg l^{-1} benzylaminopurine (BAP) and 0.3 mg l^{-1} casein hydrolysate (CH) was superior in shoot multiplication and 0.5 mg l^{-1} indole-3-butyric acid (IBA) for rooting of *P. capensis* micro-shoots. For somatic embryogenesis, three quarter strength MS medium with 0.05 mg l^{-1} thidiazuron (TDZ) and 0.3 mg l^{-1} CH, or 0.2 mg l^{-1} BAP with 0.3 mg l^{-1} CH, were effective in germination of *P. capensis* somatic embryos.

For *U. kirkiana* lateral shoot explants, shoot multiplication was superior on three quarter strength MS medium with 0.1 mg l^{-1} BAP and 0.3 mg l^{-1} CH. Rooting of micro-cuttings (36%) was achieved on ½ MS with 2.5 mg l^{-1} IBA. RP-HPLC, fluorescence microscopy and callus fusion studies showed that phenolic compounds play a major role in *U. kirkiana* graft incompatibility. Less graft compatible combinations showed an increase in phenol deposits above the union and graft incompatibility was more pronounced above the union than below the union. Proliferation of parenchymatous tissues was better below the union than above the union. Fluorescence microscopy showed presence of flavonoids and polymers above the union of less graft compatible combinations. The chromatograms showed that ferulic acid was abundant and responsible for wood discolouration. The chromatograms also isolated *para*-coumaric acids which were predominant above the union of the less compatible combinations. Therefore, *para*-coumaric acids, flavonoids and polymers were implicated in graft incompatibility of *U. kirkiana* trees.

Key words: decontaminants, embryogenesis, graft compatibility, Miombo woodland, organogenesis, phenolics, rejuvenation, seed germination

GENERAL INTRODUCTION

Trees contribute significantly to the livelihood of people in many different ways. They are sources of food, timber, poles and medicines and are grown for ornamental purposes such as for shade and protection. Wild trees have become potential commercial crops because they have multiple uses and imminent value. Edible fruits and oils are sources of food and income, and hence becoming important to many countries. Fruit trees can be utilized in different forms, for example *Uapaca kirkiana* Müell Arg. fruits can be eaten raw and made into refreshing drinks and a variety of wines (Kwesiga & Mwanza, 1995). The fruits have, therefore, become important to people in rural communities, especially in the arid and semi-arid countries of Africa where production of staple food has declined due to erratic rainfall, infertile soils and lack of farm inputs (Akinnifesi, Simons & Kwesiga, 2000a). Consequently, many households are food insecure and suffer from chronic malnutrition, of which children are the most vulnerable group (Akinnifesi *et al*., 2000b). Jacket plum (*Pappea capensis* L.) is another important wild tree which is rich in seed oil (74%) and this oil is usually used to make soap and for oiling guns (Venter & Venter, 1996). This oil is also potential source of bio-diesel (Le Roux, 2004). The tree also produces edible fruits which are made into jelly, vinegar and beverages (Venter & Venter, 1996).

People in rural communities can generate substantial income from wild fruit trees. For example, *U. kirkiana* fruits are gathered from the wild to be sold. They also serve as food reserves during seasonal food shortages (Maghembe & Seyani, 1992; Akinnifesi *et al*., 2006). *U. kirkiana* trees grow in symbiotic association with mushrooms, which is also a cheap but rich source of nutrients (Maghembe, Simons, Kwesiga & Rarieya, 1998). In

many rural areas, women and children collect edible mushrooms for food and extra income (Okafor & Lamb, 1992; Saka, Mwendo-Phiri & Akinnifesi, 2002; Akinnifesi *et al*., 2004). Moreover, fresh fruits are good sources of vitamins (e.g. vitamin C). *P. capensis* seed oil can be a viable source of income to the rural communities as it is a potential source of bio-diesel fuel.

Food shortage in arid and semi-arid countries is prevalent but this problem can be reduced through food diversification. Many countries of southern Africa are hit by human immunodeficiency virus (HIV) / acquired immunodeficiency syndrome (AIDS) pandemic, and hence poor nutrition aggravates the plight of HIV/AIDS infected people. Therefore, fruits as nutritional and dietary supplements are vital and would fill in the food shortage gaps as well as being a source of income. The HIV/AIDS pandemic has a serious impact on agriculture as there is a low labour force, and hence loss in productivity (White & Robinson, 2000). Fruit trees are perennial and do not require much labour once they are established unlike annual crops where labour is required annually (Le Roux, 2004).

Incorporating potential wild fruit tree species onto farmland or managing fruit tree species in their natural habitats is important, especially tree species in demand (Simons, 1997). This will reduce continuous harvesting from the wild and allow these trees to be managed as renewable resources. However, incorporating wild fruit trees onto farmland would demand developing reliable propagation methods and protocols that maintain desirable traits of the high valued wild fruit tree species (Akinnifesi *et al*., 2000b).

Major challenges to the rural masses of southern Africa include poverty, poor health and food insecurity and yet the region has some important wild tree species that can provide alternative sources of income and food (Akinnifesi *et al*., 2000b). Availability of markets for *U. kirkiana* and other wild fruits in countries of southern Africa indicates the need for domestication and subsequent commercial production of these wild fruit trees. Many wild tree seeds have valuable oil which can be an alternative source of diesel fuel such as *P. capensis*. These trees can be produced locally and create income-generating activity for the rural communities. Currently, there is lack of knowledge and a shortage of improved planting stocks. Furthermore, cultivation and domestication processes are hampered (Akinnifesi *et al*., 2000b). Rapid propagation would speed up cultivation and commercialisation of potential fruits and reduce food insecurity and poverty prevailing in many rural communities of southern Africa. Therefore, efforts are needed to provide reliable planting materials and adequate information on management of wild trees.

Grafted fruit trees address the fruiting precocity problem and enable the capture of proven superior fruit traits. However, scion/stock incompatibility in certain grafted fruit trees is a major constraint that may cause high losses in planting stock and established trees (Errea, Felipe, Treutter & Feucht, 1994b). Simons (1987) estimated that about half a million grafted peach trees died in southeast USA due to scion/stock incompatibility. Therefore, selection of compatible scion/stock combinations is important to ensure successful orchard establishment and productivity. Such selection of compatible scions and stocks has been neglected previously in many orchards and do not exist in domestication of wild fruit trees. It is against this background that the present study focused on development of propagation

protocols for *U. kirkiana* and *P. capensis* tree species. The general objectives for the study are:

1. To develop propagation protocols that enable rapid and mass production of *Uapaca kirkiana* and *Pappea capensis* planting materials

2. To evaluate the graft compatibility within *Uapaca kirkiana* tree clones, provenances and species

In order to test these general objectives, the following hypotheses were developed:

1. *Pappea capensis* and *Uapaca kirkiana* tree species are amenable to different propagation methods to achieve mass production of planting materials

2. Graft compatibility exists within *Uapaca kirkiana* tree clones, provenances and species

Approach:

Topics are organised into chapter format. A general synopsis of the entire thesis and the two indigenous fruit tree species studied are presented in the general introduction. The literature review (Chapter 1) provides the available information on *P. capensis* and *U. kirkiana* tree species. These include existing propagation methods, graft compatibility and the influence of polyphenols on *U. kirkiana* scion/stock combinations.

Major constraints to the micro-propagation of mature stock plants are rejuvenation and decontamination of plants associated with endophytic or cryptic microbes. Chapter 2 presents methods used to decontaminate *U. kirkiana* explants excised from grafted trees and this is followed by micro-propagation techniques employed (Chapter 3).

There are many unresolved questions on the main cause of graft incompatibility and methodologies to recognize graft incompatibility at an early stage are very limited. Secondary metabolites (phenols) occurring in vascular plants have been implicated in graft incompatibility. The amounts of phenols in plant organs vary with age, developmental state and growth conditions (Muofhe & Dakora, 1999). This provides an opportunity to time the grafts, and hence avoid accumulation of phenols at the graft unions. Chapters 4-6 aim at establishing the role of phenols on graft compatibility and potential methods to identify early scion/stock incompatibility in *U. kirkiana* trees. Histological studies, *in vitro* callus fusion, HPLC, fluorescence microscopy and Folin-Ciocalteau reagent procedures were used to improve our understanding of the role that phenols play in graft compatibility.

P. capensis is an unknown tree crop and is unexploited in terms of its commercial potential. No scientific research has yet been published on *P. capensis* propagation. Therefore, Chapters 7 - 8 focus on different propagation methods of *P. capensis*. Available literature documents that *P. capensis* seed germination is erratic and seedling growth is very slow (Venter & Venter, 1996). Organogenesis and somatic embryogenesis have been achieved in a few tree crops but many are still recalcitrant to *in vitro* propagation. No research has yet

been conducted in this field. The aim is, therefore, to improve germination and achieve mass multiplication of different plant sections taken from *P. capensis*.

Chapter 9 provides a general discussion of all the chapters and some general information related to the present study. Literature citation is found at the end of Chapter 9.

From this background information, it is clear that some wild fruit trees need special attention as they contribute significantly to food and income sources for rural community dwellers. Therefore, research is required on propagation techniques of the above mentioned valuable tree species. The results of research presented in various chapters provide the needed information to be utilised for the ultimate aim of improving productivity of the two wild fruit trees of southern Africa.

CHAPTER 1

LITERATURE REVIEW

1.1 *Uapaca kirkiana* fruit trees

1.1.1 Botany and ecological distribution

Uapaca kirkiana Müell Arg. is a small to medium sized tree with an evergreen or semi-deciduous growth habit depending on the prevailing environmental growth conditions. In Malawi, it is locally known as 'masuku', belongs to the Euphorbiaceae family and has multiple and spreading branches that form a dense crown. Leaves are simple, large and alternate, leathery, strongly ribbed, dark green and with rounded tips. Young leaves are covered with curly hairs on the under surface. Its wood is light with white sapwood and has reddish brown heartwood (Storrs, 1995). It is dioecious with staminate flowers borne in dense clusters while female flowers are solitary. It is difficult to distinguish male from female trees when they are juvenile. The male and female flowers are greenish-yellow and inconspicuous (Palgrave & Drummond, 1983). Fleshy fruits (3 - 4 cm diameter) contain edible pulp which is rich in dietary nutrients and these fruits ripen towards the end of the dry season, October – December (Ngulube *et al*., 1995). The pulp is yellowish and sweet-tasting (Storrs, 1995). The fruit contains three to five recalcitrant seeds germinating within three months after harvest during the rainy season, January – March (Mwamba, 1995). Recalcitrant seeds are defined as seeds that do not tolerate desiccation injury and have a short storage period. They must be stored at the lowest temperatures that are non-injurious

(Berjak *et al*., 2004). The tree has a long juvenile phase and fruits ripen towards the end of dry season or during the rainy season (Ngulube, Hall & Maghembe, 1995). Figure 1.1 shows *U. kirkiana* tree in fruit (Figure 1.1A); a two-year old grafted *U. kirkiana* tree (Figure 1.1B); *U. kirkiana* tree with a heavy fruit load (Figure 1.1C) and *U. kirkiana* fruits (Figure 1.1D).

U. kirkiana trees are common to Miombo woodland (Figure 1.2). The Miombo eco-region is composed of open canopy and broad-leaved forest trees. It is found on heavily populated plains and the lower mountain slopes and covers about 3.8 million km^2 of the Zambezian phyto-region (Chidumayo, 1997). The area extends over seven countries, namely Angola, Malawi, Mozambique, Tanzania, Zimbabwe, Zambia, Namibia and parts of the Democratic Republic of Congo. *Brachystegia, Julbernardia* and *Isoberlinia* genera dominate the Miombo eco-region (Chidumayo, 1997). The Miombo eco-region has the highest rate of deforestation in the tropics as a result of increasing human population and economical dependence on natural resources (Roy *et al*., 1996).

1.1.2 Importance and commercial potential

Uapaca kirkiana fruit trees contribute significantly to food security, especially to the rural community dwellers. They offer considerable scope for enhancing economic security in the region (Akinnifesi *et al*., 2006). Fruit is available when other foods are scarce (Akinnifesi *et al*., 2004). Consequently, they serve as food reserves during seasonal food shortage periods most of the rainy season, November – February (Maghembe & Seyani, 1992; Saka *et al*., 2002; Ngulube *et al*., 1995; Akinnifesi *et al*., 2004, 2006). The fruits are traded widely, eaten fresh and processed into juice, jam and a variety of wines in the Southern

Africa Development Community (SADC) countries. They are highly valued indigenous fruit trees of the Miombo woodlands and the most preferred fruit by communities in southern Africa (Maghembe *et al*., 1998; Ramadhani, 2002). Surprisingly, there is no known commercial cultivation of *U. kirkiana* and all current market of fruit comes from wild populations. Recent studies in Malawi and Zimbabwe showed that the availability of indigenous fruits, especially *U. kirkiana*, reduces the probability of household poverty by 33% during a seasonal food shortage period (Mithöfer, Waibel & Akinnifesi, 2006).

The *U. kirkiana* trees are also hosts of edible fungi (mushrooms) living in symbiotic association with these trees (Maghembe *et al*., 1998). The tree and its fruits are also sources of income for the rural communities of southern Africa. In Malawi and Zambia, the fruit is used to make wines and gins and is sold along the roadside stalls and in some local markets (Maghembe & Seyani, 1992). The fruit can be eaten raw, made into jams or used to produce fruit juices (Ngulube *et al*., 1995). The wood of *U. kirkiana* trees also has a high market value for making bee hives (Storrs, 1995).

1.1.3 Production and cultivation

The germplasm of 16 *U. kirkiana* tree provenances have been collected, characterised and established in multilocational trials in five of southern African countries, namely Malawi, Mozambique, Tanzania, Zimbabwe and Zambia (Kwesiga *et al*., 2000). This is with the ultimate aim of domesticating *U. kirkiana* trees. Domestication, according to Simons (1997), is defined as a 'human-induced evolution that brings wild plants into wider cultivation through a farmer-driven or market-led process'. Domestication of *U. kirkiana* is required, but a number of processes are needed. These include selecting and breeding

superior tree provenances, developing reliable propagation protocols, multiplying and disseminating germplasm as well as developing orchard management techniques (Akinnifesi *et al*., 2000b). These processes are necessary in order to capture superior germplasm onto farm land. Domesticating *U. kirkiana* fruit trees will benefit subsistence farmers through income generation and improved nutrition.

The wide cultivation of *U. kirkiana* is limited by farmers' lack of knowledge on the biology, ecology, propagation and management of the tree (Maghembe *et al*., 1998; Kwesiga *et al*., 2000). Research on domestication at the World Agroforestry Centre in southern Africa has addressed most of the factors relating to tree selection, establishment and management both on-farm and post harvest and for market development. Propagation has relied on conventional techniques which include seedling, grafting and air-layering (Akinnifesi *et al*., 2004). A need to develop micro-propagation, to allow for mass multiplication of the superior cultivars, has been identified.

1.2 Jacket plum (*Pappea capensis*) tree species

1.2.1 Botany and ecological distribution

Jacket plum (*Pappea capensis* L.) trees belong to the Litchi family (Sapindaceae). *Pappea capensis* is named after Ludwig Pappe (Fivaz & Robbertse, 1993; Venter & Venter, 1996). The tree grows up to 3.9 m high and can be deciduous or evergreen depending upon the climate. It grows tall in areas with heavy rainfall (Anonymous, 1997; van Wyk & Gericke, 2000) and bears alternate leaves forming a rosette at the ends of a small drooping branch. The stem is grey and often lichen-covered in arid areas. According to Fivaz & Robbertse

(1993), jacket plum tree is monoecious. Inflorescences are short and found in leaf axils and the staminate male flowers are borne on a lateral panicle, while the carpellate female flowers are scented and form a raceme (Palmer & Pitman, 1972). The male flowers have 8-10 stamens. Bees are the main pollinators. The trees flower from October to March and set their round to oval fruits in December to May (Anonymous, 1997). Figure 1.3A shows a mature *P. capensis* tree Figure 1.3B shows fruits. *P. capensis* trees are widely distributed throughout southern Africa, only absent in the western Kalahari and northern Namibia. They are common in KwaZulu-Natal, Swaziland, Mpumalanga and tropical Africa and are fairly adapted to a wide range of ecological areas (van Wyk & Gericke, 2000).

1.2.2 Importance and commercial potential

Jacket plum (*P. capensis*) tree bears fleshy fruits, which can be processed into vinegar, jelly and jam (Palmer & Pitman, 1972). The seeds are rich in edible, non-drying and contain fairly viscous oil (about 74%) used for making soap and oiling guns (Palmer & Pitman, 1972; Venter & Venter, 1996; van Wyk & Gericke, 2000). This oil can be exploited as an alternative source of bio-diesel and such diesel-fuels are renewable and emit less greenhouse gasses to the atmosphere (Ramadhas, Jayaraj & Muraleedharan, 2005; Canoira *et al*., 2006). Moreover, vegetable oil yielding trees that are grown locally and would contribute to lower amounts of net greenhouse gasses to the atmosphere than fossil diesel does (Bouaid *et al*., 2005).

1.2.3 Production and cultivation

P. capensis tree species are still growing in the wild and the known method of propagation is by seeds. However, seedling growth is extremely slow (Palmer & Pitman, 1972;

Anonymous, 1997). There has been no scientific research done on seed germination and vegetative propagation of jacket plum trees. Developing a reliable propagation protocol for mature *P. capensis* tree species would be challenging. Managing wild tree species in their natural habitat or on farm land requires reliable propagation knowledge to achieve domestication and optimal productivity.

1.3 Tree domestication process

Germplasm collection and evaluation for tree crop improvement, product quality and market research of *U. kirkiana* fruit trees have been carried out with the ultimate goal of domestication. However, domestication hinges on availability of good quality planting materials that result in precocious fruiting (Akinnifesi *et al*., 2006). Tree domestication involves a number of processes as outlined in Figure 1.4. For *P. capensis*, germplasm collection and improvement, and product quality enhancement are yet to be done since this tree species has been recently identified as a potential tree crop for bio-diesel fuel. Although germplasm collection of *P. capensis* tree species has not been carried out, it is still important to develop efficient and reproducible propagation protocols in order to have adequate germplasm for evaluation and selection.

1.4 Propagation methods

1.4.1 Sexual propagation

P. capensis and *U. kirkiana* tree species are mainly propagated by seeds. Available literature indicates that seed germination is not a problem for *U. kirkiana* and 95% seed germination has been achieved with seeds from fresh fruit (Maliro, 1997). For *P. capensis*,

slow seedling growth has been singled out as the main problem (Venter & Venter, 1996). Even though *P. capensis* seed coats seem to be relatively weak, it appears that they can still impede water imbibition, and hence lead to poor seed germination is obtained (Mng'omba & du Toit, 2006).

1.4.2 Vegetative propagation

Rooting mature *U. kirkiana* stem cuttings is not feasible (Akinnifesi *et al*., 2004) and this is a typical characteristic of many tropical woody trees (Kwapata *et al*., 1999). Some successes in grafting (80% graft take) and marcotting (63%) of *U. kirkiana* trees were achieved at Makoka Research Station in Malawi, but there was poor graft survival and slow growth in the field (Akinnifesi *et al*., 2006). Grafting of fruit trees offers a viable option to propagate mature plants, but stock selection is important, especially for graft compatibility. To my knowledge, there has been no scientific research done on *U. kirkiana* scion/stock combinations, and hence compatibility phenomenon is not well understood in grafted *U. kirkiana* trees. Graft incompatibility has an impact on orchard productivity (Simons, 1997). Therefore, a major challenge is to develop methodologies that diagnose early signs of graft incompatibility. This will allow selection of compatible scion/stock combinations for stable orchard productivity.

(a) Propagation by tissue culture

Tissue culture techniques enable regeneration of plants through organogenesis or embryogenesis. The latter method is useful for crop improvement through gene transfer techniques. Furthermore, somatic embryogenesis enables synthetic seeds to be developed, breeding cycles to be shortened and genetic transformation to be achieved (Singh & Chand,

2003). Somatic embryogenesis has been reported in a number of woody trees (Singh & Chand, 2003; Robichaud, Lessard & Merkle, 2004), but major constraints encountered in somatic embryogenesis include embryo maturation, germination and conversion to plantlets (Robichaud *et al.*, 2004). Medium components such as sugars, plant growth regulators, agar and other treatments have been manipulated to regenerate plants through somatic embryogenesis. Unfortunately, failures in somatic embryogenesis are not reported.

The advantage of embryogenesis over organogenesis is the ability for the embryos to develop functional roots within a short period. The shoot multiplication and root regeneration can occur simultaneously and this enables rapid regeneration of emblings (Bajaj, 1986), which is often not possible when plantlets are regenerated through organogenesis. Indirect embryogenesis occurs when embryos are regenerated through callus and according to Bajaj (1986), the number of plantlets that can be regenerated through embryogenesis surpasses those regenerated through organogenesis.

Reliable propagation protocols for mass production and precocious fruiting of *U. kirkiana* fruit trees are needed. *U. kirkiana* trees have long juvenile phase when propagated sexually and this frustrates many potential fruit tree growers. According to Parfitt & Arulsekar (1987), micro-propagation of mature trees is preferred over the embryos or seedlings since it is not always possible to determine if the embryos or seedlings have the genetic potential to develop the desired qualities later in their development. Furthermore, micro-propagation of mature trees is preferred when the gender of the trees needs to be assured. This is important for *U. kirkiana* fruit trees since they are dioecious (i.e. male and female flowers

are found on separate individual trees), and hence the proportion of female and male trees in an orchard plays a major role in terms of orchard productivity.

1.4.3 Culture contamination

Culture contamination is a problem to *in vitro* propagation due to rapid proliferation of pathogens (Enjalric, Carron & Lardet, 1998). With the exception of cryptic contaminants, many are visible at primary initiation. Generally, axenic cultures are preferred at any stage, and hence contaminated cultures are often discarded (George, 1993). Contaminants cause death of explants by exuding toxins or overgrowing the explants. Consequently, contaminants out compete and many adversely affect the growth of explants. Many plants are associated with symbiotic microbes that are contaminants in the growth media. Endogenous or endophytic microbes are often difficult to decontaminate although some are beneficial for the growth of explants (Herman, 1990). According to Cassells (1991), culture asepsis is important in any micro-propagation protocol.

Studies on *U. kirkiana* micro-propagation have been carried out (Maliro, 1997; Chishimba *et al*., 2000; Nkanaunena, 2002), but without success where explants have been excised from adult stock plants partly due to high contamination. *In vitro* propagation protocols of *U. kirkiana* plants were only developed using seedlings (juvenile plant materials) as stock plants (Maliro, 1997; Chishimba *et al*., 2000; Nkanaunena, 2002). However, as indicated above, it is not possible to ascertain the gender and future characters of *U. kirkiana* plantlets regenerated from the seedlings. There have been no scientific results available for micro-propagation of *P. capensis* tree species and successful protocols are yet to be developed.

P. capensis trees are monoecious (Fivaz & Robbertse, 1993), and hence determining the gender of the seedlings or embryos is not as critical as for *U. kirkiana* tree species. Consequently, use of seeds or embryos as planting materials for *P. capensis* will not affect the gender of the trees.

1.5 Mycorrhizae

Many tree species of the Miombo woodlands host mycorrhizal fungi, therefore, introducing them onto farmland is often difficult. This is because mycorrhizal flora is eliminated through continuous cropping and other land uses (Högberg, 1982). Mwamba (1995) reported vigorous growth of *U. kirkiana* seedlings when inoculated with symbiotic fungi. It was observed that the fungal hyphae formed mycelia and increased the volume of soil exploited. This increased nutrient and water uptake. Seven fungal species were isolated from the cultures of *U. kirkiana* seedlings. However, individual isolates were not identified. Högberg (1982) reported that *U. kirkiana* trees host both ecto- and endomycorrhizal fungi apart from other 'specialised' parasites belonging to different fungal groups. Multiple infections on a single *U. kirkiana* host were associated with both symbiotic and parasitic fungi depending on prevailing conditions (Mwamba, 1995). He further reported a significant growth of new roots when the fungal isolates were inoculated on fresh seedlings.

1.6 Effects of phenolics on graft compatibility

Phenolics are secondary metabolites which occur in vascular plants, but quantities vary with plant age, developmental stage and growth conditions (Muofhe & Dakora, 1999;

Wink, 1999). Phenols have aromatic ring structures with one or more hydroxyl (OH) bonds (Fry, 1988; Waterman & Mole, 1994) and polyphenols are compounds with many phenolic substitutes. Furthermore, the composition of phenols depends on the genetic constitution of the plant species, and hence some plants accumulate more than others. Phenols have multiple functions which include plant-insect, plant-pathogen and plant-plant (allelopathy) interactions (Waterman & Mole, 1994; Wink, 1999). Some plants store water-soluble phenols in large amounts (200 – 500 mM) to deter the feeding of herbivores (Wink & Schimmer, 1999). Plants are able to convert some phenolic compounds into different forms. A simplified shikimate pathway for phenolic compounds is shown in Figure 1.5. According to Neish (1964) cinnamic acid derivatives especially, *para*-coumaric, caffeic and ferulic acids are found in many plants. Furthermore, these are involved in biosynthesis of lignin and flavonoids. Plants use *para*-coumaric acids are precursor to lignin, but this biosynthetic reaction is irreversible (Neish, 1964).

Plants release phenolics in response to wounding as a defensive mechanism against pathogen attack (Waterman & Mole, 1994). Wounding occurs during grafting of trees and numerous reports indicate that phenols are implicated in graft incompatibility (Errea *et al.*, 1994b; Errea, 1998; Pina & Errea, 2005). Cell walls of some plants contain phenolic compounds including lignin and non-growing cells such as wood cells contain up to 30% lignin (Fry, 1988). Phenolics such as *para*-coumaric acids (Figure 1.6A) are esterified while ferulic acids (Figure 1.6B) are etherised to lignin in the cell walls (Xu *et al.*, 2005). These authors further reported that both *para*-coumaric acid and ferulic acid are the precursors of lignin, anthocyanin, phytoalexins and flavonoids. According to Méndez *et al.* (1968), *para*-coumaric acids are inhibitors of cell elongation, and hence they can reduce

parenchymatous cell growth. Consequently, graft compatibility can be reduced due to the actions of *para*-coumaric acids. Usenik *et al*. (2006) isolated *para*-coumaric acid in incompatible scion/stock combinations of apricot. Therefore, presence of this compound at the graft union can indicate a graft combination problem.

Phenols are important in lignification and protein binding (Pina & Errea, 2005), but have been implicated in graft incompatibility. Differences in quantity and the presence of specific phenols above and below the graft union area reduce graft compatibility (Facteau, Chestnut & Rowe, 1996; Usenik *et al*., 2006). When the wounding stress is over, many soluble phenols could be polymerised and deposited in cell walls (Swain, 1979) and might play an important role in graft compatibility.

1.6.1 Methods of separation for phenolic compounds

There are a number of methods used to separate, quantify and identify various phenolics from plant samples. The methods include fluorescence microscopy, Folin-Ciocateau reagent and high performance liquid chromatography (HPLC), among many others. The Folin Ciocateau method provides an estimate of the total phenols in a sample. Fluorescence microscopy technique is based on colour dye associated with specific phenols. However, it only provides an indication that different phenols are present in a sample but the method is not quantitative. HPLC is used to separate and quantify the types of phenolic compounds, and hence a reliable method to quantify phenolic compounds in plant samples.

1.7 Summary

From the literature review, it is clear that *U. kirkiana* and *P. capensis* tree species of southern Africa make significant contributions to food (fruits and edible oil) and income sources. However, there has been limited research done to develop reliable propagation protocols for these tree species. Therefore, there is an urgent need to develop such methods and new management protocols for accelerating domestication of these wild fruit trees to improve production and utilization. Research work is needed to solve these problems for conservation and on farm management of these wild tree species. The various chapters presented in this thesis add new knowledge and improve our understanding of the ways in which these trees can be successfully propagated.

