TOWARDS A NOVEL FRUIT CROP: MICROPROPAGATION AND GENETIC

TRANSFORMATION OF THE INDIGENOUS FRUIT TREE MARULA,

SCLEROCARYA BIRREA SUBSP. *CAFFRA*

BY

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ABSTRACT

The marula tree (*Sclerocarya birrea* subsp. *caffra*), an indigenous, multipurpose, drought tolerant tree of Africa harbors great economic potential. Acceptance of marula-derived products internationally will directly increase the demand for marula resource. Rapid multiplication of marula trees of superior quality forms the basis of sustainable export growth. *In vitro* propagation and genetic improvement offer the opportunity for accelerated multiplication of selected tree material as well as to dramatically increase production, quality and efficiencies.

The objectives of the study were therefore to develop a protocol for *in vitro* multiplication of marula and to determine the feasibility of *Agrobacterium*-mediated transformation of the marula tree. Nodal sections with axillary bud (s) were cultured on Murashige and Skoog (MS) medium supplemented with 4.8μM BA and 2.4μM KN and 0.1% polyvinylpyrrolidone (PVP) to obtain on average 2.5 microshoots per responding explant. The proliferated microshoots were elongated on MS medium supplemented with 1.2μM BA and 1.0μM KN. Elongated microshoots were rooted in MS medium at half salts strength supplemented with 10μM IBA and 0.3% activated charcoal (AC). On average 82% of the shoots rooted. Survival of acclimatized plantlets was 90%. RAPD analysis confirmed intraclonal genetic stability between parent plants and their clones within the limits of the technique.

Nodal sections cocultivated with *Agrobacterium tumefaciens* for 3 days on MS multiplication medium supplemented with 100μM acetosyringone resulted on average in transient expression of 52.5% of the explants with 1.6 blue stained zones per explant. Cocultivated explants on MS selection medium containing 300mgl^{-1} kanamycin resulted in 1.5% chimeric putative transgenic shoots.

This is the first report on the micropropagation and genetic transformation of marula, *Sclerocarya birrea* subsp *caffra*.

DECLARATION

I, Margaret Huruma Naftali Mollel, declare that the thesis submitted to the University of Limpopo for the degree of Doctor of Philosophy (Biotechnology) has not been previously submitted by me for a degree at any other university; that it is my own work and design and that all materials contained therein have been duly acknowledged.

Signed

Date

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DEDICATION

To my father, late Fabiano Shani and my mother, Wankembeta Shani, my brother Nalogwa Shani who laid the solid foundation for my education, to my husband Prof Naftali and my children Nailejileji, Ulumbi and Lotang'amwaki for their patience, moral and material support during the course of my study.

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ABBREVIATIONS

AC   Activated charcoal

AFLP   Amplification fragment length polymorphism

BA   Benzyladenine

B5   Gamborg medium

bt   Gene encoding the crystal protein of *Bacillus thuringiensis*

Chv   Chromosomal virulence protein

chv   Chromosomal virulence gene

DNA   Deoxyribonucleic acid

GA_3 Gibberellic acid

GM   Genetically modified

GUS   β-glucuronidase

IAA   Indole-3-acetic acid

IBA   Indole-3-butyric acid

ISSR   Inter simple sequence repeats

KN   Kinetin

MS   Murashige and Skoog

MSAP   Methylation sensitive amplification polymorphism

NAA   Napthalene acetic acid

NLS   Nuclear localization signal

*npt*II   Neophosphotransferase II gene

PCR   Polymerase chain reaction

PGR   Plant growth regulator(s)

PVP   Polyvinylpyrrolidone

PVP1/2D7   MS PVP jars of which the bottom was covered with catering foil for 7
days

RAPD   Random amplified polymorphic DNA

RFLP   Restriction fragment length polymorphism

T-DNA   Transfer DNA

TDZ   Thidiazuron

Ti   Tumor inducing

Vir   Virulence protein

vir   Virulence gene

X-Gluc   5-bromo-4-chloro-3-indolyl glucuronide

ZR   Zeatin riboside

2,4-D   2,4-Dichlorophenoxyacetic acid

2iP N^6-(2-Isopentenyl)adenine

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

1.1 Marula

The marula tree is an indigenous, drought tolerant and multipurpose tree, which belongs to the family of *Anacardiaceae*. The marula tree is widely distributed in Africa, from Ethiopia and Tanzania to the east, Zambia to the central and Botswana and South Africa to the south. It occurs mainly in low to medium altitudes (100-1600 meters above sea level). It is found in warm, dry, frost-free areas. The tree grows in different soil types, even in sandy soils (Palmer and Pitman, 1973; Mbuya *et al*., 1994; Simute *et al*., 1998). The marula tree is a dioecious plant where male and female flowers are borne on different trees, although trees bearing both female and male flowers have been observed (Dr. Holtzhausen; Winter P; Mollel M, personal observation). The marula tree harbors many indigenous uses. The fruit is used as food in different ways; eaten as fresh fruit, prepared as fruit jam, fruit juice, and liquor among others. The nutritional value of the fruit is high in terms of vitamin C (67.9mg/100g) (Venter and Venter, 1996) and protein content (60%) and thus presents a good source of nutrients and vitamins for people and animals. The nuts are eaten raw or cooked and pressed to extract marula oil which is stable and of high quality (Shone, 1979). The bark is used as a dye. The wood/timber is used for furniture, paneling and as firewood. The bark, roots and leaves are used as medicine. The marula tree is further important to a variety of animals for shade, feed and shelter. Recent exploitation of the novel flavor of marula fruit and the oil of its nut has led to the development of a variety of food products such as amarula, swarula, marula wines, beers, soft drinks, ice creams, dried fruits, marula oil, soaps and creams several of

which are exported. International market growth and long-term competitiveness require resource expansion of marula of a consistent high quality at concomitant increased production efficiencies for long-term sustainability of these important and expanding foreign exchange earners.

1.2 Options for marula propagation

Plant propagation is the multiplication of plants by both sexual (seed) and asexual (vegetative parts) means. Seed, due to ease of production and handling is still the preferred vehicle for the propagation of most agronomic and forest tree crops. However, many horticultural crops are vegetatively propagated, for a variety of reasons, including genetic self-incompatibility, difficulty or inability to produce or utilize uniformly homozygous seeds. In these cases, plants are propagated vegetatively by grafting (fruit, nut, ornamental trees), budding (fruit, nut, ornamental trees), layering (fruit, rubber, ornamental plants), runners (fruit, ornamental plants), division (rhizome and tuber plants) and *in vitro* culture systems (shoot-tip culture, adventitious shoot formation, micrografting, tissue and protoplast culture) (Hartmann *et al*., 1990; Bornman, 1998). Seedlings, cuttings and truncheons of about 10 cm in length (Mbuya *et al*., 1994; Simute *et al*., 1998) and grafting (Holtzhausen, 2001) are conventional methods for propagating marula.

1.2.1   Importance of vegetative propagation in agriculture

Vegetative propagation *in vivo* plays an important role in agriculture in cases where seed cannot be used to provide homogeneous crops which are prerequisite for uniform quality and production enabling standardization of growth, harvesting and processing.

Important roles of vegetative propagation include:

Convenience and ease of propagation. Some important food crops can be propagated more easily, more conveniently or more economically by vegetative propagation methods than by seed. Examples include grape, olive, banana, potatoes and sugarcane (Hartmann *et al*., 1990).

Selection and maintenance of clones. Large genetic advances can be made in a single step by selecting a single unique superior plant from a seedling population and reproducing it asexually by vegetative means. The resulting population of plants has the same basic genotype as the original plant and is called a clone. Since clones are genetically uniform, they are also highly uniform phenotypically. The plants will have the same uniform appearance, size, time to flower, fruit maturity, etc. This uniformity is the basis for the highly standardized production practices that are characteristic of modern commercial horticulture (Hartmann *et al*., 1990).

High heterozygosity and long breeding cycle. Most woody and herbaceous perennials in cultivation have long breeding cycles and are highly heterozygous. Vegetative propagation is important for these crops because their genetic improvement by breeding and back crossing to attain a homozygous population for seed propagation is slow. Many clones of horticultural interest have been discovered and perpetuated as cultivars; for example, sultana and grapes have been perpetuated as millions of plants for more than 2000 years with little change. The "delicious" apple originated about 130 years ago in Jesse Hiatt's apple orchard in Iowa, and has been vegetatively propagated to give

millions of identical "delicious" apple trees that are grown throughout the world (Hartmann *et al*., 1990).

Combination of more than one genotype into a single plant. Vegetative propagation by grafting makes it possible to unite more than one genotype and combine desirable features of both genotypes into a composite plant. Most fruit orchards are a combination of a seedling rootstock chosen for root characteristics combined with a cultivar chosen for its fruiting characteristics. Grafting can also result in special growth forms such as dwarfed trees (Hartmann *et al*., 1990).

Shortening the time to reproductive maturity. Vegetative propagation of plants in the adult growth phase reduces the long nonflowering juvenile characteristics of most seedling plants. Vegetatively propagated fruit and nut cultivars will flower at a younger age than comparable seedling plants (Hartmann *et al*., 1990).

Control of growth phases and morphology. Plants in the juvenile phases of many species have unique characteristics that differ distinctly from comparable plants in the mature phases. Most forest tree species benefit from a long juvenile period, which confers delayed flowering, better tree growth and better lumber yields than those of seedling plants. In other species such as citrus, plants in the juvenile phase are thorny; excessively vigorous and produce poor fruit quality. Vegetatively propagated citrus plants from the mature phase do not exhibit these characteristics (Hartman *et al*., 1990).

1.2.2 Micropropagation

Micropropagation of plants is generally referred to as *in vitro* propagation. It is the application of tissue culture techniques to the propagation of plants starting with very small plant parts grown aseptically in a test tube or other container (Yeoman, 1986; Hartmann *et al*., 1990). *In vitro* propagation offers a larger number of cloned materials in a controlled environment in a shorter time starting from fewer sources of plant material and ensures availability of cloned material throughout the year. In addition to becoming an important research tool, micropropagation has important practical applications in plant breeding and production. New plants are produced from very small structures (embryos, shoot tips, meristems, protoplasts etc.) in aseptic culture. Associated with this technology are novel methods of plant breeding including the ability to isolate and transfer fragments of DNA representing specific genes from one vegetative cell to another or to combine two somatic cells together outside of the normal plant's reproductive process (Hartmann *et al*., 1990; Bornman, 1998).

Micropropagation is based upon two major principles. The first one is the principle of totipotency that is; every living cell in a plant has the genetic potential to reproduce the entire organism (Hartmann *et al*., 1990). The second major principle is the regulation of shoot and root regeneration by the use of plant growth regulators (PGR) mainly cytokinin and auxin (Hartmann *et al*., 1990).

Micropropagation is achieved by empirical development of three main procedures:

-The selection of explants of conventionally grown plants, their surface sterilization and inoculation onto a suitable nutrient media (Yeoman, 1986; Hartmann *et al*., 1990).

-The transfer to media or sequence of media that promote induction and/or proliferation of shoots and roots. (Yeoman, 1986; Hartmann *et al*., 1990)

-The transfer of plantlets into the glasshouse or the field (Yeoman, 1986; Hartmann *et al*., 1990).

Although micropropagation is a well-developed procedure, the specific conditions under which a plant species in this case marula will propagate are determined empirically by varying the media composition especially the PGR. Often methods developed to a closely related species form a starting point of research and development. The successful micropropagation of cashew nut (Das *et al*., 1996; Boggetti *et al*., 1999), a close relative of marula, hints towards the feasibility of micropropagation of marula.

1.2.3   Approaches for *in vitro* multiplication of shoots

Multiplication of shoots *in vitro* falls in two main categories namely, the multiplication of existing axillary meristems by branching activity of the original shoot and axillary apices and, by the formation of new adventitious meristems, in the form of either shoot apices or embryos, from the cells and tissues of the plant body. *In vitro* techniques enable these categories to be exploited with very high efficiency either by releasing the natural controls on axillary meristem or by providing more critical conditions for the regeneration of adventitious meristems from original or cultured somatic cells (Yeoman, 1986). The distinction between the two main pathways of vegetative production is

important with regard to the genetic stability of the propagules during multiplication and their susceptibility to induced mutation or transformation (Yeoman, 1986; Gamborg and Phillips, 1995).

The multiplication of existing meristems is the most widely used method in commercial micropropagation of plants, since it mostly results in genetically uniform clones (Gamborg and Phillips, 1995).

The *de novo* formation and proliferation of adventitious meristems from plant organs (leaves, stems, roots and embryos) is often used in genetic transformation of plant species (Gamborg and Phillips, 1995).

1.3 Options for genetic improvement of marula

Genetic improvement of plants is a constant requirement for the development of better quality and higher yielding crop varieties. Conventional breeding uses naturally existing genetic differences by crossing selected parental lines containing desired characteristics. Once this has been achieved, subsequent growth and selection is carried out until genetic uniformity, agronomic stability and maximum reproductive ability are obtained (Walden, 1988; Puonti-Kaerlas, 1998). However, sometimes, desirable traits are linked to other non-desirable ones. Also not all desirable characteristics can be naturally found nor do they exist in sexually compatible plant species, which poses limitations to plant improvement. In addition, the several backcrosses required to establish new breeding lines make conventional breeding a time-consuming process. For trees, plant breeders often choose one superior line after a cross which they propagate vegetatively instead of

establishing a pure homozygous line which due to the long regenerative cycles of trees, would take several human life times. Thus far no conventional breeding has been done for marula. However, four cultivars (pharulani, mhalarula, chopperula and nqawanarula) have been selected based on yield per tree, fruit size and juice content and tree size (Holtzhausen, 2001).

At the molecular level, plant breeding exploits naturally occurring or induced mutations and recombinations. Genetic engineering uses recombinant DNA techniques combined with gene transfer to plants. It can be used to broaden the gene pool of plants by interspecies, intergenera and interkingdom gene transfers. Transgenes encoding traits such as insect and virus resistance, herbicide and pest tolerance, high beta-carotene and modified oils have been integrated in plant genomes (Douches *et al*., 1998; Fulton and Keyowski, 1999; table 1). Genetically modified (GM) plants can subsequently be incorporated into the traditional breeding programs.

Table 1. Approved GM crops and current advancements in key GM research

Sector	New trait	Crop
Agronomic	Longer shelf life; insect tolerance	Tomato[1]
	Insect and herbicide tolerance	Cotton[1]
	Herbicide tolerance	Soya[1]
	Insect and herbicide tolerance; male sterility	Maize[1]
	Insect and virus tolerance	Potato[1]
	Virus tolerance	Papaya[1]
	Virus tolerance	Squash[1]
	Herbicide tolerance; male sterility	Canola[1]
	Herbicide tolerance	Beet[1]
	Herbicide tolerance	Chicory[1]
	Herbicide tolerance	Flax[1]
	Herbicide tolerance	Rice[1]
	Virus resistance	Cassava[2]
	Herbicide tolerance	Wheat[2]
	Pest tolerance	Fruit trees[2]
Nutrition and health care	High beta-carotene	Rice[2], canola[2]
	Reduced allergens	Rice[2]
	Edible vaccines	Banana[2]
New products	Altered oil profile	Soya[1], canola[1]
	Altered lignin content	Forestry trees[2]

GM-genetically modified, [1]- approved GM crop, [2]- currently advanced key GM research
Source: Kitch *et al*., 2002

Some GM crops are grown commercially; others are undergoing advanced field trials while others are still in the laboratory research stage. New traits address post-harvest losses, pollen control, salt and drought tolerance, vitamin A-enrichment, reduced allergens and vaccine production (Kitch *et al*., 2002).

1.3.1 Methods for genetic transformation

Practical methods of plant transformation can broadly be divided into two categories, indirect and direct transfer of DNA. *Agrobacterium tumefaciens*-mediated gene transfer is considered to be an indirect method because the gene of interest has first to be transferred to the bacterium. The *Agrobacterium tumefaciens* or *A. rhizogenes* cells subsequently infect plant cells *in vitro* and transfer the gene of interest into the plant

genome (Han *et al*., 2000; Zhu *et al*., 2001). Direct gene transfer occurs when the foreign DNA is delivered directly into the plant cell either by converting the target plant cell to protoplasts followed by DNA uptake by electroporation or chemical treatment (polyvinyl alcohol or polyethylene glycol) (Bornman, 1998) or by subjecting intact plant tissue in a particle gun to a shower of high velocity particles coated with DNA (Mathews *et al*., 1993; Yao *et al*., 1996; Chowdhury *et al*., 1997). Historically, direct gene transfer methods were used more often on monocotyledonous crops whereas the indirect method was most popular for transforming dicotyledonous crops (de Villiers, 1998).