Figures

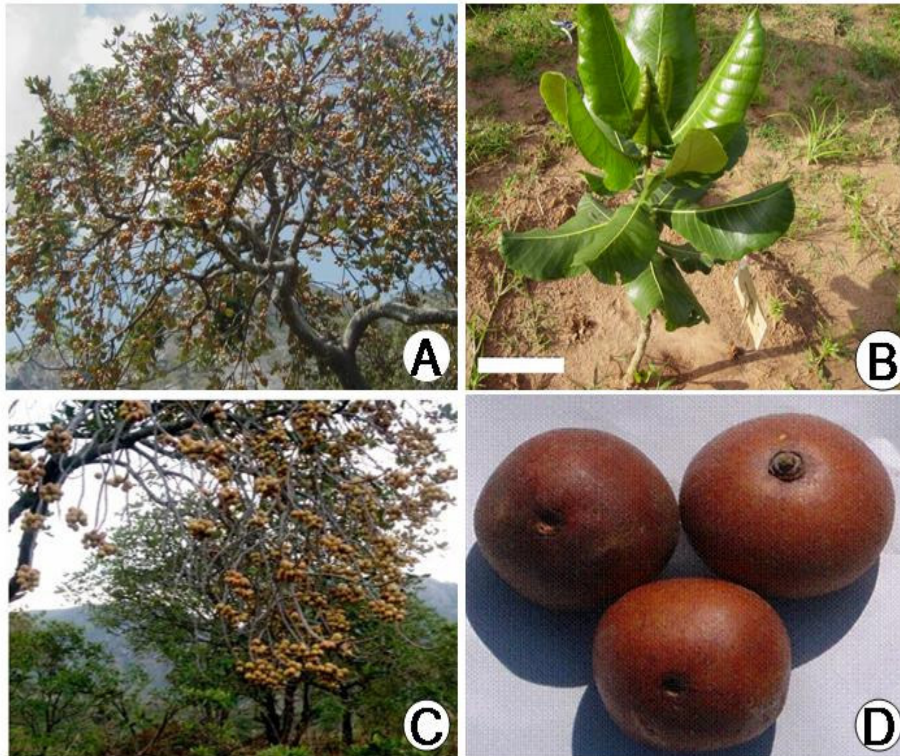

Figure 1.1 *Uapaca kirkiana* (A) tree in fruit; (B) grafted tree growing at Makoka Research Station in Malawi (two years old after grafting); (C) a tree with heavy fruit load; (D) mature fruits

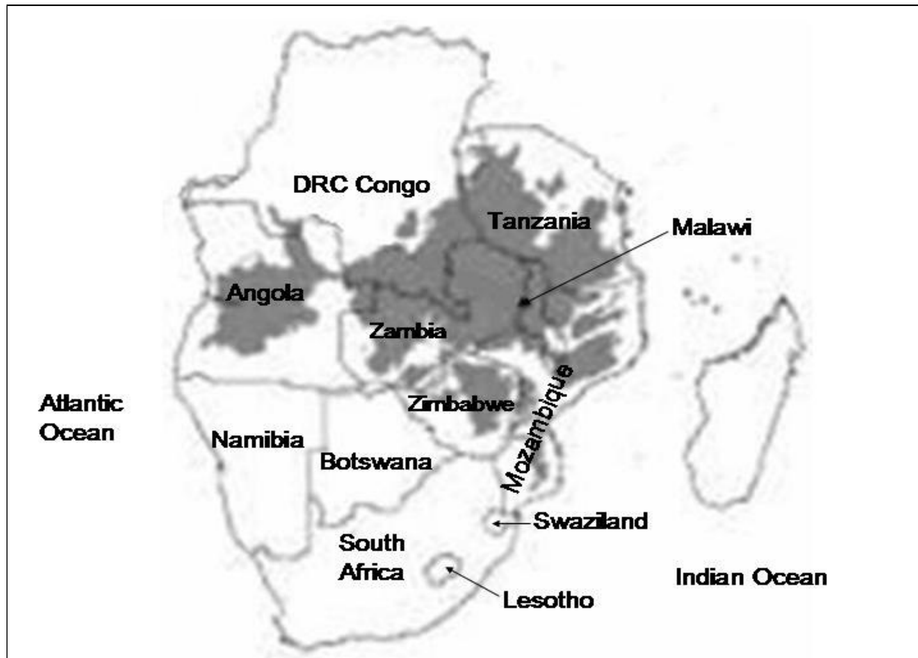

Figure 1.2 Map of southern Africa showing the distribution of Miombo woodlands (shaded areas) (adapted from White, 1983)

Figure 1.3 Jacket plum (*Pappea capensis*) (A) adult tree; (B) mature fruits and a few shattered pods

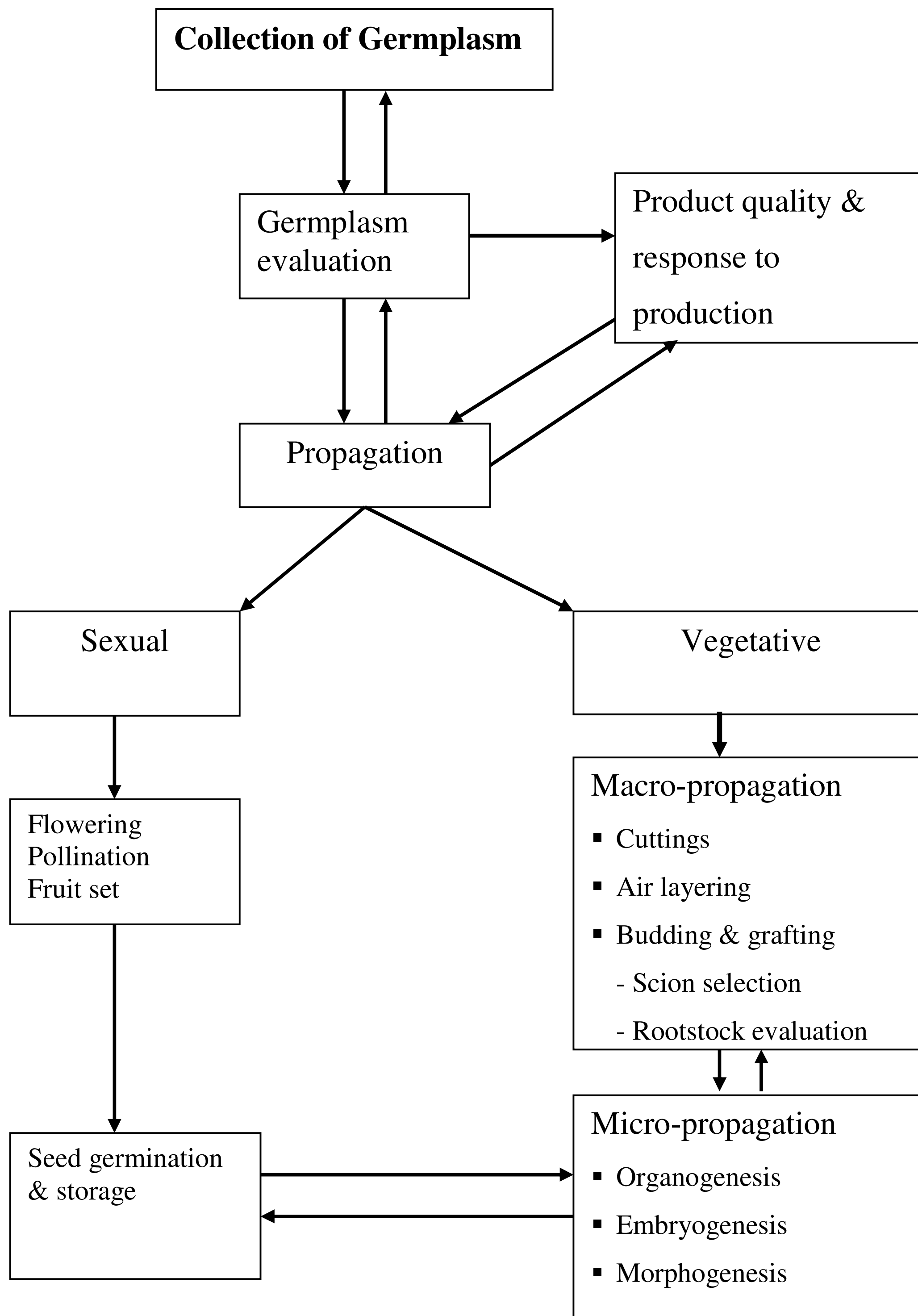

Figure 1.4 A schematic diagram for domestication of wild tree species

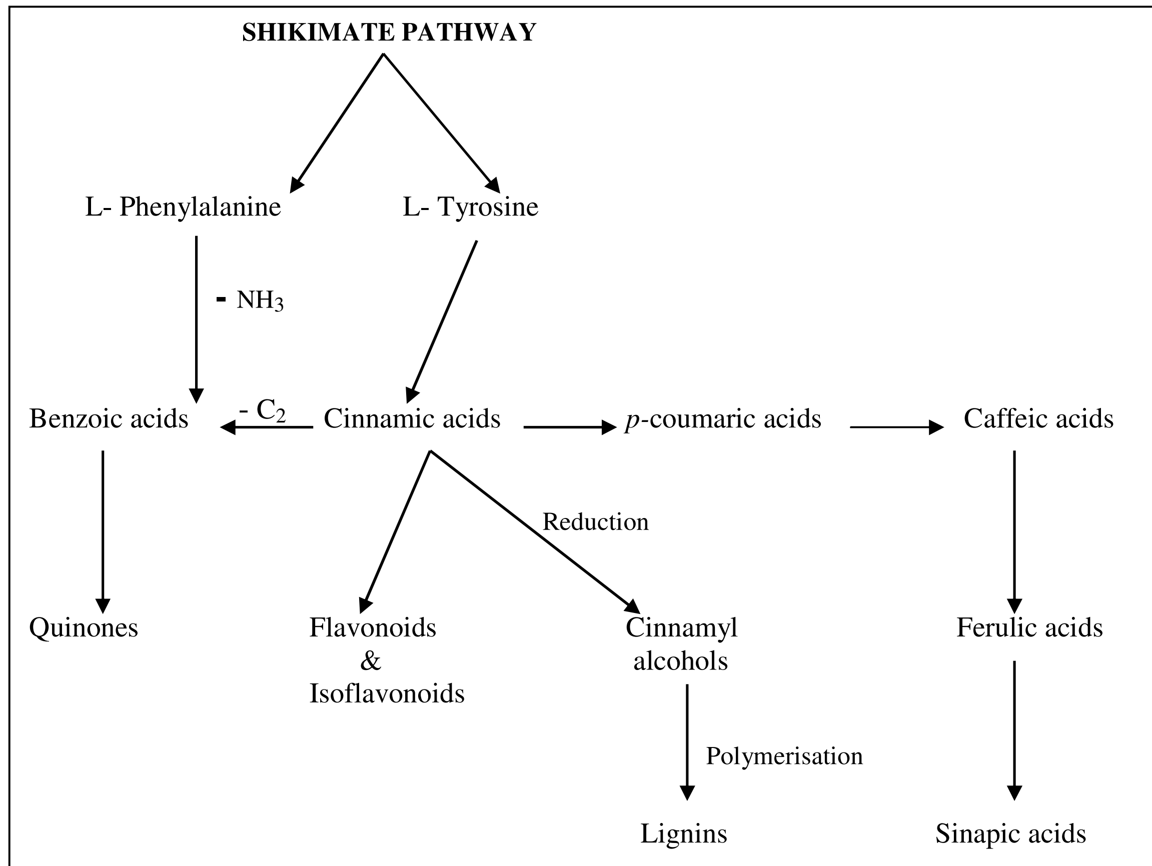

Figure 1.5 Formation of cinnamic acid derivatives from phenylalanine and tyrosine in plants (Neish, 1964; Harborne, 1989) (Only key intermediates and products of interest are shown).

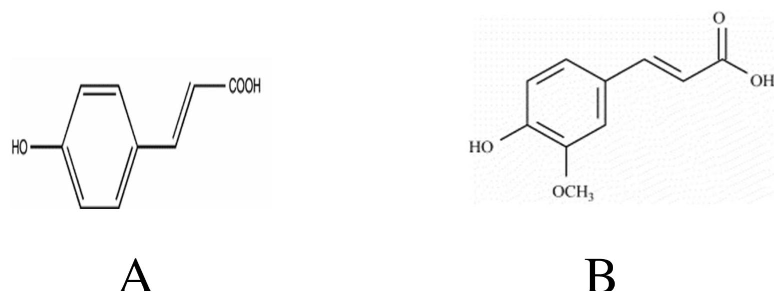

Figure 1.6 Structure of (A) *para*-coumaric acid and (B) ferulic acid (Liu, 2006)

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° 19' S and longitude 34° 16' E (Ngulube *et al*. 1997). Makoka lies at 1029 m above sea level, latitude 15° 30'S and longitude 35° 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° 45' S; 28° 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

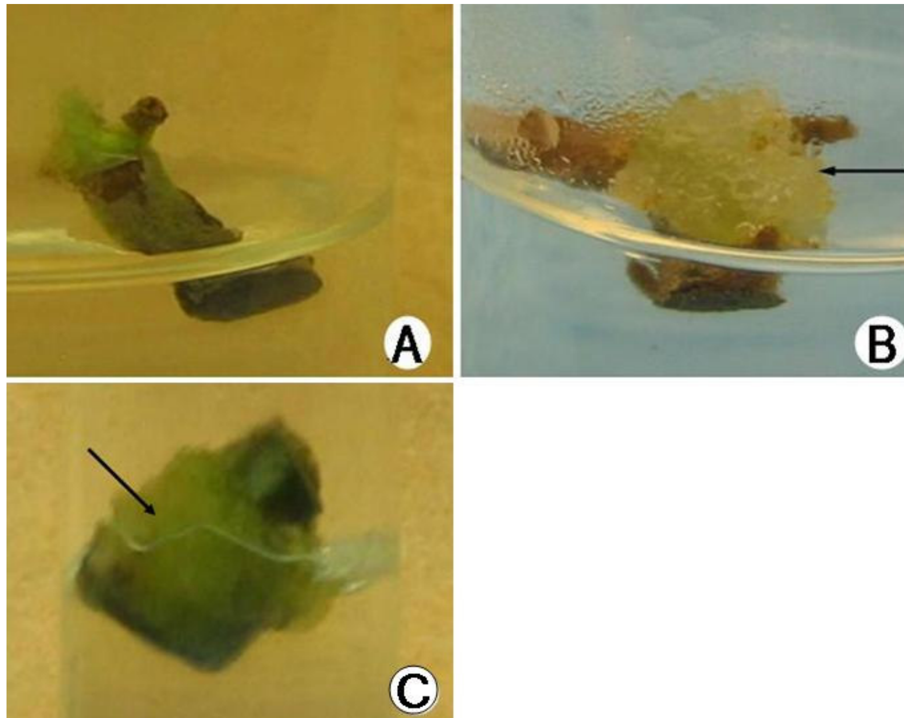

Figure 2.1 *Uapaca kirkiana* explants on three quarter strength Murashige and Skoog medium supplemented with (A) 0.1 mg l^{-1} benzylaminopurine (BAP), 0.04 mg l^{-1} α-naphthaleneacetic acid (NAA) and 0.3 mg l^{-1} casein hydrolysate (CH); (B) callusing on 0.2 mg l^{-1} thidiazuron (TDZ) and 0.3 mg 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

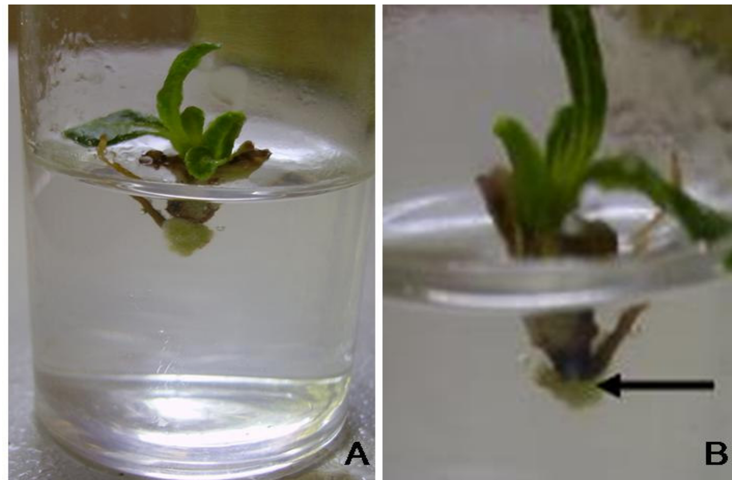

Figure 2.2 *Uapaca kirkiana* root regenerated on half strength Murashige and Skoog medium supplemented with 2.5 mg l^{-1} indole-3-butyric acid (IBA). Arrow shows base callusing of a plantlet

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

CHAPTER 3

HISTOLOGICAL EVALUATION OF EARLY GRAFT COMPATIBILITY OF SCION/STOCK COMBINATIONS IN *UAPACA KIRKIANA* Müell Arg. TREE PROVENANCES

3.1 Abstract

Graft compatibility is important for a stable orchard, and hence a trial was carried out with the objectives of determining graft compatibility and possible causes of scion/stock incompatibility in *U. kirkiana* fruit trees. Field observations of old grafted trees (at least five-years old) and a histological study using one-year old grafts (young grafts) were carried out. Stem diameters at different positions were measured and thin layers of graft union sections were examined under a light microscope. Results indicated considerable growth irregularities, which included overgrowth of stocks, constricted unions and cracks in the bark across the union. Anatomical studies showed phenol compound accumulation and lack of vascular tissue continuity at the scion side of the union. Continuity in wood and bark tissues was evident below the union of partially compatible partners while isolated parenchymatous tissues at the union might be present at the incompatible partners. Callus tissues could be breaking up phenols from the lower side of the union. Necrotic tissues and lacuna areas were present above the union. Accumulation of phenols, poor amounts of callus formation at the graft union and possibly other factors contributed to graft incompatibility in grafted *U. kirkiana* trees.

3.2 Introduction

U. kirkiana trees show wide genetic diversity and variations in geographical adaptation (Akinnifesi *et al*., 2004). Consequently, a participatory clonal selection of *U. kirkiana* from the wild has been initiated in the region to identify superior cultivars for multiplication and wider cultivation (Akinnifesi *et al*., 2006). According to Maghembe *et al*. (1998) and Akinnifesi *et al*. (2004), tree dwarfing and precocious fruiting are desirable characters that potential growers of *U. kirkiana* trees would like to see being addressed in domestication and improvement initiatives spearheaded by the World Agroforestry Centre in southern Africa.

Fruit tree grafting is important to achieve horticultural benefits which include early fruit bearing (precocity), tree dwarfness and improved fruit traits. The early bearing character is particularly important for *U. kirkiana* which is known to have a long juvenile phase when propagated sexually. It is estimated that *Uapaca* require 10-12 years before first fruiting in the wild when grown from seeds (Akinnifesi *et al*., 2004). Different vegetative propagation methods such as air-layering, budding, rooting stem cuttings and grafting have been evaluated at SADC-ICRAF Makoka Station. Budding and rooting stem cuttings have yielded poor results, while air-layering showed some promise, but root development after tree establishment was problematic (Mhango, Akinnifesi & Chilanga, 2000). Grafting using the splice method has been the most promising propagation method for *U. kirkiana* trees (Akinnifesi *et al*., 2004, 2006). It is a fact that rootstocks often impart desirable traits on the scions of different fruit crops, such as dwarfing due to reduced growth rate, improved

fruiting (e.g. fruit sweetness, size, load and colour) and fruiting precocity when they are available (Ferree & Carlson, 1987; Webster, 2001).

Improved graft take (80%) has been achieved in *U. kirkiana* trees by using skilled grafters (Akinnifesi, *et al.*, 2004). However, growth irregularities, possibly due to graft incompatibility, have been observed in some grafted trees in the nursery and the field, sometimes with suspected early or late rejection. Graft incompatibility occurring some years after grafting in normal growing trees (Errea, 1998) is a major concern in many grafted trees. Therefore, early evaluation of scion and stock combinations is important for successful orchard establishment. However, there is no known scientific research devoted to graft incompatibility in *U. kirkiana* trees to date. Therefore, evaluating compatibility of scion and stock combinations at an early stage would ensure stable grafted *U. kirkiana* tree production in clonal fruit orchards. Early diagnosis of phenols accumulating at the graft union is important since they adversely affect cell division and new cambium differentiation (Errea, 1998). The main objectives of this study were to determine the graft compatibility of different scion and stock combinations and the possible causes of scion and stock incompatibility in *U. kirkiana* trees.

3.3 Materials and methods

3.3.1 Field study

The field study was carried out at SADC-ICRAF Makoka Research Station in Malawi (refer to section 2.3.2 for site description). Two *U. kirkiana* orchards, one three years or more after grafting and another one-year old orchard (one year after grafting) were used for

the study. With the three-year old orchard (hereafter referred to as 'old graft'), visual observations were carried out and photographs were taken. The stem diameters of scions, stocks and graft unions were measured from the younger trees (one-year old after grafting, hereafter referred to as 'young graft') using a pair of callipers (model: Mitutoyo, OE7343). Bark thickness for both scions and stocks was also measured.

3.3.2 Histological and anatomical studies

Ten young grafts of *U. kirkiana* trees, grafted by the splice method, were randomly selected at Makoka nursery and the identity, sources (locations) and codes are shown in Table 3.1. Samples (at least three trees per graft combination) were collected by cutting approximately 4-5 cm below and above the scion/stock graft union. These stem sections were immediately immersed in formalin acetic acid (FAA; 5% formalin, 5% acetic acid and 90% ethanol) and later rinsed in sterile water to remove the acid. The samples were then mounted on a slide microtome stage (model: E-Leitz Wetzlar, 17815) using high pressure freezing carbon dioxide gas. Several thin layer transverse sections were cut at a right angle to the graft union and the thin layer tissues (approximately 10µm) were then mounted on microscope slides. Specimens were viewed under a light microscope (Olympus microscope Model: ach 1x, SZX7) connected to a digital camera and microphotographs of union interfaces were taken using low power magnification.

Visual scoring for graft compatibility included a visible union line in the bark and wood (scale of 1 to 4: 1 = visible, 2 = faint, 3 = very faint, 4 = absent); browning intensity of deposits at the union interfaces (scale of 1 to 4: 1 = visibly high, 2 = medium, 3 = low, 4 = visibly absent) and amount of callus proliferation (scale of 1 to 4: 1 = high, 2 = medium, 3

= low, 4 = absent). Visual scores were converted to percentages (0 - 25% = absent, 26 - 50% = very faint/low, 51 - 75% = medium/faint, 76 -100% = high). There were three samples scored per graft combination.

3.3.3 Statistical analyses

Data on diameters of scions, stocks and graft unions, and bark thickness of scions and stocks were arranged in a completely randomised design before subjecting the data to analysis of variance (ANOVA). Data on visual scores for the graft unions were analysed using correspondence analysis (GenStat 4.24DE, Rothamsted Experimental Station). Variables (union line, callus proliferation and deposit intensity) were used to discriminate the compatible from incompatible combinations and to show the distribution of these grafted trees (Lebart, Morineau & Warwick, 1984).

3.4. Results and discussion

3.4.1 Field study

There was a wide range of growth disorders at the graft unions in the old grafts of *U. kirkiana* trees (Figure 3.1A-B). Stem diameters of the graft unions were visibly greater than either the scions or stocks. This could be attributed to accumulation of necrotic tissues or deposits or metabolites (presumably phenols and carbohydrates) as there might be partial cambial continuity at the union. In some trees, there were grooves at the union area. Morphologically, a graft combination of MW26/22 (Figure 3.1A) showed good rate of callusing and healing at the graft union, and hence this combination shows good compatibility at the union. MW1/61 (Figure 3.1B) graft combination showed growth

irregularities (swellings and cracking of the bark) at the unions. Morphologically, these irregularities are indicative of incompatibility. Generally, old *U. kirkiana* trees have cracks in bark running almost vertical to the tree axis and this is attributed to genotypic traits. However, horizontal cracks across the union were visible and could be implicated in graft incompatibility.

Figure 3.2 shows contrasting growth irregularities at the unions of the old grafted *U. kirkiana* trees and these could be indicators of graft incompatibility. Their growth differences (Figure 3.2A-C) suggest variations in specific reactivity or growth rates of the grafted partners. Matching scions and stocks in *U. kirkiana* trees is a problem due to scions, which are always thicker than stocks (Akinnifesi, *et al*., 2004) but an overgrowth of stocks and constricted graft unions are least expected.

The graft union swelling could be due to a number of factors including the accumulation of phenols, but this can be temporary as changes may occur as trees grow old, as evidenced by Figure 3.2B-C. Further, the lacuna might get filled up with parenchymatous (callus) tissues and hence this would increase the area of vascular continuity at the union. Differences in diameter between scions and stocks might also be due to differential growth rates, especially after grafting, as evidenced by an overgrowth of the rootstock in Figure 3.2B. Constriction of the union in older grafted partners might be due to degeneration of vascular cambium.

For the young grafted trees, there were significant differences ($P \leq 0.05$) with respect to stem diameter and bark thickness (Table 3.2). There was a significant increase in diameter at the

unions (1.50 cm) compared to the scions (1.10 cm) and stocks (1.21 cm). Significant differences in diameter between the scions and stocks were also obtained. Tshokoeva and Tsonev (1995) reported marginal differences between scion and stock diameters in grafted apricot trees, but a significant increase in diameter at the union. An increase in stem diameter at the union could be attributed to metabolite accumulation (presumably phenols and carbohydrates) as a result of partial cambium continuity at the union. Errea (1998) reported that translocation constraints caused accumulation of some compounds. Moreover, a high amount of callus forming into the undifferentiated parenchymatous cells could also cause the union to swell.

The stocks had significantly thicker barks (0.25 cm) than scions (0.18 cm) and this could be attributed to differences in growth and amount of callus formation of the scion/stock partners after grafting. According to Akinnifesi *et al*. (2004), matching the cambial cells between scion and stock has been a challenge in grafting *U. kirkiana* trees since scions are usually thicker than the stocks. Therefore, correct matching depends on selecting scions and stocks with almost similar stem diameter and bark thickness. This is to improve proximity of vascular tissues of the scions and stocks. Bark thickness at the union was not measured, but this could be a factor contributing to an increase in union diameter since the presence of non-functional tissues can increase the union diameter. Simons and Chu (1981) reported an overgrowth of the union due to radial growth of vascular tissues.

3.4.2 Histological and anatomical studies

Figure 3.3 illustrates an external view and longitudinal section of the graft unions of *U. kirkiana* trees. There are variations in the amounts of callus proliferation and union line

visibility although these trees were alive. There were no perfect unions for all of the ten *U. kirkiana* tree provenances sampled except some formed a good union below the graft (Figure 3.3B) and this was termed a 'partial' graft union (Ünal, 1995). Graft partners with partial union showed a good amount of callusing at the union (both external view and longitudinal section of the union). Therefore, a poor union might be associated with poor amounts of callus formation at the union. Figure 3.4 illustrates incompatible (A) and partially compatible (B) grafted *U. kirkiana* partners.

MW84 scion on MW57 (MW84/57) stock shows incompatibility, possibly due to wide unfilled areas (poor amounts of callusing) at the union (Figure 3.4A). This poor union is illustrated by a visible line between partners showing no continuous bark and wood tissues. Survival of such partners could be attributed to the presence of some portions of undifferentiated tissues (parenchymatous tissues) into cambium and vascular tissues. Errea, Felipe & Herrero (1994a) reported presence of some parenchymatous tissues in incompatible combinations that made the unions in *Prunus* species weak. In the present study, the bark tissues at the upper part of the union were dead and this was also observed in partially compatible trees. MW26/22 stock (Figure 3.4B) showed partial continuity in bark tissues and a small area of necrotic tissues at the pith and the upper part of the union. This combination might form a good graft union with time.

Data in Table 3.3 shows mean separation of different graft combinations with respect to the four attributes (absence of visible line in bark and wood, callus proliferation and phenol accumulation). Mean separation was done using Student-Newman-Keuls test (SAS, 1999). Graft combinations are in three main groups (Table 3.4) with respect to the absence of a

visible line in the bark, namely (i) MW26/26, (ii) MW7/10, MW84/57 and MW12/57, and (iii) MW 1/61, MW2/U, MW28/32, MW57/49 and MW71/U. For callus proliferation, the graft combinations are in two groups, namely (i) MW26/22 and (ii) MW12/57. Graft combinations, MW2/U and MW7/10, are also in two groups with respect to phenol accumulation. With respect to absence of visible line in the bark, there are three main groups, namely (i) MW26/26, (ii) MW26/22 and (iii) MW1/61, MW7/10, MW2/U, MW84/57, MW12/57 and MW57/49. From this statistical analysis, it is difficult to group graft combinations into a compatible or incompatible category when all the four attributes are simultaneously considered. Moreover, it is difficult to interpret histological sections because of variability induced during grafting and variations in incompatibility symptoms (Ermel *et al*., 1995).

Figure 3.5 shows a simultaneous representation and descriptive summary of data from the correspondence analysis output. MW26/22, MW26/26 and MW7/10 have been grouped together indicating compatibility. Correspondence analysis is based on transformation of Chi-square values and produces dimensions which represent the Chi-square distances (Lebart, Morineau & Warwick, 1984). In this trial, the principal inertias were 0.06 (79.5%) and 0.14 (18.0%) at one- and two-dimensions, respectively. A two-dimensional correspondence analysis was appropriate since it represents 97.5% of the profiles (i.e. 97.5% simultaneous representation and descriptive summary of the data).

According to Lebart, Morineau & Warwick (1984) and Greenacre (1984), correspondence analysis gives a simultaneous perceptual map showing relationships between the objects (rows) and variables (columns) of a data matrix. It provides an informative and descriptive

summary of a data set containing many interrelationships, which are difficult to interpret with other statistical methods. It is used in several fields including compatibility of tissues (Greenacre, 1984). It is clear that correspondence analysis (Figure 3.5) improves interpretation and provides a descriptive summary of the graft combination data. In this trial, different graft combinations were categorised into a compatible or incompatible group with respect to all the attributes. According to Ermel *et al*. (1997), correspondence analysis offers a better procedure to discriminate compatible from incompatible graft combinations.

Time of grafting of *U. kirkiana* trees could have played a role with respect to accumulation of phenols since the trees were grafted in different months of the year. However, incompatibility might be compounded by variability in imperfect grafting although skilled grafters were used to graft these trees. Using a visual classification described by Ünal (1995), there was no perfect union observed in this study. It is only suspected that partially compatible partners might form a perfect union with time.

Figure 3.6 shows a common trend for callus cell proliferation at the graft unions observed under a light microscope. In all the combinations, callus cells were prolific below the graft union where a good union had been formed. However, tissues above the union were necrotic and highly stained, and hence there was no continuity in the bark and wood. Observations showed that *U. kirkiana* plants exude a lot of metabolites (phenols) in response to wounding. Hamisy (2004) reported high amounts of phenols in *U. kirkiana* leaves during DNA extraction. Therefore, it is suspected that phenols could play a role in graft incompatibility of *U. kirkiana* trees since grafting involves wounding of plant tissues. Figure 3.6A shows more lacuna or unfilled areas than the other scion/stock combinations

(Figures 3.6B-C). According to Errea (1998), phenols have been implicated in union formation processes, which include insufficient callus proliferation, cell necrosis and metabolic interactions. These are known to bring about disorders and damage at the graft unions. Therefore, phenol accumulation observed in this trial might play a role in graft incompatibility.

Poor callus formation was observed in some partners (e.g. MW84/57) and possibly, phenols were oxidised to other forms (such as quinones) which were toxic and eventually disrupted chemical reactions (Errea, 1998). According to Errea *et al*. (1994b), a significant amount of flavanol (phenol) in phloem was found in apricot as a response to graft incompatibility. Phenols prevent cambial connection continuity formation and a high accumulation occurs at the union of incompatible or less compatible combinations (Errea *et al*., 1994b). They induce cell damage and alter phloem cambium around the graft union. Our study showed a high phenol accumulation above the union where the bark and phloem tissues were both dead. This agrees with the findings by Ermel *et al*. (1997) where cell necrosis and discontinuity of vascular connections at the union were the main indicators of incompatibility. Figure 3.7A shows accumulation of phenols at the union, and hence prevents continuity in the tissue connection. Gebhardt & Feucht (1982) reported that a high concentration of phenols above the union is the cause of graft incompatibility. Furthermore, some incompatible combinations may grow without any external indication of incompatibility, but the presence of phenols accumulating at the union serves as an indicator of problems in graft combinations (Considine, 1983; Errea, 1998).

Phenol accumulation above (high) and below (low) the graft unions are shown in Figure 3.7A-B. There were unfilled areas and necrotic tissues at the unions (Figure 3.7A) and callus tissues broke up the phenols from the lower side of the union (Figure 3.7C). The quantity of phenols is high above the union and coincidentally there was no continuity of cambial connections. A good connection was found below the union in partial compatible partners. An accumulation of phenols, especially above the union has been implicated in reduced graft compatibility in many heterogenetic grafts (Usenik & Štampar, 2001).

Hartmann, Kester & Davies (1990) reported that maintaining a film of water at the union during grafting is necessary for callus formation. This water could possibly dilute some phenols as they accumulate below the union, especially water-soluble phenols. This could aid in breaking up of phenols by prolific callus tissues and consequently, grafted partners are able to establish cambial continuity. High accumulation of deposits (phenols) at the union of some *U. kirkiana* trees indicates that such partners might take time for phenols to be broken up completely and form complete vascular tissue continuity. This could be the reason that the union diameter was larger than either the scions or stock in young grafted trees. Figure 3.8A shows a section of a partial MW26/22 compatible combination (Figure 3.8B). Union line visibility increases above the graft union. Figure 3.8A shows small pockets of deposits and invisible union line. Figure 3.8C shows a line of deposits and necrotic layers along the union line.

Graft set in November - December period was found to be the best for *U. kirkiana* trees (Akinnifesi *et al*., 2004). This could suggest seasonality in phenol accumulation and the

quality of phenols at a particular time of the year. Trees used in this trial were grafted during the months of June, August and early October.

3.5 Conclusion

Indicators of graft incompatibility in *U. kirkiana* trees include growth irregularities at the union, poor callus formation, presence of necrotic tissues and accumulation of phenols. Such findings confirm existence of graft union problems despite the fact that some trees were surviving in the nursery and the field. For graft incompatible partners, portions of parenchymatous tissues supported the graft unions. MW26/26, MW26/22, MW7/10 and MW28/32 were partially compatible. However, phenolic compounds were major factors influencing graft incompatibility.

Tables

Table 3.1 Tree identification (ID) of *Uapaca kirkiana* stocks and scions from different districts and locations (natural forest or cultivated field) in Malawi

Tree ID	Accession name	District	Area	Fruit trait
MW1	ICR02NkhumbaMW1	Zomba	Forest	sweetness
MW2	ICR02KanyotaMW2	Zomba	Forest	sweetness
MW7	ICR02MalemiaMW7	Zomba	Forest	sweetness
MW10	ICR02MalemiaMW10	Zomba	Forest	sweetness
MW12	ICR02SitolaMW12	Zomba	Forest	sweetness
MW22	ICR02ElsoniMW22	Dedza	Forest	sweetness
MW26	ICR02HardwickMW26	Dedza	Field	sweetness, load, size
MW28	ICR02HamiyoniMW28	Dedza	Field	sweetness
MW32	ICR02YesayaMW32	Dedza	Forest	sweetness, size
MW49	ICR02NkhumbaMW49	Phalombe	Forest	sweetness, fruit load
MW57	ICR02NkhumbaMW57	Phalombe	Forest	sweetness, fruit early
MW61	ICR02MigowiMW61	Phalombe	Forest	sweetness, fruit early
MW71	ICR02NkhumbaMW71	Phalombe	Forest	sweetness, fruit load
MW84	ICR02NazombeMW84	Phalombe	Forest	sweetness

Table 3.2 Average scion, stock, and graft union diameters and bark thicknesses of young *Uapaca kirkiana* fruit trees (one-year old after grafting). Measurements taken approximately 5 mm below and above the graft union and means are calculated with standard errors (N = 40)

Plant parts	Stem diameter (cm)	Bark thickness (cm)
Scion	1.10 ± 0.04^{c}	0.18 ± 0.01^{b}
Stock	1.21 ± 0.06^{b}	0.25 ± 0.02^{a}
Union	1.50 ± 0.07^{a}	-
CV (%)	13.3	20.5
$LSD_{0.05}$	0.08	0.04

Numbers with different letters within a column are significantly different ($P \leq 0.05$)

- Not measured since one side of the graft union is a scion and on the other is a stock

Table 3.3 Mean scores of *Uapaca kirkiana* graft combinations with respect to absence or presence of visible line in the bark and wood, callus proliferation and phenol accumulation

Graft combination	Bark	Wood	Callus	Phenol
MW1/61	45.0^{c}	5.0^{c}	60.0^{ab}	70.0^{ab}
MW26/22	80.0^{ab}	30.0^{b}	80.0^{a}	65.0^{ab}
MW2/U	48.3^{c}	5.0^{c}	60.0^{ab}	80.0^{a}
MW26/26	90.0^{a}	50.0^{a}	65.0^{ab}	60.0^{ab}
MW71/U	48.3^{c}	5.0^{c}	65.0^{ab}	65.0^{ab}
MW7/10	70.0^{b}	30.0^{b}	60.0^{ab}	50.0^{b}
MW84/57	65.0^{b}	5.0^{c}	50.0^{ab}	60.0^{ab}
MW12/57	70.0^{b}	5.0^{c}	45.0^{b}	65.0^{ab}
MW28/32	45.0^{c}	40.0^{ab}	75.0^{ab}	65.0^{ab}
MW57/49	48.3^{c}	5.0^{c}	50.0^{ab}	60.0^{ab}
Probability	0.0001	0.0001	0.0245	0.0001
CV (%)	11.88	34.02	18.51	13.98

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

Figures

Figure 3.1 Morphology of *Uapaca kirkiana* graft unions (A) HardwickMW26 (scion) on ElsoniMW22 and (B) NkhumbaMW1 (scion) on MigowiMW61 (stock)

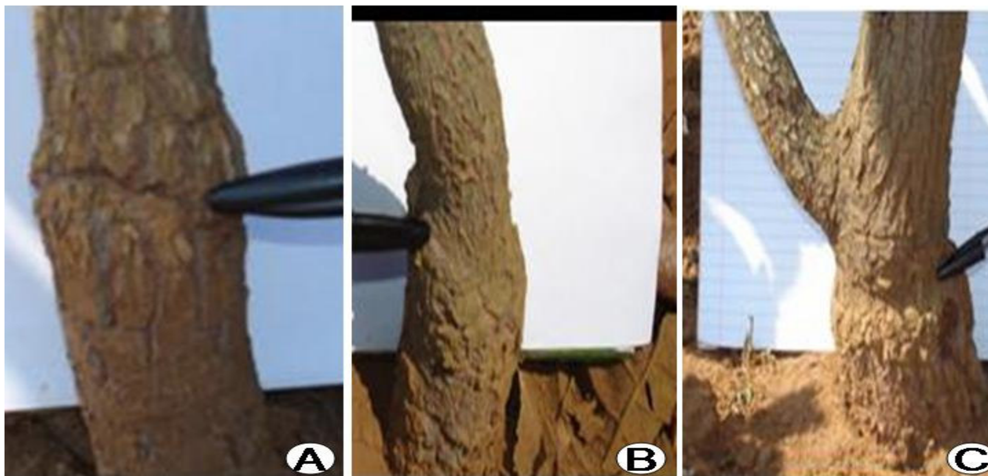

Figure 3.2 Growth irregularities at the graft unions of *Uapaca kirkiana* fruit trees (A) a groove across the union; (B) a small scion on an overgrown stock; (C) a constriction at the union (pen points at the union)

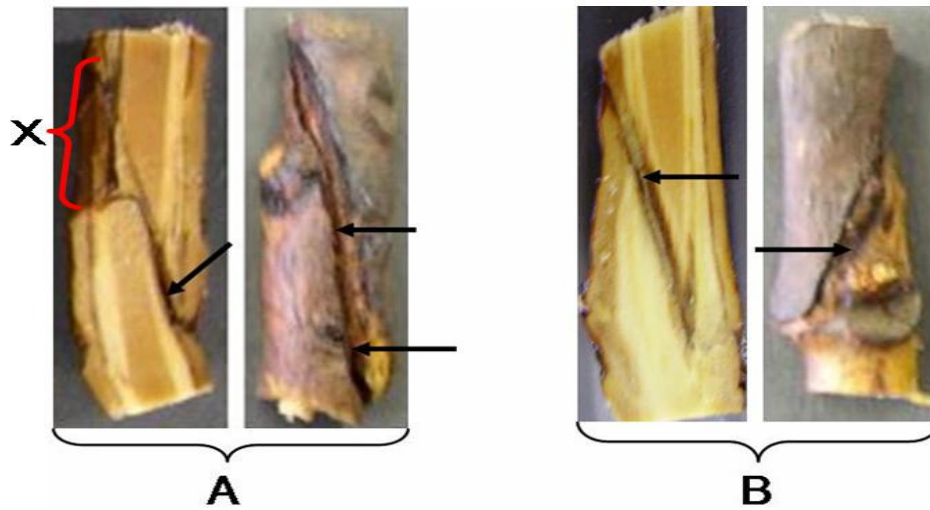

Figure 3.3 Longitudinal sections and external view of the graft unions of *Uapaca kirkiana* trees showing (A) poor rate of callusing at the union interface; (B) good amount of callus formation at the union. (Arrows show necrotic tissues for the internal sections and differences in callus proliferation for the external sections, X = dead bark area of the incompatible graft combination)

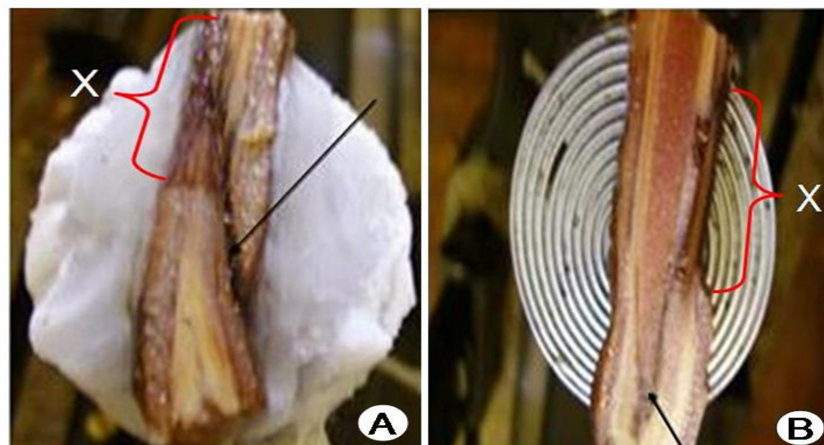

Figure 3.4 *Uapaca kirkiana* scion/stock combinations (A) a visible line between the scion (NazombeMW84) and stock (NkhumbaMW57); (B) a faint line between HardwickMW26 and ElsoniMW22 (X = death of bark and vascular tissues, arrows show visible union lines)

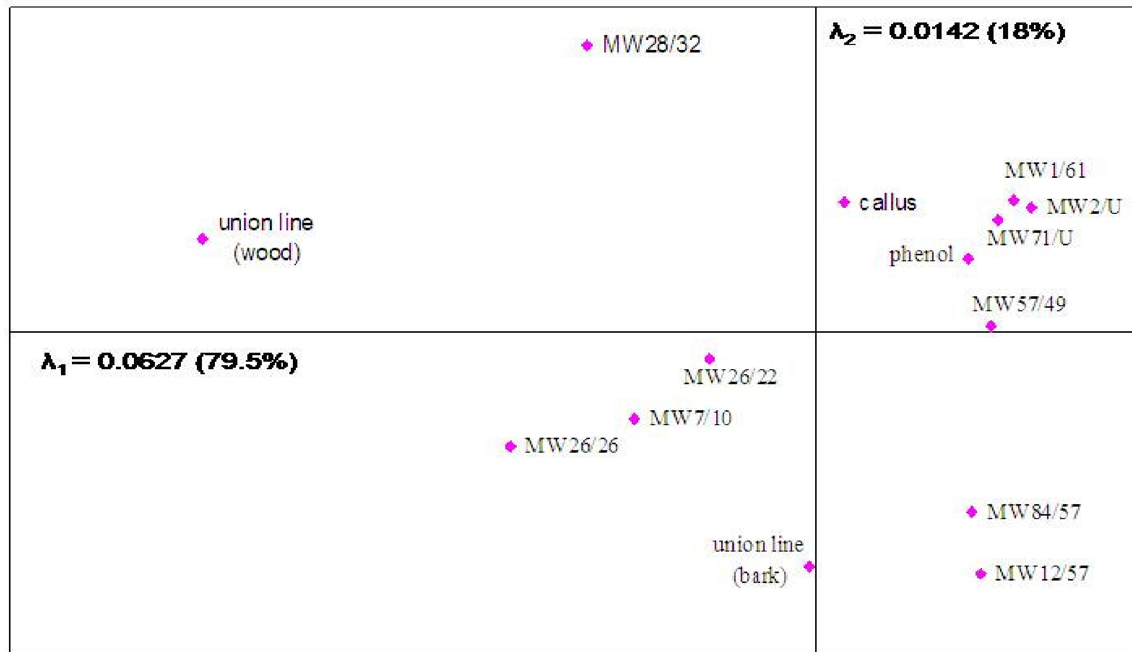

Figure 3.5 Two-dimensional correspondence analysis showing distribution and association of different *Uapaca kirkiana* graft combinations with respect to union line in the bark and wood, presence of phenols and callus proliferation at the union (U = unknown stock, λ = inertias)

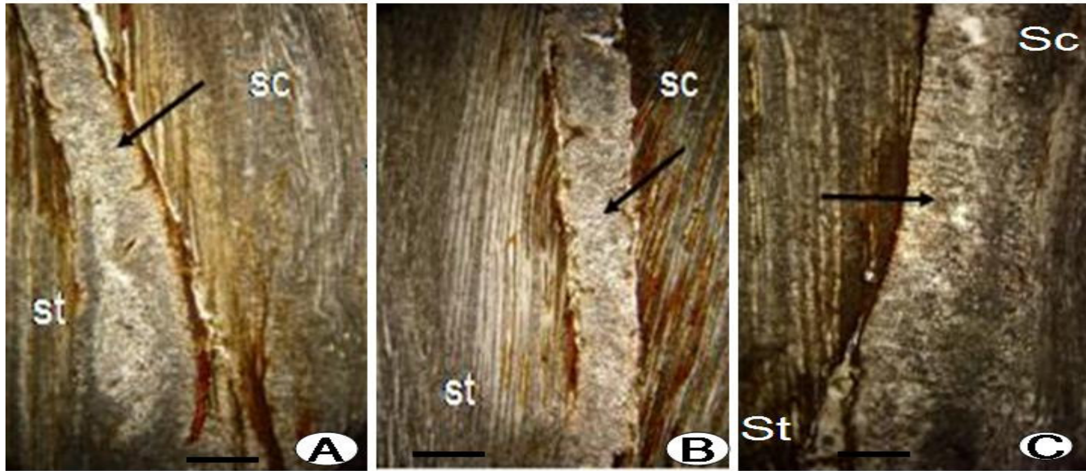

Figure 3.6 Anatomical observations of callus tissues very prolific below the union area (A) numerous unfilled (lacuna) areas; (B) a few unfilled areas; (C) absence of unfilled areas (arrows indicate callus tissues and the stains at the union interface are phenols, St = stock, Sc = scion, bar = 5 µm)

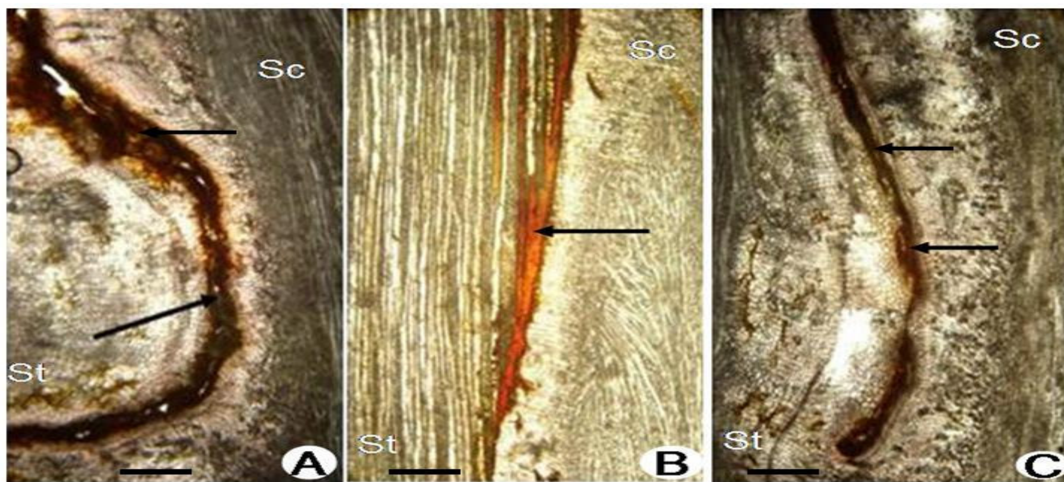

Figure 3.7 Deposits at *Uapaca kirkiana* scion/stock union (A) incompatible partner (NkhumbaMW1/MigowiMW61) with high amounts of deposits; (B) partial compatible union with high amounts of deposits above and at the union; (C) callus cells breaking up deposits from below the union area (arrows indicate deposits, St = stock, Sc = scion, bar = 5 µm)

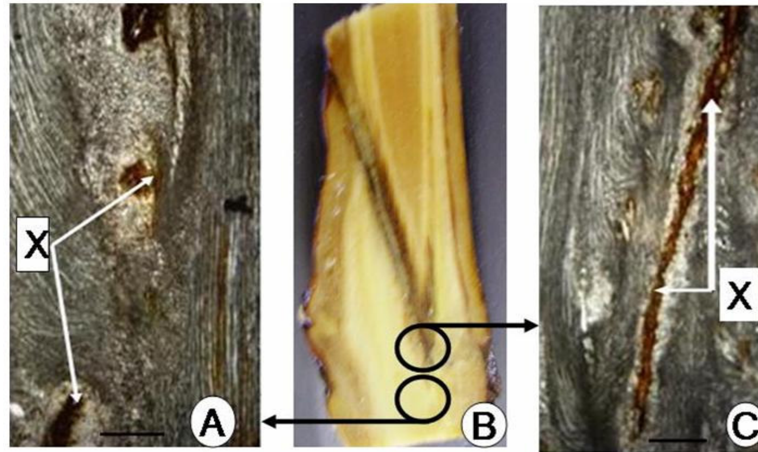

Figure 3.8 Sections below the union of a compatible HardwickMW26/ElsoniMW22 *Uapaca kirkiana* combination (A) invisible union line; (B) HardwickMW26/ElsoniMW22 section; (C) a faint union line (X = pocket/line of deposits, bar = 5 µm)

CHAPTER 4

EARLY RECOGNITION OF GRAFT COMPATIBILITY IN *UAPACA KIRKIANA* Müell Arg. FRUIT TREES

4.1 Abstract

Early signs of graft incompatibility in *Uapaca kirkiana* tree clones and provenances, *U. nitida* and *Jatropha curcas* were studied using *in vitro* callus fusion technique. Calli was induced from leaves explanted on Murashige and Skoog (MS) medium with different supplements. Two pieces of calli were co-cultured on MS medium with 0.1 mg l^{-1} thidiazuron (TDZ) and 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA). Co-cultured calli were embedded in paraffin wax, sections were stained in safranin and fast green and then examined under a light microscope. Results showed that 0.1 mg l^{-1} TDZ in combination with 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} dichlorophenoxyacetic acid (2,4-D) with 0.5 mg l^{-1} NAA were effective in callus induction. There were no necrotic layers at the unions within *U. kirkiana* clones and provenances. Differential growth between *U. kirkiana* and *U. nitida* was observed. Accumulation of phenol deposits in cells was observed in all *U. kirkiana* combinations and more accumulation was found on callus cells from mature explants. Phenols were absent in *J. curcas* callus tissue heterografts but necrotic layers developed on *U. kirkiana* and *J. curcas* unions indicating a symptom of graft incompatibility. This experiment showed that phenol accumulation impeded callus proliferation, and hence inhibited graft compatibility in *U. kirkiana* grafts.

4.2 Introduction

Early signs of graft incompatibility, often only detected after several years in the field, can be identified using *in vitro* callus fusion (Jonard *et al*., 1990; Errea, Garay & Marin, 2001). Formation of a successful graft union involves many processes, but the main mechanism is still not clear. However, cell recognition is the first step in graft compatibility (Pina & Errea, 2005). Callus cells are able to reject partner cells at an early stage and hence bring about an incompatible response. Cell necrosis and vascular connection discontinuity at graft unions are reported to be the main indicators of graft incompatibility (Ermel *et al*., 1997). Cell division and differentiation take place after cell recognition (Considine, 1983). Thereafter, callus cells initiate cell continuity in compatible partners, but necrosis as a result of incompatible partners (Pina & Errea, 2005). Cell necrosis is usually seen when callus tissues grow together for some time (Ermel *et al.,* 1997), but can also occur at any stage, especially during callus proliferation (Moore, 1986). It is clear that graft compatibility is complex and involves a number of processes.

U. kirkiana tree provenances show wide genetic diversity and variations in geographical adaptation (Akinnifesi *et al*., 2004). Random amplified polymorphic DNA (RAPD) has shown genetic variation within *U. kirkiana* provenances (Agufa, 2002). Early fruiting of two to three years has been reported from fruit orchards established from clonal propagation using grafting and marcotting in Malawi and 80% graft take has been achieved with skilled grafters at Makoka in Malawi (Akinnifesi *et al*., 2006). However, as with most fruit trees, this success in graft take may not always result in high field survival due to graft incompatibility (Nito, Han & Katayama, 2005). Furthermore, grafted *U. kirkiana* trees have

not been evaluated for scion/stock compatibility and some trees have shown growth irregularities between the scions and stocks in the nursery and field at Makoka in Malawi. Selection of graft partners based on desirable fruit or stock traits is important, but scion and stock compatibility needs to be evaluated for stable orchard productivity.

Early recognition of graft compatibility is, therefore, important and assessing callus compatibility of *U. kirkiana* tree clones and provenances and of different *Uapaca* species would aid in early diagnosis and selection of compatible scion and stock combinations. The objective was to determine the early signs of callus compatibility within *Uapaca kirkiana* tree clones and provenances, *Uapaca* species and other trees from the same family.

4.3 Materials and methods

4.3.1 Plant material

U. kirkiana leaves from different juvenile tree clones and provenances were collected at Makoka Research Station in Malawi (see section 2.3.2 for site description). Other *U. kirkiana* provenances originally from Tanzania, Zambia and Zimbabwe were also collected at Makoka from a mature provenance field trial. *U. kirkiana* trees from which leaf samples were collected in January and October 2005, included NkhumbaMW49 (MW49), NkhumbaMW57 (MW57), NazombeMW84 (MW84), HardwickMW26 (MW26), YesayaMW32 (MW32), Phalombe and Dedza from Malawi; Chipata and Choma from Zambia; Mapazure, Murewa and Nyamakwaar from Zimbabwe, and Mpwapwa from Tanzania. Since *U. kirkiana*, *U. nitida* and *Jatropha curcas* all belong to the Euphorbiaceae family, graft compatibility within this family was also tested. Leaf samples of *U. nitida*

from Malawi and *J. curcas* from South Africa were collected (Table 4.1). Plant materials were taken to the tissue culture laboratory at the University of Pretoria for callus induction and compatibility evaluations.

4.3.2 Decontamination and callus induction

Leaves were cut into 1 cm^2 sections, stirred for 20 minutes in Benomyl solution (0.14 g l^{-1}) with a few drops of detergent and then washed under running tap water (30 min). They were then stirred in 3.5% sodium hypochlorite (5 min) and washed off under running tap water (1 h). Under the laminar airflow cabinet, explants were sterilized in 0.1% w/v mercuric chloride ($HgCl_2$, 8 min) and rinsed in sterile distilled water for six consecutive times.

Leaf sections were explanted either abaxially or adaxially on 25 ml aliquot of Murashige and Skoog (Murashige & Skoog, 1962) dispensed in 9-cm Petri dishes. The MS medium supplements were: (i) 1.0 mg l^{-1} indole-3-butyric acid (IBA) and 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA); (ii) 0.1 mg l^{-1} thidiazuron (TDZ) and 0.5 mg l^{-1} NAA; (iii) 1.0 mg l^{-1} NAA and 0.5 mg l^{-1} benzylaminopurine (BAP); or (iv) 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l^{-1} NAA.

This experiment was laid out as a completely randomised block design in 4 × 2 factorial arrangement with four treatments (plant growth regulators) and two sources of explants (juvenile and mature tissues). Five Petri dishes per treatment were used and each Petri dish contained eight leaf explants. Petri dishes were covered and sealed with parafilm strips

before incubation (see section 2.3.8 describes incubation conditions). *In vitro* contaminated cultures were discarded and data collected included the number of contaminated callus cultures, a measure of callus induction and proliferation.

4.3.3 Callus co-culture and fixation

Two pieces of calli from different explants were excised and put together in a thermal labile polythene ring and then placed on MS medium supplemented with 0.1 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA. This medium supplement was selected because it was superior in promoting callus proliferation compared to other treatment combinations (refer to section 4.3.2). *U. kirkiana* clonal homografts (grafts from the same mother tree), inter-specific homografts (grafts from different mother trees but of the same kind e.g. MW49/49), heterografts (grafts from different trees in the same population), tree provenances, *U. nitida* and *J. curcas* combinations were evaluated (Table 4.1). All callus co-cultures were maintained on the same medium for at least two months with a culture (with intact ring) transfer interval of three to four weeks onto fresh medium. This was done carefully, for callus combinations with good unions and prolific growth in the rings, to avoid disturbing the combinations.

Callus co-cultures were removed from the rings and placed in polythene tubes containing formalin acetic acid (FAA; 5% formalin, 5% acetic acid and 90% ethanol) for fixation. Samples were placed on a shaker and dehydrated in a series of graded ethanol (30, 50, 70, 100 and again 100%) followed by a series of xylene (30, 50, 70, 100 and again 100%). Xylene was evaporated and then specimens were embedded in paraffin wax using thermolyne sabron equipment (Histo-Center II-N). Specimens were dissected using Stereo Star Zoom autocut 2040 (model: Rechert-jung 0.7x to 4.2x 570) and transversal sections at

a right angle to the callus union were made. They were then dipped in water maintained at 40 °C in an Electrothermal basin (Model: Cat No. MH8504). Specimens were mounted on microscope slides and staining was done in safranin and dehydrated in a series of graded ethanol (30, 50, 70, 100 and again 100%) and dipped in fast green followed by a series of graded xylene (30, 50, 70, 100 and again 100%). Specimens were viewed under a light microscope (Olympus microscope, ach 1x, SZX7) connected to a digital camera and microphotographs of callus union interfaces were taken.

4.3.4 Statistical analyses

Data on contamination were subjected to analysis of variance (ANOVA) after arcsine transformation (Steel & Torrie, 1980). Visual scoring for the graft union line (scale of 1 to 3: 1 = visibly high, 2 = faint, 3 = absent), necrosis (scale of 1 to 3: 1 = visible, 2 = faint, 3 = absent) and phenolics (scale of 1 to 4: 1 = high, 2 = medium, 3 = low, 4 = absent) was carried out. The scores (percentages) were subjected to correspondence analysis (GenStat, Rothamsted Experimental Station). A perceptual map was drawn to show distribution and association of various callus co-cultures.

4.4 Results and discussion

4.4.1 Decontamination of explants

Although 0.1% $HgCl_2$ is effective in surface decontamination of many explants, *U. kirkiana* culture contamination was high and some explants were completely lost due to this problem. There were significant differences between means ($P \leq 0.05$) with respect to the source of explants, but no significant differences were obtained with respect to the medium

supplement treatments and the interaction between medium supplements and source of explants. Explants excised from mature stock plants were highly contaminated irrespective of the MS medium supplements used (Table 4.2). *In vitro* contamination was observed at any stage, but placing a few explants per Petri dish reduced the risks of losing explants due to contamination. Table 4.2 shows that explants from mature stock plants were difficult to decontaminate (8.9%) unlike those explants excised from the juvenile stock plants (49.5%). Similar observations were also made in Chapter 2 for *U. kirkiana* shoot explants excised from mature trees growing in the forest.

4.4.2 Callus induction

Data in Table 4.3 shows that 0.1 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA, and 0.1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} NAA MS medium supplements were effective in callus induction. Callus was induced on the mid ribs, especially when explants were abaxially placed on the medium and implying that position of explants on the medium was important. No callus was induced on the cut surfaces and some explants curled away from the medium. Sita & Swamy (1993) reported easy callus induction along the cut surfaces of rosewood leaf disc cultures. This could be due to specific differences amongst species. There was rapid callus proliferation on fresh medium supplemented with 0.1 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA, and hence adequate callus mass was achieved by subculturing pieces of calli on this medium. Explants from mature trees resulted in poor callus induction and browning of the MS medium due to exudates. Frequent transferring of excised calli onto fresh medium improved the callus quantity.