1.3.2 Opportunities for genetic manipulation of marula

Among many others, priorities for marula transformation include pest tolerance, fruit ripening control and shortening the time to flowering. Pests such as fruit flies often attack the unripe and ripe marula fruits damaging up to 40% of the fruit (De Lange *et al*., 2001). This reduces the quality of the fruit for fresh consumption and the productivity of marula processing. At such an infestation rate, an insect resistance strategy may offer a viable alternative to avert the problem.The insecticidal crystal protein (delta-endotoxin) of the bacterium *Bacillus thuringiensis (bt)* has been used as a specific, effective and safe insect control agent in traditional and organic farming for several decades. The *bt* genes have been cloned, codon-modified and introduced into various crop species to specifically kill problem insects in the crop ecosystem. For example, in potato an introduced modified *bt* gene synthesizes Cry1 protein that offers resistance against tuber moth (Douches *et al*., 1998) and expression of the Cry1A protein in maize offers effective control against the European corn borer (Von Broock and Spanakakis, 1996). The Cry1B protein is effective to control insects that belong to the order of Diptera to which the fruit flies (family:

Tephritidae) belong. Cry1B has been reported to confer resistance against the olive fly, *Bactrocera oleae.* Bioassays with Cry1B resulted in death of both larvae and adult olive fly (Alberola *et al*., 1999). The mango or marula fruit fly, *Ceratitis cosyra*, is a serious pest in small holder and commercial mango across subsaharan Africa (Steck, 2003) where it is more destructive than the Mediteranean fruit fly, one of the most polyphagous and widespread species of the Tephritidae (Liquido *et al.*, 1991). The genera *Bactrocera* (tribe Dacini) and *Ceratitis* (tribe Ceratitidine) both belong to the subfamily of Dacinae (family: Tephritidae). The broad spectrum against many species of the Diptera including species of the subfamily of Dacinae hints that Cry1B may be effective against the marula fruit fly. Experimental bioassays are needed to substantiate a transgenic approach to fruit fly resistance in marula.

The marula fruit is perishable and has a short shelf life. The fruits fall off the tree, ripen on the ground and start to decay 16 days after falling off (De Lange *et al*., 2001). This necessitates rapid utilization, which in turn leads to cheap disposal price of fruits or cold storage, which increases the cost of the product. The shelf life of other climacteric fruits has been successfully extended by antisense constructs technology (Giovannoni *et al*., 1989; Chrispeels and Sadara, 1994; Stearns and Glick, 2003). Control of fruit ripening and increased shelf life have been achieved by regulation of ethylene biosynthesis using antisense constructs of aminocyclopropane carboxylic acid (ACC) oxidase or synthase (Giovannoni *et al*., 1989; Chrispeels and Sadara, 1994; Stearns and Glick, 2003). In tomato, expression of antisense transgene of ACC synthase suppressed ethylene synthesis; some 99.5% fruit did not ripen until treated with exogenous ethylene whereafter they ripened into normal fruit (Stearns and Glick, 2003). Expression of the

antisense transgene of ACC oxidase reduced ethylene synthesis to 5% in tomato and 1% in cantaloupe. Fruit ripening was delayed and blocked, respectively (Chrispeels and Sadara, 1994; Ayub *et al*., 1996).

The marula tree like all trees is characterized by a long juvenile phase, which in turn lengthens the breeding cycle. The long vegetative phase prolongs the time needed to analyze traits such as those of the flower and fruit, that only manifest in mature trees. Tree generation time can be reduced through genetic manipulation using the flower-meristem-identity genes (Pena *et al*., 2001; Martin-Trillo and Martinez-Zapater, 2002; Campbell *et al*., 2003). The flower-meristem-identity genes *Arabidopsis LEAFY* (*LFY*) or *APETALA*1 (*AP*1) have been successfully used to shorten the time to flowering in citrus (Pena *et al*., 2001), poplar (Rottmann *et al*., 2000) and aspen (Weigel and Nilsson, 1995). Flowering was observed only 7 months after germination instead of 8 to 20 years in transgenic aspen that constitutively expressed *LFY* (Weigel and Nilsson, 1995). In transgenic citrus, constitutive expression of either *LFY* or *AP*1 drastically reduced the length of the juvenile phase and accelerated reproductive competence. The juvenile phase of citrus is normally 6 to 7 years. Transgenic citrus initiated flowering 12 to 20 months after transfer to the greenhouse in contrast to 5 years for non transgenic and T-DNA vector transgenic controls. Flowers were regular and fertile and the trait was inheritable. Transgenic seedlings flowered in their first season. The AP1 transgene was more effective than LFY in the reduction of juvenile traits and the promotion of flowering and did not cause abnormal leaf developmental effects which were observed in LFY transgenic plants (Pena *et al*., 2001). The number and type of GM crops being planted globally has increased exponentially since its onset. Tree transformation methodologies,

first reported in the late 1980s, lag five to ten years behind those reported for other crops. One hundred sixteen confirmed field trials, involving at least 24 tree species, in 17 countries have been undertaken between 1988 and 1999 (Owusu, 1999) less than 1% of the total number of GM field trials in 38 countries during the same period. No commercial plantings of transgenic trees have as yet been reported (IUFRO, 2005) and commercialization of GM trees lags behind that of other crops (Pena and Seguin, 2001). The long generation time slowing research and field testing, the biodiversity debate in particular the potential impact on indigenous forests and the large distances of pollen travel (e.g. of pine) have delayed commercial planting of transgenic trees. The International Union of Forestry Research Organizations (Strauss *et al*., 1999) however declared that GM trees can be evaluated according to similar principles as other GM crops. In particular cases where pollen dispersal cannot be contained, alternative tools must be developed prior to commercial planting. Notwithstanding, research achievements include among others, herbicide and pest resistance (Schuler *et al*., 1998; Dandekar *et al*., 1998), improvement of agronomic, forestry, fruit and industrial properties (Li *et al.*, 2003; Pilate *et al.*, 2002), reduction of generation time (Weigel and Nilsson, 1995; Rottmann *et al*., 2000; Pena *et al*., 2001), new traits such as biodegradation of explosives (French *et al.*, 1999), phytoaccumulation and bioremediation of toxic compounds (Stomp *et al*., 1993; Watanabe, 2001). Many of these are undergoing field trials and commercial transgenic tree plantations may soon become a reality.

1.3.3   GM plants and public perception

Although genetic modification technology provides a powerful tool in assisting conventional plant breeding, public perception is divided (Kitch *et al*., 2002; Heritage,

2005). Concerns regarding genetically modified (GM) plants group into environmental, food/feed safety, economic and social classes. Environmental issues include decrease of biodiversity, invasiveness, gene flow, weediness, ecological impact on non-target organisms, toxicity to living things and long-term transgene instability (Kitch *et al*., 2002; Heritage, 2005). Food and feed safety concerns include toxicity, allergenicity, nutritional changes, possible transfer of genes conferring antibiotic resistance and unexpected effects (Kitch *et al*., 2002; Netherwood *et al*., 2004; Heritage, 2005). Economic concerns include multinational control, globalization, trade issues, income inequality and intellectual property rights (Kitch *et al*., 2002). Social issues pertain to dietary preference, ethics, farmers' right to replant seed and labeling (Kitch *et al*., 2002; Heritage, 2005). GM technology cannot be applied in isolation, it offers only one of the technologies applied in agriculture and forestry and like other technologies, it will require continuous innovation in order to remain relevant and offer competitive advantage. E.g., appropriate and integrated management and continuous improvement of GM technologies are required to avoid the development of pest resistance in particular in large-scale GM plantings. Groups strongly opposed to GMO push the precautionary principle and call for a ban on GM: since there may be potential negative impacts which cannot be known at this point in time, we should not engage. They often disseminate unbalanced and inaccurate information and use controversy in the media to push their agenda. Those who support genetically modified plants believe that all technologies possess an inherent risk and that risks associated with GMO are appropriately managed. A strong biosafety framework has been established in order to assess, prevent and limit the impact of potentially negative effects associated with the general release of GM crops

to the public and the environment on a case by case basis. These rather extensive investigations and risk assessments reduce risks associated with GM crops well below those of crops and products produced by conventional non-GM technologies. Dale *et al*. (2002) can find no compelling scientific arguments that GM crops and their impact on the environment are innately different from non GM crops. More novel transgenic crops such as those used for "biopharming" however may pose new challenges and opportunities for environmental management. Transgenic food and feed crops are often safer than their nontransgenic counterparts as they may contain lower amounts of pesticides and phytotoxins (Kitch *et al*., 2002; Heritage, 2005; Chrispeels and Webster, 2002).

Eighty one million ha in 17 countries are under GM crop cultivation, a 20% increase in acreage over 2003 (James, 2004). Global adoption of GM crops is accelerating as both commercial (Pena and Seguin, 2001; Kitch *et al*., 2002; James, 2002) and small-scale farmers (Kitch *et al*., 2002; Chrispeels and Webster, 2002; Thomson, 2002; GMO indaba, 2004) aim to reap the benefits. Small-scale farmers in South Africa enjoyed net profit increases by cultivating *bt* crops of 29% in cotton (Van der Walt, 2000; Bennet *et al*., 2002) and 27% in maize (GMO indaba, 2004). In addition, reductions in insecticide applications from 6 to 0.2 times enabled small-scale farmers to increase the acreage under cultivation several-fold thereby dramatically improving their livelihoods (Van der Walt, 2000).

1.4 Problem statement

Limitations to traditional breeding are more pronounced in trees due to their long regeneration cycle. Especially in trees such as marula where there are needs for resource

expansion and improved properties, biotechnology offers many opportunities to shorten the breeding and production cycles through genetic transformation and micropropagation. Recombinant DNA technology can introduce one gene or trait in an existing variety and thus shorten the breeding cycle; micropropagation enables amplification of the superior selected plant or the genetically modified marula. Many reports document protocols of plant transformation. However, few have been reported on woody plant transformation. Some of the success stories of woody plant transformation where *Agrobacterium* was used as a method for delivering DNA into plant cells include apple (Maheswaran *et al*., 1992), rhododendron (Ueno *et al*., 1996), pear (Mourgues *et al*., 1996), Spring-glory (Rosati *et al*., 1996), cocoa (Sain *et al*., 1994), plum (Scorza *et al*., 1995), aspen (Tuominen *et al*., 2000), poplar (Han *et al*., 2000; Delledonne *et al*., 2001) and apple rootstock (Zhu *et al*., 2001). Reports on woody plant transformation where particle bombardment was used as the means for DNA transfer include oil palm (Chowdhury *et al*., 1997) and citrus (Yao *et al*., 1996). Mathews *et al*. (1993) authored a preliminary report on the genetic transformation of somatic embryos of mango (*Anacardiaceae*). However no transformation of explants either by *Agrobacterium* mediation or by particle bombardment has been reported for other members of the *Anacardiaceae*.

1.5 Objectives of the study

The aim of this study was to develop a micropropagation protocol for the marula tree and to determine feasibility of *Agrobacterium*-mediated transformation for the marula tree, *Sclerocarya birrea* subsp *caffra*. The specific objectives were:

- Micropropagation

16

- To develop a surface sterilization protocol

- To optimize concentrations of plant PGR for shoot amplification and elongation

- To root the shoots

- To harden and establish plants in the growth room

- To evaluate somaclonal variation of micropropagated material and parent plants

- Transformation

 - To determine the kanamycin concentration for selection

 - To optimize cocultivation protocols using transient expression

1.6 Importance of the study

Results obtained from this study will add to the existing knowledge on woody plant micropropagation and transformation. In addition, the findings will offer opportunities to address problems associated with development of marula as a novel fruit tree crop, such as rapid clonal multiplication of elite material and introduction of, among others, insect resistance, ripening and flowering control.

1.7 Limitation of the study

<u>Unsatisfactory growth room conditions.</u> Frequent power interruption switched off the cooling system, which needed to be manually restarted. This resulted in frequent growth

room temperatures above 45°C. High temperatures affected growth of cultures, often resulting in necrosis and death.

CHAPTER 2. LITERATURE REVIEW

The literature review reports on the state of the art in micropropagation and genetic transformation of woody plants. Woody plants are trees and shrubs, whose stems live for a number of years and increase in diameter each year by addition of woody tissue (Kung and Wu, 1993; Thorpe, 1995). Since very little has been reported on micropropagation and genetic transformation of the *Anacardiaceae* family, relevant literature on other members of woody plants will be presented.

2.1 Micropropagation

2.1.1 Choice of explants

The most suitable part of the plant for the initiation of tissue culture will depend on the species and the type of shoot proliferation that is considered the most appropriate. Other factors that are considered in the selection of explants include the organ that is to serve as explant tissue source, the physiological stage and age of the organ, the season in which the explant is obtained, the size of the explant and the overall quality of the plant from which explants are obtained. These factors determine the cell division potential and morphogenic plasticity and thus the reaction obtained from the explant in tissue culture (Vasil, 1980; Yeoman, 1986; Stafford and Warren, 1991). Immature/juvenile growing tissues usually satisfy these criteria. Table 2 shows explant sources and their morphogenic potentials.

Table 2. Source of angiosperm explants and their relative morphogenic potential *in vitro*

Explant type	Morphogenic potential/availability[a]			
	Dicotyledons		Monocotyledons	
	Herbaceous	Perennial	Herbaceous	Perennial
Shoot tip	High/abundant	High/abundant	High/abundant	High/abundant
Lateral bud	High/abundant	Variable/abundant	High/abundant	Variable/abundant
Inflorescence	High/abundant	Variable/abundant	High/abundant	Variable/abundant
Rachis	High/abundant	Low/abundant	Variable/abundant	Low/abundant
Mature leaf	High/abundant	Variable/abundant	Variable/abundant	Variable/abundant
Petiole	High/abundant	Low/abundant	High/abundant	Low/abundant
Root	High/abundant	Low/abundant	High/abundant	Low/abundant

[a]-Morphogenic potential is used to describe the ability of the explant to regenerate plants *in vitro.* Availability refers to the relative number of explants obtainable from a single plant. Source: Dixon (1985)

In woody plants, it is often difficult to obtain juvenile materials, unless the plant is rejuvenated. Rejuvenation can be manipulated by micro-grafting to seedlings, meristem culture, isolation of zones which are still juvenile, pruning severely to stimulate the production of lateral juvenile shoots and spraying with cytokinin (Pierik, 1987; Narayanaswamy, 1994).

Different explants in use for micropropagation of woody plants include shoot tips (Thomas and Ravindra 1987; Mereti *et al*., 2002), nodal segments containing axillary buds (Boggetti *et al*., 1999; Naik *et al*., 1999; Mhatre *et al*., 2000), leaves (Herve *et al*., 2001), callus (Sahoo *et al*., 1997), cotyledonary nodes (Das *et al*., 1996; Naik *et al*., 2000) and others. Nodal sections (having axillary buds) and shoot tips which harbor-preformed meristems are the most widely used explants in commercial micropropagation. As these bypass the callus stage, they show the least somaclonal variation among propagated plants when compared to micropropagation techniques that include callus,

somatic embryo or adventitious plantlet stages (Gamborg and Phillips, 1995; Bornman, 1998).

2.1.2 Surface sterilization of plant materials

In principle, there are four sources of infections of plant material namely the plant (internal as well as external), the nutrient medium (insufficiently sterilized), the air and the research worker (inaccurate work). The most important of these is the plant itself (Pierik, 1987). Materials whether brought from the field, the green house or the growth room need to be decontaminated of bacteria and fungi before they are used to establish *in vitro* aseptic cultures.

Surface sterilization involves several steps. Harvested plant material is first washed with a disinfecting solution and/or tap water followed by a 70% ethanol wash. This is normally followed by submersion in a sterilizing solution with or without a surfactant and finally with several washes in sterile distilled water to remove traces of the sterilizing agents.

Sterilizing agents include sodium hypochlorite (NaOCl), calcium hypochlorite $(Ca(OCl)_2)$, mercuric chloride $(HgCl_2)$ and silver nitrate $(AgNO_3)$. Calcium hypochlorite and NaOCl solutions are mostly used and are suitable for many tissues (Narayanaswamy, 1994). They are harmless and effective in bacterial decontamination. Their low toxicity and treatment does not show any growth inhibitions as long as the optimal concentration and exposure time are determined (Narayanaswamy, 1994).

For woody species, underground tubers and heavily infected plants from the field, treatment with $HgCl_2$ is recommended. $HgCl_2$ however is very toxic and especially if it is not completely removed by multiple water washes may cause erratic growth or affect cell proliferation (Narayanaswamy, 1994; Gamborg and Phillips, 1995).

Internal contaminants cannot be removed by surface sterilization; this necessitates the incorporation of antibiotics such as penicillin, tetracycline or rifampicin and fungicides in the tissue culture media (Narayanaswamy, 1994).

2.1.2a *Anacardiaceae* explants

Surface sterilization of members of the *Anacardiaceae* including cashew nut, mango and pistachio is presented in table 3. Explant materials obtained from the field were mostly decontaminated by 0.05 to 0.1% $HgCl_2$ solution followed by 1.05% NaOCl. Explant materials obtained from the green house were decontaminated with 1 to 2% NaOCl or 9% $Ca(OCl)_2.$

Explants materials collected from the field were difficult to decontaminate as indicated by the use of $HgCl_2$ or 20% (v/v) NaOCl (table 3). In mango, shoot tip explants from the field remained clean for only 2 to 3 weeks, after which fungal contamination appeared on the explants (Thomas and Ravindra, 1997). Further attempts to decontaminate mango shoot tip explants included increasing the concentration of pretreatment solution, wrapping of shoots with gauze and harvesting of emerging shoots for surface sterilization. This procedure resulted in 80% healthy cultures (Thomas and Ravindra, 1997).

Table 3. Surface sterilization of *Anacardiaceae*

Plant	Explant type	FD, GH	Source	Pretreatment-period (h, min)	SS method-period (h, min)	Results % S	Reference
C	Immature/ mature nuts	FD	Mature tree	70% EtOH-1min flamed	0.1% $HgCl_2$-1h, EtOH flamed	NI	Das *et al*., 1996; 1999
C	Mature seeds	FD	Mature tree	NI	0.05% $HgCl_2$	NI	Phillip, 1984
C	Mature nuts	FD	Mature tree	NI	0.1% $HgCl_2$- 0.1% SLS-10min	NI	D'Silva and D'Souza, 1992
C	Mature nuts	FD	Mature tree	NI	8% NaOCl	NI	Leva and Falcone, 1990
C	Juvenile twigs	FD	Mature tree	Fungicide-1h	0.1% $HgCl_2$.10-12min	3-21	Das *et al*., 1996
C	Microcutting/ Leaves	GH	Seedling	NI	1% NaOCl-15min	NI	Leva and Falcone, 1990
C	Nodal segments	GH	Seedling	Fungicide-10min	9% $Ca(OCl)_2$-25min	74	Lievens *et al*., 1989
C	Nodal segments	GH	Seedling	NI	1% NaOCl-30min	NI	Boggetti *et al*., 1999
C	Nodal segments	GH	Young tree	NI	1% NaOCl	NI	Boggetti *et al*., 1999
M	Shoot tips	FD	Tree	Disinfectant-30min 0.1% cetrimide-20min	0.1% $HgCl_2$-T-10min	Clean for 2-3wks	Thomas and Ravindra, 1997
M	Shoot tips	GH	Rootstock	NI	2% NaOCl- 0.1% T-15min-repeat-10min	NI	Yang and Lüdders, 1993
P	Mature seeds	FD	Tree	NI	1.05% NaOCl-Tw-20min	NI	Abousalim and Mantell, 1992
P	Mature nuts	FD	Mature tree	NI	20% (v/v) domestos - 25min	NI	Barghchi and Alderson, 1983
P	Shoot tips	GH	Seedling	NI	20% (v/v) bleach-10-15min	NI	Barghchi and Alderson, 1985
P	Shoots, forced sprouting	GH	Mature tree	NI	20% (v/v) NaOCl – 10min	NI	Onay, 2000

FD-field, GH-green house, h-hour(s), min-minute(s), SS-surface sterilization method, S-sterile, C-cashew, M-mango, P-pistachio, T-Tween 20, SDS-sodium dodecyl sulphate, NI-not indicated

Only Das *et al*. (1996) and Lievens *et al*. (1989) reported on the aseptic culture rates, 3 to 21% and 74% after decontamination of cashew explants from the field and greenhouse, respectively. Although 3 to 21% aseptic cultures of cashew were recorded, cultured explants turned brown and died (Das *et al*., 1996). Also for non-*Anacardiaceae* explants, $HgCl_2$ or 1 to 10% NaOCl were used (grape, Mhatre *et al*., 2000; olive, Rama and

Pontikis, 1990; pomegranate, Naik *et al*., 1999). Aseptic culture rates of 70-80% and 95% were reported for olive and pomegranate, respectively.

2.1.3   Plant/Tissue nutrition

The healthy growth of a plant requires a well-balanced mineral salt solution. A soil lacking in essential elements does not support healthy growth. Plants grown on such soils exhibit deficiency symptoms and so will explants grown on deficient media in culture (Taiz and Zeiger, 1991; Narayanaswamy, 1994). Plants are autotrophic with regard to organic nutrition because of their photosynthesizing ability whereas explants in culture are heterotrophic and thus require sugar. Green plants require 16 elements, of which 4 (C, H, O and N) are derived from the atmosphere and the other 12 (K, Ca, Mg, P, S, Fe, Cu, Mn, Zn, Mo, B, Cl) from the soil, forming the major groups of inorganic macro and micronutrients.

Nutrition of cells and tissues in culture is based on the mineral nutrition of green plants growing in soil. Nutrient media are based on the principle of mobile metabolites within the plant, which are sufficient for the growth of most isolated cells and tissues (Yeoman 1986; Narayanaswamy, 1994). The nutrient solution for most plant tissue cultures consists of five groups of major ingredients. These are inorganic macro and microelements (in the form of mineral salts), a carbon source usually sucrose, vitamins, PGR and organic supplements. The choice of a nutrient medium is an important consideration and is based on its mineral composition. A comparison of the constituents on the main tissue culture media can be found in Gamborg and Phillips (1995).

MS medium was widely used for propagation of *Anacardiaceae* explants (table 4). Non-*Anacardiaceae* explants were propagated on MS (grape, Mhatre *et al*., 2000; pomegranate, Naik *et al*., 1999; guava, Amin and Jaiswal, 1987; jack fruit, Roy *et al*., 1990; macadamia, Mulwa and Bhalla, 2000; lavender, Sanchez-Gras and Calvo, 1996) and WPM (strawberry, Mereti *et al*., 2002; soursop, Lemos and Blakes, 1996; bay laurel, Gavidia *et al*., 1996) media. The choice of media was based on best performance of cultures at optimum PGR concentrations (table 4; grape, Mhatre *et al*., 2000; pomegranate, Naik *et al*., 1999; guava, Amin and Jaiswal, 1987; jack fruit, Roy *et al*., 1990; macadamia, Mulwa and Bhalla, 2000; lavender, Sanchez-Gras and Calvo, 1996; strawberry, Mereti *et al*., 2002; soursop, Lemos and Blakes, 1996; bay laurel, Gavidia *et al*., 1996).

2.1.4   PGR for shoot amplification and elongation

In order to trigger cell division and elongation and to sustain growth of an explant, endogenous PGR levels have to be supplemented in the complete basal media of inorganic salts, sucrose and vitamins. The growth and development of the plants is controlled by gradients of endogenous PGR (Narayanaswamy, 1994), which in the case of explants *in vitro* have to be incorporated at appropriate levels in the media to effect different growth forms. Traditionally, there are five known classes of PGR namely auxins, cytokinins, gibberellins, ethylene and abscisic acid. Recently, a sixth class, brassinosteroids has been described (Creelman and Mullet, 1997; Chory, 2000; Schuster *et al*., 2000). Auxins are required for induction of cell division, cell expansion, formation of adventitious roots, phototropism, geotropism, promotion of apical dominance, flower formation and fruit set and growth. Cytokinins have an essential role in shoot induction

and plant regeneration in most plant species and may also stimulate cell division. Ethylene induces ripening and abscission; causes leaves to droop (epinasty) and promotes senescence. Gibberellins stimulate cell division and elongation; break seed dormancy and speed up germination. Abscisic acid is generally a plant-growth inhibitor, induces dormancy and prevents seed germination, causes abscission of leaves, fruits and flowers and causes stomata to close (Narayanaswamy, 1994). Brassinosteroids are implicated in cell division and elongation, stem elongation, pollen tube growth, leaf bending and root growth inhibition (Creelman and Mullet, 1997; Chory, 2000; Schuster *et al*., 2000).

Auxin and cytokinin are mostly used in plant tissue culture (Pierik, 1987; Narayanaswamy, 1994; Gamborg and Phillips, 1995). The ratio of cytokinin to auxin in the culture media determines the tissue differentiation pathway. Generally a high concentration of cytokinin alone or a relatively high cytokinin to auxin ratio stimulates axillary and/or adventitious bud proliferation on the explant. Low cytokinin concentration favors shoot elongation. A relatively high concentration of auxin to cytokinin stimulates callus formation whereas auxin alone or a high auxin to cytokinin ratio favors root induction. These responses form the basis of micropropagation (Pierik, 1987; Stafford and Warren, 1991; Narayanaswamy, 1994; Chrispeels and Sadava, 1994; Gamborg and Phillips, 1995). Both fruit and forest trees have been micropropagated in different media supplemented with high cytokinin concentration alone or high cytokinin to auxin ratios (table 4).

2.1.4a   PGR for *Anacardiaceae* explants multiplication

Reports on micropropagation of *Anacardiaceae* plants include cashew, mango and pistachio (table 4). MS medium was widely used for propagation of trees belonging to the mango family (table 4). Shoot multiplication was achieved in media supplemented with BA alone (pistachio, Onay, 2000; Barghchi and Alderson, 1985; Martinelli, 1988, cashew, D'Silva and D'Souza, 1992; Lievens *et al*., 1989) or in combination with KN and ZN (cashew, Das *et al*., 1996) or 2iP alone (cashew, Boggetti *et al*., 1999) or ZR alone (cashew, Leva and Falcone, 1990) or in combination with NAA (Barghchi and Alderson, 1983). Bud break, buds per explant and shoots per explant ranged from 56 to 90%, 12 to 40 and 0.89 to 6, respectively (table 4). Microshoots were elongated on the same media (shoot multiplication media) (cashew, Das *et al*., 1996; Lievens *et al*., 1989, pistachio, Onay, 2000; Barghchi and Alderson, 1985; 1983). Incorporation of GA_3 in the media (cashew, Boggetti *et al*., 1999) or reduction of cytokinin concentration (cashew, D'Silva and D'Souza, 1992) resulted in shoot elongation. Shoot length ranged from 3 to 7.8 cm (table 4).

Explant browning of adult field-obtained material was reported by all authors whereas only some authors reported that polyphenols affected explant survival (Das *et al*., 1996; Lievens *et al*., 1989; Thomas and Ravindra, 1997). Different measures were used to alleviate the polyphenol linked explant necrosis such as inclusion of activated charcoal (AC) (Das *et al*., 1996; Thomas and Ravindra, 1997) or ascorbic acid (Lievens *et al*., 1989) in the medium. Frequent transfers of explants to new medium and dark incubation during the first week of culture also alleviated the problem (Das *et al*., 1996). However,

Boggetti *et al*. (1999) reported that browning never inhibited growth of more than 20% of explants. Inclusion of AC and dark incubation favored shoot elongation but suppressed bud sprouting (Boggetti *et al*., 1999). Optimum levels of cytokinin (2iP 1μM) in media stimulated bud break and shoot multiplication whereas BA, ZN and KN (20μM) as well as TDZ (1 to 20μM) led to callus formation (Boggetti *et al*., 1999). Low cytokinin concentrations alone or in combination with GA_3 promoted shoot elongation. Juvenile materials (cotyledonary nodes) resulted in a higher number of shoots per explant (Das *et al*., 1996) than nodal explants, which sometimes lead to branching rather than multiple shoots (Boggetti *et al*., 1999). High cytokinin (often including BA 4 to 9μM) media also promoted budbreak and shoot multiplication for non-*Anacardiaceae.* Seventy seven to 100% budbreak (Mulwa and Bhalla, 2000; bay laurel, Gavidia *et al*., 1996) were reported and shoots per explant varied from 1.5 to 16 (16 in gum kadaya, Purohit and Dave, 1996; 10.7 in macadamia, Mulwa and Bhalla, 2000; 8-10 in apple, Van Nieuwkerk *et al*., 1986) in comparison to reports of 56 to 94% budbreak and 0.89 to 6 shoots per explant for *Anacardiaceae* (table 4).

Table 4. Optimal PGR for shoot multiplication and elongation of *Anacardiaceae*

Plant	Explant	Medium	PGR (µM)	bbg	C/p	% bb	b/e	s/e	sl (cm)	Reference
C	Nodal	½MS	2iP1, GA₃20	NI	NI	70	NI	2	3	Boggetti *et al*., 1999
C	Nodal	L	BA8.9	bbg	NI	80	NI	6	NI	Lievens *et al*., 1989
C	Cotylnode	MS	BA4.4, KN2.32, ZN9.12	NI	NI	90	12	NI	6.5	Das *et al*., 1996
C	Cotylnode	SH	NAA32.2, BA3.55	NI	NI	NI	NI	NI	NI	Leva and Falcone, 1990
C	Cotylnode	LS	KN2.32, IAA 2.64	NI	P	NI	NI	NI	NI	Philip, 1984
C	Cotylnode	MS	BA22.2, BA4.4	NI	NI	NI	40	NI	7.8	D'Silva and D'Souza, 1992
C	Microcutt	MS	ZR14.2	bbg	NI	NI	NI	NI	NI	Leva and Falcone, 1990
C	Leaves	MS	NAA32.2, BA3.55	NI	C	NI	NI	NI	NI	Leva and Falcone, 1990
M	Shoot tip	½MS	NI	NI	NI	70	NI	NI	NI	Thomas and Ravindra, 1997
M	Shoot tip	G	BA4.4, 2iP5.9, IBA2.5, IAA2.6, ZN4.6	NI	NI	94	NI	NI	NI	Yang and Lüdders, 1993
P	Shoot tip	MS	BA8.8	NI	NI	NI	NI	3.7	5.5	Onay, 2000
Pk	Shoot tips	MS	BA17.75	NI	NI	82	NI	2	0.75	Barghchi and Alderson, 1985
Po	Shoot tip	MS	BA17.75	NI	NI	82	NI	1.14	0.95	Barghchi and Alderson, 1985
Pv	Shoot tip	MS	BA17.75	NI	NI	56	NI	0.89	0.22	Barghchi and Alderson, 1985
P	Shoot tip	MS	BA17.75 NAA1.34	NI	NI	NI	5.5	NI	5.4	Barghchi and Alderson, 1983
P	Nodal	MS	BA17.75 NAA1.34	NI	NI	NI	NI	NI	NI	Barghchi and Alderson, 1983
*P*i	Nodal	MS	BA4.44	NI	NI	NI	NI	NI	NI	Martinelli, 1988
Pa	Nodal	MS	BA3.1	NI	NI	NI	NI	NI	NI	Martinelli, 1988
Pv	Nodal	MS	BA8.87	NI	NI	NI	NI	NI	NI	Martinelli, 1988

bbg-bud break and growth, c/p-callus or plantlet, bb-bud break, b/e-buds per explant, s/e-shoots per explant, sl-shoot length, C-cashew, M-mango, P-pistachio, Pk-pistachio cv kalleghochi, Po-pistachio cv ohadi, Pv-*Pistacia vera,* Pi-*Pistacia integerrima,* Pa-*Pistacia atlantica,* cotylnode-cotyledonary nodes, microcutt-microcuttings, MS-Murashige and Skoog medium (1962), 1/2MS, MS at half salts concentration, L-Levoipre medium (Lievens *et al*., 1989), SH-Schenk and Hildebrandt medium (1972), LS-Lin and Staba medium (1961), Yang *et al*., medium (1984), NI-not indicated, PGR abbreviations are listed in table 5

Table 5. Abbreviations of reported auxins, cytokinins and gibberellins

Cytokinins	
BA	Benzyladenine
2iP	N^6-(2-Isopentenyl) adenine
KN	Kinetin
ZN	Zeatin
ZR	Zeatin riboside
TDZ	Thidiazuron
Auxins	
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPA	Indole-3-propionic acid
NAA	Naphthalene acetic acid
Gibberellins	
GA$_3$	Gibberellic acid

2.1.5 Rooting of microshoots

Most plant species require the presence of auxin for efficient root regeneration. External addition of auxin noticeably increases adventitious rooting. As long as the time of application and dose are suitable, stem cuttings or shoots generally respond favorably to external auxin addition. However, the reaction is dependent on the plant type, the auxin nature, the contact duration and the application time (Nemeth, 1986). Commonly used rooting auxins include IAA, IBA and NAA, although the former two seem more active (Nemeth, 1986). The auxinic stimulation is generally at its maximum when high concentrations are applied immediately or just after the cutting process. When auxin is added at too low doses rhizogenesis is not stimulated (Moncousin, 1991). Apart from auxin, plants prefer a low salt concentration for the formation of adventitious roots (Moncousin, 1991). The basal mineral fraction of the media is usually diluted. Sugar is necessary for adventitious root formation (Pierik, 1987). Light generally has a negative effect on root formation, with higher irradiances inhibiting root formation more than

lower irradiances (Pierik, 1987). High temperatures generally promote adventitious root formation (Pierik, 1987; Moncousin, 1991).

2.1.5a   Rooting of *Anacardiaceae*

PGR requirements for rooting of microshoots of *Anacardiaceae* are shown in table 6. Most shoots were rooted on full strength MS media (Das *et al*., 1996; D'Silva and D'Souza, 1992; Leva and Falcone, 1990; Onay, 2000) followed by half strength MS media (Barghchi and Alderson, 1983, 1985) and WPM (Boggetti *et al*., 1999). IBA at different concentrations was generally used. Shoots inoculated on IBA were either incubated at high concentrations for several hours and later transferred to PGR free media (Das *et al*., 1996; Boggetti *et al*., 1999), at intermediate concentration for 16 to 120hours (Lievens *et al*., 1989) or were incubated until root establishment in a low auxin media (Barghchi and Alderson, 1983, 1985; Leva and Falcone, 1990; D'Silva and D'Souza, 1992). Rooting efficiency for cashew and pistachio ranged between 25 and 80% with 1 to 5 roots per plantlet. Rooting efficiencies for many non-*Anacardiaceae* shoots (lavender, 100%, Sanchez-Gras and Calvo, 1996; jack fruit, 95%, Roy *et al*., 1990; strawberry, 92% Mereti *et al*., 2002; pomegranate, 86%, Naik *et al*., 1999; pear, 84%, Baraldi *et al*., 1995) were reported higher than those observed for *Anacardeaceae* (table 6). Rooting efficiencies of other woody shoots such as macadamia (Mulwha and Bhalla, 2000), soursop (Lemos and Blake, 1996) bay laurel (Gavidia *et al*., 1996) and kadaya gum (Purohit and Dave, 1996) however were only 20, 58, 58 and 60%, respectively.