4.4.3 Callus union interface between *U. kirkiana* clones

There were some deposits (phenols) at callus union interfaces of *U. kirkiana*. There was no visible union line in the MW32/32 clonal homograft (Figure 4.1A), but a faint union line in the MW49/49 inter-specific homograft (Figure 4.1B). Phenols have been implicated in graft incompatibility (Pina & Errea, 2005), but Errea *et al*. (1994a) reported existence of phenols in both compatible and incompatible *Prunus* tree species. High amounts of phenols have been reported in *U. kirkiana* leaves during DNA extraction (Hamisy, 2004). However, the quantity and nature of phenols at the union might be important factors in determining graft compatibility. Nito *et al*. (2005) found accumulation of deposits (suspected to be phenols) at the callus union of species of the orange subfamily which were not closely related. In the present study, perhaps the nature of plant material used contributed a lot to the presence of phenols even within homografts (Figure 4.1A-B). Furthermore, observations made during transportation of *U kirkiana* planting materials (shoots and leaves) from Malawi (Makoka Station) to South Africa (University of Pretoria) showed accumulation of phenols in the containers used.

Growth irregularities exhibited by some grafted *U. kirkiana* trees in the field might be attributed to accumulation of phenolics which are known to hinder graft union formation and reduce the graft success (Considine, 1983). Browning of MS medium, attributed to phenolic accumulation, was a major problem encountered. This confirms that *U. kirkiana* mature explants release a lot of phenols, so too the juvenile explants. There was no accumulation of phenols at the union lines of the *J. curcas* clonal heterograft (Figure 4.2A), while cells of *U. nitida* heterograft (Figure 4.2B) showed a lot of deposits. Generally, *U.*

kirkiana clonal homografts and inter-specific heterograft had faint union lines, but there were no necrotic layers observed, and hence they were considered to be compatible clones.

4.4.4 Callus union interface within *U. kirkiana* provenances

Some callus combinations showed visible lines at the unions (Figure 4.3A), but these might disappear with time since some areas along the union formed complete cell continuity. The thick arrow in Figure 4.3A (Dedza/Mpwapwa) indicates union areas with complete cell continuity (no line) despite a visible union line in other parts of the culture (thin arrow). For Phalombe/Dedza combination (Figure 4.3B), the union line is very faint while Phalombe/Murewa (Figure 4.3C) provenance combination shows a visible union line and significant deposits (phenol accumulation). Generally, all calli from mature planting materials were stained brown or purple and this indicates a high accumulation of phenols (Figure 4.3C). Dedza/Chipata (Figure 4.3D) combination shows a visible union line and deposits along the union interface. According to Errea *et al*. (1994a), phenols are vital in initiating tissue lignification, an important process resulting in a successful graft union. Therefore, quantification and structural analysis of phenols present at the union would be useful in determining graft incompatibility (Errea *et al.*, 1994b).

Figure 4.4A-B (Dedza/Choma) shows callus from mature explants (Choma) covered in polyphenols, degenerated and dead (MC) while callus from the juvenile plants (JC) was growing profusely. There was no union between the two partners due to a heavy accumulation of phenols which suppressed callus growth of mature explants. In this study, we hypothesise that the use of callus from mature and juvenile trees as a better option to simulate the reality in grafted fruit trees. However, callus co-cultures from juvenile plants

are also used in assessing *in vitro* callus compatibility. Nito *et al*. (2005) used callus from seedlings to assess compatibility among species of the orange subfamily. In practice, grafting of fruit trees is often between scions form mature trees and rootstocks from either young (seedlings) or mature trees, especially to achieve precocious fruiting. Therefore, use of callus from both juvenile partners might mask the effect of phenols on the graft union formation that is normally displayed in grafted trees. Mature woody scions release more phenols than juvenile scions and this might be critical for woody trees that are known to exude a lot of phenols such as *U. kirkiana*. Therefore, in this trial, *U. kirkiana* explants excised from both juvenile and mature planting materials were used.

Calli from both juvenile partners might not capture the effect of deposits (phenols) on graft compatibility in woody trees, but it might be easy to assess cell recognition in the absence of deposits. Dedza/Choma (Figure 4.4B) showed degeneration of callus from mature source (Choma) to an extent that there was no growth of callus from the mature source (MC), and hence no union between the two partners. Consequently, many such partners were weakly joined and easily separated when removed from the rings. This phenomenon depicts the effect of excess phenols on callus growth and union formation. Therefore, excessive phenols can dictate the success of a graft union due to the impact on callus proliferation.

In some provenance combinations, phenols suppressed the growth of the other partner. Figure 4.5A shows that Phalombe/Nyamakwaar callus was brown, possibly due to phenols from the mature partner (Nyamakwaar). There are also callus projections, which are necessary in grafted trees since they interlock tissues of the scion and stock strongly together upon differentiation and thus form a strong graft union. Figure 4.5B shows prolific

and green callus tissues from the juvenile planting materials (Dedza explants) while the mature partner tissues (Chipata) are brown. Calli from the juvenile plant materials have occupied a bigger volume than those from mature plant materials. This observed growth difference could be attributed to high quantities of phenols present in the mature plant tissues. Consequently, the phenols suppressed callus growth as evidenced in Figure 4.5A-B. Furthermore, there was death of callus from the mature planting (MC).

4.4.5 Callus union between *Uapaca kirkiana* and *Uapaca nitida*

There were faint union lines between *U. kirkiana* and *U. nitida* callus combinations (Figure 4.6A) but with some deposits (phenols) accumulated at the union area. This combination seems to be compatible. However, it was observed that there were differential growth irregularities between the two partners. *U. kirkiana* callus was rapidly increasing in volume unlike *U. nitida* and this could be an indicator of a weak combination that might eventually bring poor union. Based on this differential growth, faint union line and accumulation of deposits at the callus union, this exhibits a delayed incompatibility.

4.4.6 Callus unions between *Uapaca kirkiana* and *Jatropha curcas*

There were necrotic layers with dead cells between *U. kirkiana* and *J. curcas* callus combination. Furthermore, accumulation of phenols was seen on *U. kirkiana* but absent on *J. curcas* cells (Figure 4.6B). This indicates cell rejection and hence outright incompatibility. Cell walls of *U. kirkiana* calli had a thicker appearance (Figure 4.6B). This observation agrees with Errea *et al*. (1994a) for incompatible grafts of *Prunus* tree species. We hypothesise that cell recognition is very important when assessing compatibility in trees, especially for those that are distantly related.

Figure 4.7 shows the distribution and association of various callus combinations of *U. kirkiana*, *U. nitida* and *J. curcas* with respect to presence of phenols, union line and cell necrosis attributes. MW84/84 (PP), MW32/32 (DD), *U. nitida* (UN) clonal homografts, *J. curcas* (JJ) and MW49/49 (NK) inter-specific homografts are close together (top right quadrant) indicating a strong association and compatibility. Axis 2, accounting for 29.4% of the total inertia, shows *U. kirkiana*/*J. curcas* (DJ) combination being isolated and closely associated with cell necrosis (bottom left quadrant). Ermel *et al*. (1997) reported that cell necrosis is the main factor for graft incompatibility and our results agree with their finding as this is outright incompatible. *U. kirkiana* and *U. nitida* (NN and PN) combinations are closely associated with each (bottom right quadrant) and display less compatibility. This could be due to the differential growth irregularities that were observed.

It is difficult to declare if provenance combinations are incompatible based on presence of phenols alone because accumulation of phenols in the cells could be seasonal. Success of the grafted partners would also depend on the physiological state of the plants. Moreover, differences in quantity and quality of phenols between grafted partners are known to affect the union and hence the need for structural analysis and recognition of phenols at the onset of graft establishment (Errea, 1998). This observation necessitated quantification and identification of phenolics at the graft union, as presented in subsequent chapter.

4.5 Conclusion

Presence of a visible union line, accumulation of deposits (polyphenols) and cell necrosis were main indicators of graft incompatibility. Due to excessive phenols, compatibility was

not clear-cut within *U. kirkiana* provenances. Callus fusion within *U. kirkiana*, *U. nitida* and *J. curcas* clones were compatible, but compatibility evidence was less so between *U. kirkiana* and *U. nitida*. Callus combinations between *J. curcas* and *U. kirkiana* showed graft incompatibility. Despite accumulation of phenols that suppressed callus growth, callus fusion is a promising technique for assessing early graft compatibility or incompatibility in species that are not closely related.

Tables

Table 4.1 Callus co-cultures of *Uapaca kirkiana*, *Uapaca nitida* and *Jatropha curcas*

No.	Callus combinations	Tree origin	Type of combination
1.	MW84/MW84	Malawi	clonal homograft
2.	MW32/MW32	Malawi	clonal homograft
3.	MW49/MW49	Malawi	inter-specific homograft
4.	MW26/MW57	Malawi	heterograft
5.	*U. nitida/U. nitida*	Malawi	heterograft
6.	*J. curcas/J. curcas*	South Africa	heterograft
7.	*U. kirkiana/U. nitida*	Malawi	species
8.	*U. kirkiana/J. curcas*	Malawi/South Africa	family
9.	Dedza/Phalombe	Malawi	provenance
10.	Phalombe/Nyamakwaar	Malawi/Zimbabwe	provenance
11.	Phalombe/Murewa	Malawi/Zimbabwe	provenance
12.	Dedza/Chipata	Malawi/Zambia	provenance
13.	Phalombe/Mapazure	Malawi/Zimbabwe	provenance
14.	Phalombe/Choma	Malawi/Zambia	provenance
15.	Phalombe/Nyamakwaar	Malawi/Zimbabwe	provenance
16.	Dedza/Mpwapwa	Malawi/Tanzania	provenance
17.	Dedza/Murewa	Malawi/Zimbabwe	provenance
18.	Dedza/Chipata	Malawi/Zambia	provenance
19.	Dedza/Choma	Malawi/Zambia	provenance

Table 4.2 Percentage contamination of young and old *Uapaca kirkiana* leaves explanted on different Murashige and Skoog medium supplemented with benzylaminopurine (BAP), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and thidiazuron (TDZ). Means are calculated with standard deviations.

MS medium	Culture asepsis (%)		
supplements (mg l^{-1})	Young leaves	Old leaves	Mean
1.0 IBA + 0.1NAA	12.5	48.5	30.5 ± 8.8^{a}
0.1 TDZ + 0.5 NAA	6.9	52.4	29.7 ± 12.3^{a}
0.5 BAP + 1.0 NAA	7.6	47.1	27.4 ± 9.3^{a}
0.1 2, 4-D + 0.5 NAA	8.3	50.0	29.2 ± 12.1^{a}
Mean	8.9 ± 1.5^{b}	49.5 ± 5.3^{a}	

CV (%) = 48.6

LSD (5%) Medium supplements (M) = 17.6^{ns}

Leaves (L) = 12.4*

M × L = 24.9^{ns}

* Means with different letters are significantly different at $P \leq 0.05$

ns = no significant difference at $P \leq 0.05$

Table 4.3 Callus induction of *Uapaca kirkiana* explants on Murashige and Skoog (MS) medium supplemented with benzylaminopurine (BAP), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and thidiazuron (TDZ). Means are calculated with standard errors

MS medium supplements (mg l^{-1})	Callus formation (%)
1.0 IBA + 0.1NAA	21.7 ± 6.0^{b}
0.1 TDZ + 0.5 NAA	58.3 ± 4.4^{a}
0.5 BAP + 1.0 NAA	0.9 ± 0.6^{c}
0.1 2, 4-D + 0.5 NAA	53.3 ± 4.4^{a}
CV (%)	22.4
$LSD_{0.05}$	15.9

Means with the same letters within a column are not significantly different at P≤0.05

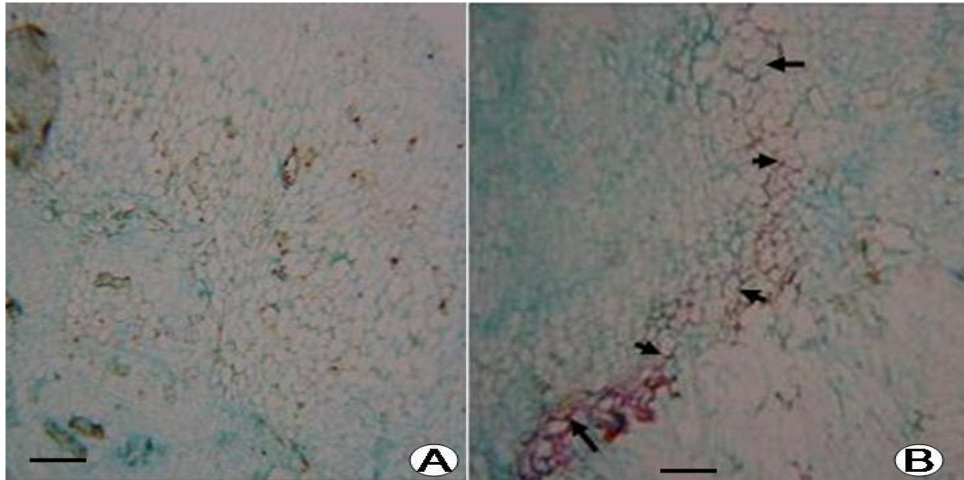

Figure 4.1 Callus union interfaces within *Uapaca kirkiana* clones (A) MW32/32 clonal homograft showing no visible union line; (B) MW49/49 inter-specific homograft. The brown stains on the cells are due to deposits (polyphenols) and arrows show faint union line for MW49/49 inter-specific homograft (bars = 10 µm)

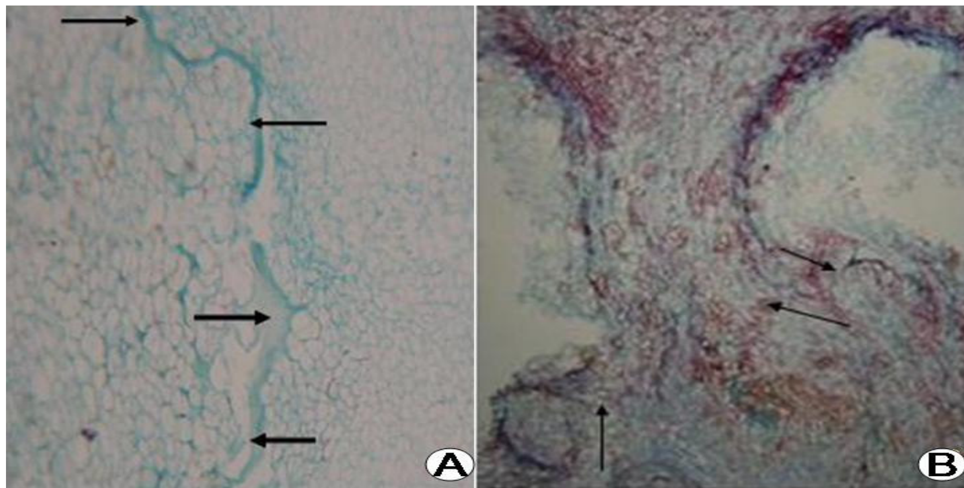

Figure 4.2 Sections of callus union interfaces of (A) *Jatropha curcas* heterograft; (B) *Uapaca nitida* heterograft (arrows indicate the union interfaces). No major phenolic compound staining was present in *J. curcas* cells, but it was present in *U. nitida* cells

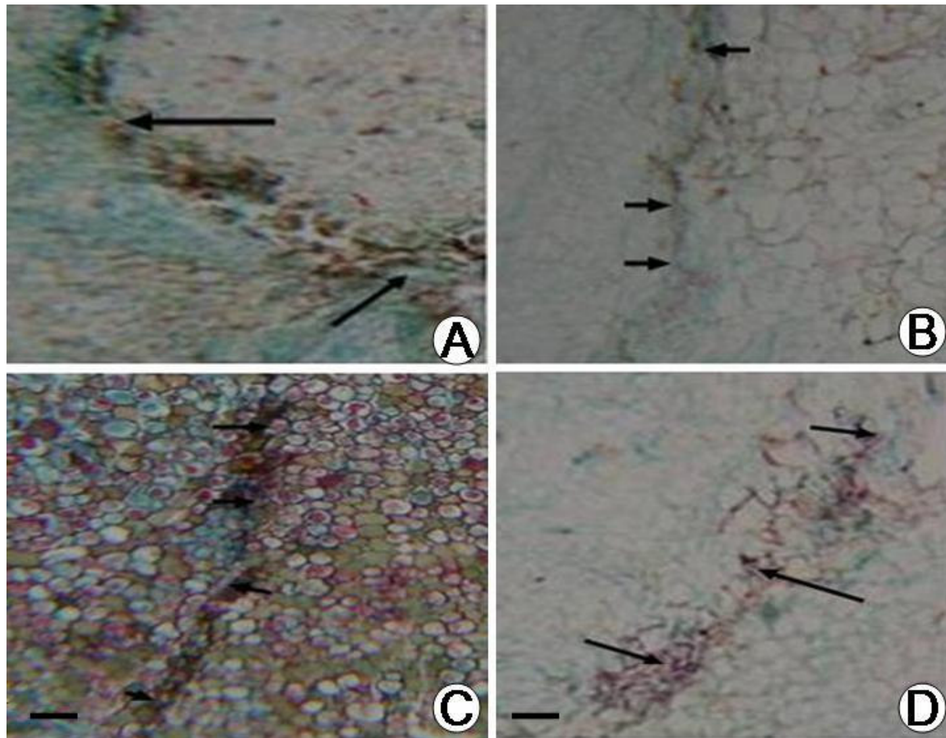

Figure 4.3 Callus union interfaces of *Uapaca kirkiana* tree provenances (A) Dedza/Mpwapwa; (B) Phalombe/Dedza; (C) Phalombe/Murewa; (D) Dedza/Chipata (Arrows show the union line, bars = 10 µm)

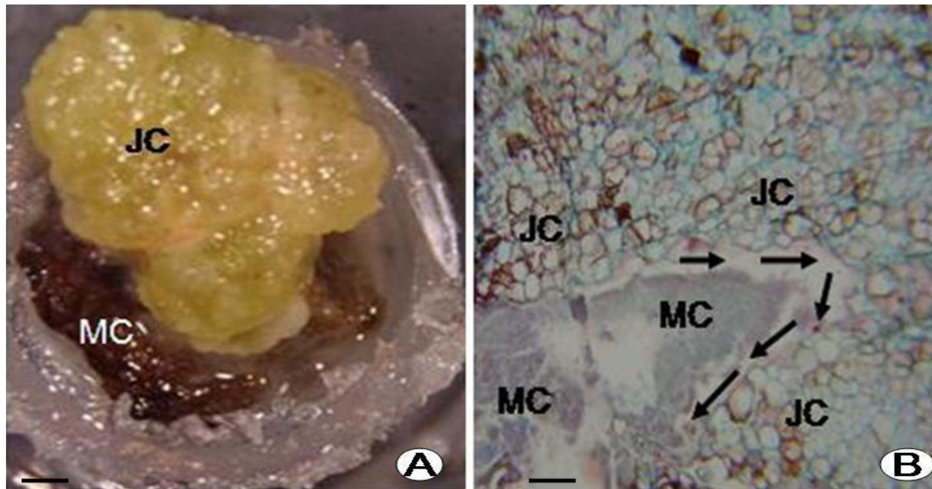

Figure 4.4 Callus union interface of *Uapaca kirkiana* tree provenance (A) a surface view of Dedza (juvenile) and Choma (mature) callus union in a polythene ring; (B) same callus combination section showing callus degeneration at the union (JC = callus from juvenile planting materials, MC = callus from mature planting materials). Arrows indicate union area between calli from mature and juvenile stock plants (bar = 10 μm)

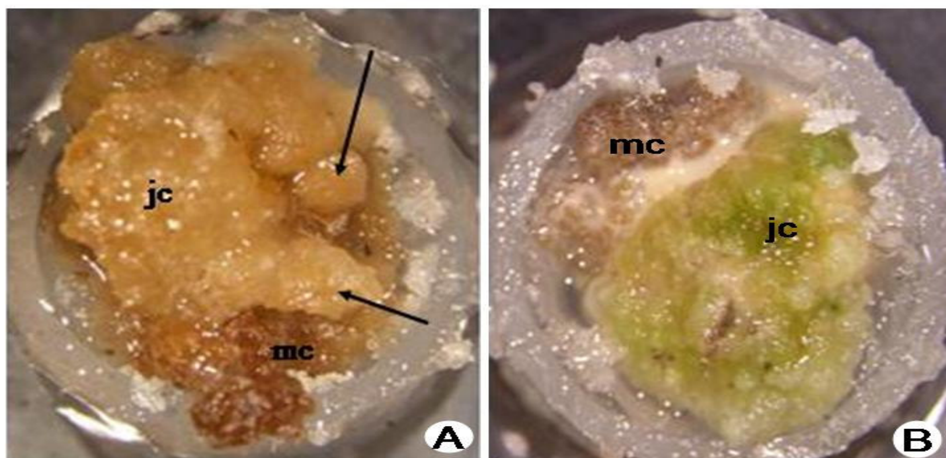

Figure 4.5 Callus co-cultures of *Uapaca kirkiana* tree provenances in thermal labile rings (A) Phalombe/Nyamakwaar; (B) Dedza/Murewa (jc = callus from juvenile tissues, mc = callus from mature tissues). Arrows show bead-like projections

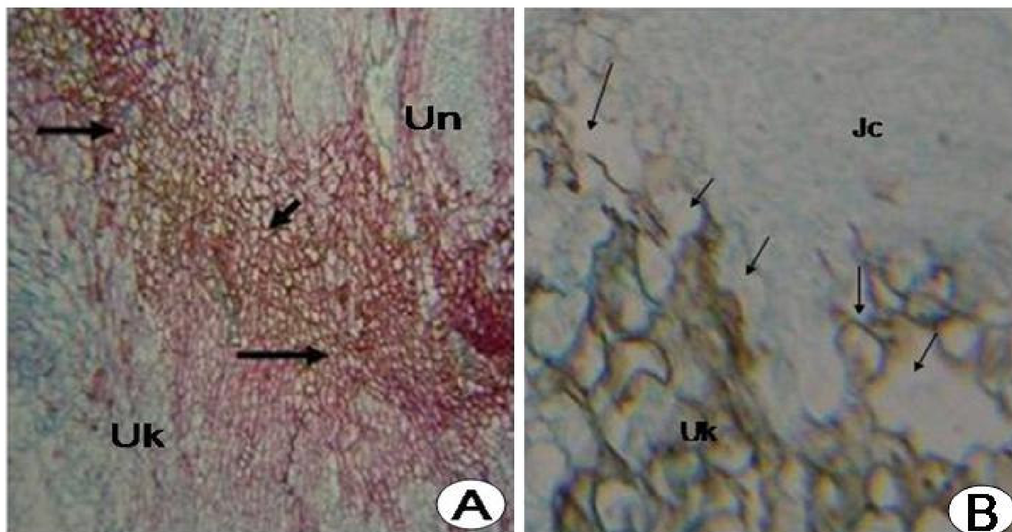

Figure 4.6 Callus union interface sections between: (A) *Uapaca kirkiana* (Uk) and *Uapaca nitida* (Un); (B) *Uapaca kirkiana* (Uk) and *Jatropha curcas* (Jc) showing phenol accumulation on the *U. kirkiana* cells. Arrows show callus union

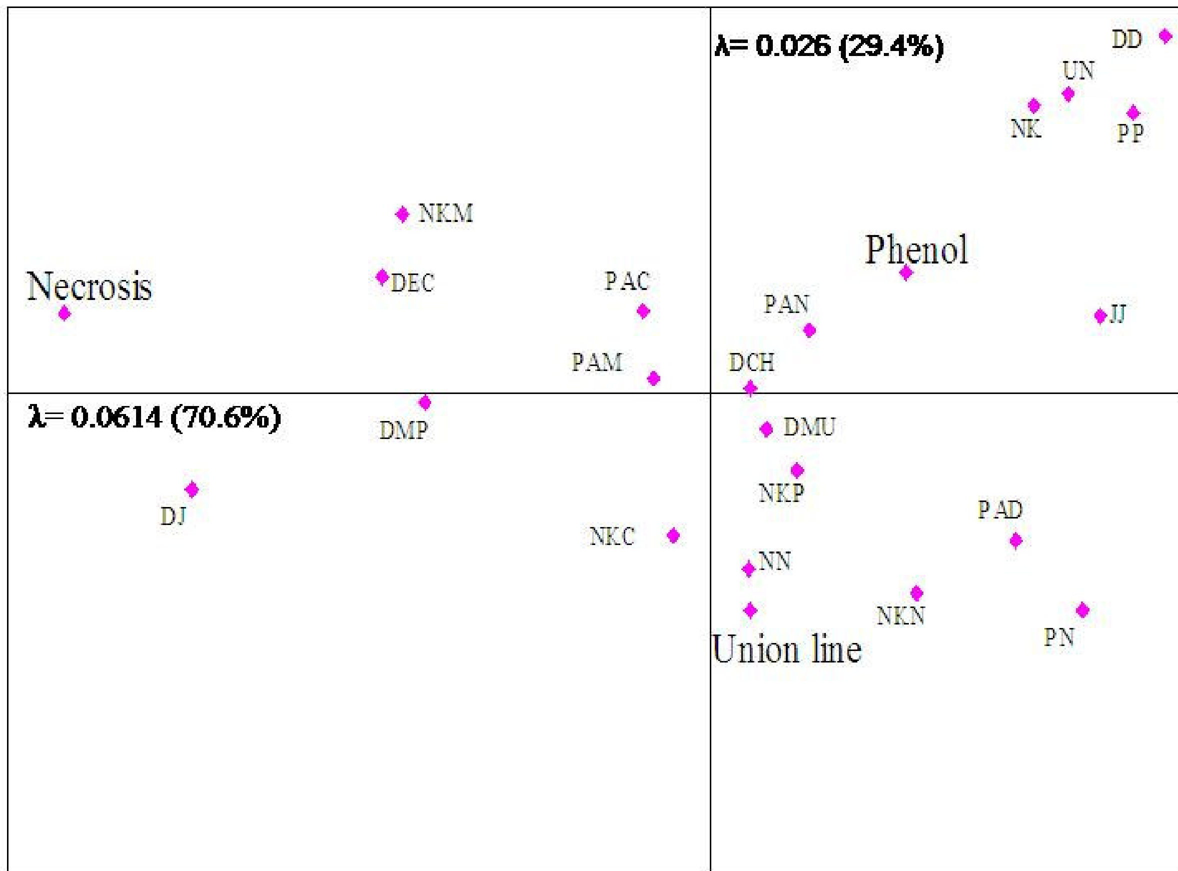

Figure 4.7 Distribution and association of *Uapaca kirkiana* tree clones and provenances, *Uapaca nitida* and *Jatropha curcas* callus combinations with respect to phenol, union line and cell necrosis attributes from correspondence analysis output (PP = MW84/84, DD = MW32/32, NK = MW49/49 inter-specific homograft, PAN = MW26/57, NN = *U. nitida* heterograft, JJ = *J. curcas* heterograft, NKP = MW57/26, PN = *U. kirkiana*/*U. nitida*, DJ = *U. kirkiana* (Dedza)/*J. curcas*, PAM = Phalombe/Murewa, PAC = Phalombe/Chipata, PAD = Phalombe/Dedza, NKM = MW57/Murewa, NKC = Phalombe/Chipata, NKN = Phalombe/Nyamakwaar, DMP = Dedza/Mpwapwa, DMU = Dedza/Murewa, DCH = Dedza/Chipata, DEC = Dedza/Choma) (λ = inertias)

CHAPTER 5

DIAGNOSIS OF PHENOLIC COMPOUNDS IMPLICATED IN GRAFT INCOMPATIBILITY OF *UAPACA KIRKIANA* Müell Arg. TREES

5.1 Abstract

The objective of the trial was to determine the influence of phenolics on the graft union of *U. kirkiana* trees. Stem diameters were measured before sample collection. Soluble phenols were extracted with methanol-acetone-water followed by Folin-Ciocalteau reagent procedure and quantity assessed spectrophotometrically. Cell wall bound phenols were extracted and the precipitate was resuspended in 80% methanol before HPLC analysis. Thin (10 µm) layer sections were viewed under UV, blue and white light with or without Vanillin-HCl staining using fluorescence microscopy. Results showed significant differences in stem diameters. The unions were thicker than the scions and stocks. Both soluble and insoluble phenols at the unions were significantly high and homospecific combinations showed a high quantity of soluble phenols above the union. Fluorescence microscopy indicated the presence of flavonoids, anthocyanins or their derivatives above the union. HPLC indicated ferulic acid as the major component responsible for wood discolouration. For a less compatible combination (MW12/12), high peaks of *para*-coumaric acid were found above the union, and hence *para*-coumaric acid, flavonoids or polymers were implicated in graft incompatibility of *U. kirkiana* tree species.

5.2 Introduction

Grafted fruit trees perform better than sexually propagated fruit trees and some desirable attributes include precocious fruiting, improved fruit quality (fruit size) and tree dwarfness. Furthermore, dwarf trees are easier and cheaper to manage than large trees (Usenik & Štampar, 2001; Webster, 2001). However, graft incompatibility has been reported in some fruit trees and this has a negative effect on orchard productivity (Simons, 1987; Facteau *et al.*, 1996; Errea *et al.*, 2001).

Graft incompatibility has been attributed to many factors which include growth regulators and peroxidases, lack of formation of plasmodesmata and vascular tissue connection and inherent incompatibility at cellular level (Usenik *et al.*, 2006). Micromolecules such as proteins, RNA and IAA transport are also involved (Pina & Errea, 2005). For *U. kirkiana*, successful grafting (80% graft take) has been achieved with skilled grafters at Makoka in Malawi (Akinnifesi *et al.*, 2004). However, this does not mean long term scion/stock compatibility and field observations have revealed signs of graft incompatibility (Thomson Chilanga, personal communication). Similar observations have been made in *Eucalyptus* trees (Decooman *et al.*, 1996). Recent assessment of the field survival of *U. kirkiana* grafted trees showed a decline from 100% after 6 months down to 67% after 13 months of establishment (Akinnifesi *et al.*, in preparation). Usenik & Štampar (2001) reported that reduced graft compatibility is manifested by differences in the quantity of phenols at the graft union and the concentration of phenols is often high above the unions of heterografts.