Table 6. Optimal PGR for rooting *Anacardiaceae* shoots

Plant	Media	PGR (µM)	Exposure time (h)	RE (%)	r/p	Reference
Cashew	MS	IBA2460	48	40	2	Das *et al*., 1996
Cashew	WPM	IBA100	120	43	3	Boggetti *et al*., 1999
Cashew	NI	IBA19.68, IBA9.84	16	30	2-3	Lievens *et al*., 1989
Cashew	MS	IAA2.9, IBA4.9	NI	80	3.5	D'Silva and D'Souza, 1992
Cashew	MS	NAA26.85	NI	25	1	Leva and Falcone, 1990
Pistachio	MS	IBA9.8	NI	80	5	Onay, 2000
Pistachio	1/2MS	IBA12.3 /14.7/17.2	NI	80	3	Barghchi and Alderson, 1985
Pistachio	1/2MS	IBA 12.3	NI	57	NI	Barghchi and Alderson, 1983

h-hours, RE-rooting efficiency, r/p-roots per plantlet, NI-not indicated, PGR abbreviations are listed in table 5, MS-Murashige and Skoog medium (1962), WPM-Lloyd and McCown medium (1980)

2.1.6 Transfer from nutrient media to soil

Micropropagated plants do not survive direct transfer from *in vitro* conditions to a greenhouse or field environment. *In vivo* conditions exhibit substantially lower humidity, higher light levels and septic environments that are stressful to micropropagated plants compared to *in vitro* conditions that provide little physiological stress, moreover a carbon source is provided, reducing the need for photosynthesis (Preece and Sutter, 1991).

Plants that have originated *in vitro* differ from the ones produced *in vivo*. Poorly developed cuticle (wax layer), stomata that do not work properly, thin, soft and photosynthetically less active leaves and roots with few or no root hairs characterize *in vitro* grown plants (Pierik, 1987; Preece and Sutter, 1991). Due to the high relative humidity (90-100%) in culture vessels the wax layer is poorly developed and upon transfer to soil extra water will be lost through cuticular evaporation in the lower *in vivo* humidity. Open stomata in tissue culture plants cause the most significant water loss during the first few hours of acclimatization (Pierik, 1987; Preece and Sutter, 1991). Leaves of an *in vitro* plant are not well adapted for the *in vivo* climate. Test tube plants

have smaller and fewer palisade cells to use light effectively and have larger mesophyll air space. *In vitro* plants have been raised as heterotrophs, while they must be autotrophic *in vivo*. Sugar assimilation must be replaced through photosynthesis (Pierik, 1987; Preece and Sutter, 1991). Roots that have originated *in vitro* appear to be vulnerable and not to function properly *in vivo* (few or no root hairs) (Pierik, 1987; Preece and Sutter, 1991). They may quickly die off and must be replaced by newly formed subterranean roots. The poorly developed root system makes *in vivo* growth for such plants very difficult, especially when there is high evaporation. Poor vascular connections in tissue culture plants between the shoots and roots reduce water conduction (Pierik, 1987; Preece and Sutter, 1991).

In vitro plants should be given time to get used to the *in vivo* climate or be allowed to acclimatize and become hardened-off. Acclimatization can take place by allowing the *in vitro* plants to gradually adapt to the lower *in vivo* relative humidity. Keeping the relative humidity high *in vivo* by covering plants for a period with beakers, polyethylene bags or incubate plants in plastic humidity trays results in wax formation in the cuticular layer and cuticular evaporation is reduced (Pierik, 1987; Preece and Sutter, 1991). During this time, a stomatal closure mechanism develops which is another important component of acclimatization (Pierik, 1987). Alternatively, acclimatization is achieved by gradually opening the tube or flask in a sterile environment over a few days to adjust to *in vivo* conditions (Pierik, 1987).

During acclimatization, it is important to avoid infections by fungi and bacteria. The agar (with sugar) should be well rinsed and the soil should be sterilized. Potential insects and

pathogens (bacteria and fungi) should be eliminated, as the *in vitro* plant is often weak (Pierik, 1987; Preece and Sutter, 1991).

2.1.6a   Acclimatization of *Anacardiaceae*

Survival rates for acclimatization of cashew and pistachio plantlets ranged between 28% and 80% (table 7).

Table 7. Acclimatization of *Anacardiaceae* plantlets

Plant	AM (Tb, Pe)	Survival rate (%)	Reference
Cashew	NI	40-50	Boggetti *et al*., 1999
Cashew	NI	NI	Lievens *et al*., 1989
Cashew	NI	28	Das *et al*., 1996
Cashew	Pe	68	D'Silva and D'Souza, 1992
Pistachio	Tb	50	Onay, 2000
Pistachio	NI	70-80	Barghchi and Alderson, 1985

AM-acclimatization method, Tb-transparent beakers, Pe-polyethylene bags, NI-not indicated

Cashew plantlets were weaned in 1:1vermiculite: peat mixture (Boggetti *et al*., 1999) or 1:1:0.25 sand: soil: powdered coconut husk mixture (D'Silva and D'Souza, 1989). Pistachio plantlets were transferred to 1:1 sterile peat: perlite mixture (Onay, 2000) or peat-based compost (Barghchi and Alderson, 1985). Only D'Silva and D'Souza (1989) and Onay (2000) incubated plantlets under polyethylene sheet and transparent beakers, respectively. While all authors (table 7) weaned plantlets in pots, Lievens *et al*. (1989) acclimatized cashew plantlets on Jiffy-7s plugs and sprayed the plantlets with a 1% solution of thirame (TMTD) to prevent fungal infection. Similar sterile soil compositions and acclimatization procedures were reported for other woody non-*Anacardiaceae.* Acclimatization rates varied from 50 to 100% (lavandin, 50-55%, Jordan *et al*., 1998;

apple, 70-100%, Bolar *et al*., 1998; guava, 90%, Amin and Jaiswal, 1987) Fertilization and biological protectants were included occasionally (Bolar *et al*., 1998).

2.2 Somaclonal variation

Since micropropagation is a worldwide-applied tool in the horticultural industry, genetic as well as epigenetic uniformity is important. When propagating using nodal explants somaclonal variation is limited (Gamborg and Phillips, 1995), but stressful conditions and long periods in tissue culture do impact (Skirvin *et al*., 1994). Somaclonal variation can involve small changes such as point mutations to gross ploidy changes and is influenced by intrinsic and extrinsic factors such as nature of explants (Pescheke and Phillips, 1992; Van den Bulk *et al*., 1990), the degree of dedifferentiation the tissues undergo in culture (Snowcroft, 1984; George and Sherrington, 1984; Smith, 1988; Peschke and Phillips, 1992; Skirvin *et al*., 1994), culture age (Smith, 1988; Lee and Phillips, 1988; Peschke and Phillips, 1992; Skirvin *et al*., 1994), culture medium (Snowcroft, 1984; George and Sherrington, 1984; Smith, 1988; Lee and Phillips, 1988; Phillips *et al*., 1994; Skirvin *et al*., 1994) and proliferation rate (Snowcroft, 1984; George and Sherrington, 1984; Skirvin *et al*., 1994). Somaclonal variation has been observed in a number of cases such as in among others, banana (Vuylsteke and Ortiz, 1996; Peraza-Echeverria *et al*., 2001), oil palm (Rival *et al*., 2002), sugar beet (Munthali *et al*., 1996) and wheat (Brown *et al*., 1993) where vegetative apex of the sucker and floral apex of the male inflorescence, somatic embryo, leaf and protoplasts explants, respectively were used. No somaclonal variation after axilary bud or nodal explant proliferation was reported in kiwifruit (Rugini *et al*., 2000) and oak (Barrett *et al*., 1997) whereas in

contrast, nodal explant propagation of tea resulted in DNA polymorphisms between propagated clones and their source plants (Devarumath *et al*., 2002).

2.2.1 Characteristics of plant genomes

Plant cells contain comparatively large amounts of DNA due to polyploidization. Even *Arabidopsis thaliana* with the smallest plant genome contains approximately 125Mb DNA (The *Arabidopsis* Genome Initiative, 2000), which is larger than that of the soil nematode (90Mb) (Cavalier-Smith, 1985). The genetic material carried within plant cells is far in excess of that required to code for all the proteins synthesized during the growth and differentiation of the whole organism. For instance in *Arabidopsis* only 39% and in mung bean only 4% of the total genome are estimated to code for protein and 61 and 96%, respectively are non-coding (The *Arabidopsis* Genome Initiative, 2000; Stafford and Warren, 1991). About 60% of the genome of a "typical" plant is composed of repeated DNA, which is either low or moderately repetitive (Stafford and Warren, 1991). In addition, transposable elements make up a significant proportion of the genomes of higher plants (Okamoto and Hirochika, 2001). These characteristics of plant genomes play a key role in the changes that occur in tissue culture (Stafford and Warren, 1991).

2.2.1a Molecular mechanisms for somaclonal variation

At least four molecular mechanisms account for somaclonal variation. These include chromosomal aberrations (Finnegan, 2001), DNA level aberrations, transposable elements (Finnegan, 2001) and methylation changes (Finnegan, 2001; Peraza-Echeverria *et al*., 2001; Rival *et al*., 2002). Molecular changes associated with tissue culture involve alterations in heterochromatic (late replicating) regions of chromosomes or regions

composed of repetitive DNA sequences (Okamoto and Hirochika, 2001). Changes in late replicating regions lead to breakage of separating chromatids (Kidwell and Osborn, 1993; Phillips *et al.*, 1994; Okamoto and Hirochika, 2001), which in turn affect the cell cycle (Lee and Phillips, 1988; Stafford and Warren, 1991; Phillips *et al.*, 1994). Breakage could also lead to activation of transposable elements, aneuploidy, methylation changes and further chromosome breakage (Peschke and Phillips, 1992; Phillips *et al.*, 1994). Activation of transposable elements can have a range of effects including alterations in gene expression, gene deletions and insertions and chromosome rearrangements (Okamoto and Hirochika, 2001) and it has been associated with loss or gain of gene expression in tissue culture (Groose and Bingham, 1986; Peschke and Phillips, 1992). DNA level aberration could lead to increased synthesis of a specific gene product or to disturbances in developmental timing of gene activity (Stafford and Warren, 1991). However, the position in the DNA where mutation occurred might not impact on the phenotype. For example a mutation in the intron may not change a phenotype. DNA methylation on deoxycytidine residues has been shown to be involved in the regulation of gene expression at transcriptional level (Finnegan, 2001), particularly during the differentiation and dedifferentiation processes and as a response to a variety of environmental stresses (Phillips *et al.*, 1994; Sheldon *et al.*, 1999; Finnegan *et al.*, 2000; Steward *et al.*, 2000), and especially PGR treatments often used in micropropagation protocols (Phillips *et al.*, 1994; Kaeppler *et al.*, 2000; Rival *et al.*, 2002). In palm oil, DNA hypomethylation was recorded in abnormal calli and leaves from "mantled" regenerants upon comparison with their normal counterparts (Jaligot *et al.*, 2000). Peraza-

37

Echeveria *et al*. (2001) recorded 23% DNA hypermethylation in micropropagated banana plants compared to 18.4% in conventionally propagated ones.

2.2.2   Screening for somaclonal variation

Phenotypic analysis, karyological analysis, *in situ* hybridization, measurement of DNA content and molecular techniques have been used to screen for somaclonal variation. Each of these methods poses its own limitations and opportunities.

Phenotypic analysis of regenerants remains the most conclusive and easiest method of screening for somaclonal variation. However, limitations include the time required to discover the change and that the appearance of a normal phenotype under one or few particular environments is no guarantee that molecular changes have not occurred and that such changes would not lead to a phenotype in another environment. The karyological analysis of plants can very often reveal significant chromosomal changes such as alterations in ploidy levels and structural re-arrangements. Molecular techniques such as restriction fragment length polymorphism (RFLP) (Colombi *et al*., 1987; Devarumath *et al*., 2002), random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990; Brown *et al*., 1993; Munthali *et al*., 1996; Rival *et al*., 1998; Linacero *et al*., 2000; Devarumath *et al*., 2002), amplification fragment length polymorphism (AFLP) (Vos *et al*., 1995; Polanco and Ruiz, 2002) and methylation sensitive amplification polymorphism (MSAP) (Reina-Lopez *et al*., 1997; Xiong *et al*., 1999; Peraza-Echeverria *et al*., 2001; Rival *et al*., 2002) detect changes in a DNA sequence and its methylation. RFLP is time-consuming and the results of such analysis are limited only to the gene sequence used as a probe. As several hypervariable regions are being identified, RFLP

may become more useful for detecting somaclonal variation. Among the PCR-based techniques, RAPD is the simplest and fastest; the amount of starting material required is small and a very large number of arbitrary primers are commercially available (Brown *et al.*, 1993). The more technically challenging AFLP and MSAP result in a multitude of markers with few primer combinations and like RAPD, they do not require prior knowledge of sequence information. Each method only analyses a subset or particular aspect of the DNA.

2.2.2a Use of RAPD to detect somaclonal variation

Table 8 lists reports where RAPD analysis was used to determine somaclonal variation. Many authors reported the absence of intraclonal variation between *in vitro* and source plants in the presence of interclonal variation. Munthali *et al.* (1996), Brown *et al.* (1993), Rival *et al.* (1998), Linacero *et al.* (2000) and Devarumath *et al.* (2002) detected polymorphisms between *in vitro* and source plants in sugar beet, wheat, oil palm, rye and tea, respectively.

Table 8. The use of RAPD analyses to assess somaclonal variation

Plant	Explant type	Bands scored	Number of markers	Remarks	Reference
Sugar beet	L	5607	31	Polymorphic	Munthali *et al*., 1996
Wheat	P	NI	NI	Polymorphic	Brown *et al*., 1993
Oil palm	SE	8900	259	Polymorphic	Rival *et al*., 1998
Rye	SE	NI	NI	Polymorphic	Linacero *et al*., 2000
Tea*	N	NI	NI	Polymorphic	Devarumath *et al*., 2002
Ginseng	SE	NI	NI	Monomorphic	Shoyama *et al*., 1997
Japanese pine	L	4824	134	Monomorphic	Goto *et al*., 1998
Poplar	SE	NI	NI	Monomorphic	Rani *et al*., 1995
Spruce	SE	900	10	Monomorphic	Isabel *et al*., 1993
Begonia*	L	8000	200	Monomorphic	Bouman and De Klerk, 2001
Asparagus*	SE	12089	157	Monomorphic	Raimondi *et al*., 2001
Norway spruce*	SE	2154	5	Monomorphic	Fourre *et al*., 1997
Oak*	N	4260	142	Monomorphic	Barrett *et al*., 1997
Oak*	SE	NI	NI	Monomorphic	Wilhem, 2000
Kiwifruit	AB	NI	NI	Momomorphic	Rugini *et al*., 2000

*-Other methods like DNA content measurement, cytological and morphological were also used, L-leaf, P-protoplasts, SE-somatic embryo, N-nodal section, AB-axillary bud, NI-not indicated

Munthali *et al*. (1996) reported that RAPD analysis revealed two and one band polymorphisms for the first and second-generation regenerants, respectively. In oil palm, polymorphism between clones was detected, however, no intraclonal variability was identified between source and regenerated plants, despite the presence of "mantled" phenotypes (Rival *et al*., 1998). This mutant phenotype correlated with changes in DNA methylation at CCGG sites as detected by MSAP but undetectable by RAPD (Rival *et al*., 2002). MSAP also detected 3% and 1.7% polymorphisms of micropropagated banana plants from male inflorescences and sucker explants (Peraza-Echeverria *et al*., 2001). In tea, parents (U3 and U27) and their clones were genetically similar as detected by RAPD, RFLP and inter simple sequence repeats (ISSR) (Raina *et al*., 2001; Devarumath *et al*., 2002) analyses. RFLP, ISSR and RAPD analyses concurred on 7.7% polymorphism among U26 parents and clones (Devarumath *et al*., 2002).

2.3 *Agrobacterium tumefaciens*-mediated transformation

The most widely used method for the introduction of new genes into plants is based on the natural DNA transfer capacity of *Agrobacterium tumefaciens*. In nature, this gram-negative soil bacterium causes tumor formation (crown galls) on a large number of dicotyledonous as well as some monocotyledonous plant species and gymnosperms (Stafford, 2000). Development of crown galls is dependent on the genetic transfer from *Agrobacterium tumefaciens* of a section of DNA (the T-DNA i.e., transfer-DNA) present on a large extrachromosomal plasmid, called the Ti (tumor-inducing) plasmid to the plant cells (Van Larebeke *et al*., 1975). The wild type T-DNA harbours plant-like genes that encode the production of PGR (auxins and cytokinins) responsible for rapid proliferation resulting in tumours.

Three genetic elements are required for T-DNA transfer to plants. The first of these elements are the T-DNA border sequences that consist of 24- or 25-base pair (bp) direct repeats flanking and defining the T-DNA. T-DNA transfer is independent of the DNA sequence located between its borders and usually all DNA sequences between the borders are transferred to the plant (Gheysen *et al*., 1997; de la Riva *et al*., 1998; Stafford, 2000). The second element consists of the virulence (*vir*) genes encoded by the Ti-plasmid in a region outside of the T-DNA. Vir proteins interact with plant signal molecules, process the T-DNA from the tumor-inducing plasmid, transfer the T-DNA from the bacterium to the plant nucleus and interact with plant host nuclear proteins to effect T-DNA integration in the plant genome (Gheysen *et al*., 1997; de la Riva *et al*., 1998; Stafford, 2000). The third bacterial element necessary for T-DNA transfer consists of a number of

chromosomal genes of which some are important for attachment of the bacterium to the plant cell. Plant signal molecules also play a role in the T-DNA transfer process (Gheysen *et al*., 1997; de la Riva *et al*., 1998; Stafford, 2000).