Phenols have been implicated in graft incompatibility (Errea, 1998). Histological study of *U. kirkiana* tree (Chapter 4) revealed the presence of deposits and death of tissues above the union as the main causes of graft incompatibility. Differences in quantity or specific phenols at the union could play a role for reduced graft compatibility. However, when the wounding stress is over, many soluble phenols could be polymerised to other tannin-like compounds (Swain, 1979) and then deposited in the cell walls. Du Plooy (2006) who studied lenticels on mango fruits reported that cell wall bound phenols formed red colouration and that soluble phenols were polymerised into cell walls. Therefore, evaluation of cell wall bound phenols in grafted trees would be interesting, especially when the wounding is complete. Moreover, many studies on graft incompatibility have not focused on cell wall bound phenols, which might play an important role in graft incompatibility. The objective of this study was to determine the influence of phenolic compounds at the graft union of *U. kirkiana* trees.

5.3 Materials and methods

5.3.1 Plant material

Three-year old grafted *U. kirkiana* tree samples were collected at Makoka (see section 2.3.2 for site description) in February 2006. The trees were growing normally in the field and some were already fruiting. Scion, stock and union diameters of forty trees were measured using a pair of digital callipers (Mitutoyo, OE7343) before sample collection. The trees were propagated by skilled grafters using splice grafting method. Table 5.1 shows identification (ID) of the ten *U. kirkiana* tree species sampled. They were placed in cooler

box containing ice blocks and then transported within two days to the University of Pretoria for phenol analysis.

Samples were freeze-dried (Edwards, Modulyo, Pirani 10) to prevent oxidation and then dissected into segments from the union towards either the scion or stock (Figure 5.1). The segment at the union was 1 cm while the other segments were about 0.5 cm. The bark samples comprising the periderm, phloem and the vascular cambium, were ground using a mortar and pestle. The wood samples were ground using a motor grinder (Willey mill, 20 mesh). The fine powder (0.05 g) per sample was placed in Eppendorf tubes for extraction of phenol.

5.3.2 Phenol extraction

Samples were extracted with 1 ml of methanol-acetone-water solution (7:7:1 v/v/v) three times and then evaporated. The mixture was made up to 1 ml with deionised water and mixed in ultrasonic cleaner VWR™ (USC900TH, VWR International bvba/spri, B-3001 Leuven) for 4 min. Samples were centrifuged fro 4 minutes with a bench centrifuge (Combi-spin, type: FVL-2400N, Rochelle Chemicals & lab equipment, Germany). The supernatants were mixed and 1 ml was collected. Since the concentration was high, 100 µl supernatant was added to 900 µl deionised water for soluble phenol quantification.

5.3.3 Quantification of total soluble phenol

Folin-Ciocalteau reagent (Sigma), based on reduction of phospho-molybdene/phospho-tungstate (de Ascensao & Dubery, 2003), was used. Deionised water (175µl) was dispensed in ELISA plate wells, then the sample extract (5 µl), followed by Folin-Ciocalteau reagent

(25 µl). Sodium carbonate (50 µl of 20% w/v) was added and a blank in which water replaced the sample was used as a control. Four wells (replicates) were used per sample and incubated at 40 ^{o}C for 30 minutes. Absorbance was read using Multiskan Ascent spectroscopy (V1.24 345-00007T) set at 690 nm. Gallic acid was used as a phenol standard to construct a standard curve. The concentration of phenols in the extracts was calculated from the standard curve, y = 1.3527x - 0.0109 and multiplied by 10 since the initial dilution was in the ratio of 1: 9. The total soluble phenol quantity (mg) was expressed as gallic acid equivalent per g dry weight (DW) of sample. Data analysis was done using GenStat 4.24DE (Rothamsted Experimental Station).

5.3.4 Fluorescence microscopy

Four samples (MW12/12, MW26/26, MW32/28 and MW7/10) were used and these samples were fixed in formalin acetic acid (i.e. 5% formalin, 5% acetic acid and 90% ethanol). Thin (10 µm) layer sections were dissected at a right angle to the graft union using a sliding microtome (Leitz Wetzlar, 17815). The sections were fixed onto the microscope slides using glycerol and viewed with a Zeiss Axiovert 200 (Zeiss, Göttingen, Germany) microscope fitted with a sensys camera. Digital images of emission at 397 nm, 515 nm and 565 nm were captured with Nikon ACT-1 version 2. The sections were viewed under UV, white and blue light then stained with Vanillin-HCl (1% of concentrated HCl) and viewed again under UV, white and then blue light. Vanillin used was purchased from Sigma Aldrich Chemie (Steinheim, Germany). Blue light did not provide a good contrast between compatible and incompatible combinations. Therefore, the results under blue light will not be presented.

5.3.5 Phenol analysis using HPLC

Cell wall bound phenols were extracted from MW26/26, MW7/10 and MW12/12 samples according to De Ascensao & Dubery (2003) methodology. The reason why these three graft combinations were chosen was because MW26/26 graft combination was partially compatible, MW12/12 showed reduced compatibility (Chapter 4) and MW7/10 was an intermediate. Precipitates, obtained after total soluble phenol extraction, were dried and 0.5 M NaOH added to 0.01 g of precipitate. The mixture was suspended in a water bath (Julabo V, Labotec Pty Ltd, model 101) for 1 hour at 96 ^{o}C. The supernatant was acidified with HCl to a pH of 2.6, centrifuged fro 10 minutes and then extracted with 1 ml anhydrous diethyl ether. The mixture was dried and the precipitates suspended in 200 µl 80% aqueous methanol (MeOH). Folin-Ciocalteau reagent was used to determine the total cell wall-esterified phenolic acids and the remaining solution was separated using a reverse phase high performance liquid chromatograph (RP-HPLC).

The RP-HPLC (Hewlett Packard Agilent 1100 Series) was equipped with 20 µl loop injection value, DAD detection (diode array detector, 280, 325, 340 nm), Luna 3u C-18 (Phenomenex®) reverse phase column (150 mm by 2 mm, µm particle size, Ref. 550) and fitted with solvent deggaser (G1322A). A gradient elution was performed with HPLC water acidified with orthophosphoric acid (pH = 2.6) and acetonitrile (ACN) as follows: 0 min, 7% ACN; 0 - 20 min, 20% ACN; 20 - 28 min, 23% ACN; 28 - 40 min, 27% ACN; 40 – 45 min, 29% ACN; 45 - 47 min, 33% ACN. The flow rate was 0.6 ml min^{-1}. After each sample, the column was stabilized for 5 minutes and 40 µl of each sample was injected through a 20 µl loop. The UV detector was set at 280 nm but this is not optimal for ferulic

acid and its derivatives (Du Plooy, 2006). Identification of phenols was carried out by comparing their retention times (t_R) and UV apex spectrum to those of authentic standards purchased from Sigma Chemical Company, USA.

5.4 Results and discussion

5.4.1 Diameters of scion, stock and graft union

There were significant differences ($P \leq 0.05$) in mean diameters (Figure 5.2) amongst the scions, stocks and the graft union. The graft unions showed thick diameters compared to the scions and stocks. Selection of matching *U. kirkiana* scions and stocks is always difficult because scions are usually thicker than the stocks (Akinnifesi *et al*., 2006). In this experiment, stocks (18 mm) were thicker than scions (15 mm) after grafting (Figure 5.2). This could be attributed to vegetative growth restriction imposed by the stocks (dwarfing stock effect). According to Andrews and Marquez (1993) cytokinins are produced by root tips and acropetally translocated to promote the scion growth. The thin scions observed after grafting could be an indication of problems at the union. Generally, differences in stem diameters impose poor scion/stock alignment (Figure 5.3A). Consequently, wart-like projections of the wood cause poor continuity of the bark, and hence many cracks along the union.

Wood discoloration (Figure 5.3B) and swelling of the union (Figure 5.3C) are attributed to accumulation and oxidation of phenols. With such growth irregularities in the wood, overbearing in the early years might impose stress at the union and the ultimate survival (graft failure risk) of the trees. The lacuna areas in the wood can adversely affect the

support and water movement in the xylem as the trees grow (i.e., increase in size and mass). Presence of lacuna layers and wood discoloration above and at the union indicated that graft incompatibility was intense at these positions.

5.4.2 Total soluble phenol quantity in the wood

Table 5.2 shows total soluble phenols for individual grafted *U. kirkiana* samples and at different positions of the union in the wood. Forty percent of both homografts (MW32/32 and MW26/26) and heterografts (MW80/82 and MW84/57) had similar total soluble phenol above and below the union. The small variations within the homografts are attributed to genetic differences since the rootstocks were raised from seed. Different pollen sources in *U. kirkiana* trees are possible as they are dioecious, and hence mixed pollen on one female tree may occur. MW32/32 (Figure 5.4A) shows graft compatibility while MW32/28 (Figure 5.4B) shows signs of incompatibility in the wood. Pina & Errea (2005) reported that heterograft combinations often show graft incompatibility. There are lacuna or holes and wood discoloration, especially above the graft union. Despite these growth irregularities, all the trees were growing normally in the field.

There was no clear relationship between soluble phenol quantity and the graft union morphology. This could be attributed to the fact that phenols could be modified or polymerised into different forms. They might be deposited in the cell walls (cell wall bound phenols), and hence Folin reagent procedure could not quantify phenols which were bound in the cell walls. According to Pina & Errea (2005), small quantities of phenols could limit cell functions at the union and quantitative differences in phenols between the graft partners could cause metabolic problems. Furthermore, phenols play vital physiological roles during

the early stages of graft establishment. Therefore, effective proliferation of parenchymatous cells during the early stages to break up accumulated phenols is important for vascular continuity.

5.4.3 Total soluble phenol quantity in the bark

Table 5.3 shows total soluble phenols at different positions of the graft union in the bark of the individual graft combinations. Collectively, the graft unions showed significantly high accumulation of soluble phenols. There were no significant differences ($P \leq 0.05$) between above and below the union of 80% of the homograft combinations (MW32/32, MW13/13, MW12/12 and MW76/76). Furthermore, 60% of the heterograft combinations (MW7/10, MW32/28 and MW84/57) showed no significant difference between below and above the union. Gebhardt & Feucht (1982) and Usenik & Štampar (2001) reported that homografts show similar amounts of phenols above and below the union. In this experiment, it is suspected that isolated vascular continuity in the bark enabled tree growth of incompatible combinations. However, poor continuity in wood could adversely affect tree survival as the tree matures.

Table 5.4 shows soluble phenols in the bark and wood. There are significant differences ($P \leq 0.05$) between positions of the union and sample section (bark and wood), but no significant interaction. The data show significantly higher soluble phenol in the bark (87.9 mg g^{-1}) than in the wood (66.0 mg g^{-1}). This is because phenols are produced in the bark (phloem) and stored in the cell vacuoles. They are released in response to wounding or other stimuli. Total soluble phenol was significantly high at the union (85.4 mg g^{-1}) and low both below and above the union (75.2 mg g^{-1}).

5.4.4 Cell wall bound phenols

Figure 5.5 shows the quantity of cell wall bound phenols (mg equivalent of gallic acid per g of dry weight) at different positions (above, below and at the union) in the bark. There were significant differences ($P \leq 0.05$) between positions for MW12/12 and MW26/26. However, MW26/26 shows no significant differences in the quantity of phenol above and below the union. Morphologically, this graft combination showed partial compatibility. MW12/12 shows a higher quantity of cell wall bound phenols in the bark at all the positions compared to MW26/26 and MW7/10. Morphologically, this graft combination (MW12/12) showed incompatibility above, below and at the union. Therefore, this incompatibility is attributed to high quantity of cell wall bound phenols. De Ascensao & Dubery (2003) reported that cell wall bound phenols increased more than other phenols after 36 hours of *Fusarium* elicitor interaction.

Phenol deposition such as lignin and tannins occurs in plants and the former contributes to the stiffening and rigidity of cell walls and other internal structures while the latter helps in inhibiting microbial attack (Swain, 1979). Plants are also able to modify phenols deposited at the wounded site. De Ascensao & Dubery (2005) reported an increase in cell wall bound phenolics at the wounded site. In this trial, the death of a large portion of bark above the union of MW12/12 affected cell activities such as phenol polymerization and modification. This possibly, resulted in more phenol conversion into cell walls below the union (11.02 mg g^{-1}) than above the union (9.11 mg g^{-1}) of the bark.

There were no significant differences ($P \leq 0.05$) between the different positions of the union in the wood for MW7/10 and MW26/26 (Figure 5.6), but there were significant differences

for MW12/12 combination. MW12/12 also shows a significantly higher quantity of phenols above the union (4.40 mg g^{-1}) than below the union (2.54 mg g^{-1}). The same trend was obtained in Table 5.2 for this graft combination. Morphologically, MW26/26 and MW7/10 combinations had good vascular continuity in the wood below the union and showed better compatibility than MW12/12. Lack of continuity in wood tissues can be severe set-back because the wood provides the support. Absence of wood continuity at the graft union interface can cause graft failure.

5.4.5 Fluorescence microscopy

Phenols in the *U. kirkiana* tissues were detected using Vanillin-HCl as a fluorescent dye and Table 5.5 shows the colour characteristics of some phenolics. Fluorescence was observed in both unstained and stained sections (Figures 5.7 - 5.10). A blue staining was pronounced, especially below the union, indicating the presence of ferulic acid (Figures 5.7- 5.10). The darkened and blackened parts show cell death due to crushed cells during grafting. Cell death is pronounced above the union in all the graft combinations and this is attributed to accumulation of phenols. Brown or red staining under the white light for MW32/28 (Figure 5.7A-F) combination indicates presence of flavonoids (quinones). There were also zones of purple fluorescence and this indicates the presence of anthocyanins or terpenoids. Traces of cell wall bound red fluorescence were also present.

Visible observations indicated that MW7/10 and MW26/26 were partially compatible, but MW32/28 and MW12/12 were incompatible. Red or purple staining indicates the presence of flavonoids (Dixon & Paiva, 1995) or anthocyanins (Kangatharalingam *et al*., 2002). Presence of flavonoids has been implicated in graft incompatibility (Errea *et al*., 1994b)

and, from the results presented here, can also be implicated in *U. kirkiana* graft incompatibility. According to Errea *et al*. (1994b), a high amount of flavanoids (red) was found in the phloem of apricot as a response to graft incompatibility. Phenols can be oxidized to quinones which are toxic due to chemical reaction disruption (Errea, 1998).

MW32/28 (Figure 5.7A-F) shows that the cells fluoresced red though not very bright with an orange to brown colour under white light and above the union. This indicates the deposition of polymeric phenolics. According to Du Plooy (2006), polymeric phenolics often disguise the flavonoids (red). There are traces of yellow spots and bright blue at the union, under the UV and white light. There were blue colour lines below the union when *U. kirkiana* sections were viewed under UV and white light (Figure 5.7C-F), indicating ferulic aids. MW7/10 (Figure 5.8A-F) showed a pale brown colour (flavonoid or its derivatives) above the union, but a predominant blue colour (ferulic acid) below the union. Darkened (dead tissues) and brown areas were present at the union. MW26/26 sections (Figure 5.9A-F) show faint purple colour above the union, but a blue staining or colour is predominant below the union. MW12/12 sections (Figure 5.10A-F) show a purple colour (polymers or terpenoids) which is predominant below the union when viewed under the white light. Under the UV light, there are many dead cells (black or dark areas) above the union, purple colour (Figure 5.10E-F) and a few white spots in certain parts of the section. Therefore, cell death and presence of flavonoid or its derivatives could be implicated in graft incompatibility for this combination. Presence of dead tissues, holes and phenols could have prevented vascular continuity formation, caused cell damage and altered phloem cambium around the union.

5.4.6 Phenol analysis using RP-HPLC

Bark: RP-HPLC results indicated that ferulic acid was prominent in all the samples and at all the union positions (Figures 5.11 - 5.13). This also agrees with the fluorescence microscopy results in that ferulic acid was abundant in many sections. Ferulic acids eluted from the column within 14 minutes (t_R = 14) for MW12/12 (Figure 5.11A-B) at all the positions of the union. There were higher peaks (directly related to higher concentrations) of *para*-coumaric acids (t_R = 12.5) above the union for MW12/12 (Figure 5.11A), but low peaks for MW26/26 (Figure 5.12A-B). According to Méndez *et al*. (1968), *para*-coumaric acids strongly hinder cell elongation. Ramina & Masia (1982) reported that *para*-coumaric acids, isolated in peach fruits, were inhibitory to cell elongation and cress seed germination. Furthermore, cell wall bound *para*-coumaric acids were related to pit hardening in peach fruits. In this study, the presence of *para*-coumaric acids at the union might have adversely affected graft compatibility since it is possible that cell elongation and plasticity were inhibited. Generally, higher peaks were obtained below than above the graft unions in the in all the combinations (Figures 5.11 - 5.13). This could be attributed to the presence of many living tissues below the union that were able to carry out cell metabolism unlike the non-functional tissues presence above the union.

There was an unknown phenol (t_R = 44) indicated as phenol 44 (Figures 5.11A-B and 5.12A-B). The peaks for phenol 44 were high for MW12/12 compared to MW26/26 combinations, but this unknown phenol cannot be implicated in graft incompatibility for *U. kirkiana*. This is because it has higher peaks below the union of both MW12/12 and MW26/26 combinations. Morphologically, MW26/26 showed compatibility below the union despite the presence of a high peak of this unknown phenol (Figure 5.12B). This

indicates that its presence has no adverse effects on graft compatibility. Moreover, this unknown phenol was absent in MW7/10 combination (5.13A-B). Therefore, the presence of this phenol is dependent on the tree provenance or the source of a tree provenance.

Figure 5.11 shows the presence of 3, 4 dihydroxybenzoic acid ($t_R = 3$) only below the graft unions of MW12/12 (Figure 5.11B) and MW7/10 (Figure 5.13B). Its influence on graft compatibility is not adverse since it occurred where the union showed compatibility. For MW7/10 combination, another unknown phenol ($t_R = 47$) (Figure 5.13A-B) was obtained. This phenol shows also high peak below the union where there was more continuity in the bark than above the union. Furthermore, phenol 44 was absent in the other combinations. Therefore, it is assumed that this phenol had no impact on graft compatibility.

Wood: Generally, there were low peaks for all phenols in the wood and this made identification of peaks difficult (Figures 5.14 - 5.16). Ferulic acid was predominant and this indicates that ferulic acids were bound to the cell walls (Du Plooy, 2006). Vanillin ($t_R = 7$) was present for M12/12 combinations (Figure 5.14A-B). Phenol 44 was also obtained in the wood of MW12/12 with higher peaks above the union. Comparatively, small peaks were obtained from MW26/26 (Figure 5.15A-B). Low peaks in the wood indicate that many phenols were produced in the epidermal cells of the phloem (bark). Therefore, phenol analysis of the bark tissues (phloem vascular tissues) provided a clearer indication of the available phenols in *U. kirkiana* tree species. Although the quantities (mg g^{-1}) of phenols might be higher below than above the union, differences in accumulation of water could dilute the water-soluble phenols. This might reduce adverse effects on graft compatibility.

RP-HPLC results indicate that wood discoloration observed in all the graft combinations under a light microscope was largely due to ferulic acids bound to cell walls. High concentrations of *para*-coumaric acid above the union of MW12/12 in the bark could be implicated in graft incompatibility. DeCooman *et al*. (1996) reported accumulation of *para*-coumaric acid in less compatible *Eucalyptus gunnii* and Usenik *et al*. (2006) found *para*-coumaric acids to be high above the union of all the Betinka/stock combinations in apricot fruit trees. Betinka/stock was found to be less compatible even within homospecific combinations (Usenik *et al*., 2006). The predominance of ferulic acids in all of the samples provides evidence that ferulic acids were bound to the cell wall structures. More incompatibility problems can be expected with MW12 in heterospecific combinations. However, this needs to be verified since there is seasonality for phenol production.

5.5 Conclusion

Graft incompatibility in *U. kirkiana* is attributed to the presence of *para*-coumaric acids, flavonoids and polymers. MW12/12 was incompatible, while MW26/26 was partially compatible. Wood discoloration was due to ferulic acid which was abundant in all the combinations. Soluble phenols peaked at the union in many combinations in wood and bark. Flavonoids and anthocyanins were observed above the union under the fluorescence microscope on MW12/12, and hence implicated in graft incompatibility. Cell wall bound phenol analysis showed significant concentrations below the union of a less compatible combination (MW12/12). There were also high peaks of *para*-coumaric acids for MW12/12. Therefore, cell wall bound phenol analysis from the bark tissues provides useful information, especially when the wounding stress is complete.

Tables

Table 5.1 Tree identification (ID) of *Uapaca kirkiana* graft combinations collected at Makoka Agricultural Research Station in Malawi

Tree ID	Scion accession name	Tree ID	Stock accession name
MW76	ICR02NkhumbaMW76	MW76	ICR02NkhumbaMW76
MW7	ICR02MalemiaMW7	MW10	ICR02MalemiaMW10
MW12	ICR02SitolaMW12	MW12	ICR02SitolaMW12
MW13	ICR02SitolaMW13	MW13	ICR02SitolaMW13
MW26	ICR02HardwickMW26	MW26	ICR02HardwickMW26
MW32	ICR02YesayaMW32	MW28	ICR02HamiyoniMW28
MW56	ICR02NkhumbaMW56	MW49	ICR02NkhumbaMW49
MW32	ICR02YesayaMW32	MW32	ICR02YesayaMW32
MW84	ICR02NazombeMW84	MW57	ICR02NazombeMW57
MW80	ICR02NazombeMW80	MW82	ICR02NazombeMW82

Table 5.2 Total soluble phenol quantity (mg gallic acid equivalent per g of dry weight) in the wood of ten grafted *Uapaca kirkiana* trees measured above, below and at the union

Graft combinations	Gallic acid equivalent of dry weight (mg g^{-1})			CV (%)
	above the union	at the union	below the union	
Homografts				
MW26/26	61.5^{b}	73.9^{a}	67.3^{ab}	7.1
MW12/12	66.0^{a}	67.5^{a}	52.7^{b}	11.6
MW13/13	30.0^{c}	62.0^{a}	36.9^{b}	6.9
MW76/76	80.9^{a}	70.3^{b}	58.6^{c}	4.4
MW32/32	82.0^{a}	78.6^{a}	81.5^{a}	25.5
Heterografts				
MW84/57	52.5^{b}	65.6^{a}	51.4^{b}	34.7
MW56/49	64.3^{b}	83.7^{a}	40.5^{c}	14.0
MW32/28	71.0^{a}	49.0^{b}	40.1^{b}	20.3
MW80/82	90.8^{a}	87.0^{a}	77.9^{a}	15.9
MW7/10	57.5^{b}	95.0^{a}	83.7^{a}	10.7

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

Table 5.3 Total soluble phenol quantity (mg gallic acid equivalent per g of dry weight) in the bark of *Uapaca kirkiana* grafted trees measured above, below and at the unions

	Gallic acid equivalent of dry weight (mg g^{-1})			
Graft combinations	above the union	at the union	below the union	CV (%)
Homografts				
MW26/26	97.0[b]	159.4[a]	159.3[a]	13.0
MW12/12	79.7[b]	105.2[a]	81.5[b]	15.1
MW13/13	71.4[a]	71.0[a]	84.3[a]	37.0
MW76/76	110.0[a]	90.0[a]	109.9[a]	31.8
MW32/32	79.2[a]	91.2[a]	84.0[a]	26.2
Heterografts				
MW84/57	70.8[ab]	93.8[a]	64.3[b]	17.1
MW56/49	25.2[b]	65.9[a]	62.3[a]	38.1
MW32/28	62.7[b]	105.3[a]	90.4[ab]	15.0
MW80/82	59.9[b]	100.2[a]	90.8[a]	19.5
MW7/10	94.0[a]	93.1[a]	85.5[a]	21.4

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

Table 5.4 Total soluble phenol quantity (mg gallic acid equivalent per g of dry weight) at different positions of *Uapaca kirkiana* graft unions in the wood and bark. Data calculated with standard errors

Position of the graft union interface	Sample sections		Mean
	bark	wood	
Above the union	75.0	65.6	70.3 ± 4.6^{b}
At the union	97.5	73.3	85.4 ± 5.2^{a}
Below the union	91.2	59.1	75.2 ± 6.2^{ab}
Mean	87.9 ± 4.8^{a}	66.0 ± 3.1^{b}	
LSD (P≤0.05)	Part of the graft union		11.1*
	Sample section		9.1*
	Interaction		15.8^{ns}
CV (%)			22.7

* = significantly different, ns = not significantly different at P≤0.05

Table 5.5 Table of fluorescence colours and the associated phenol groups (Regnier & Macheix, 1996; Du Plooy, 2006)

Fluorescence colour	**Group of Phenols**
Strong blue	Ferulic acid
White	Gallic acid/Caffeic acid
Blue-white	Coumaric acid
Light Blue	Hydroxycinnamic acid and Ferulic acid derivatives
Red	Flavonoids
Brown	Quinone
Deep blue / Purple	Not phenolic compounds – polymer, Terpenoids

Figures

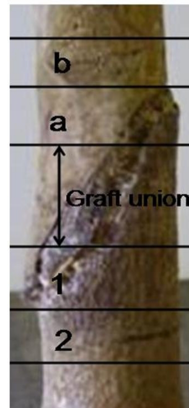

Figure 5.1 Stem segments dissected from a three-year old *Uapaca kirkiana* grafted tree (1 and 2 = segments towards the stock, a & b = segments towards the scion)

Figure 5.2 Diameters of scions, stocks and graft unions of *Uapaca kirkiana* trees (three years old after grafting) pooled together and measured approximately 5 mm above and below the union area

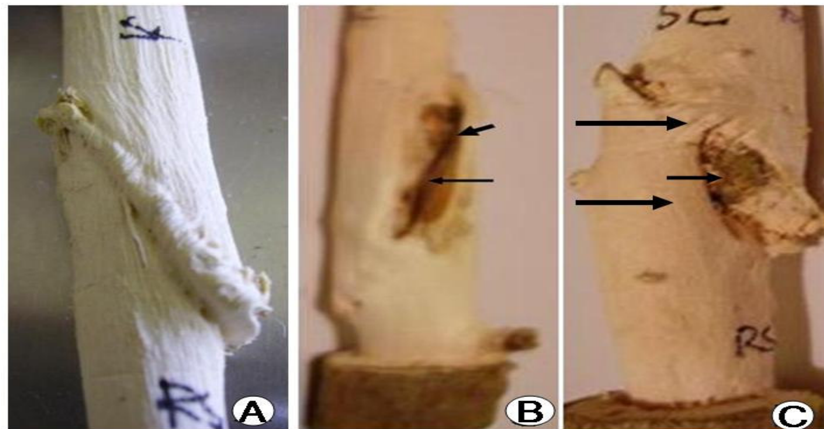

Figure 5.3 Morphological growth structure of *Uapaca kirkiana* wood at the union (A) MW80/82 showing wart-like projections at the union; (B) lacuna or holes (thin arrows) at the wood pith; (C) a swollen graft union (thick arrows) with some projections (Rs = rootstock and Sc = scion)

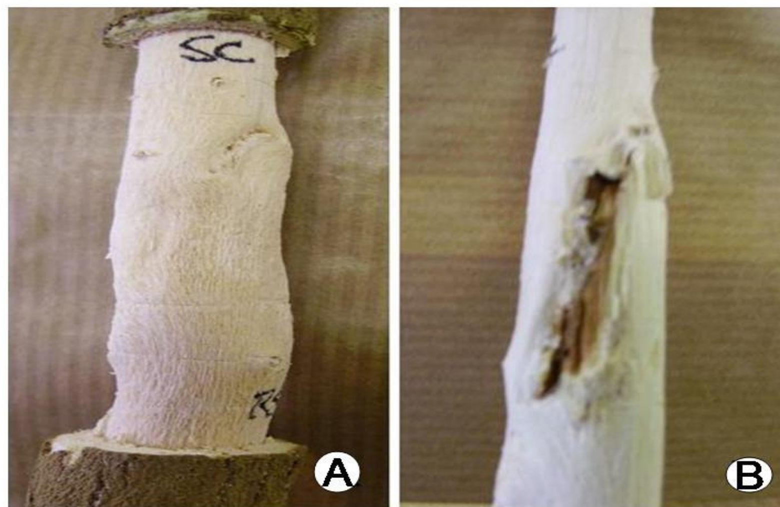

Figure 5.4 Morphology of MW32 *Uapaca kirkiana* scions on two different stocks (A) MW32/32 clonal homograft without wood discoloration or holes; (B) MW32/28 heterograft with lacuna in wood

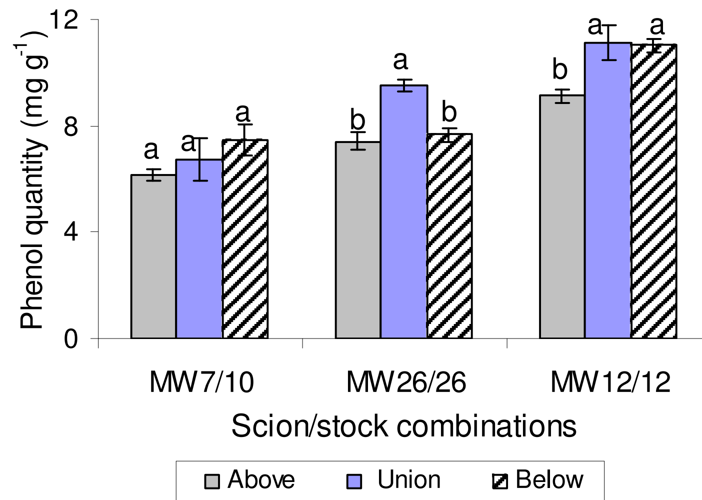

Figure 5.5 Cell wall bound phenol quantity (mg gallic acid equivalent per g of dry weight, DW) in the bark measured above, below and at the union (bars with the same letters within a column are not significantly different at P≤0.05)

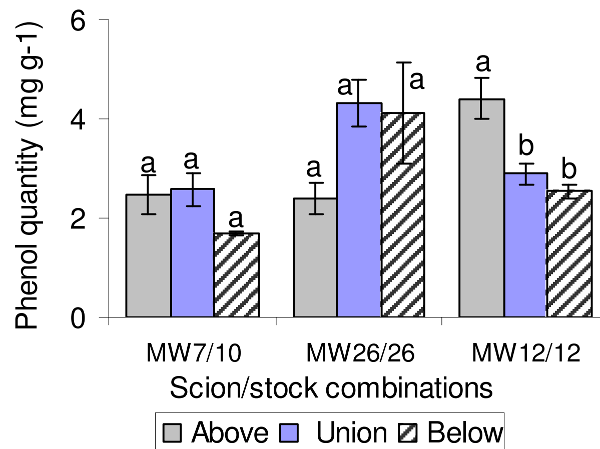

Figure 5.6 Cell wall bound phenol quantity (mg gallic acid equivalent per g of dry weight, DW) in the wood measured above, below and at the union (bars with the same letters within a column are not significantly different at P≤0.05)

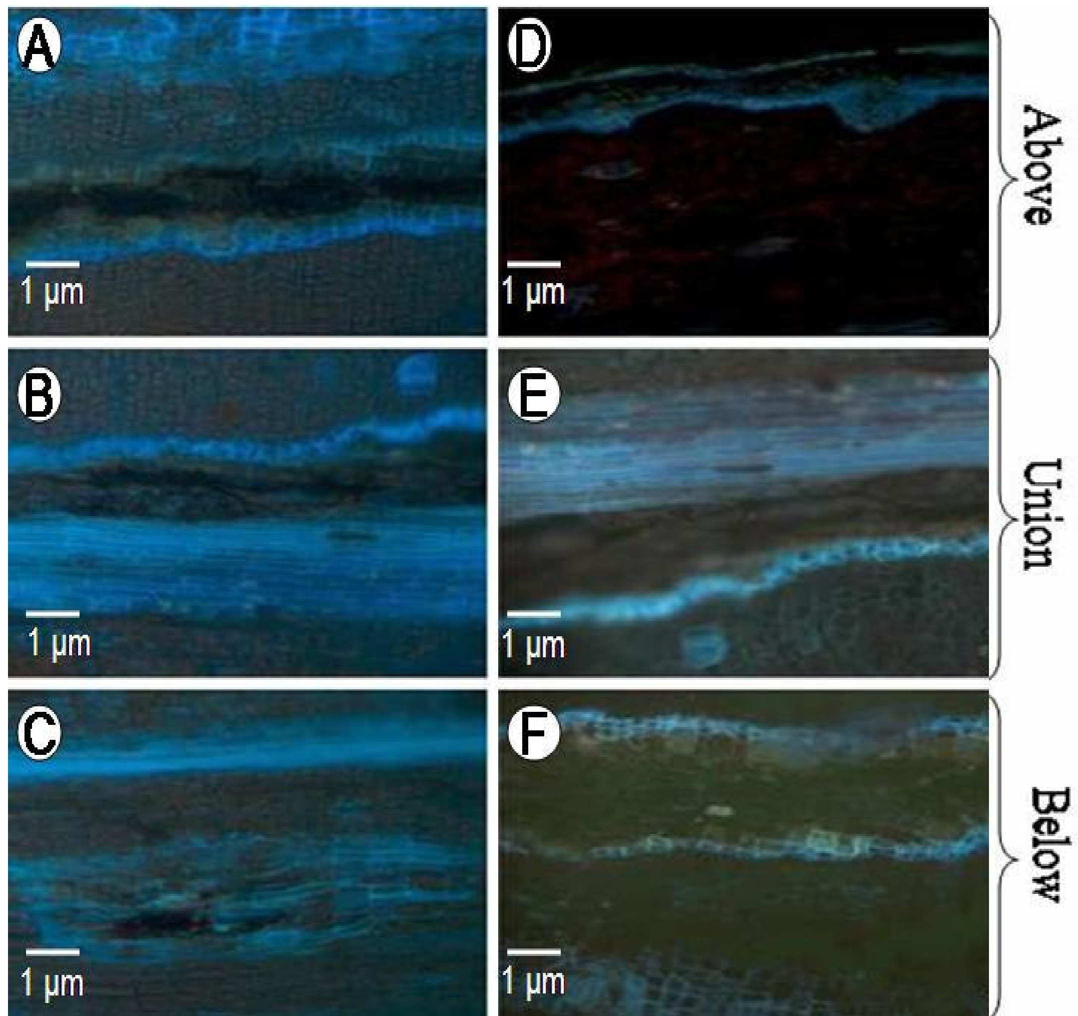

Figure 5.7 MW32/28 *Uapaca kirkiana* sections (above, below and at the union) viewed under a fluorescence microscope using (ABC) UV light and (DEF) Vanillin-HCl and white light

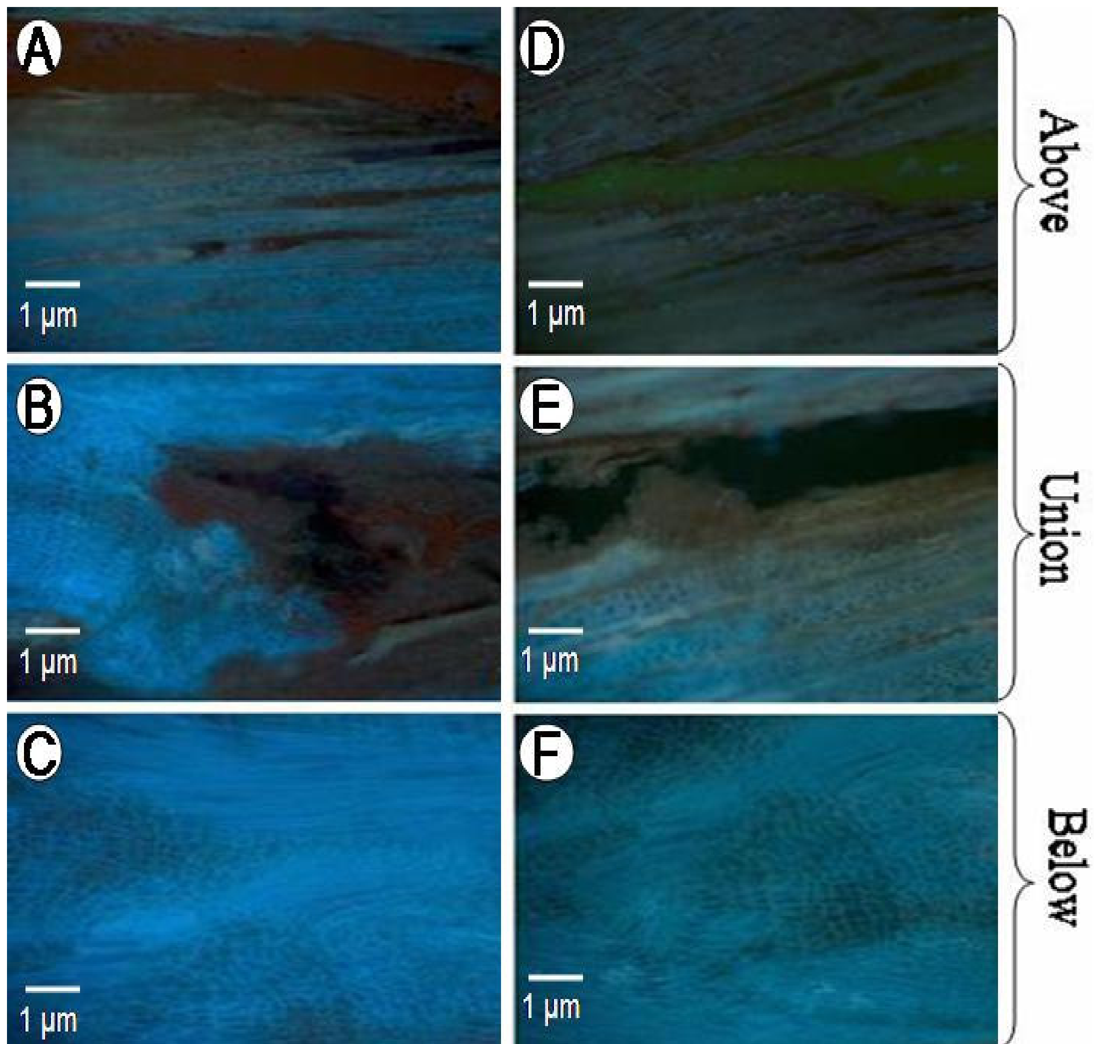

Figure 5.8 MW7/10 *Uapaca kirkiana* sections (above, below and at the union) viewed under fluorescence microscope using (ABC) UV light and (DEF) Vanillin-HCl and white light

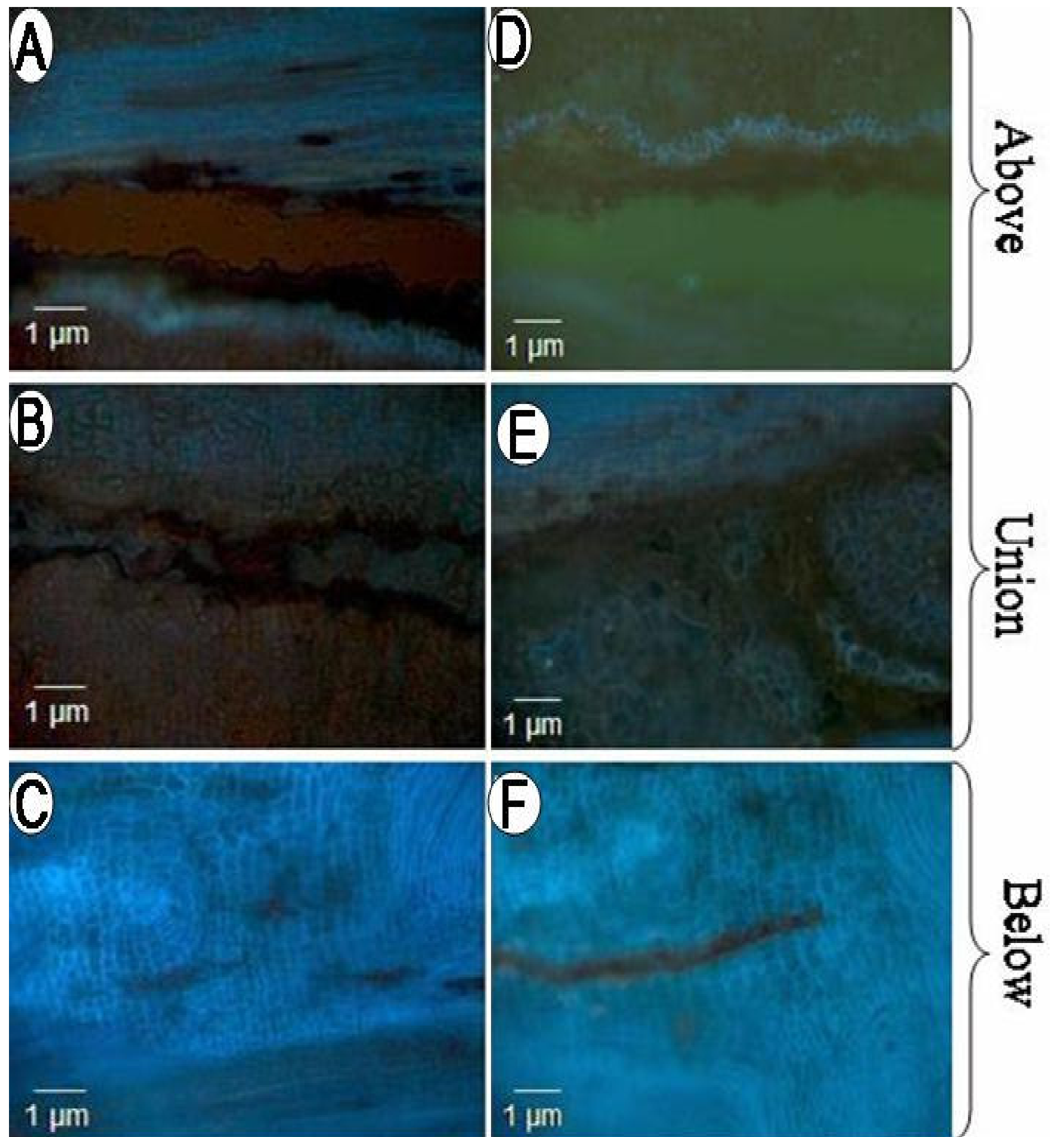

Figure 5.9 MW26/26 *Uapaca kirkiana* sections (above, below and at the graft union) viewed under a fluorescence microscope using (ABC) UV light and (DEF) Vanillin-HCl and white light

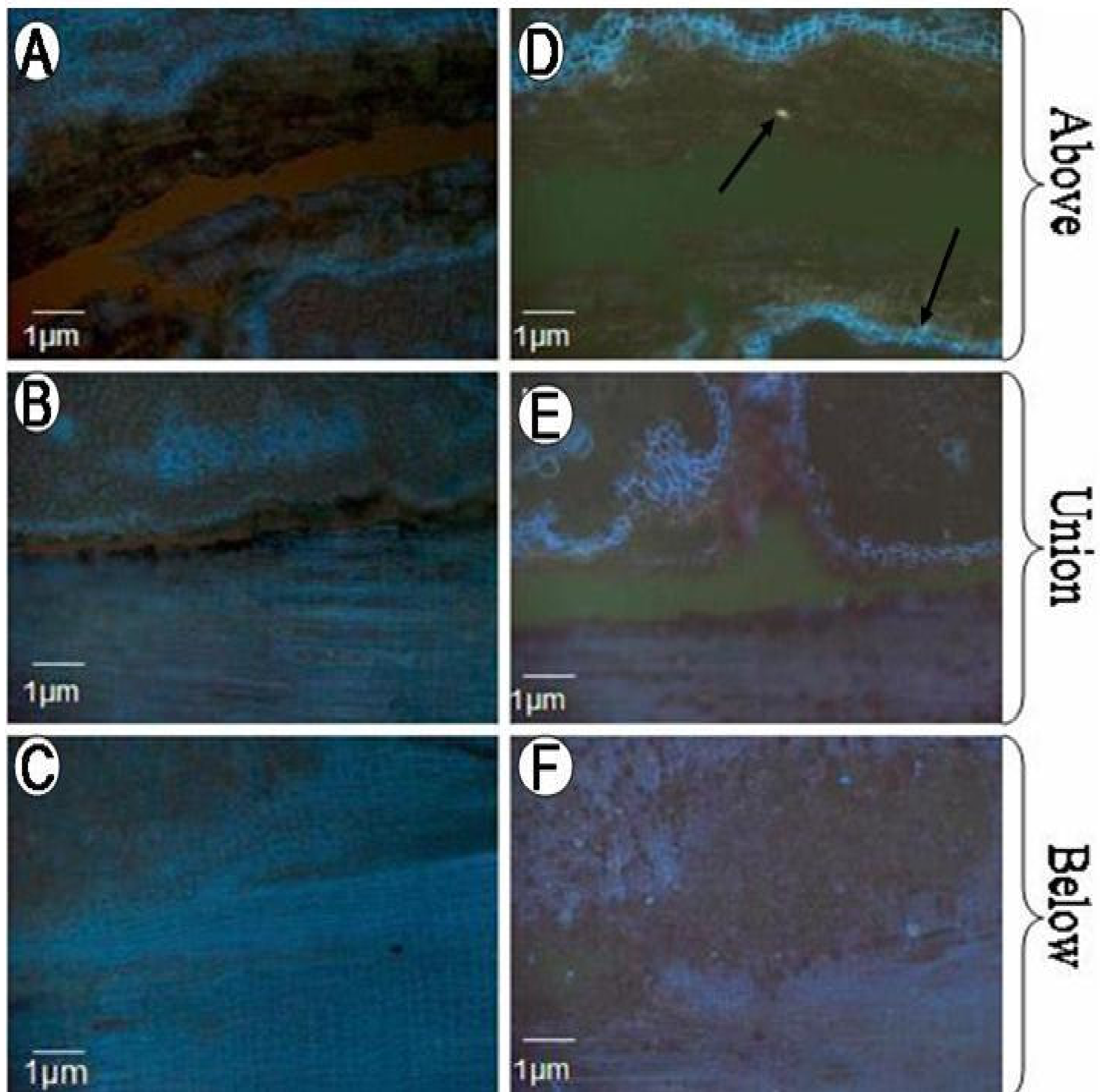

Figure 5.10 MW12/12 *Uapaca kirkiana* sections (above the union, at the union and below the union) viewed under fluorescence microscope using (ABC) UV light and (DEF) Vanillin-HCl and white light (arrows show isolated white spots)

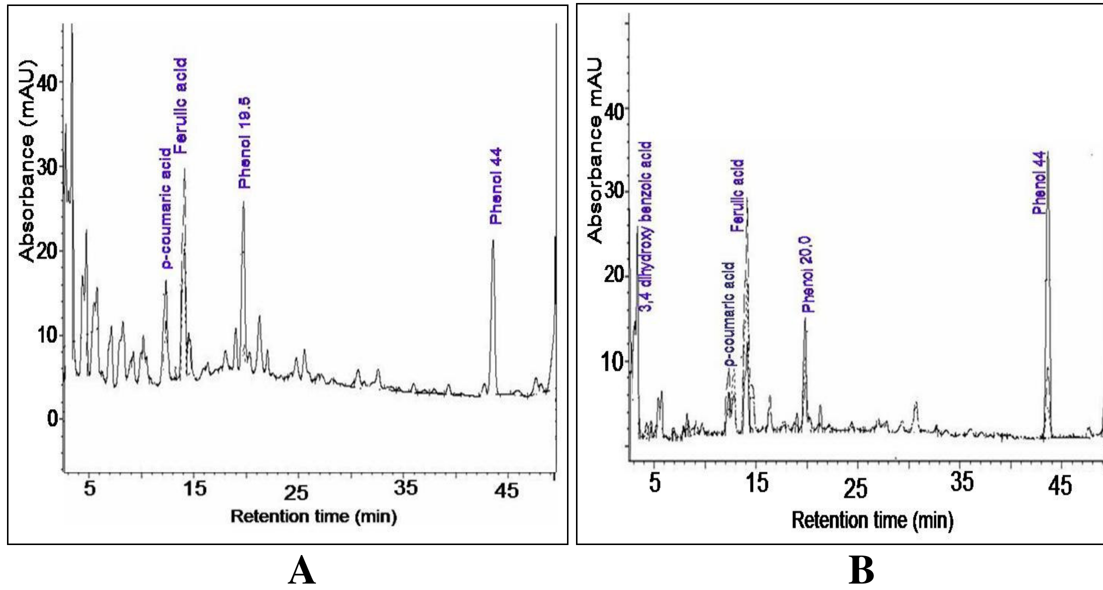

Figure 5.11 Chromatograms of cell wall bound phenols extracted from MW12/12 *Uapaca kirkiana* in the bark (A) above the union and (B) below the graft union

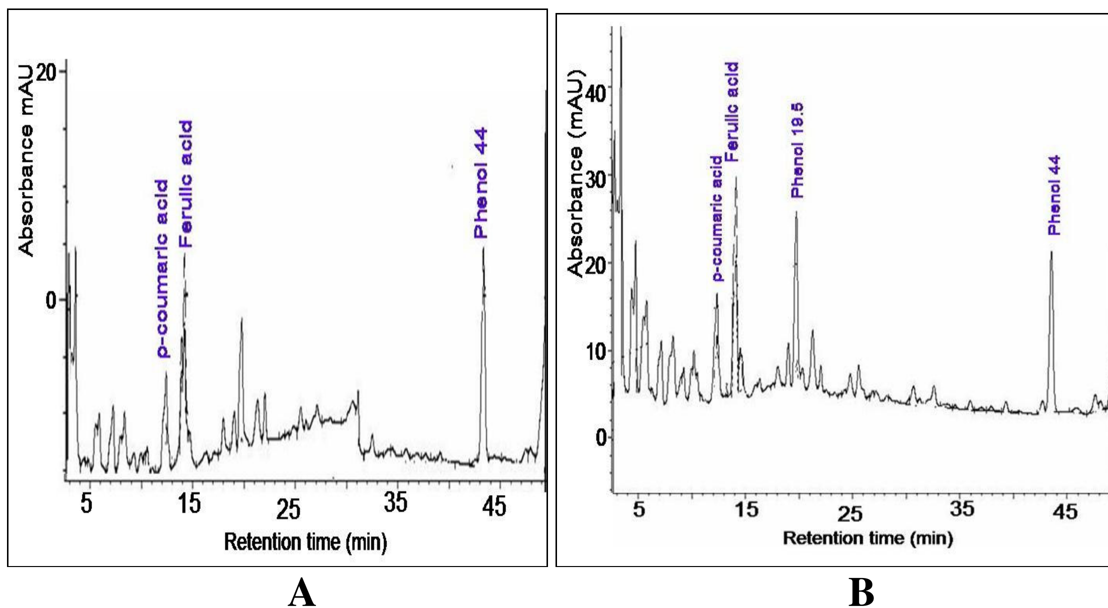

Figure 5.12 Chromatograms of cell wall bound phenols extracted from the bark of MW26/26 *Uapaca kirkiana* combination (A) above the union and (B) below the graft union

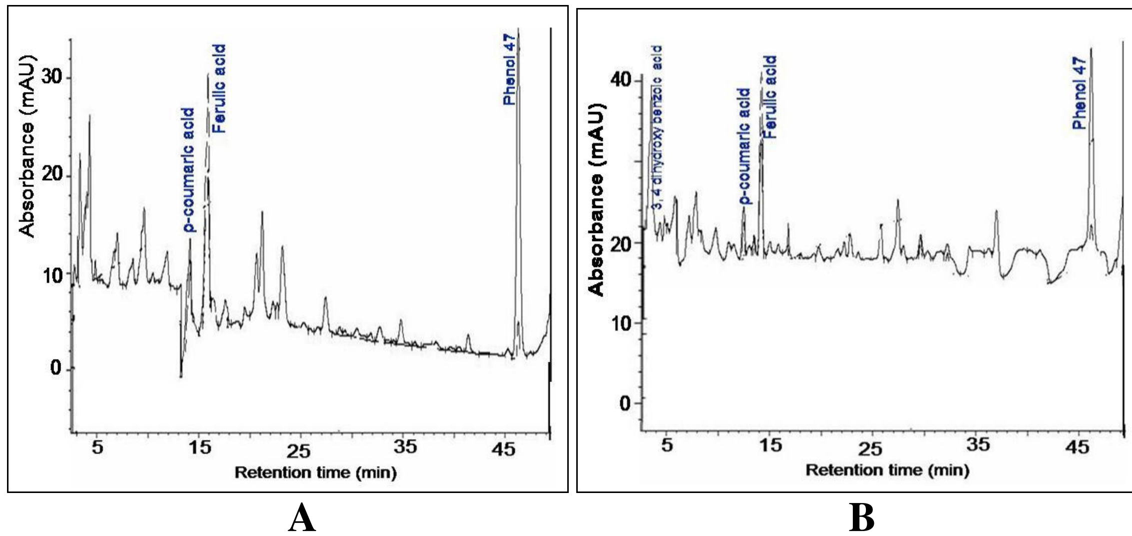

Figure 5.13 Chromatograms of cell wall bound phenols extracted from the bark of MW7/10 *Uapaca kirkiana* combination (A) above the union and (B) below the graft union

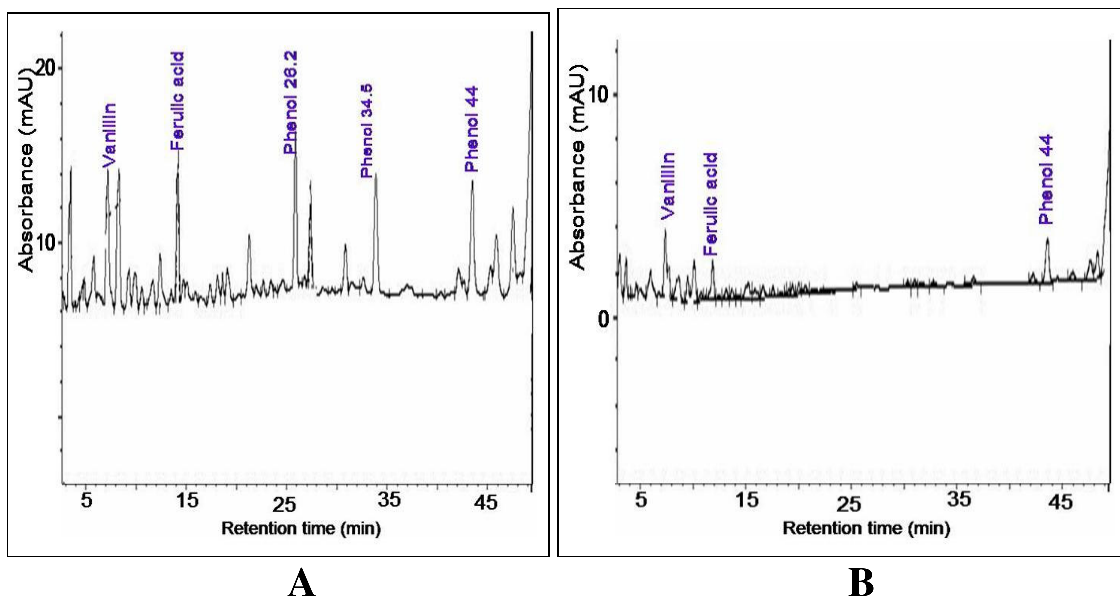

Figure 5.14 Chromatograms of cell wall bound phenols extracted from MW12/12 *Uapaca kirkiana* in the wood (A) above the union and (B) below the graft union

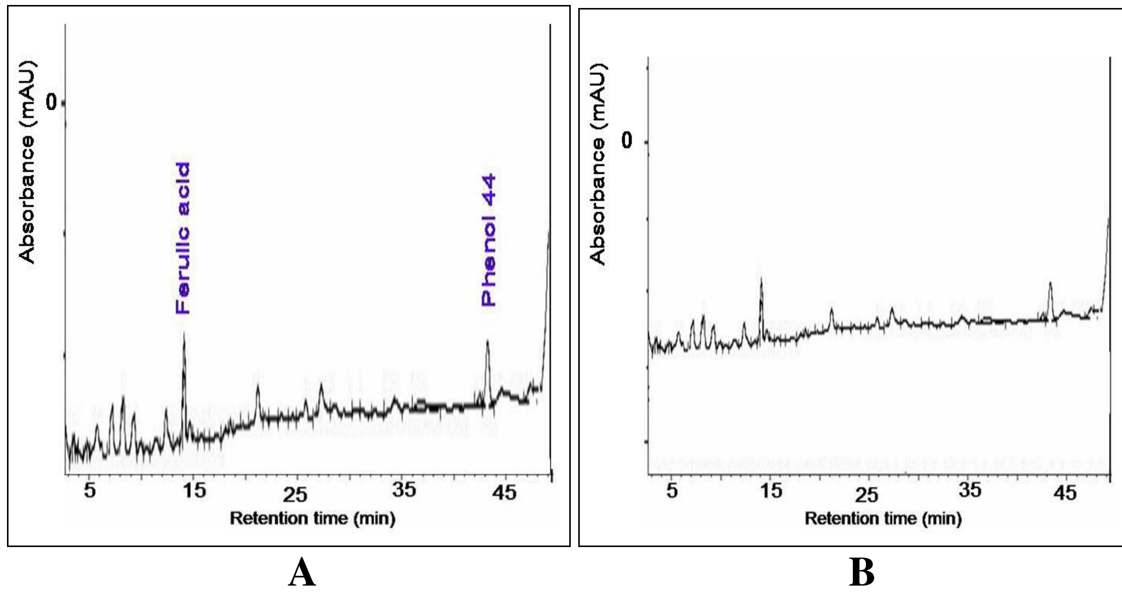

Figure 5.15 Chromatograms of cell wall bound phenol extracts from the wood of MW26/26 *Uapaca kirkiana* combination (A) above the union and (B) below the graft union

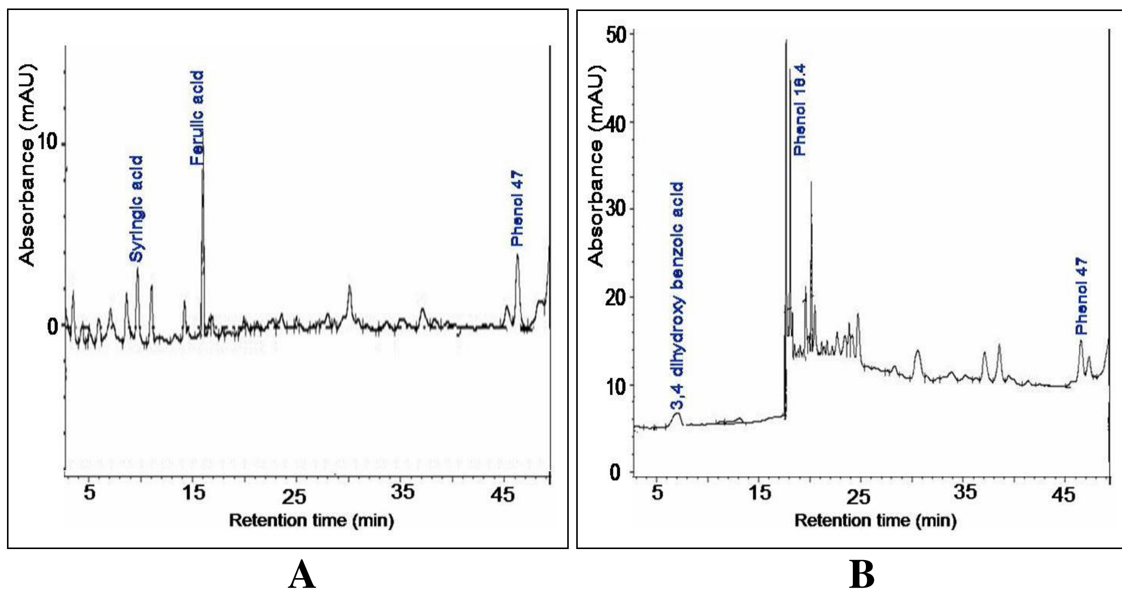

Figure 5.16 Chromatograms of cell wall bound phenol extracts from the wood of MW7/10 *Uapaca kirkiana* combination (A) above the graft union and (B) below the union

CHAPTER 6

PRE-TREATMENT METHODS FOR IMPROVING JACKET PLUM (*PAPPEA CAPENSIS*) SEED GERMINATION

6.1 Abstract

A trial was carried out with the objective of determining effective methods to improve *Pappea capensis* seed germination and culture asepsis. Seed germination tests were carried out using different growth chambers with 12 °C and 24 hours of darkness (D), 17 °C and 24 hours of light (L), 20 °C and D, 25 °C and D, 25 °C and L, or 32 °C and L. Seed germination was assessed using water floatation (sinking or floating seeds). They were primarily scarified in 98% H_2SO_4 and seed decontamination was assessed using 70% ethanol, 0.1% mercuric chloride ($HgCl_2$) or 1.75% NaOCl. The results indicated high seed germination (80%) with 25 °C and D followed by 20 °C and D (60%) treatments, but 12 °C and D, 17 °C and L, 25 °C and L, or 32 °C and L treatments inhibited seed germination (0%). Dark conditions with temperature ranges of 20 ^{o}C to 25 ^{o}C stimulated seed germination, but low temperatures (<20 ^{o}C) adversely affected seed germination. High seed germination (76%) was observed from submerged seeds. NaOCl decontamination method improved *in vitro* seed germination to 76%. $HgCl_2$ improved asepsis (89%), but with many abnormal seedlings.