Genetic colonization of plant cells by *A. tumefaciens* and subsequent crown gall development proceeds in a set manner. Firstly the plant cells must be wounded to allow for entrance of the bacterium and to secrete compounds that induce its virulence system. According to Gheysen *et al*. (1997) and Stafford (2000) the infection process starts with the recognition of wounded plants by *Agrobacterium* in which chemotaxis plays an important role. The bacterium is chemotactic to a wide range of sugars and amino acids as well as to specific secondary plant metabolites such as phenolics produced by damaged plant cells. A second step in the infection is the attachment of the bacterium to the plant cell walls. The bacterial chromosomal virulence (*chv*) genes are mainly involved during this stage. Hooykaas and Schilperoort (1984) reported that ChvA and ChvB proteins are associated with the binding of the bacterium to the host-cell surface receptors. A third step is the interaction of ChvE with VirA thereby making VirA supersensitive for activation by phenolic compounds secreted by wounded plant cells. Binding of phenolic compounds to VirA-ChvE complex leads to autophosphorylation of VirG, which in turn activates transcription of other *vir* genes (Stafford, 2000). The next step is the production of a T-DNA transfer intermediate. VirD1 and VirD2 recognize the T-DNA border sequences and produce nicks. These nicks determine the initiation and termination sites for T-strand formation. This T-strand travels through prokaryotic and eukaryotic membranes and cellular compartments by means of association with certain Vir proteins. VirD2 at the 5$^{'}$ end of the T-strand acts as a pilot protein, whereas VirE2

42

binds to this single-stranded T-DNA-VirD2 unit, protecting it from plant nucleases and forming the so-called T-complex (Cook *et al*., 1997; de la Riva *et al*., 1998). The T-complex is then transferred through the nuclear pore aided by nuclear-localization signals (NLS) in VirD2 and VirE2, which interact with the endogenous NLS machinery, followed by integration of the T-DNA into the plant genome (Stafford, 2000). Specific interaction between host DNA repair proteins, the T-complex and possibly other Vir proteins may underpin the high frequency and integration of the T-DNA in the plant genome (de la Riva *et al*., 1998; Stafford, 2000).

2.3.1 *Agrobacterium* transformation methods

Agrobacterium-mediated transformation can be accomplished through either a regeneration-dependent or a regeneration-independent transformation.

2.3.1a Regeneration-dependent transformation

Regeneration-dependent transformation involves *de novo* regeneration of explant tissues (leaf, root, stem section, etc.). *De novo* regeneration is the ability of some somatic plant cells to dedifferentiate, proliferate and reorganize into shoot meristems (organogenesis) or embryos with shoot and root meristems (somatic embryogenesis) (Tisserat, 1985; Stafford and Warren, 1991; Gamborg and Phillips, 1995; Bornman, 1998). Regeneration is usually indirect where a callus phase precedes shoot or embryo formation (Gheysen *et al*., 1997). Direct organogenesis requires a relatively high cytokinin to auxin ratio whereas indirect organogenesis needs a high concentration of auxin and low concentration of cytokinin to promote callus formation (Tisserat, 1985; Stafford and Warren, 1991; Gamborg and Phillips, 1995). Callus is transferred to a medium with

lowered auxin concentration and a similar or higher cytokinin concentration to promote shoot development (Tisserat, 1985; Stafford and Warren, 1991; Gamborg and Phillips, 1995). In both cases, shoots are transferred to a different culture medium for root formation (Tisserat, 1985; Stafford and Warren, 1991; Gamborg and Phillips, 1995). Explants used for the direct shoot regeneration of woody plants include cotyledonary leaves (Asian pea pear, Kaneyoshi *et al*., 2001) and leaves (high bush blueberry, Callow *et al*., 1989; apple, Predieri and Malavasi, 1989; Maheswaran *et al*., 1992; spring glory, Rosati *et al*., 1996; plum, Yancheva *et al*., 2002; fig, Yancheva *et al*., 2004). Explants were cultured on media supplemented with BA and NAA (22 and 1 μM, Maheswaran *et al*., 1992), TDZ and IBA (7.49 and 2.46 μM, Yancheva *et al*., 2002; 9.08 and 9.84 μM, Yancheva *et al*., 2004), BA and IAA (4.44 and 2.85 μM, Rosati *et al*., 1996), 2iP alone (25 μM, Callow *et al*., 1989) or TDZ alone (10 μM, Predieri and Malavasi, 1989). Indirect regeneration from petioles of kiwifruit was on media supplemented with 4.5 ZN and 0.1 μM IAA (Gonzalez *et al*., 1995) and of eucalyptus nodes was on 0.04 μM picloram and 2.25 μM BA supplemented media (Herve *et al*., 2001) and both involved a callus stage. Indirect regeneration via somatic embryogenesis requires a high concentration of auxin to induce proembryos (Tisserat, 1985; Stafford and Warren, 1991; Gamborg and Phillips, 1995). Callus containing proliferated proembryos is then transferred to PGR-free medium to induce bipolar embryo formation and development (Tisserat, 1985; Stafford and Warren, 1991; Gamborg and Phillips, 1995). Nucellar tissue was used to induce somatic embryos in cashew (Cardoza and D'Souza, 2002; Das *et al*., 1999) and mango (Litz *et al*., 1984, 1998; Mathews *et al*., 1993) by supplementing media

with 2,4-D alone (9.05 μM, Litz *et al*., 1984; 4.52 μM Litz *et al*., 1998) or picloram (2.07 μM, Cardoza and D'Souza, 2002).

Explants obtained from the field have been used for regeneration. *In vitro* derived explants however were preferred as it eliminated the need for surface sterilization that could lead to tissue damage (Predieri and Malavasi, 1989).

Although *de novo* regeneration forms the basis of the vast majority of transformation procedures, it is often a bottleneck for plant species that lack a suitable regeneration method and thus results in failure to develop a transformation procedure (Gheysen *et al*., 1997).

2.3.1b Regeneration-independent transformation

Regeneration-independent (meristems-shoot apices, axillary buds) transformation forms an attractive target for transformation as it contains the meristematic cells from which all aerial parts of a plant derive during the normal course of plant development. Targeting these cells for transformation has, therefore, the advantage that transformed cell lineages, forming part of an organized shoot can be obtained without the empirical development of a *de novo* regeneration pathway. Transformation can be applied *in situ* (on meristems that are still part of a plant), on excised meristems or shoot tips. *In vitro* development of these explants into plantlets can be accomplished with minimum tissue culture manipulations (Smith and Murashige, 1970; Gheysen *et al*., 1997). Moreover, plants originating from the culture of apical or axillary meristems are considered to show no somaclonal variation (Karp, 1991; Gheysen *et al*., 1997).

Since meristems are multicellular, primary transformants from a meristem transformation procedure are expected to be chimeric, consisting of transformed and untransformed sectors. Several reports (Knittel *et al.*, 1994; Park *et al.*, 1996; Bean *et al.*, 1997; Gould and Magallanes-Cedeno, 1998; Han *et al.*, 2000) clearly prove the validity of the meristem transformation approach for obtaining transgenic plants. The reports demonstrate the development of (chimeric) primary transformants from meristems present at the time of transformation and give conclusive evidence for stable transformation of the offspring of these primary transformants. Important crop species transformed with *Agrobacterium*-mediated meristem transformation include sunflower (*Helianthus annus*) (Malone-Schoneberg *et al.*, 1994) and rice (*Oryza sativa*) (Park *et al.*, 1996).

Various ways to enhance the efficiency of meristem transformation have been devised. These methods usually consist of treatments favoring the formation of larger transgenic sectors in the primary transformants combined with the use of screenable or selectable markers to visualize or enrich for transgenic tissue. Both mechanical such as bombardment with uncoated particles (Knittel *et al.*, 1994; May *et al.*, 1995), bisection and wounding with a needle or scalpel (Park *et al.*, 1996; Bean *et al.*, 1997) and hormonal treatments (Gheysen *et al.*, 1997) have been used to promote the formation of large transgenic sectors in chimeric shoots. The damage caused to the plant tissue probably results in meristem reorganizations. Descendants from a single meristematic cell may form clonal sectors larger than normal and/or invade other meristem cell layers (McCabe and Martinell, 1993). Another approach to obtain shoots with a higher proportion of transformed cells from meristems is the induction of axillary buds. Since axillary buds

are generally smaller than apical meristems, a cell lineage derived from a single transformed cell is expected to contain a relatively large portion of such buds (Gheysen *et al*., 1997). Treatments favoring chimeric shoots with large transgenic sectors only improve the efficiency of meristem transformation if one can enrich for these shoots or if they can be visualized or selectively cultured. The use of selective agents at sublethal concentrations has been used successfully to enrich for transformed cells in shoots (Malone-Schoneberg *et al*., 1994; Knittel *et al*., 1994; Lowe *et al*., 1995). The advantages of regeneration-independent *in vitro, in vivo* and *in planta* transformation procedures have also been exploited in trees (Van Beveren *et al.,* 2003)

2.3.2   Cocultivation with *Agrobacterium*

Cocultivation refers to the period that explants and *Agrobacterium tumefaciens* are cultivated together and during which gene transfer takes place (de la Riva *et al*., 1998). The conditions during the precultivation of the *Agrobacterium* and plant material as well as during cocultivation affect the efficiency of transformation. *Agrobacterium* strain, concentration, vector system, inclusion of PGR, *vir*-inducing compounds such as acetosyringone and wounding of explants also influence the transformation frequencies (de Villiers, 1998; Gheysen *et al*., 1997). Wounding may enhance the accessibility of plant cells to bacteria, stimulate the production of *vir* gene inducers and enhance plant cell competence for transformation (Gheysen *et al*., 1997).

Table 9 lists some empirically determined cocultivation conditions for transformation of woody plants.

Table 9. Cocultivation period and conditions in woody plant transformation

Plant	Acetosyringone (µM)	Wounding +, -	Cocultivation time (d)	GUS+ ve (%)	R (%)	Reference
Tamarillo	20	-	2	35	NI	Atkinson and Gardner, 1993
Spruce	25	-	2	90	NI	Wenck *et al*., 1999
Grape	50	+	3	NI	NI	Torregrosa and Bouquet, 1997
Tea	10-50	+	5	10	NI	Mondal *et al*., 2001
Pine	50	-	2	60	NI	Wenck *et al*., 1999
Apple	100	-	2	NI	NI	Bolar *et al*., 1999
Guayule	100	+	3	NI	<1	Pan *et al*., 1996
Poplar	200	-	3	NI	13	Confalonieri *et al*., 1995
Tea	500	-	NI	NI	NI	Matsumoto and Fukui, 2002

+-wounding of explants, --no wounding, d-days, GUS+ve-GUS-positive cocultivated explants, R-selective agent resistant plants/shoots, NI-not indicated

In grape (Torregrosa and Bouquet, 1997), pine (Wenck *et al*., 1999) and rice (non-woody) (Park *et al*., 1996) transformation, 50µM acetosyringone was included in the cocultivation media and *Agrobacterium* suspension. Atkinson and Gardner (1993) reported that addition of acetosyringone resulted in 35% transformation efficiency of tamarillo. Whereas Confalonieri *et al*. (1995) reported that acetosyringone increased the transformation efficiency of poplar callus and plantlets from 19.8 and 3% to 55.5 and 13.3%, respectively. However, in spruce, higher levels of acetosyringone (100µM or greater) were not beneficial as a phytotoxic effect was observed (Wenck *et al*., 1999). Explant wounding was used in guayule (Pan *et al*., 1996), poplar (Confalonieri *et al*., 1995), and grape (Torregrosa and Bouquet, 1997). In contrast, Mondal *et al*. (2001) reported that neither acetosyringone nor wounding enhanced the efficiency of transformation in tea (10% compared to 40% for control). The authors suggested that the inherent prevalence of high amounts of phenolics generally observed upon wounding woody plant tissues and the high rate of tissue browning are deleterious to explants survival and reduced transformation efficiency.

2.3.3   Transient expression

Optimization of transformation protocols can be achieved by monitoring transgene expression in the period immediately after cocultivation before integration of the transgene (T-DNA) into the plant genome (Janssen and Gardner, 1993; Pounti-Kaerlas, 1998). The expression lasts only as long as there are copies of the transgene in the plant cell nuclei (Pounti-Kaerlas, 1998). Advantages of using transient expression in the development of a gene transfer system are; experiments can be cycled weekly and cells that are "competent" for gene transfer can be localized. If the location of the cells that can regenerate is also known then gene transfer can be optimized specifically for these cells. Finally, transient expression allows gene transfer to be assayed separately from regeneration. In difficult-to-regenerate species, this feature greatly assists in obtaining the first transgenic tissue, a crucial step in developing a gene transfer system (Janssen and Gardner, 1993). Vancanneyt *et al*. (1990), reported first on the use of intron-containing β-glucuronidase (GUS) encoding *uid*A gene in monitoring early events in *Agrobacterium*-mediated plant transformation of *Arabidopsis* cotyledons and roots. The intron-containing GUS gene allows monitoring of GUS activity in transgenic plants without background bacterial expression. Transgenic plants containing the chimeric gene efficiently splice the intron, giving rise to GUS enzymatic activity whereas no GUS activity is detected in *Agrobacterium* due to the lack of an eukaryotic splicing apparatus in prokaryotes (Vancanneyt *et al*., 1990). The most widely used substrate for histochemical localization of GUS activity in tissues and cells is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The product of GUS action on X-Gluc is colourless, but it undergoes an oxidative reaction to form the GUS characteristic insoluble blue dye. The oxidation process is

stimulated by atmospheric oxygen and enhanced by an oxidation catalyst (potassium ferricyanide and ferrocyanide) present in the X-Gluc solution (de Villiers, 1998).

Several authors subsequently reported on GUS transient expression (Uematsu *et al*., 1991; Janssen and Gardner, 1993; de Jong *et al*., 1993; Atkinson and Gardner, 1993; Knittel *et al*., 1994; Dronne *et al*., 1999; Wenck *et al*., 1999; Rao and Rohini, 1999; Droste *et al*., 2000; Chateau *et al*., 2000; Mondal *et al*., 2001; Amoah *et al*., 2001; Khanna and Daggard, 2002). GUS-positive efficiencies varied among plants, with 35% observed in tamarillo (Atkinson and Gardner, 1993), 50% in kiwifruit (Janssen and Gardner, 1993), 1 to 40% dependent on cultivar in lavender (Dronne *et al*., 1999), 41% and 50% in sunflower (Knittel *et al*., 1994; Rao and Rohini, 1999), 60% in pine and 90% in spruce (Wenck *et al*., 1999), 51.7% and 35 to 63% in wheat (Amoah *et al*., 2001; Khanna and Daggard, 2002) and 14 to 69% in different *Arabidopsis thaliana* genotypes (Chateau *et al*., 2000), respectively. Dronne *et al*. (1999), Chateau *et al*. (2000) and Amoah *et al*. (2001) observed 3 to 6 spots per lavender explant, 0.5 to 10.1 spots per *Arabidopsis thaliana* explant and 33.8 spots per wheat explant, respectively. Although the number of blue spots or zones was not presented, some authors reported on the location of GUS-expressing cells. In *Arabidopsis* explants, blue staining cells were observed at the cut surface, in the neighborhood of vascular tissue, and in epidermal cells (Vancanneyt *et al*., 1990). Janssen and Gardner (1993) observed small zones of or individual blue-staining cells in kiwifruit rather than the large blue zones in petunia. Blue-stained cells of kiwifruit or petunia were found near veins and were evenly distributed on all sides of the leaf (Janssen and Gardner, 1993). In chrysanthemum and tea most blue spots were observed on the cut margins rather than the central-wounded

region and on the surface of somatic embryos, respectively (de Jong *et al*., 1993; Mondal *et al*., 2001). Yancheva *et al*. (2004) observed large blue sectors on leaf explants of fig. In transgenic citrus (Cervera *et al*., 2000) and sorghum (non-woody) (Emani *et al*., 2002) the intensity of staining was different between explants due to transgene copy number (Cervera *et al*., 2000) and transgene/promoter methylation (Emani *et al*., 2002).

2.3.4 Selection of putative transformants

The most widely used selectable marker genes include the neomycin-phosphotransferase-II gene (*npt*II), which encodes resistance to aminoglycoside antibiotics like kanamycin, neomycin, geneticin and paromycin; hygromycin phosphotransferase gene (*hpt)*, which encodes resistance against the antibiotic hygromycin and phosphinothricin acetyltransferase gene (*bar)*, which encodes resistance against the herbicide phosphinothricin (Puonti-Kaerlas, 1998; Kunert, 1998; Brasileiro and Aragao, 2001). The presence of selectable marker genes in the transgenes renders resistance of putative transformed plant cells, tissues or plants to the antibiotic or herbicide. The optimum concentration of the selective agent must kill non-transformed explants and prevent regeneration of untransformed cells. This concentration is genotype-dependant and determined empirically (Puonti-Kaerlas, 1998; Kunert, 1998). Optimal selective agent concentration either leads to explant browning (Bean *et al*., 1997), bleaching (Knittel *et al*., 1994; Gould and Magallanes-Cedeno, 1998; Mondal *et al*., 2001) or death (Matsumoto and Fukui, 2002; Joung *et al*., 2001) of non-transformed tissues. Selective agents, apart from being useful in identifying putative transformants, have been implicated in inhibition of shoot formation and elongation (Xie and Hong, 2002),

regeneration (Yancheva *et al*., 2002) and have lead to the production of phenotypicaly

abnormal plants (Bean *et al*., 1997).