6.2 Introduction

Jacket plum (*Pappea capensis* L.) tree species produce seeds which are rich in edible oil (74%) and this seed oil is used for making soap (Palmer & Pitman, 1972; Swart, 1991). They are alternative sources of bio-diesel fuel, and hence scientific research trials have been initiated at the University of Pretoria. *P. capensis* tree species are mainly propagated by seeds, but seedling growth is extremely slow (Anonymous, 1997; Venter & Venter, 1996; Van Wyk & Gericke, 2000). Seed morphology shows a relatively tough seed coat (testa) which is hard to crack or break by hand. According to Venter & Venter (1996), *P. capensis* seeds do not require any pre-treatment when sown soon after collection from trees. However, there have been no scientific reports on the percentage germination of fresh or stored seeds. Moreover, there have been no reports on identification of any seed treatment that would lead to an improvement in seedling growth.

P. capensis trees are widely distributed in the tropical ecological areas (Venter & Venter, 1996). According to Venter & Venter (1996), tree fruiting occurs from December to July period, while Fivaz & Robbertse (1993) reported April - May as a fruit development period. Differences in fruiting time or duration can be attributed to the prevailing environmental conditions within a particular ecological area. *P. capensis* tree species can be either deciduous or evergreen depending upon the prevailing environmental conditions (Palmer & Pitman, 1972; Venter & Venter, 1996). Furthermore, it is believed that late rains promote *P. capensis* seed germination. However, we observed no seed germination or seedling growth under the trees or in proximity during the dry season (August – October).

Many planting materials derived from *in vitro* culture of tree species have exhibited rapid growth (George, 1993), but *in vitro* propagation for mass multiplication of planting materials requires aseptic explants. Therefore, there is a need to select a pre-treatment that effectively decontaminates the seeds before germination. Moreover, the seed coats might harbour *in vitro* contaminants.

Many environmental factors such as temperature, light and water have a major influence on seed germination (Abdul-Baki & Anderson, 1972). According to Naidu, Rajendrudu & Swamy (1999), temperature has a strong influence on breaking seed dormancy in some plant species. For example, incubating *Prosopis juliflora* seeds at 60 ^{o}C for 12 – 24 hours improved seed germination and 30 - 100 ^{o}C temperature regimes improved *Sapindus trifoliatus* (soapnut) seed germination (Naidu *et al*., 1999). *S. trifoliatus* plants belong to the family Sapindaceae. Furthermore, it is known that high temperature treatments improve water imbibition and gaseous exchange rates. Generally, manipulation of such factors might provide an optimal condition for seed germination. However, a propagation method to enhance rapid *P. capensis* seedling growth has not been established. Therefore, the objective of the study was to determine effective pre-treatment methods that improve *P. capensis* seed germination and culture asepsis.

6.3 Materials and methods

6.3.1 Site description

P. capensis seeds were collected from Pretoria National Botanical Gardens in August 2005. This site lies at an altitude 1360 m above sea level, latitude 25° 44’S, longitude 28° 16’E

and an annual rainfall of 750 mm (Botha, Willis & Winter, 2000). Matured seeds were collected from the ground under the trees, but these seeds could be lying on the ground for more than a month after dropping from *P. capensis* trees. They were kept in paper bags and taken to the laboratory for different seed germination experiments.

6.3.2 Effects of temperature and light on seed germination

P. capensis seeds were stored at room temperature in paper bags for four weeks. They were soaked in a warm-bath (28 °C for 12 h) in order to weaken the seed coat, and hence improving water permeability. The seeds were then disinfected in 3.5% sodium hypochlorite (NaOCl, 8 min) and rinsed three consecutive times in sterile water. Ten seeds were placed on moist two layer-filter papers (Whatman No. 1) in each Petri dish (9-cm diameter), sealed and placed in transparent plastic bags. They were then exposed to different temperature and light regimes (treatments) in growth chambers maintained at (i) 12 °C with 24 hours of darkness (D), (ii) 17 °C with 24 hours of light (L), (iii) 20 °C with D, (iv) 25 °C with D, (v) 25 °C with L or (vi) 32 °C with L. There were six Petri dishes per treatment and the experimental design was a complete randomized block with six treatments and three replicates.

6.3.3 Germination of floating and submerged seeds

The reason for this experiment was to determine methods to separate viable from non-viable seeds. It was suspected that some seeds could have lost their viability due to a long storage period and perhaps the condition of storage. *P. capensis* seeds were stored in paper bags at room temperature for 12 or 16 weeks after collection. They were then placed in a

basin of water and floating seeds were separated from submerged seeds. Treatments were (i) sank seeds (ii) floating seeds or (iii) a mixture of sank and floating seeds. The seeds were sown in seedling trays filled with a mixture of fine sand and coconut bark growing medium and placed on a mist bed. Water (mists) was supplied for eight seconds every four minutes. The conditions in the mist propagation chamber were maintained at 70- 90% relative humidity and 23 ^{o}C to 26 ^{o}C temperature. There were 200 seeds per treatment and the experiment was laid out in a complete randomised block design with four replicates.

6.3.4 Seed decontamination

The reason for carrying out this experiment was due to high *in vitro* contamination (>50%) observed when seeds were only decontaminated in 98% H_2SO_4 (3 min). This experiment was laid out in a completely randomised design with three replicates. Seed coats were easily removed when the seeds were scarified in 98% H_2SO_4. Seed decontamination treatments were (i) 70% ethanol (40 sec), (ii) 0.1% w/v $HgCl_2$ (8 min) or (iii) 1.75% NaOCl (10 min). These seeds were rinsed in sterile water for four consecutive times and germinated on 10 ml aliquot of hormone-free half strength MS media (see section 6.3.4).

6.3.5 Incubation condition

Refer to section 2.3.8 for incubation conditions.

6.3.7 Statistical analysis

Data on seed germination and culture asepsis were expressed in percentages and then transformed where necessary (Steel & Torrie, 1980). Mean germination time (MGT) was calculated using the formula: $MGT = \sum(\eta \times d)/N$, where η is the number of seeds germinated

each day, d is the number of days from the beginning of the test and N is the total number of seeds germinated at the end of the experiment. The data were subjected to analysis of variance (ANOVA) using GenStat 4.24DE (Rothamsted Experimental Station) and mean separation was done using LSD test.

6.4. Results and discussion

6.4.1 Effects of temperature and light on seed germination

Significant differences ($P \leq 0.05$) in percentage germination of *P. capensis* seeds were obtained (Figure 6.1). An effective seed pre-treatment was 25 °C with dark (D) (80%) followed by 20 °C with D (60%). Seed germination was inhibited (0%) at 12 °C with D, 17 °C with L, 25 °C with L and 32 °C with L. *P. capensis* seeds that failed to germinate were still intact (there were no indications of rotten or damaged seeds) (Figure 6.2A). These results show that low temperatures (≤17 °C) and light inhibited seed germination. Observations showed that 25 °C with D treatment resulted in more vigorous seedling growth than 20 °C with D treatment (Figure 6.2B-C).

No significant differences were obtained in root and shoot lengths between 20 °C with D and 25 °C with D treatments. Roots were longer (9.4 cm) than shoots (2.6 cm). 20 °C with D treatment resulted in seedlings with thin roots and shoots (Figure 6.2). These results demonstrate that 25 °C with D was an optimal condition for *P. capensis* seed germination.

The calculated mean germination time (MGT) shows that early germination (16.3 days) was at 20 ºC with D treatment and late seed germination was at 25 ºC with D (19.3 days) treatment. Apart from this rapid seed germination obtained at 20 ºC with D treatment, the final seed germination at 25 ºC with D treatment was high (80%).

Generally, seed germination responses to temperature and light vary with species. Pandey & Palni (2005) reported that dark conditions inhibited *Parthenium hysterophorus* seed germination (8%) while 12 h of light and dark conditions improved germination (56%). In this trial, dark conditions promoted *P. capensis* seed germination. This could be genetically controlled or an ecological adaptation to avoid exposure to dehydration above ground which may limit natural multiplication of *P. capensis* trees.

6.4.2 Germination of floating and submerged seeds

Significant differences ($P \leq 0.05$) in percentage seed germination were obtained amongst floatation treatments (Figure 6.3). Submerged seeds sown 12 weeks (November) after collection resulted in 65% germination. Light or floating seeds resulted in 9% germination while the mixture resulted in 37% germination. There was 20% germination for submerged seeds sown 16 weeks (December) after collection, but none of the floating seeds germinated (Figure 6.3). Low germination (3%) was obtained from a mixture of floating and submerged seeds. The findings from 16 weeks stored seeds could be attributed to deteriorating embryos due to storage or that stored seeds entered some form of dormancy. Seed germination occurred once this speculated dormancy was broken, but this needs further research to confirm the speculated embryo deterioration or dormancy phenomenon.

Piña-Rodrigues & Figliolia (2005) reported 69% germination for *Virola surinamensis* seeds that sank and 85% germination for those that floated. This was attributed to ecological adaptation in that these trees were found in flood prone areas. Therefore, light-weight seeds enabled these species to survive the floods while heavy seeds were buried deep and hence failed to emerge. Consequently, this reduced germination chances of *V. surinamensis* seeds. In the case of *P. capensis*, the opposite is true due to the fact that these tree species are found on a high ground without floods.

Limitation in soil moisture as the dry season progresses does not seem to support *P. capensis* seedling growth. Many seeds falling onto the ground might be dehydrated and embryos of such seeds might be deteriorated if collected late in the dry season. Therefore, water floatation method is important to separate deteriorated *P. capensis* seeds (floating) from viable seeds (submerged). This method is cheap, easy and can be used to improve germination of stored seeds and can save labour and ensure seed germination uniformity.

6.4.3 Seed decontamination

H_2SO_4 (98%) was not effective as a primary seed decontaminant. Its use is still justified because it was easy to remove the seed coats. Figure 6.4 shows effects of ethanol, $HgCl_2$ and NaOCl on *P. capensis* percentage seed decontamination and germination. There were significant differences ($P \leq 0.05$) between treatments with respect to asepsis and germination. Seed germination was significantly higher for NaOCl (76%) than either the $HgCl_2$ (23%) or ethanol (60%) treatment. Clear MS media were observed for seeds decontaminated either in NaOCl or ethanol and seedlings were normal (Figure 6.5A). In contrast, $HgCl_2$ was effective in seed decontamination (87%), but resulted in low

percentage seed germination and stunted seedlings. MS medium turned brown indicating presence of $HgCl_2$ residues which were detrimental to seed germination and growth. There was no clear distinction between roots (radicle) and shoots (plumule) (Figure 6.5B).

$HgCl_2$ exposure time and concentration can be optimised, but $HgCl_2$ is toxic to aquatic life, especially fish (Crompton, 1997). Its use is not justified if other less toxic sterilants can decontaminate explants. Efficacy of a sterilant depends on contact with the contaminated area, size and choice of explants (Hammerschlag, 1986). A sterilant can be ineffective if it does not reach the contaminated area. In this trial, seed testa removal facilitated good contact between the seed and sterilant. Consequently, this improved culture asepsis, but loss of a single cotyledon (seed leaf) delayed or caused seed germination failure as shown with $HgCl_2$. Mild stirring of seeds was important to avoid breaking the union of the two cotyledons. Generally, *P. capensis* seed germination started six days after culturing on the MS media for all the treatments except where $HgCl_2$ was used as a sterilant.

6.5 Conclusion

Improved *P. capensis* seed germination was achieved at 25 ºC with 24 hours of darkness. Germination was high when seeds were sown soon after collection (fresh seeds). 1.75% NaOCl improved seed asepsis while 0.1% $HgCl_2$ was detrimental to seed germination. Submerged seeds exhibited high germination rate and seedling growth uniformity. Based on these results, *P. capensis* seeds should be scarified with 98% H_2SO_4 and sterilised with 1.75% NaOCl and incubated at 25 ^{o}C with 24 hours darkness in order to obtain high germination rates.

Table

Table 6.1 Effect of temperature (°C) and darkness (D) on mean root length (cm) and shoot length (cm) of jacket plum (*Pappea capensis*) seedlings

Treatments	seed length	seed width	root length	shoot length
20 °C and D	0.45	0.40	9.08	2.52
25 °C and D	0.40	0.40	9.72	2.70
Mean	0.43	0.40	9.40	2.61
Probability	ns	ns	ns	ns

ns = not significantly different at $P \leq 0.05$

Figures

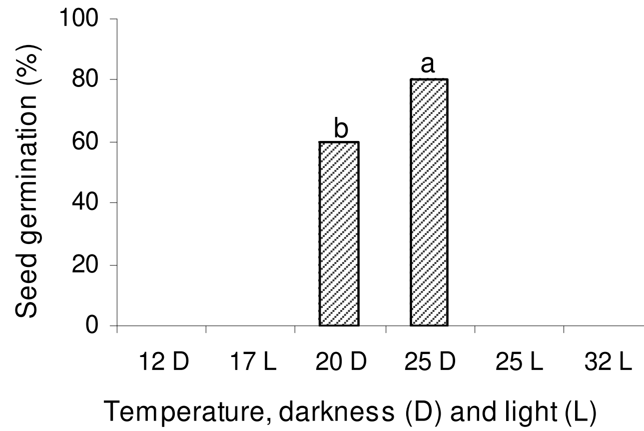

Figure 6.1 Percentage germination of jacket plum (*Pappea capensis*) seeds exposed to different temperatures (^{o}C), darkness (D) and light (L) four weeks after sowing. (Bars with different letters are significantly different at P≤0.05)

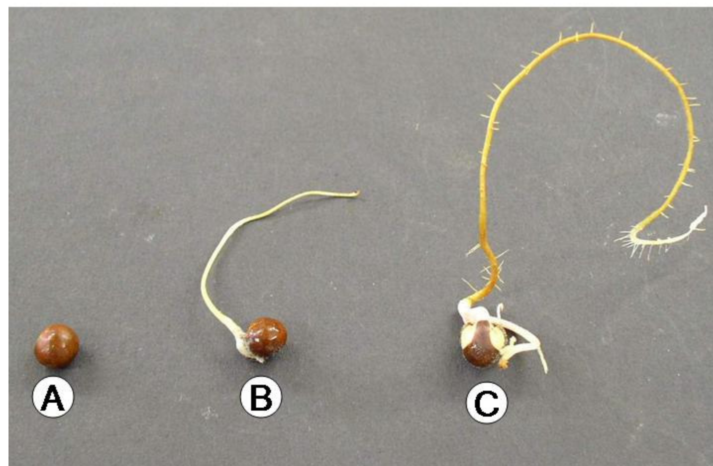

Figure 6.2 Jacket plum (*Pappea capensis*) seed/seedlings exposed to 24 hours of darkness and at (A) 12 ºC (B) 20 ºC and (C) 25 ºC temperatures (note that vigorous seedling growth was obtained at 25º C)

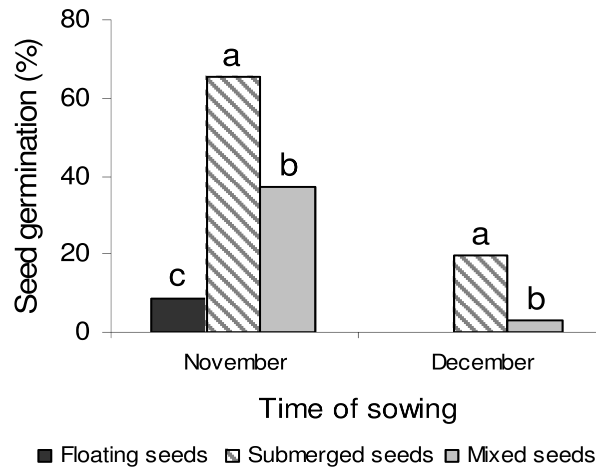

Figure 6.3 Jacket plum (*Pappea capensis*) seed germination (%) separated by water floatation method into floating, submerged and a mixture of submerged and floating seeds. Different letters within the same column indicate significant differences (P ≤0.05)

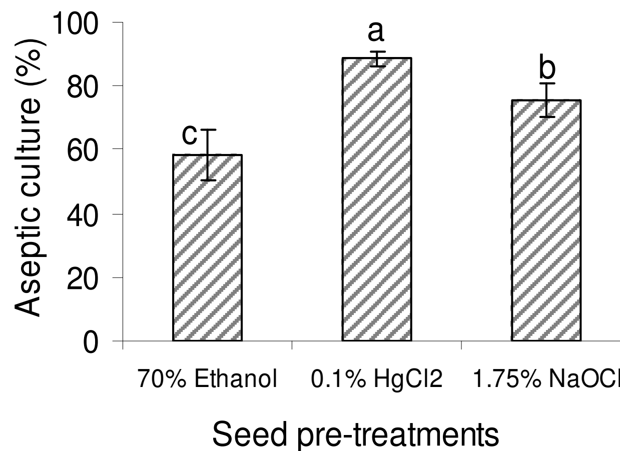

Figure 6.4 Percentage asepsis of jacket plum (*Pappea capensis*) seeds to 70% ethanol, 0.1% $HgCl_2$ or 1.75% NaOCl pre-treatments. Bars with the different letters are significantly different at P≤0.05

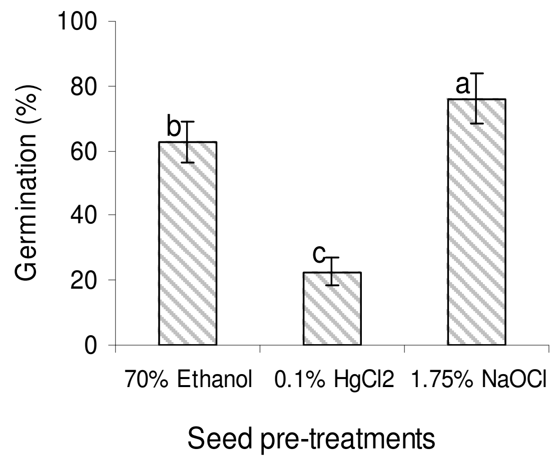

Figure 6.5 Percentage germination of jacket plum (*Pappea capensis*) seeds to 70% ethanol, 0.1% $HgCl_2$ or 1.75% NaOCl pre-treatments. Bars with the different letters are significantly different at $P \leq 0.05$

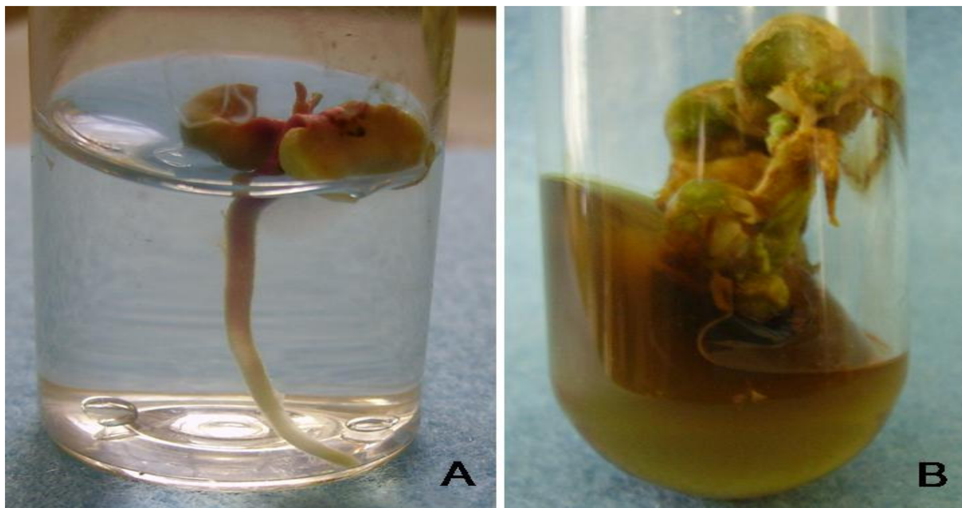

Figure 6.6 Jacket plum (*Pappea capensis*) seedlings in test tubes (A) normal seedling six days old; (B) abnormal seedling four weeks old (note brown culture medium in test tube B)

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 ^{o}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

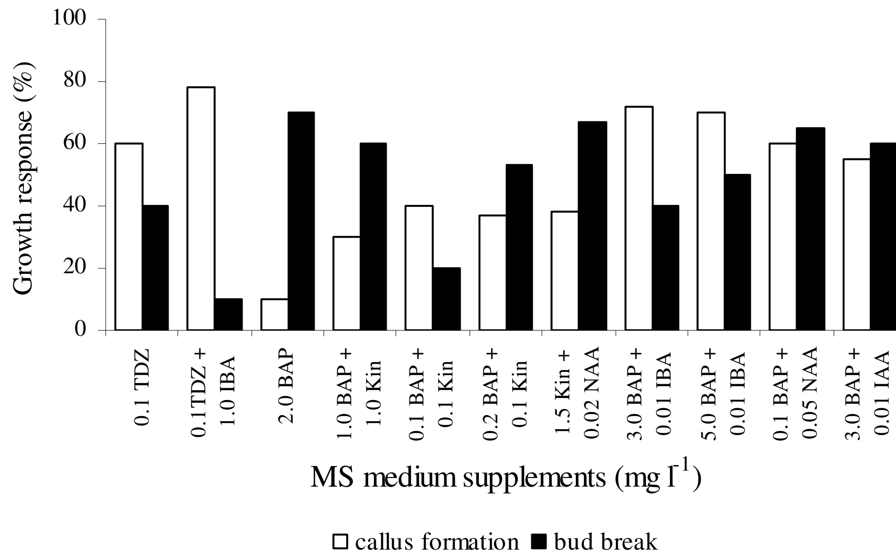

Figure 7.1 Callus formation and bud break (%) of jacket plum (*Pappea capensis*) micro-shoots explanted on Murashige and Skoog medium supplemented with different concentrations (mg l^{-1}) and combinations of benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), Kinetin (Kin),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

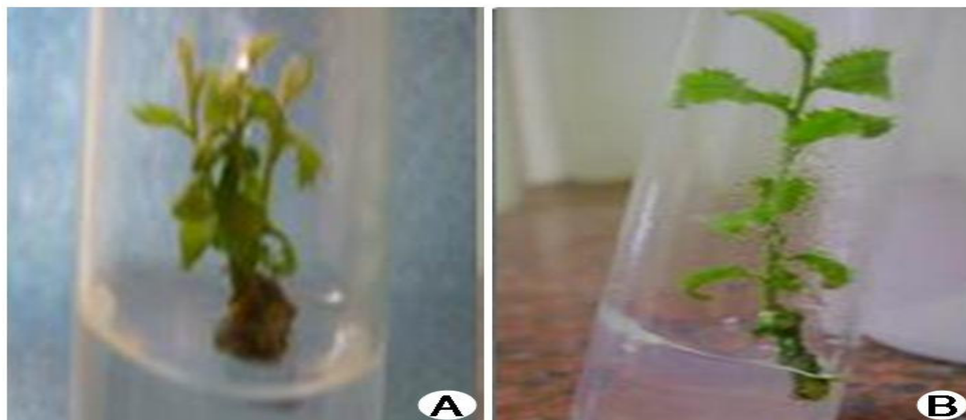

Figure 7.3 Jacket plum (*Pappea capensis*) shoot multiplication on Murashige and Skoog medium supplemented with 2.0 mg l^{-1} benzylaminopurine (BAP) after two weeks: (A) three healthy micro-cuttings; (B) single shoot produced after subculturing the ortet explant

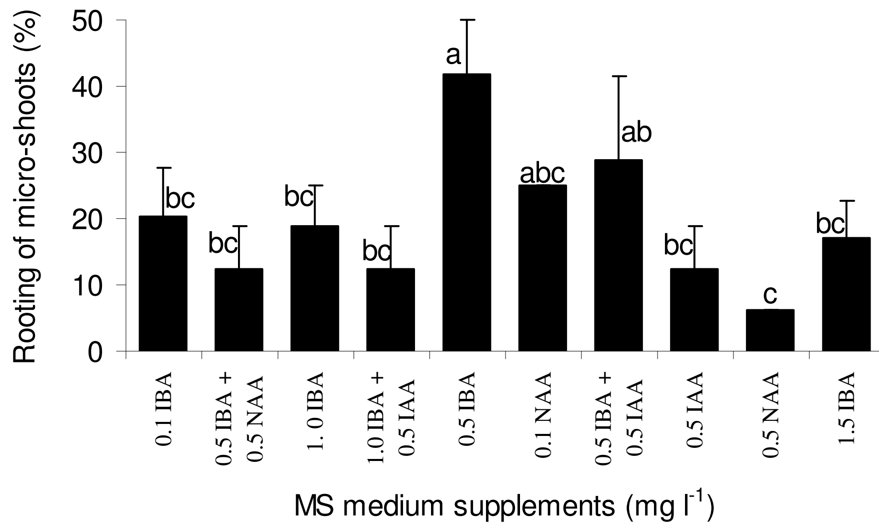

Figure 7.4 Rooting percentage of jacket plum (*Pappea capensis*) on half strength Murashige and Skoog medium supplemented with different concentrations (mg l^{-1}) and combination of indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) and indole-acetic acid (IAA) three weeks after explanting

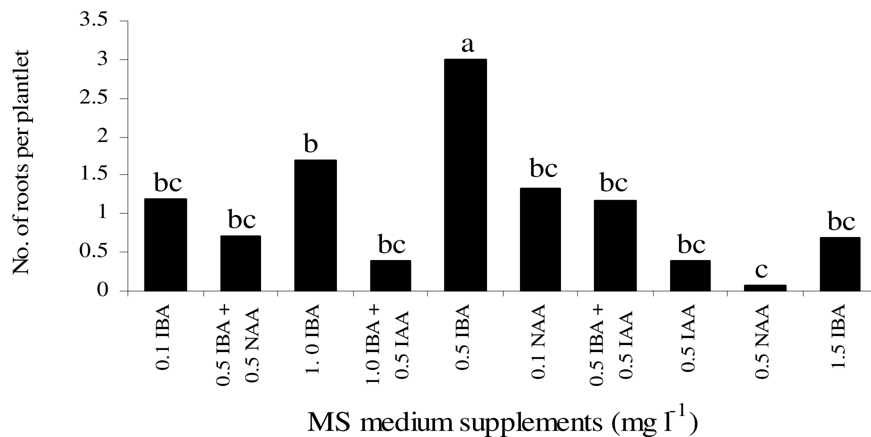

Figure 7.5 Mean number of roots regenerated on jacket plum (*Pappea capensis*) micro-cuttings rooted on different concentrations (mg l^{-1}) and combinations of indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) and indole-acetic acid (IAA)

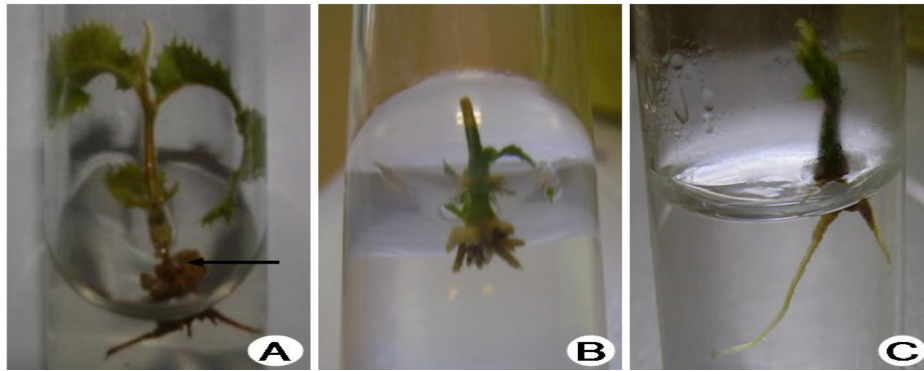

Figure 7.6 Rooted jacket plum (*Pappea capensis*) micro-cuttings on half strength Murashige and Skoog medium supplemented with either (A) 0.5 mg l^{-1} indole-3-butyric acid (IBA) and 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA) after three weeks or (B) 0.5 mg l^{-1} IBA or (C) 0.5 mg l^{-1} IBA and 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)

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°).