Several authors reported on different agents used to select putative transformants.

Kanamycin at concentrations from 25 to 400mgl^{-1} was mostly used (table 10).

Table 10. Selection agents for putative transformants

Plant	SA (Kn, Pp, G)	Co (mgl^{-1})	TE (%)	Reference
Apple	Kn	50	NI	Bolar *et al*., 1999
Cotton	Kn	50	NI	Gould and Magallanes-Cedeno, 1998
Guayule	Kn	50	NI	Pan *et al*., 1996
Bellflower	Kn	50	7	Joung *et al*., 2001
Plum	Kn	50	2.7	Yancheva *et al*., 2002
Sunflower	Kn	50	NI	Knittel *et al*., 1994
Tea	Kn	50	NI	Mondal *et al*., 2001
Apple	Kn	100	NI	Sriskandarajah *et al*., 1994
Cottonwood	Kn	100	NI	Han *et al*., 2000
Kiwifruit	Kn	100	NI	Jansen and Gardner, 1993
Tamarillo	Kn	100	NI	Atkinson and Gardner, 1993
Tea	Kn	200	NI	Matsumoto and Fukui, 2002
Mango	Kn	400	NI	Mathews *et al*., 1993
Rice	Pp	0.5	2.8	Park *et al*., 1996
Peas	Pp	2.5	NI	Bean *et al*., 1997
Mandarin	Pp	50	NI	Li *et al*., 2002
Acacia	G	20	NI	Xie and Yong, 2002

SA-selection agent, Kn-kanamycin, Pp-phosphinothricin, G-geneticin, Co-concentration,
TE- transformation efficiency, NI-not indicated

Phosphinothricin at 0.5mgl^{-1}, 2.5mgl^{-1} and 50mgl^{-1} were used to select putatively

transformed rice (Park *et al*., 1996), peas (Bean *et al*., 1997) and mandarin (Li *et al*.,

2002), respectively. Transformation efficiencies were recorded as 2.8% and 0.4 to 13.8%

in rice (Park *et al*., 1996; Muniz de Padua *et al*., 2001), 2.7% in plum (Yancheva *et al*.,

2002) and 7% in bellflower (Joung *et al*., 2001), respectively.

CHAPTER 3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Source of explant materials

Marula seeds gathered from the wild and obtained from Mirma Products, a marula fruit processor in Phalaborwa, South Africa, were dried and germinated in sterile vermiculite or soil and maintained at $25^{\circ}C$, $81\mu molm^{-2}s^{-1}$ and 12h day/night cycle. Seedlings between 2 and 24 months were used as a source of explant materials. During the dry season (February-March) and the rainy season (October-November) old shoots (more than 5 months old) and actively growing 2-month-old shoots, respectively were collected from wild marula trees growing at the University of the North, main campus.

3.1.2 *Agrobacterium* strain and plasmid

Agrobacterium tumefaciens strain LBA4404 harboring the binary vector p35SGUSintron, a kind donation from the Agricultural Research Council (ARC)-Roodeplaat Vegetable and Ornamental Plant Institute, South Africa was used for genetic transformation of marula nodal explants. The construct (p35SGUSintron) (Vancanneyt *et al*., 1990) contained a nopaline synthase (NOS) promoter- neomycin phosphotransferase (*npt*II) selectable marker gene- NOS terminator poly-A sequence and a β-glucuronidase (*uid*A) reporter gene with a portable plant intron under the regulatory control of the cauliflower mosaic virus (CaMV) 35S promoter and CaMV35S terminator sequence (figure 1)(Vancanneyt *et al*., 1990). To prevent background expression of the marker gene in *A. tumefaciens*, the GUS gene is interrupted by a plant intron that can be spliced from the

primary transcript in plants but not in bacteria thus restricting GUS activity to transformed plant cells only (Vancanneyt *et al*., 1990).
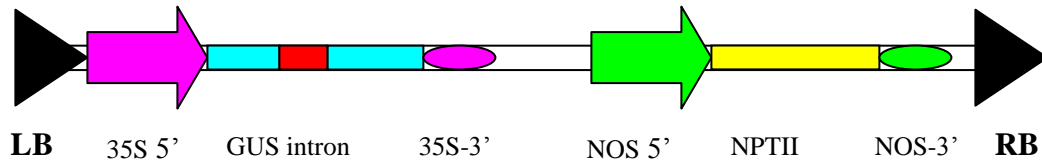
Figure 1. T-DNA region of plasmid p35SGUSintron.

LB Left border of T-DNA. RB right border of T-DNA. 35S 5', 5' promoter region of CaMV35S gene. GUS-intron, *iudA* gene separated by plant intron. 35S3', 3' terminator region of the CaMV35S gene. NOS 5', 5' promoter region of the nopaline synthase gene. NPTII, *NPT*II gene. NOS 3' 3' terminator region of nopaline synthase gene.

3.1.3   Media, buffer and enzymes

All media salts, PGR, vitamins, buffers, molecular weight markers and molecular biology chemicals were purchased from Sigma Aldrich. *ExTaq*™ was purchased from *TaKaRa* and primers (table 11) were purchased from Genosys. SEDADIX NO.2 rooting powder was purchased from Sterkloop Garden Pavilion, Polokwane, South Africa. X-Gluc substrate (Research Organics 11-77-B) was kindly donated by Dr C. Koncz, Max-Planck-Institute for Plant Breeding, Cologne, Germany.

Table 11. Genosys primers and their sequences

Primer name	GC%	Sequence
Gen 1-60-04	60	GTCCTTAGCG
Gen 1-60-06	60	CTACTACCGC
Gen 1-60-08	60	GTCCTCAGTG
Gen 1-60-09	60	CGTCGTTACC
Gen 1-60-10	60	GCAGACTGAG
Gen 1-70-06	70	GGACTCCACG
Gen 1-70-09	70	TGCAGCACCG

3.1.4 Tissue culture media

Murashige and Skoog (MS) basal medium (1962) without glycine at full and half strength salts, 3% sucrose, 0.6% agar and supplements of 0.08% or 0.1% PVP or 0.3% AC was used in the research work. Lloyd and McCown (WPM) basal medium (1980) at full strength salts, 2% sucrose, 0.6% agar and 0.1% PVP was also used alongside MS medium at 4.8μM BA and 2.4μM KN. MS and WPM media were prepared from stock solutions of inorganic salts, vitamins, PGR, sucrose and agar. The media pH was adjusted to 5.8 and 5.6 for MS and WPM media, respectively dispensed in baby food jars and autoclaved at 121°C for 20 minutes. Heat labile components were filter sterilized (0.22μm) and added to the autoclaved media (Sigma bioscience™, 1996).

3.2 METHODS

3.2.1 Surface sterilization of explant materials

Eight centimeter long shoots harvested from growth room seedlings and field-grown trees were defoliated and kept in water or fungicide (some of field materials) during transfer to the laboratory.

Seedling explant materials (nodal sections with axillary buds, hypocotyl, shoot tips, leaves and roots) were washed several times in running tap water and rinsed with distilled water prior to surface sterilization. The surface sterilization methods consisted of immersion in 70% ethanol for 1 minute, followed by 10%, 20% or 30% (v/v) bleach (JIK) solution containing 0.1% (v/v) Tween 20 for 20, 15 and 10 minutes, respectively. Explants were rinsed 5 times with sterile distilled water prior to inoculation on tissue culture media. Each treatment consisted of 10 explants repeated twice.

Explant materials from mature field plants harvested in February-March were decontaminated by immersion in 70% ethanol for 2 minutes followed by 20%, 30% (v/v) bleach (JIK) solution with or without 0.1% (v/v) Tween 20 for 5, 10, 15, 20, 30 and 50 minutes. Each treatment consisted of 10 explants repeated twice.

Explant materials from the field harvested in October-November were surface sterilized by immersion in 70% ethanol for 2 minutes followed by 30% (v/v) bleach (JIK) solution containing 0.1% (v/v) Tween 20. Additional steps for explant materials from the field harvested in October-November included:

Washing with antibacterial dish washing liquid for 10 minutes and running tap water prior to surface sterilization.

Collection and soaking in fungicide (Funginex, active ingredient- Triforine 190mg/liter) solution (20ml funginex/10 liters of water) for 2 hours, rinsing thoroughly under running tap water followed by washing with antibacterial dish washing liquid for 10 minutes and rinsing with tap water prior to surface sterilization.

Washing with antibacterial dish washing liquid for 10 minutes and tap water prior to surface sterilization followed by dipping in filter sterilized fungicide solution before tissue culture inoculation.

Each treatment consisted of 10 and 11 explants. Contamination frequencies were assessed 7 and 14 days after inoculation and were scored as the percentage clean/aseptic explants of the total inoculated.

3.2.2 Tissue culture conditions

All cultures were incubated in a growth room at 25 ± 2^oC at a 16-hour photoperiod with light at $81 \mu molm^{-2}s^{-1}$ provided by cool-white and red horticultural tube lights.

3.2.3 Microshoot multiplication and elongation

Surface sterilized nodal explants 1.5 cm long containing axillary buds were inoculated on MS medium supplemented with 0.1% (w/v) polyvinylpyrrolidone (PVP) and combinations of 1.2 to 6.0μM BA and 1.2 to 6.0μM KN. Initial experiments included all BA and KN concentrations and combinations. In subsequent experiments only those BA and KN concentrations and combinations with a bud break above 50% and more than 1 shoot per responding explant were repeated. Treatments were thus repeated 2 to 7 times with 5 and 15 explants each, respectively. Bud break, number of shoots per explant and callus formation were observed after 2 and 4 weeks, respectively.

Shoots from multiplication medium were transferred to elongation medium. MS basal medium at full strength inorganic salts supplemented with low concentrations of BA and

KN (1.2 and 1.0μM, respectively), GA$_3$ (1 and 5μM) and MS medium without PGR were evaluated. Each treatment was repeated twice with 10 and 15 explants, respectively. The number of elongated shoots was scored after 4 weeks.

3.2.4 Rooting of elongated microshoots

Elongated shoots with 2 to 3 new leaves were isolated and transferred to MS medium without PGR for 2 weeks prior to transfer to rooting medium (MS medium at half strength inorganic salts) supplemented with different concentrations of IBA (0,1, 3, 5 and 10μM), 0.3% (w/v) AC or 0.08% (w/v) PVP. In order to create dark conditions, the bottoms of some of the cultures in PVP-supplemented medium were wrapped with catering foil. Each treatment consisted of 14 shoots duplicated twice. The number of rooted shoots was scored after 4 to 6 weeks.

3.2.5 Acclimatization of the plantlets

Rooted plantlets were washed with sterile water to remove medium from roots and plantlets were transferred to baby food jars containing sterile vermiculite supplemented with MS inorganic salts at half strength concentration. After 1 week the caps were gradually lifted to lower the relative humidity in the baby food jars. After 2 week, plantlets were transferred to sterile garden soil (in pots) and covered with beakers. Beakers were gradually lifted and removed after 2 to 4 weeks. Survival of plants was scored after 4 to 6 weeks in the growth room and acclimatized plants were reported as a percentage of the total. The procedure was repeated twice for 20 plantlets each.

3.2.6 Somaclonal variation

Parents and cloned plants were assigned identifications: NS9P with clones NS9-1 to NS9-7, NS2P with its clone NS2, NS18P with its clone NS18, NS16P with its clone NS16, NS6P without clones. Leaves from hardened *in vitro* grown plants and parent plants (source of explants) were harvested in liquid nitrogen in duplicate and tested as independent samples.

The CTAB method of Saghai-Maroof *et al*. (1984) and Rogers and Bendich (1985) as modified by Doyle and Doyle (1987) was used for DNA extraction. In short, leaves were ground in liquid N_2 to a fine powder. To approximately 100mg of the ground powder, 610μl extraction buffer [2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-Cl (PH 8), 2% PVP, 2% BSA and 0.2% β-mercaptoethanol (added just before use)], 20μl 5MNaCl and 70μl 20%SDS were added and mixed. The mixture was incubated at 65°C for 60 minutes in a water bath, with occasional gentle mixing. After 1-hour incubation the extract was RNase (6ng)-treated at 37°C for 10 minutes followed by chloroform: isoamyl (24:1, v/v) extraction. The DNA was precipitated by addition of 0.6 volumes of isopropanol at $–20^{o}$C overnight and pelleted at 13000rpm for 15 minutes (Jouan microfuge model A14). The pellet was washed twice with 1ml 70% ethanol, dried and resuspended in 50μl Tris 10mM EDTA 0.1mM. DNA was size-fractionated by agarose gel electrophoresis (0.8% agarose, 1X TAE ethidium bromide $200μgl^{-1}$, 2V/cm) visualized by UV illumination at 317nm of ethidium bromide stained DNA. The DNA concentration was estimated by comparison of the band intensity with those of the λ *Hind*III molecular weight marker. The DNA was diluted with sterile distilled water to 2.5ng/μl. The RAPD PCR were carried out in 20μl total volume containing 5 and 10ng

template DNA, 2.5 units *TaKaRa Ex Taq™* polymerase, 1x *TaKaRa Ex Taq™* reaction buffer, 0.3μM genosys primer (Table 11) and 0.2μM dNTPs. Amplifications were performed in a Perkin Elmer 9700 PCR machine for 5 minutes at 94°C, 45 cycles of 94°C for 45 seconds, 36°C for 1 minute and 72°C for 2 minutes followed by 72°C for 7 minutes. PCR products were size-fractionated by gel electrophoresis (1.5% agarose, 0.5X TAE, 200μgl^{-1} ethidium bromide, 2V/cm) and visualized by UV illumination. Only reproducible bands obtained with 5 and 10ng template DNA from two independent DNA extractions were scored as either 1 (present) or 0 (absent). RAPD products were scored as primer number a-z. The total number of markers, polymorphic markers and their averages per primer were calculated.

3.2.7 Rejuvenation of adult marula truncheons

SEDADIX (hormone powder No.2 for semi-hard woody cuttings, based on 4,3-indoly-butyric acid (IBA) manufactured by Maybaker South Africa Pty Ltd) was applied to cut ends of adult marula truncheons harvested in April, excess powder taped off and the truncheons planted on a mist bed. Two hundred truncheons were tested.

Alternatively, truncheons were soaked in MS liquid medium supplemented with 4.8μM BA, 2.4μM KN overnight followed by planting on sand. One hundred truncheons were tested.

3.2.8 Regeneration

Surface sterilized growth room explants (leaves and stems) or explants (leaves, stems, roots) derived from tissue culture propagated plantlets before acclimatization were

inoculated in MS medium supplemented with different combinations and concentrations of BA (1, 1.5, 2. 2.5, 3, 4, 6, 8, 10μM), KN (1μM), IBA (0.1μM), TDZ (0.5, 1, 1.5, 2 and 2.5μM) and 2,4-D (0.5, 1, 1.5, 2 and 2.5μM). Each treatment of growth room explants consisted of 10 explants each, and 7 explants per treatment for tissue culture explants. Growth response was observed after 3 to 4 weeks.

3.2.9   Kanamycin concentration of selection

Nodal explants were inoculated on MS multiplication medium (MS medium containing 4.8μM BA and 2.4μM KN) supplemented with different concentrations of kanamycin (0, 10, 20, 30, 40, 50, 70, 100, 200, 250 and 300 mgl^{-1}). Each treatment consisted of 15 explants repeated twice. Bleached shoots were scored and reported as a percentage of the total number of emerging shoots.

3.2.10 *Agrobacterium* culture

Agrobacterium tumefaciens were grown from frozen stocks (-20°C) on solid YEB medium supplemented with 50mgl^{-1} kanamycin and 50mgl^{-1} rifampicin for 3 days at 25°C. For cocultivation experiments a single colony was grown overnight in 10ml YEB medium supplemented with 50mgl^{-1} kanamycin and 50mgl^{-1} rifampicin at 150rpm at 25°C. The culture was precipitated at 2500rpm for 2 minutes and the pellet washed twice with 10ml 10mM MgSO$_4$ before resuspension in 10ml full strength MS medium (Janssen and Gardner, 1993; Wenck *et al*., 1999). The *Agrobacterium* suspension was supplemented with or without 100μM acetosyringone.

3.2.11 Cocultivation of marula nodal explants with *Agrobacterium tumefaciens*

Surface sterilized marula nodal explants were incubated with *Agrobacterium* suspension with or without 100μM acetosyringone for 10 minutes with or without wounding the bud area with a sterile needle. Explants were blotted dry on 3MM sterile filter paper and inoculated on cocultivation medium. Cocultivation medium was multiplication medium (MS medium containing 4.8μM BA and 2.4μM KN without 0.1% PVP)) supplemented with or without 100μM acetosyringone. Cocultivation was for 3 days at 25°C and a 16-hour photoperiod at 81μmolm^{-2}s^{-1}. Two experiments of 303 and 311 explants in total were carried out. Sixty explants per treatment and 15 no *Agrobacterium* control explants were analysed for transient expression while the remaining 48 and 56 explants, 12 and 14 per treatment of the first and second experiment, respectively were subcultured on selective medium.

3.2.12 Transient expression

Four hundred and eighty cocultivated and 30 control explants of two experiments were subjected to histochemical GUS staining (Jefferson, 1987) to determine transient GUS activity. Each nodal explant was cut longitudinally in 4 pieces prior to staining. Explant sections were incubated for 24 hours at 37°C in X-Gluc 1gl^{-1} in 100mM ferro/ferricyanide according to Jefferson (1987) and Koncz and Koncz-Kalman (1998). Explant sections were then soaked in 70% ethanol for 48 hours to remove chlorophyll before enumeration of blue zones under the microscope at 625X magnification and photographed. The number of blue stained explant sections among the total number of explant sections and blue stained zones per explant section were scored and reported as the percentage of the GUS-positive explants and the number of GUS-positive zones per explant section,

respectively. Explants for GUS staining were sampled on day 1, 2, 3 and 6 after inoculation. Each sampling per treatment consisted of 15 nodal explants per experiment.

3.2.13 Selection of putative chimeric transformants

Cocultivated nodal explants were transferred to multiplication medium (MS medium containing 4.8µM BA and 2.4µM KN) supplemented with 300mgl^{-1} kanamycin (selective agent) and 500mgl^{-1} cefotaxime (a bacteriostatic used to kill gram-negative bacteria such as *Agrobacterium)*. After 4 weeks in culture, putative transformants (sprouted shoots that were completely green or with both bleached and green sectors) were scored of 60 and 70 emerging shoots, respectively. A total of 28 chimeric putative transgenic shoots (10 white green and 2 green, 15 white green and 1 green shoots from first and second experiment, respectively) were transferred to elongation medium (MS medium containing 1.2µM BA and 1.0µM KN) supplemented with 300mgl^{-1} kanamycin.

3.2.14 Data analysis

Data were entered in the Microsoft excel™ program and coded; means and standard deviations were computed. The STATISTICA software version 6 (2001) from Softstats™ was used for statistical analysis. In an effort to normalize populations, percent values were log-transformed before analysis. One-way and factorial analysis of variance (ANOVA) were used to identify factors significantly impacting surface sterilization, elongation and selection and, bud break, shoot multiplication, rooting and transient expression, respectively. Multiple comparisons of treatment means was carried out using posthoc Tukey HSD test (Winer *et al*., 1991) to determine significant differences between treatments at the 5% level of significance (p<0.05).

CHAPTER 4. RESULTS

The results presented in section 4.1 and 4.2 have been prepared for publication. The submission entitled "Micropropagation of marula, *Sclerocarya birrea* subsp. *Caffra* (*Anacardiaceae*), by axillary bud proliferation and RAPD analysis of plantlets" is included as appendix one.

4.1 Micropropagation of marula

4.1.1 Surface sterilization of marula explants

Explant materials (leaves, nodal sections, shoot tips and roots) collected from growth room seedlings and field-grown trees were subjected to different surface sterilization treatments as described in materials and methods.

4.1.1a Explants obtained from the growth room

Explants collected from the seedlings maintained in the growth room (hypocotyls, leaves, nodal sections, shoot tips and roots) were surface sterilized (table 12). The percentage of aseptic cultures ranged from 0 to 100%. Root explants scored the lowest percentage (0%) significantly different ($p<0.05$) from the percentage aseptic cultures for other explants, which ranged from 85 to 100%.

Table 12. Effect of bleach concentration and exposure time on decontamination of growth room explants

Ssm	Method	t_E, t_B	Hypocotyls A (%)	Leaves A (%)	Nodal A (%)	Shoot tips A (%)	Roots A (%)	Av (%)
1	EB10%T	1,20	85±7a	95±7a	80±0a	85±7a	0±0b	69x
2	EB20%T	1,15	90±14a	85±7a	85±7a	85±7a	0±0b	69x
3	EB30%T	1,10	95±7a	95±7a	100±0a	100±0a	0±0b	78y

Different letters (x, y) indicate significant difference at $p<0.05$ between different surface sterilization methods (factorial ANOVA posthoc Tukey test). Data represent means ± standard deviation of 2 repeats of 10 explants each (a total of 300 explants). Ssm-number referring to surface sterilization method, t_E-minutes submerged in 70% ethanol, t_B-minutes submerged in bleach with Tween 20, A-aseptic, Av-average, E-70% ethanol, B-bleach (JIK) in % (v/v), T-Tween 20

Although the aseptic percentage for hypocotyls, leaves, nodal sections and shoot tips were not significantly different, method 3 (1 minute in 70% ethanol followed by 10 minutes in 30% bleach containing 0.1% Tween 20) which resulted in routine near 100% aseptic cultures of hypocotyls, leaves, nodal sections and shoot tip explants was significantly different ($p<0.05$) from methods 1 and 2 (1 minute in 70% ethanol, 20 minutes in 10% bleach containing 0.1% Tween 20, 1 minute in 70% ethanol, 15 minutes in 20% bleach with 0.1% Tween 20, respectively). Method 3 was adopted as surface sterilization procedure for further experiments for growth room obtained explants.

4.1.1b Field explants harvested in February-March

Explants materials (leaves, nodal sections and shoot tips) were collected from field-grown adult trees in February-March and subjected to different surface sterilization methods (table 13). The surface sterilization method 3 established for explants from growth room grown-seedling was unsatisfactory (0 to 20% aseptic cultures) for field-grown tree explants.

Of 22 methods tested for surface sterilization of field grown explants harvested in February-March (table 13), only methods that resulted in more than 40% aseptic explants were repeated. Methods 8 and 23 (5 minutes in 70% ethanol, 10 minutes in 20% bleach with 0.1% Tween 20 and 2 minutes in 70% ethanol, 20 minutes in 30% bleach with 0.1% Tween 20) for leaves and nodal sections, respectively resulted in high aseptic rates (75 and 80%, respectively) that were significantly different ($p<0.05$) from all other methods that resulted in below 50% aseptic cultures. 100% aseptic culture establishment was not achieved with any of the methods tested on explants from field-grown adult trees harvested in February-March. Shoot tips required substantially longer submersion periods, 10 minutes in 70% ethanol, 30 (method 11) or 50 (method 12) minutes in 20% bleach containing 0.1% Tween 20 to achieve aseptic cultures at 40 and 50%, respectively versus methods 8 and 23 at 75 and 70% aseptic culture establishment for leaf and nodal explants, respectively.

Table 13. Effect of bleach concentration and exposure time on surface sterilization of marula explants from field-grown adult trees collected in February-March

Ssm	Method	t_E, t_B	Leaves A (%)	Nodal A (%)	Shoot tips A (%)
4	EB20%T	2,5	NR	25±21a	NR
5		1,10	NR	45±7a	NR
6		2,10	40±0a	ND	ND
7		2,15	NR	50±14ab	ND
8		5,10	75±7b	70±14ab	NR
9		5,15	40±0a	NR	NR
10		5,20	35±0a	NR	NR
11		10,30	NR	NR	40±0a
12		10,50	NR	50±14ab	50±14a
13	EB20%	2,5	60±0ab	NR	NR
14		1,10	NR	40±0a	NR
15		2,10	NR	ND	ND
16		2,15	NR	NR	ND
17		5,10	NR	NR	NR
18		5,15	45±7a	ND	NR
19		5,20	55±7ab	NR	NR
20		10,30	NR	NR	ND
21		10,50	60±0ab	NR	NR
22	EB30%T	1,10	NR	NR	NR
23		2,20	45±7a	80±0b	NR
24	EB30%	1,10	NR	NR	NR
25		2,20	55±7ab	50±14ab	NR

Different letters along the column indicate significant difference at $p<0.05$ (one-way ANOVA posthoc Tukey test). Data represent means ± standard deviation of 2 repeats of 10 explants each (a total of 400 explants). Ssm-number refering to surface sterilization method, t_E-minutes submerged in 70% ethanol, t_B-minutes submerged in bleach with or without Tween 20, A-aseptic, E-70% ethanol, B-bleach (JIK), T-Tween 20, NR-not repeated, ND-not determined

Therefore methods 8, 12 and 23 that resulted in highest aseptic cultures were adopted as surface sterilization methods for decontaminating leaf, shoot tip and nodal explants in February-March, respectively.

Aseptic cultures of different explant types (leaves, nodal sections and shoot tips) collected in February-March after the rainy season did not show growth in culture.

4.1.1c Field nodal explants collected in October-November

Nodal explants collected during October-November season were subjected to surface sterilization method 23 that proved to be effective in decontaminating nodal explants harvested during the February-March season. Only 13% of the initiated cultures were aseptic (Table 14).

Table 14. Effect of pre and post surface sterilization treatments on aseptic culture establishment from explants of field-grown trees collected during October-November

Ssm	Method	t_E, t_B	Aseptic (%)
23	EB30%T	2, 20	13±19a
26	AEB30%T	2, 20	35±17a
27	FAEB30%T	2, 20	73±0b
28	AEB30%TSF	2, 20	46±1a

Different letters indicate significant difference at $p < 0.05$ (one-way ANOVA posthoc Tukey test). Data represent means ± standard deviation of 2 repeats of 10 and 11 explants each (a total of 84 explants). Ssm-number referring to surface sterilization method, t_E-minutes submerged in 70% ethanol, t_B-minutes submerged in bleach with Tween 20, E-70% ethanol, B–bleach (JIK) 30% (v/v), T-Tween 20, A-antibacterial dish washing detergent pretreatment, F-fungicide pretreatment, SF-filter sterilized fungicide dipped before inoculation

Additional steps were included to the adopted surface sterilization method 23. These included washing of explants in antibacterial dishwashing detergent, soaking of explants in fungicide prior to the antibacterial wash or, dipping surface sterilized explants in sterile fungicide solution before inoculation. Percentage aseptic cultures are shown in table 14. Washing in antibacterial dishwashing liquid (35%) and dipping in sterile fungicide (46%) did not significantly improve the percentage aseptic cultures above the basic surface sterilization method 23 (13%). The highest percentage of aseptic cultures (73%) significantly different ($p < 0.05$) from other methods tested was obtained by including a 2-

hour soak in fungicide solution before washing in antibacterial dishwashing liquid followed by surface sterilization method 23 (method 27). Therefore method 27 was adopted for surface sterilization of nodal explants collected from the field during October-November season.

The advantage of collecting explant materials during this period is the availability of newly sprouted shoots, which are juvenile and thus amenable to grow *in vitro*.

During both collection seasons, bacteria and fungi were the contaminants on the field explants.

4.1.2   Explant browning

Surface sterilized marula nodal explants were cut into pieces of approximately 1.5 cm and were inoculated on MS medium supplemented with KN and BA. Browning of the medium and explants, a common phenomenon in woody plant tissue culture (Pierik, 1987; Debergh and Read, 1991; Nayaranaswamy, 1994), was observed within 2 days after inoculation of the nodal explants. It was first observed at the cut ends of the explant cultures, gradually spreading into the surrounding medium (Figure 2).
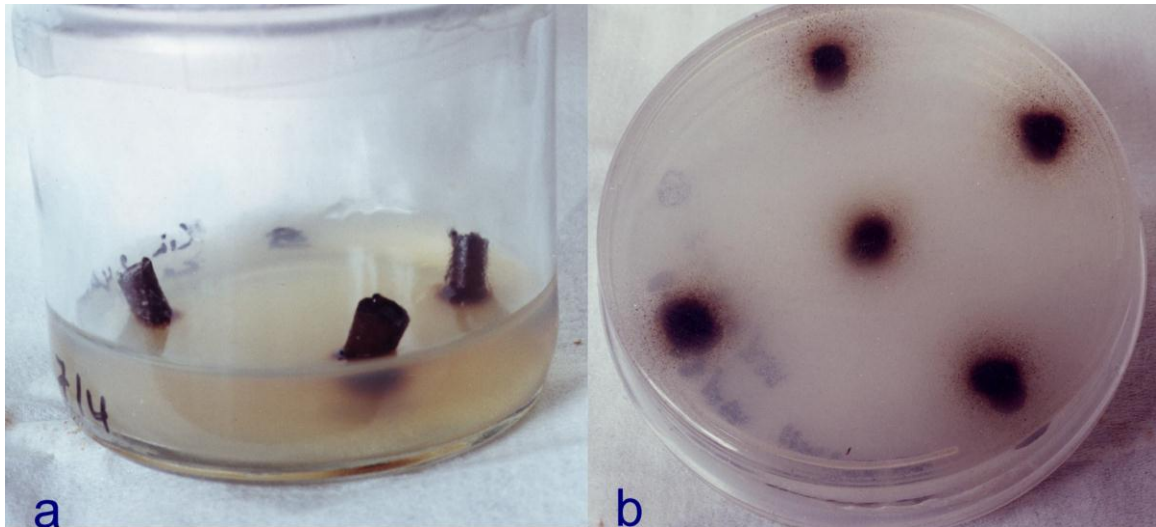
Figure 2. Browning of marula explants.

a. Browning of explants. b. Diffusion of polyphenols in the medium.

The explants did not show any signs of growth and died within 2 weeks of culture. Adsorbents, antioxidants or frequent transfers of explants to fresh media have been reported to overcome browning ascribed to polyphenols (Pierik, 1987; Debergh and Read, 1991; Nayaranaswamy, 1994). Inclusion of the adsorbent PVP at 0.1% (Gamborg and Phillips, 1995) in MS medium with and without bi-daily transfers of explants obtained from field-grown trees and growth room, respectively during the first week of culture helped to overcome browning and cell death. Nodal explants responded well to polyphenol alleviation treatment and showed growth response within 2 weeks after inoculation.

4.1.3 Marula shoot multiplication

Marula seedlings maintained in the growth room were used to develop the procedure for micropropagation, which was adopted for field-collected materials. Nodal sections with axillary buds were the choice of explants in the study since nodal sections with preformed

meristems exhibit the least somaclonal variation compared to micropropagation techniques that include a dedifferentiation stage (Phillips and Gamborg, 1995).

The widely used MS medium formulation was used as a source of macroelements, microelements, vitamins and carbon for marula explants. WPM medium showed similar growth to MS medium (figure 3).
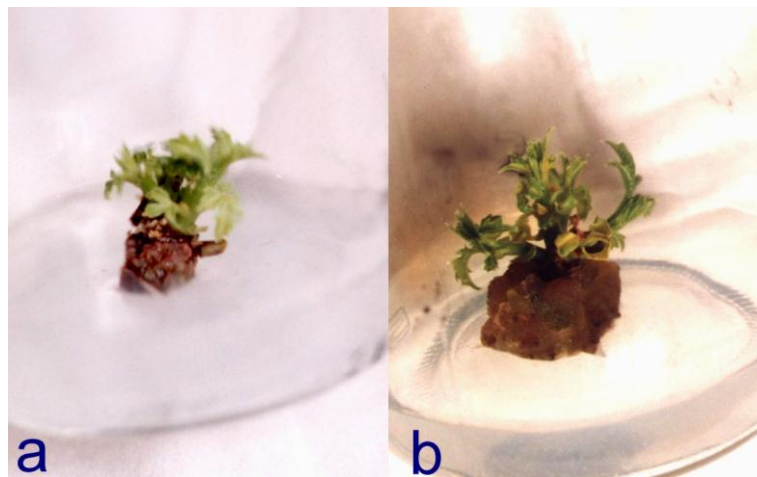
Figure 3. Marula microshoots on WPM and MS media.

a. Microshoots on WPM medium supplemented with 4.8μM BA and 2.4μM KN. b. Microshoots on MS medium containing 4.8μM BA and 2.4μM KN.

In order to stimulate axillary and adventitious bud growth of explants a high concentration of cytokinin alone or a high cytokinin to auxin ratio is incorporated in shoot multiplication media (Pierik, 1987; Narayanaswamy, 1994; Chrispeels and Sadava, 1994; Gamborg and Phillips, 1995). BA and KN at different concentrations and combinations (materials and methods) were used to induce bud break and shoot proliferation on nodal explants of marula seedlings.

4.1.3a   Bud break

Bud break on nodal explants was evident within 2 weeks of culture (figure 4a to d). The percentage bud break ranged from 20 to 72% (Table 15). Growth response on MS medium without PGR was evident with 30% bud break. Generally, low bud break was observed on MS medium with lower and higher cytokinin concentrations whereas more than 50% bud break was observed on MS medium containing intermediate cytokinin concentrations. Lower bud break (20 to 47%) was observed on MS medium supplemented with 1.2µM BA at all KN concentrations (1.2 to 6.0µM), 2.4µM BA and 1.2 to 4.8µM KN, 3.6µM BA and 1.2, 4.8 and 6.0µM KN, 4.8µM BA and 6.0µM KN and 6.0µM BA and 1.2, 4.8 and 6.0µM KN.

Bud break of explants cultured on MS medium supplemented with intermediate cytokinin concentrations (2.4µM BA and 6.0µM KN, 3.6µM BA and 2.4 and 3.6µM KN, 4.8µM BA and 1.2 to 4.8µM KN, 6.0µM BA and 2.4 and 3.6µM KN) ranged from 50 to 72.0%.