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° 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° - 40°). 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 $P \leq 0.05$

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

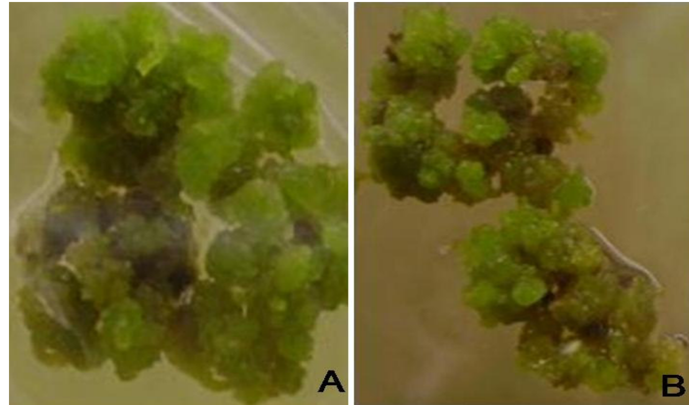

Figure 8.1 Jacket plum (*Pappea capensis*) calli induced on three quarter strength Murashige and Skoog medium with 0.05 mg l^{-1} thidiazuron and 0.3 mg l^{-1} casein hydrolysate: (A) chlorophyllous and embryogenic calli after three weeks; (B) calli after four weeks

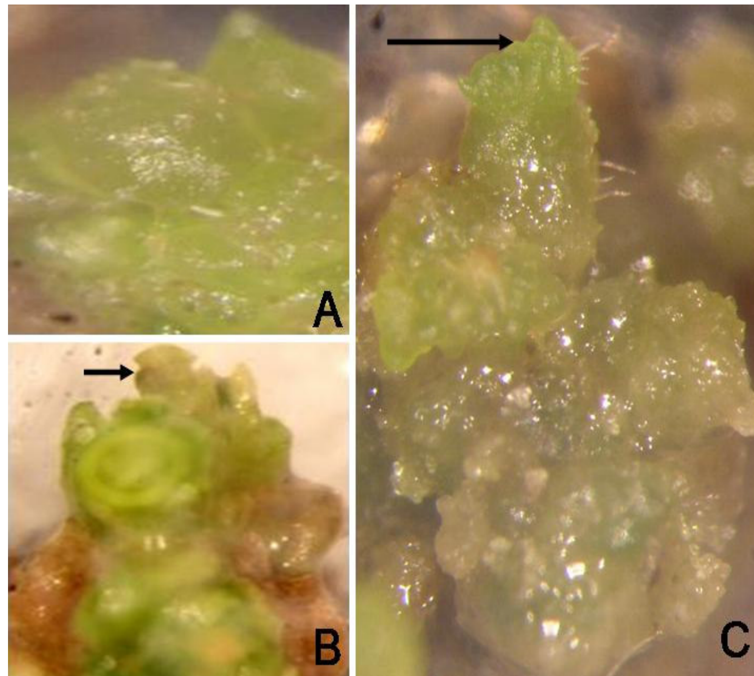

Figure 8.2 Jacket plum (*Pappea capensis*) somatic embryos on three quarter strength Murashige and Skoog medium with 0.05 mg l^{-1} thidiazuron and 0.3 mg l^{-1} casein hydrolysate: (A) early globular stage after 4 weeks; (B) open cotyledonary somatic embryo; (C) closed-up polycotyledonary somatic embryo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

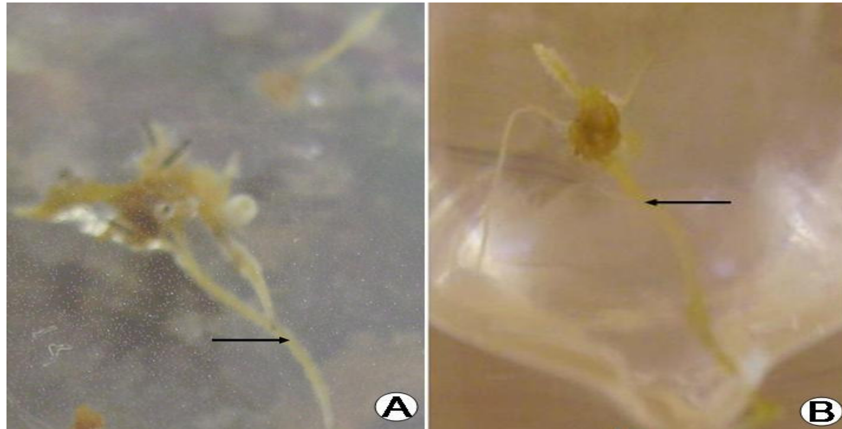

Figure 8.3 Jacket plum (*Pappea capensis*) embryos regenerated through indirect embryogenesis (A) cluster of somatic embryos on Murashige and Skoog medium with 0.05 mg l^{-1} thidiazuron and 0.3 mg l^{-1} casein hydrolysate (CH); (B) embryo germination on 0.2 mg l^{-1} benzylaminopurine and 0.3 mg l^{-1} 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

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

9.1 Propagation of woody perennial trees

Different propagation techniques for *U. kirkiana* and *P. capensis* tree species have been the main focus of the thesis. This is because mass multiplication, maintenance and conservation of superior provenances of these plant species depend on reliable propagation methods. Domesticating or managing these trees successfully in their natural habitats hinges on better propagation methods. Generally, many wild fruit trees are sexually propagated (i.e., from seeds), but a few have been successfully propagated by vegetative methods.

Results of this study (Chapter 2) demonstrate that mature *U. kirkiana* tree species are amenable to micro-propagation. The objective of the study was achieved, and hence, precocious fruiting can be achieved using the developed micro-propagation protocol. However, there were still low multiplication rates compared to micro-propagation protocols developed for *P. capensis* tree species. Effect of seasonality on rooting capacity of *U. kirkiana* micro-cuttings needs further investigation to optimise the developed protocol. Furthermore, improvement in survival of plantlets when potted or planted needs to be evaluated.

Results presented in Chapter 2 show that proper stock plant manipulations, and the use of rejuvenated and pre-conditioned stock plants, were necessary for *in vitro* propagation of

mature *U. kirkiana* tree species. Manipulation of stock plants before collection of explants and use of an effective sterilant such as $HgCl_2$ can overcome *in vitro* contamination, rejuvenation problem of explants and phenol accumulation which are the major barriers to micro-propagation of *U. kirkiana* tree species. From this study, lateral shoots from pre-conditioned stock plants are ideal for *in vitro* propagation of *U. kirkiana* tree species.

Results in Chapters 3 to 6 demonstrate that phenol accumulation at the graft union could be implicated in graft incompatibility. However, phenol production is known to be seasonal, and hence timing of macro-grafting can minimise excessive exudates (phenol deposits) which have adverse effects on graft compatibility. Furthermore, quantification of phenol deposits in different seasons would improve our understanding on the adverse effects of these phenols on graft compatibility in different seasons.

The results (Chapters 3 - 6) also demonstrate that poor callus formation, presence of *p*-coumaric acids and cell necrosis were the causes of graft incompatibility in *U. kirkiana* scion and rootstock combinations. From this study, some breakthroughs have been achieved and Figure 9.1 shows a schematic diagram which elucidates problems and possible solutions for successful grafting of *U. kirkiana* trees as described in Chapters 5 - 6.

Plant macro-propagation: this includes rooting stem cuttings, air layering, budding and grafting. Some tree crops are easily propagated by macro-propagation while others are difficult. In this study, rooting of *P. capensis* stem cuttings (Chapter 7) was difficult even with the juvenile plant materials. Therefore, macro-propagation constraints for many woody plants include low multiplication rate and scion/rootstock graft incompatibility

(Chapter 3). There has been no information on the success of grafting, air layering and budding of *P. capensis* tree species, but a low rooting percentage (11%) was obtained with epicormic shoot cuttings.

Micro-propagation techniques increase the multiplication rates of many planting materials (George, 1993; Read & Preece, 2001). Many improved micro-propagation techniques for woody plants have been established. However, there are other important tree crops that are still recalcitrant to micro-propagation techniques (Read & Preece, 2001). Micro-propagation protocol developed for a specific tree crop does not often work for all tree crops, and hence there is no recipe for all tree crops. Each tree species is unique in its requirements for successful propagation and these must be established. *In vitro* propagation protocols for *U. kirkiana* (Chapter 2) and *P. capensis* (Chapters 7and 8) tree crops reported in this study show differences in the way they responded to different growth medium formulations. Mature stock plants (*U. kirkiana*) exhibited rejuvenation problem unlike juvenile stock plants (*P. capensis*). Therefore, an efficient propagation protocol must be developed for each tree crop.

Results from different chapters have been harnessed into a schematic diagram (Figure 9.2) to summarise *in vitro* propagation problems often encountered and the possible solutions for the successful field survival and establishment of *U. kirkiana* planting material. This schematic diagram is to guide potential *U. kirkiana* tree domesticators, nurserymen and growers on the best ways to propagate and manage *U. kirkiana* planting material from the nursery to the early stages of field establishment. This is with the ultimate goal of optimising orchard productivity.

The results obtained in Chapter 8 indicate successful somatic embryogenesis for *P. capensis*. This protocol can be used for genetic improvement either through deliberate introduction of genes with superior traits or desirable somaclonal variation. *U. kirkiana* explants were also amenable to high amount of callusing, and hence high risks of somaclonal variants. This could defeat the efforts involved in selecting superior provenances from the wild. Assessing somaclonal variations would help before mass multiplication of *U. kirkiana* plantlets through indirect somatic embryogenesis was adopted.

U. kirkiana tree species accumulate secondary metabolites and this makes *in vitro* propagation difficult. These metabolites are mainly produced in response to wounding during subculturing and grafting as a healing mechanism. These compounds might reduce graft compatibility as shown in grafted *U. kirkiana* trees (Chapters 4 - 5). Histological studies (Chapter 3), *in vitro* callus fusion technique (Chapter 4), and phenol analysis (Chapter 5) have been used to provide new information about graft compatibility in *U. kirkiana*. Collectively, such studies remain useful to screen out incompatible combinations. Further studies on phenol production associated with seasonality are needed to ascertain whether or not incompatibility can be minimised at different times of the year.

The study has shown that *P. capensis* tree crops can be successfully propagated using seeds (Chapter 6), organogenesis (Chapter 7) and somatic embryogenesis (Chapter 8). The results in Chapter 6 suggest that *P. capensis* seeds exhibit intermediate germination behaviour (i.e. they are neither true recalcitrant nor orthodox). Furthermore, the results suggest that a reasonable seed storage period (six months or more) is possible with cold storage. The

study has demonstrated that organogenesis and embryogenesis are reliable propagation methods for *P. capensis* species to achieve mass multiplication of planting material.

The study has shown that *in vitro* propagation of *P. capensis* was not as difficult as with *U. kirkiana* plants, since explants from the former tree were excised from juvenile stock plants. Shoot multiplication on Murashige and Skoog (Murashige & Skoog, 1962) medium supplemented with 2 mg l^{-1} BAP was an optimal medium formulation and root regeneration was optimal on MS with 0.5 mg l^{-1} IBA (Chapter 7). Frequent subculture of *P. capensis* micro-shoots and addition of adjuvants (casein hydrolysate) minimised shoot tip necrosis. For somatic embryogenesis, continuous exposure to thidiazuron with reduced concentrations at different stages was necessary for somatic embryo induction and maturation. Therefore, reproducible propagation methods were developed for *P. capensis* trees that enable mass multiplication of planting material. Figure 9.3 is an illustration of propagation methods described in this research study for *P. capensis* tree species. This schematic illustration is aimed at guiding potential *P. capensis* tree propagators.

Each plant is unique in its regeneration requirements, and hence better propagation protocols must be identified depending upon the objective, cost and economic value of the crop plant in question. Somatic embryogenesis is more efficient in increasing the number of plantlets regenerated from a single plant.

9.2 Suggestions for future investigations

Based on the results of the present study, some areas that need further studies have been identified. *In vitro U. kirkiana* root regeneration and growth of micro-cuttings (Chapter 2) were not satisfactory and this is suspected to be due to lack of sufficient rejuvenation of stock plants and explants. Therefore, experiments on different methods to rejuvenate stock plants would bring a better insight of *in vitro* growth capacity of *U. kirkiana* tree species. In addition, *in vitro* propagation of *U. kirkiana* using mature stock plants is challenging due to heavy contamination and failure to rejuvenate explants. Furthermore, old shoot explants from grafted trees did not respond to different MS medium supplements. This suggests that there was a rejuvenation problem and possible adverse effects of phenol deposits, but this warrants further studies.

The study has outlined a method to achieve culture asepsis from mature *U. kirkiana* trees (Chapter 2). With this effective decontamination method and rejuvenated lateral shoots, it would be important to investigate micro-grafting of *U. kirkiana*. This is likely to overcome the rejuvenation problem. Moreover, use of lateral shoots will avoid the mismatching scion/stock problem for micro-grafts.

Accumulation of phenols at the union inhibited graft compatibility in *U. kirkiana* grafts. However, other factors are equally important such as activities of enzymes, proteins, starch and auxins (Andrews & Marquez, 1993). These factors have been also implicated in graft incompatibility of many fruit trees (Errea *et al*., 1994b; Ermel *et al*., 1997), but these factors have not been studied in *U. kirkiana* graft combinations. Despite the fact that

phenolics play a role in graft incompatibility of *U. kirkiana* scion and stock combinations, the main cause of graft incompatibility is still complex and requires further research.

Grafting is still a potential propagation method due to dwarfing effects imposed by the stocks. However, graft incompatibility is a threatening problem, and hence selection of compatible scion/stock combinations is needed at an early stage for stable orchard productivity. Furthermore, graft incompatibility is a complex process and phenols are part of the complex mechanism. In the present study, histological experiment provided useful information so too was callus fusion technique. However, there is need for field evaluations for scion/stock combinations that show compatibility using histological, phenol analysis and callus fusion techniques. This will cross-examine if the present graft compatibility assessment techniques simulate field observations.

Scientific research studies on different macro-propagation (vegetative propagation) methods for *P. capensis* tree species have not yet been done. There is need to evaluate air layering, budding, grafting and micro-grafting as potential vegetative propagation methods for *P. capensis* tree species. Furthermore, field survival assessment of air layers and budded or grafted *P. capensis* trees will be required. This is because *U. kirkiana* air layers and budded trees have shown poor field survival despite good rooting capacity or graft take (Akinnifesi *et al.*, 2007). Therefore, such research studies will assist propagators in selecting a feasible and reproducible propagation method for *P. capensis* tree species.

Shoot tip necrosis problem for *P. capensis* micro-shoots (Chapter 7) was not completely eliminated despite frequent subculturing and adding casein hydrolysate to the culture

medium. Although the shoot tip necrosis was not very serious, it is good to eliminate it in order to improve shoot multiplication. Therefore, evaluation of other adjuvants or procedures to completely eliminate shoot tip necrosis in *P. capensis* micro-shoots would be required so that to improve production of multiple micro-shoots.

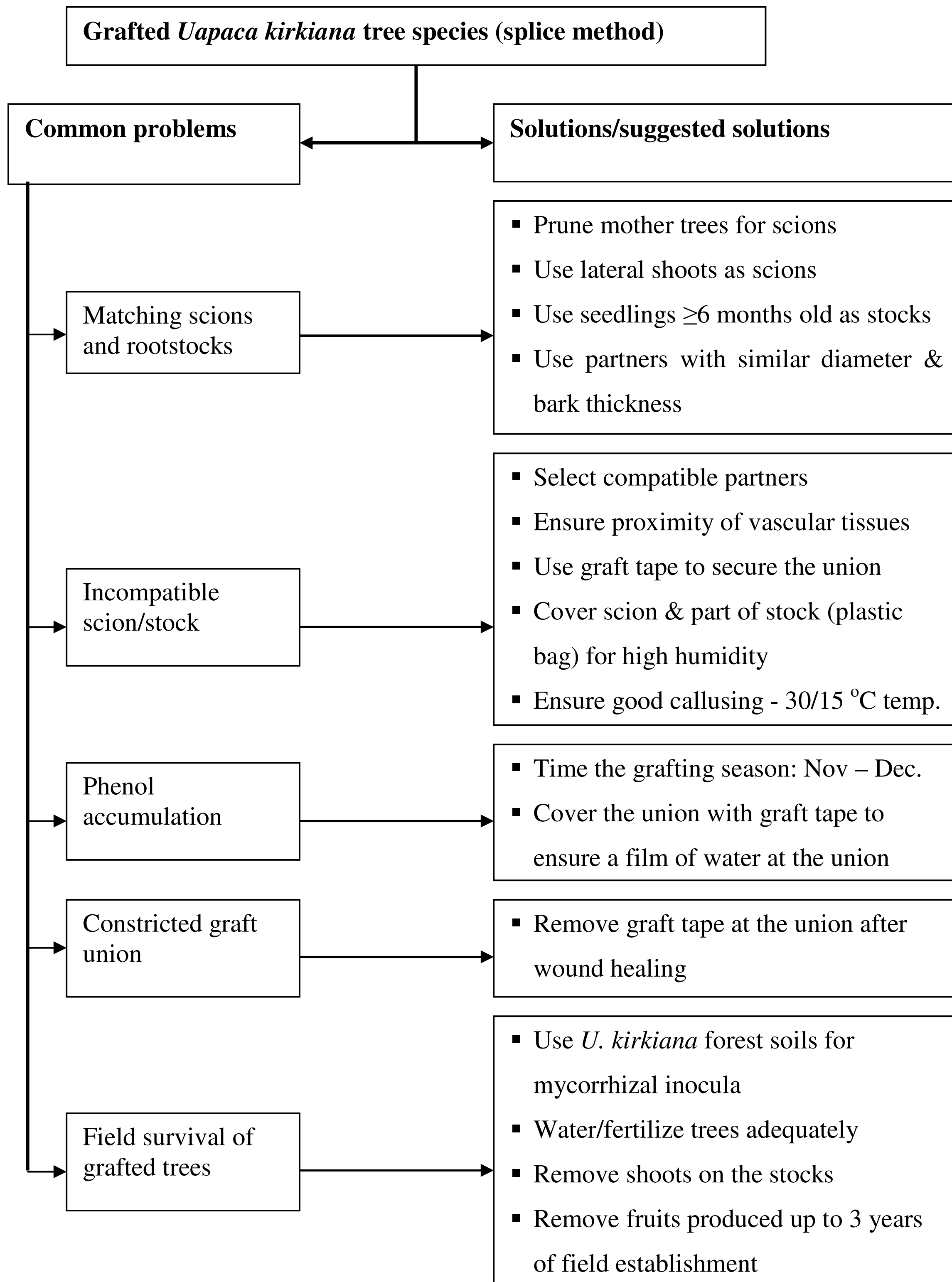

Figure 9.1 A schematic diagram for grafted (splice method) *Uapaca kirkiana* tree species

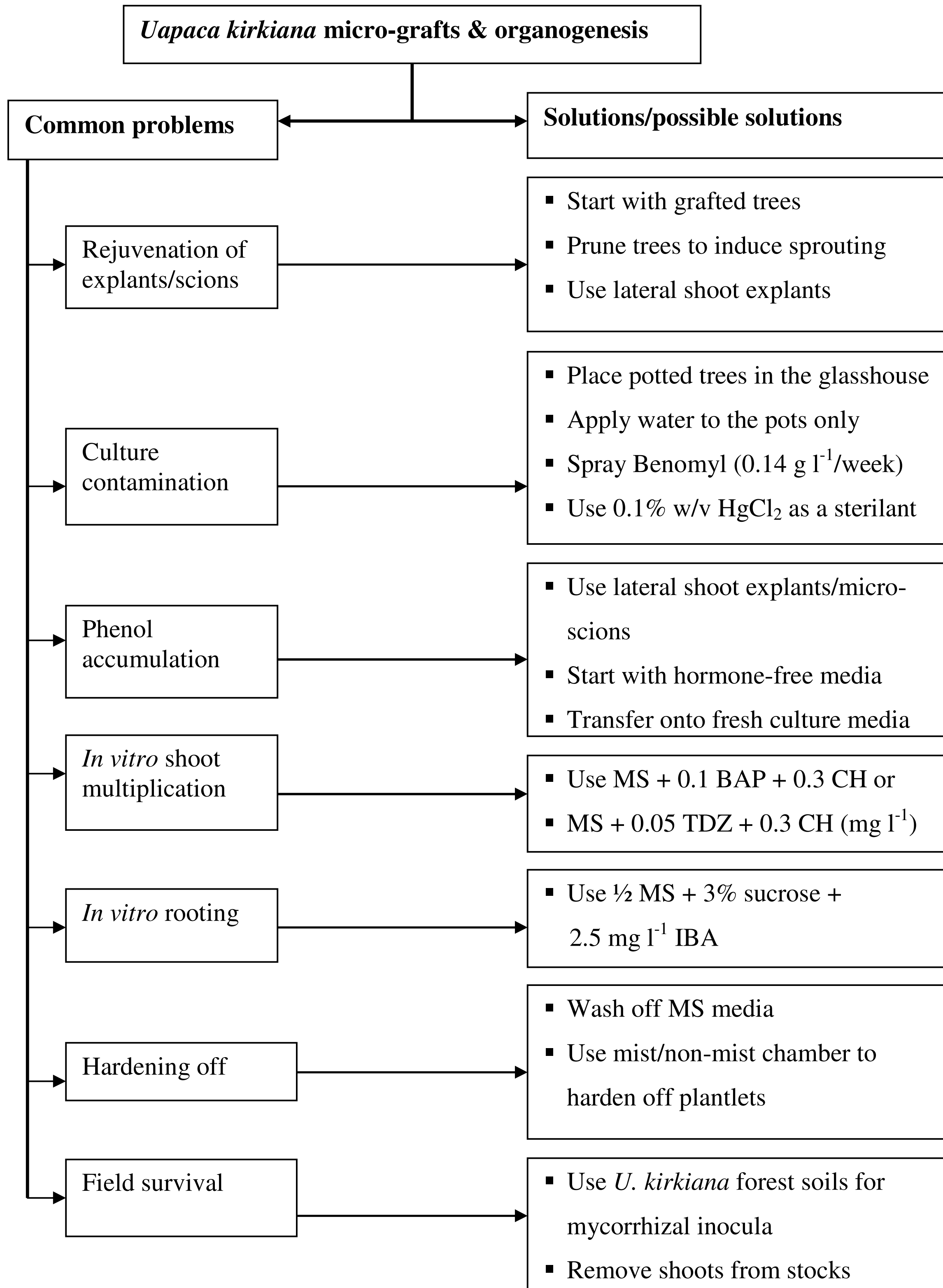

Figure 9.2 A schematic diagram for *in vitro* propagation of *Uapaca kirkiana* tree species

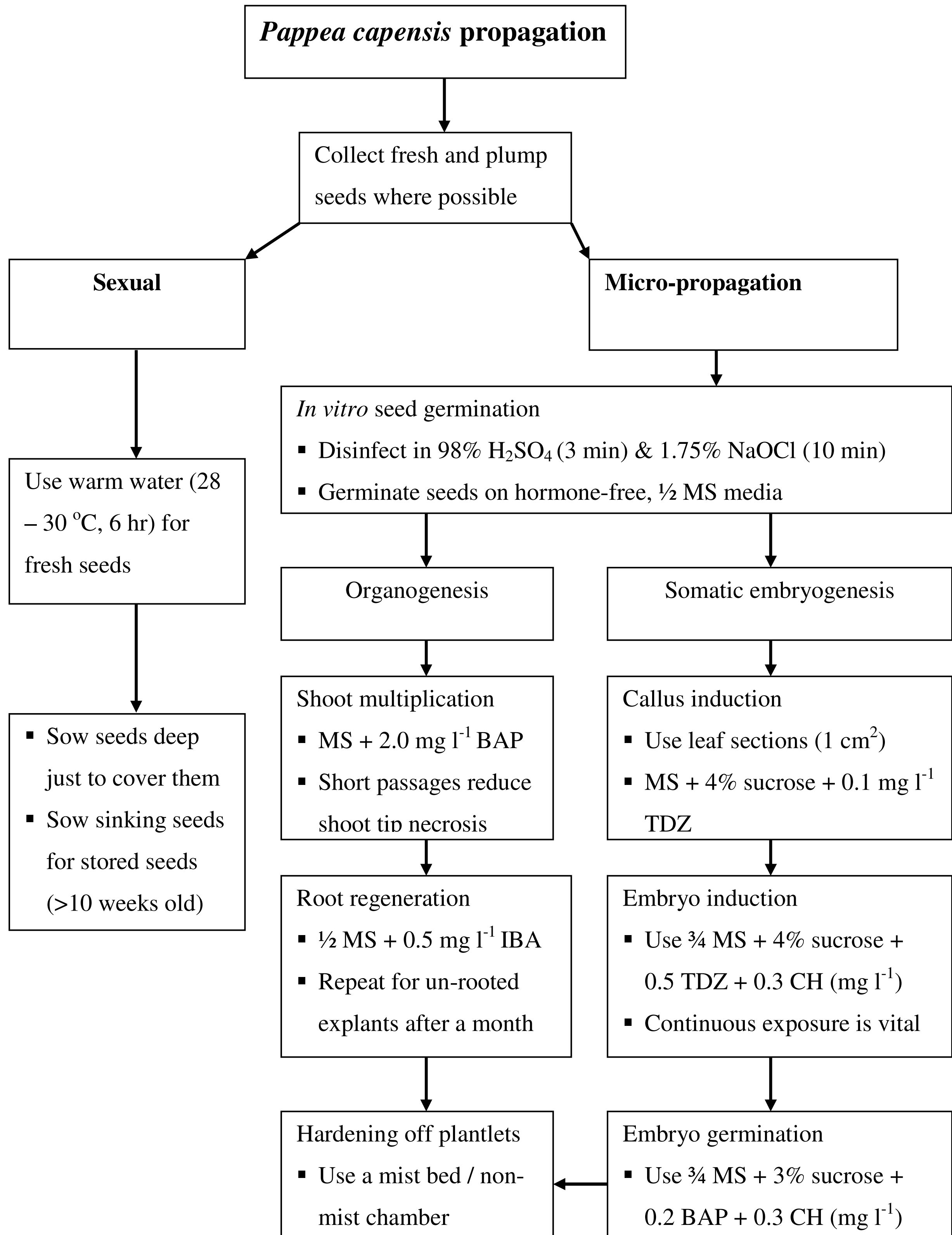

Figure 9.3 A schematic diagram for propagation methods of *Pappea capensis* tree species

SUMMARY

Uapaca kirkiana and *Pappea capensis* tree species are potential sources of income to the rural community dwellers of southern Africa. Therefore, domestication or managing these tree species in their natural habitats is warranted. Domestication, tree improvement and affordable silvicultural management of these renewal resources hinge on reliable propagation protocols. The present study was carried out with the following main objectives:

- To develop propagation protocols that enable rapid and mass production of *Uapaca kirkiana* and *Pappea capensis* planting materials

- To evaluate the graft compatibility within *Uapaca kirkiana* tree clones, provenances and species

A series of experiments were carried out and the results of the study are useful for tree improvement, domestication, conservation and to farmers and nurserymen. Significant findings from the study are as follows:

- A successful decontamination of hard-to-disinfect *U. kirkiana* explants was achieved through pre-conditioning of grafted trees and use of an effective sterilant ($HgCl_2$). Stock plant rejuvenation was achieved through pruning the trees and use of lateral shoots excised from grafted trees. A positive growth response from lateral

shoot explants was obtained on MS medium supplemented with 0.1 mg l^{-1} BAP, 0.04 mg l^{-1} NAA and 0.3 mg l^{-1} CH. Rooting was obtained on MS medium with 2.5 mg l^{-1} IBA (Chapter 2). This reproducible micro-propagation procedure enables rapid clonal multiplication of mature *U. kirkiana* tree species, and hence a major breakthrough in micro-propagation of *U. kirkiana* using mature stock plants.

- Early diagnosis of graft incompatibility is important to reduce losses due to graft incompatibility which might occur several years after a successful orchard establishment. The study (Chapter 3 and 4) has identified poor callus proliferation, cell necrosis and accumulation of phenols at the graft union as indicators of graft incompatibility. Therefore, these parameters can be assessed during early life of the young grafted trees to determine potential graft compatibility. This will enable selection of compatible scions and rootstock, and hence stable orchard establishment.

- Isolation of *ρara*-coumaric acid (with high peaks) and presence flavonoid derivatives such as quinones (Chapter 5) at the union of less compatible *U. kirkiana* graft combinations improved our understanding on the adverse effects of accumulation of these secondary metabolites on graft compatibility. Therefore, the choice of *U. kirkiana* scion/stocks combination must be done using compatible partners that do not show high accumulation of ρara-coumaric acid and flavonoid derivatives which hinder cell elongation or callus proliferation, and hence brings about graft incompatibility.

- The study has determined an affordable and easy technique for improved *P. capensis* seed germination (Chapter 6). Use of water floatation method enables farmers to identify the viable seeds after seed storage, and hence ensures good seed germination.

- The study (Chapter 7 and 8) has established new innovations in clonal propagation of *P. capensis*. The developed somatic embryogenesis protocol offers an option for tree improvement through rapid and mass multiplication of planting materials, which can be subjected to selection pressure for superior traits.

Based on the results obtained from different experiments conducted in this study, new knowledge on the causes of incompatibility in grafted scions and rootstocks; and *in vitro* shoot multiplication and root regeneration of *U. kirkiana* and *in vitro* propagation potential of *P. capensis* has been generated. This will contribute to our understanding of different trees species responses to manipulation. Also, the knowledge generated will be useful in advancing propagation and domestication of the tree species.

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