The highest bud break (72%) was only significantly different ($p<0.05$) from the lowest bud break (20%) but did not differ significantly ($p<0.05$) from other cytokinin combinations and concentrations tested. Table 16 and 17 show which of the cytokinins were critical to bud break. In contrast to KN, BA showed a significant impact on bud break.
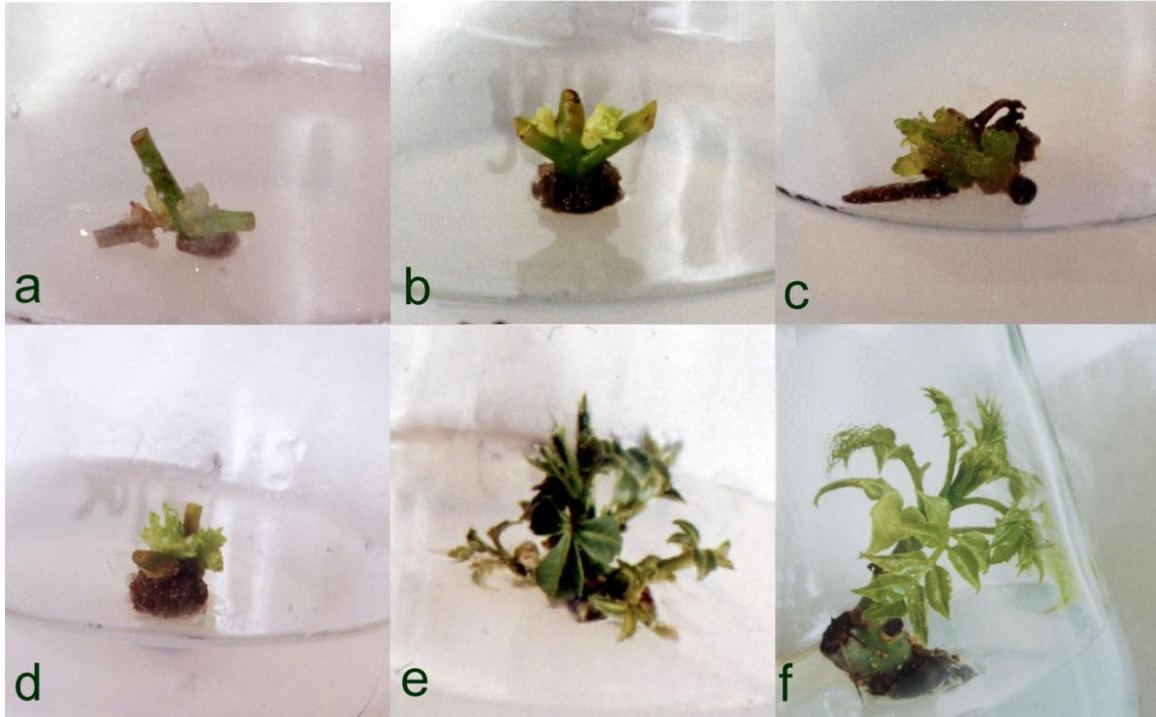
Figure 4. Marula shoot multiplication.

a. Bud break on MS medium without PGR. b. Bud break and callus on MS medium supplemented with 2.4µM BA and 1.2µM KN. c. Bud break and callus on MS medium supplemented with 4.8µM BA and 2.4µM KN. d. Bud break and callus on MS medium supplemented with 4.8µM BA and 3.6µM KN. e. Microshoots from seedling explant on MS medium supplemented with 4.8µM BA and 2.4µM KN. f. Microshoot from adult tree explant on MS medium supplemented with 4.8µM BA and 2.4µM KN.

Table 15. Effect of BA and KN concentrations on bud break and shoot proliferation

Origin of explants	BA, KN (µM)	Bud break (%)	Mean # of shoots/res expl	Mean # of shoots/st expl
GR seedlings	0.0 , 0.0	30±14ab	1.0±0.0a	0.3±0.1a
	1.2 , 1.2	40±14ab	1.5±0.0a	0.4±0.0a
	1.2 , 2.4	30±14ab	1.0±0.7a	0.3±0.1a
	1.2 , 3.6	30±14ab	1.0±0.0a	0.4±0.0a
	1.2 , 4.8	30±14ab	1.0±0.0a	0.3±0.1a
	1.2 , 6.0	20±0a	1.0±0.0a	0.2±0.0a
	2.4 , 1.2	40±0ab	1.0±0.0a	0.4±0.0a
	2.4 , 2.4	40±0ab	1.0±0.0a	0.4±0.0a
	2.4 , 3.6	30±14ab	1.0±0.0a	0.3±0.1a
	2.4 , 4.8	40±0ab	1.3±0.4a	0.5±0.1a
	2.4 , 6.0	61±14ab	1.2±0.2a	0.7±0.2a
	3.6 , 1.2	40±0ab	1.3±0.4a	0.5±0.1a
	3.6 , 2.4	64±17ab	1.3±0.2a	0.8±0.2a
	3.6 , 3.6	72±12ab	1.2±0.2a	0.9±0.2a
	3.6 , 4.8	30±14ab	1.0±0.0a	0.3±0.1a
	3.6 , 6.0*	40±0ab	1.3±0.4a	0.5±0.1a
	4.8 , 1.2	66±21ab	1.4±0.3a	0.9±0.3a
	4.8 , 2.4	72±27bx	2.5±0.4bx	1.8±0.9bx
	4.8 , 3.6	69±20ab	1.6±0.4a	1.1±0.4ab
	4.8 , 4.8*	53±12ab	1.4±0.1a	0.7±0.1a
	4.8 , 6.0*	47±12ab	1.4±0.3a	0.6±0.3a
	6.0 , 1.2	40±0ab	1.8±0.4ab	0.7±0.1a
	6.0 , 2.4	50±14ab	1.3±0.4a	0.6±0.0a
	6.0 , 3.6*	50±14ab	1.8±0.2ab	0.9±0.1ab
	6.0 , 4.8*	35±14ab	1.6±0.1a	0.8±0.3ab
	6.0 , 6.0*	40±0ab	1.8±0.4ab	0.7±0.1a
FG adult trees	4.8 , 2.4	100±0y	1.0±0.0y	1.0±0.0y

Different letters (a, b) along the column indicate significant difference at p<0.05 (factorial ANOVA posthoc Tukey HSD test). Data represent means ± standard deviation of one to 4 repeats of 5 to 8 explants (a total of 465 seedling and 71 field grown explants). Different letters (x, y) along the column indicate significant difference between the seedling and field explants at the same PGR treatment (one-way ANOVA posthoc Tukey HSD test). res expl-responding explants, st expl-sterile explants, GR-growth room grown, FG-field grown, *-BA and KN combinations and concentrations that resulted in excessive callus formation

Table 16. Effect of BA on bud break and shoot multiplication of marula seedling explants

BA, KN (μM)	Bud break (%)	Average # of shoots
0,0	20a	1.0a
1.2, 1.2-6.0	21a	1.1a
2.4, 1.2-6.0	35ab	1.1a
3.6, 1.2-6.0	42ab	1.2a
4.8, 1.2-6.0	57b	1.7b
6.0, 1.2-6.0	36ab	1.6b

Different letters along the column indicate significant difference at $p<0.05$ (factorial ANOVA posthoc Tukey HSD test). Analysis based on data of table 15

Table 17. Effect of KN on bud break and shoot proliferation of marula seedling explants

KN, BA (μM)	Bud break (%)	Average # of shoots
0,0	20a	1.0a
1.2, 1.2-6.0	38a	1.3a
2.4, 1.2-6.0	51a	1.7b
3.6 1.2-6.0	45a	1.4ab
4.8, 1.2-6.0	32a	1.2a
6.0, 1.2-6.0	37a	1.3a

Different letters indicate significant difference at $p<0.05$ (factorial ANOVA posthoc Tukey HSD test). Analysis based on data of table 15

Among the best concentrations and combinations for bud break obtained for seedling marula nodal explants MS medium supplemented with 4.8μM BA and 2.4μM KN was adopted for adult (field) explants. The bud break for nodal explants obtained from sprouted shoots of adult marula tree was 100%, a value which was significantly different ($p<0.05$) from seedling explants treated with the same cytokinin regime (Table 15).

4.1.3b Shoot multiplication

The average number of shoots per responding explant ranged from 1 to 2.5 (table 15). Average shoot numbers of 1 to 1.6 on different BA and KN concentrations were not significantly different at $p<0.05$. Whereas an average shoot number of 1.8 to 2.5 was

observed on MS medium supplemented with 4.8μM BA and 2.4μM KN, 6.0μM BA and 1.2, 3.6 and 6.0μM KN. Of these, the highest number, 2 to 4 shoots, on average 2.5 shoots per responding bud or 1.8 shoots per inoculated explant (when loss due to contamination was considered), observed on MS medium supplemented with 4.8μM BA and 2.4μM KN was not significantly different at $p < 0.05$ from the last three (MS medium 6.0μM BA and 1.2, 3.6 and 6.0μM KN). The highest average number of shoots per explant was significantly different ($p < 0.05$) from that of 21 tested cytokinin combinations (MS medium 1.2μM BA and 1.2 to 6.0μM KN, MS medium 2.4μM BA and 1.2 to 6.0μM KN, MS medium 3.6μM BA and 1.2 to 6.0μM KN, MS medium 4.8μM BA and 1.2, 3.6, 4.8 and 6.0μM KN, MS medium 6.0μM BA and 2.4, 4.8μM KN). Table 16 and 17 show which cytokinins were critical to shoot number. The average number of shoots at different BA concentrations ranged from 1.1 to 1.7. The average number of shoots on 4.8 and 6μM BA (1.7 and 1.6 respectively) was significantly different ($p < 0.05$) from other BA concentrations (1.2, 2.4 and 3.6μM) (table 16). The number of shoots per explant on different KN concentrations ranged from 1.2 to 1.7. The highest number of shoots (1.7) at 2.4μM KN was significantly different at $p < 0.05$ from that obtained at 1.2, 4.8 and 6.0 μM KN (1.2 to 1.3) (Table 17).

MS medium supplemented with 4.8μM BA and 2.4μM KN, with highest bud break (72%) and average number of shoots per explant (2.5) was adopted as the shoot proliferation medium. This medium was then used to inoculate explants from adult field-grown trees. An average of 1 shoot per responding and sterile nodal explant or 0.73 shoot per inoculated explant when loss due to contamination was considered was recorded, a

value significantly different ($p < 0.05$) from seedling nodal explants (2.5 per responding or 1.8 per sterile nodal explant) (table 15).

4.1.4 Formation of callus on marula nodal explants

Callus formation on marula explants was common on shoot proliferation medium. Callus was noticed on the explants within 3 weeks of culture (figure 4).

No callus was observed on explants inoculated on MS medium without cytokinin and MS medium containing 2.4µM total cytokinin concentration (BA concentration + KN concentration). However the extent of callus formation increased as the total cytokinin concentration increased (Table 18). Callus formation usually started at the cut ends of explants in contact with the media.

Table 18. Effect of total cytokinin concentration on callus formation

Cytokinin (µM)	Callus formation
0	-
2.4	-
3.6	+
4.8	+++
6.0	+++
7.2	+++
8.4	+++
9.6	++++
10.8	++++
12.0	++++

- no callus, + - little callus, +++ - callus, ++++ - excessive callus

Little callus was observed on explants inoculated on MS medium supplemented with 3.6µM total cytokinin concentration. At this cytokinin concentration callus was only observed at the base of the explant. There was no coexistence of callus and growing

shoots as the shoots were at the bud area, away from the base of the explant. As the total

cytokinin concentration increased the extent of callus formation also increased.

Figure 5. Excessive callus on marula explant and microshoots

Excessive callus on explant and microshoots on MS medium supplemented with 4.8μM
BA and 4.8μM KN.

More callus formed on MS medium supplemented with 4.8μM to 8.4μM total cytokinin,

but shoot quality was not affected. At higher concentrations of total cytokinin (above

8.4μM) excessive callus was observed on explants spreading to the shoots. Shoots were

affected as they were often covered with callus, especially when explants were smaller

than 1.5 cm (figure 5).

4.1.5 Elongation of marula microshoots

Marula shoots grown on multiplication medium were short. Shoots were transferred on

Figure 6. Elongated marula microshoot.

(MS medium with 1.2µM BA and 1.0µM KN).

MS medium devoid of PGR or MS medium supplemented with low cytokinin (BA 1.2µM and KN 1.0µM) or 1 or 5µM GA_3 to achieve elongation (figure 6). Generally shoot elongation was low for all treatments with results ranging from 4 to 42% (table 19).

The lowest shoot elongation responses (4-10%) were recorded for shoots on MS media supplemented with 1 or 5 µM GA_3, which did not differ significantly (p<0.05).

Table 19. Effect of PGR on marula shoot elongation

PGR (µM)	Elongated shoots (%)
0	34±2.8b
GA₃ 1	10±2.8a
GA₃ 5	4±0.0a
BA 1.2, KN 1.0	42±2.8b

Different letters indicate significant difference at p<0.05 (one-way ANOVA posthoc Tukey HSD test). Data represent means ± standard deviation of 2 repeats of 10 and 15 microshoots (a total of 100 microshoots)

Shoot elongation on MS medium supplemented with GA_3 differed significantly (p<0.05) from that on MS medium without PGR and MS medium supplemented with low concentration of cytokinin. Thirty four percent shoots achieved elongation on MS medium without PGR. The highest shoot elongation (42%) was recorded for shoots cultured on MS medium enriched with 1.2μM BA and 1.0μM KN. The recorded results from the latter two treatments were not significantly different at p<0.05. Although none of the treatments tested achieved above 50% elongation, MS medium supplemented with 1.2μM BA and 1.0μM KN was chosen as the elongation media in marula micropropagation.

No callus was observed on elongated shoots on any of the elongation media tested.

Marula shoots micropropagated from the field explants were long compared to those originating from seedlings (figure 4f and 4e). These shoots were inoculated on the elongation medium. However due to the poor cooling system in the growth room leading to recurring high temperatures, cultures were killed during two consecutive December-January holidays when field-grown cultures were in tissue culture. No cultures were successfully established from field materials harvested in February-March due to the seasonality of the primary shoots.

4.1.6   Rooting of marula microshoots

Elongated marula shoots with 2 to 3 new leaves were first transferred on MS medium without PGR for 2 weeks. This transfer acted as an acclimatization period before shoots

Figure 7. Rooted marula plantlets.

a. Plantlet in rooting medium (half strength MS medium supplemented with 10µM IBA and 0.3% AC). b. Plantlet rooted in MS medium supplemented with 3µM IBA and 0.08% PVP of which the bottom of the jar was covered with catering foil (short adventitious roots on the left) and 0.3% AC (on the right with long adventitious roots).

were transferred to auxin containing medium. After 2 weeks shoots were transferred onto

rooting media and rooting was observed after 4 weeks (figure 7).

Table 20. Effect of IBA concentration, AC, PVP and foil wrapped PVP on rooting of marula shoots

IBA (µM)	AC Rooted (%)	PVP Rooted (%)	PVP½D7 Rooted (%)	Average (%)
0	79±10a	50±10a	36±10a	55m
1	71±20a	54±15a	57±20a	61m
3	61±5a	43±10a	46±15a	50m
5	61±15a	68±15a	43±10a	57m
10	82±5a	61±15a	57±10a	67m
Average (%)	71x	55y	48y	

Different letters (a, b) along the table indicate significant difference at $p<0.05$ (factorial ANOVA posthoc Tukey HSD test). Different letters (m, n) along the column indicate significant difference between different IBA concentrations. Different letters (x, y) along the row indicate significant difference between AC, PVP and PVP1/2D7. Data represent

means ± standard variation of 2 repeats of 14 microshoots each (a total of 140 microshoots). AC-activated charcoal, PVP-Polyvinylpyrolidone, PVP1/2D7-bottom of jars covered with catering foil for 7 days

Marula shoots inoculated on half strength MS medium without IBA responded, with percentages of 36, 50 and 79 for catering foil wrapped PVP, PVP and AC, respectively (table 20). Average percentages of rooted explants on different IBA concentrations were not significantly different (p<0.05).

Table 20 (bottom row) shows the effect of AC, PVP and PVP1/2D7 treatments to rooting, independent from IBA concentration. Low rooting rates of 48 and 55% were recorded on catering foil wrapped PVP and PVP, respectively. The highest rooting response (71%) was observed on MS at half strength salts supplemented with 0.3% AC was significantly different (p< 0.05) from those supplemented with PVP (PVP and PVP1/2D7).

No callus was observed on rooted plantlets on any of the media tested.

4.1.6a Root quality and number

The quality and number of adventitious roots observed per shoot varied between IBA concentrations as well as whether AC or PVP were included in the media. Marula shoots on half strength MS medium supplemented with PVP at all IBA concentrations produced very short, stubby adventitious roots, increasing in number as the IBA concentration increased. Even after a prolonged incubation period of 6 weeks the roots remained short and stubby. Wrapping of the bottom of culture bottles of PVP cultures with catering foil for 7 days to mimic darkness (PVP1/2D7) did not improve the short stubby root growth.

Roots of shoots cultured on half strength MS medium supplemented with 0.3% AC at all IBA concentrations were elongated (figure 7). Marula shoots on rooting media containing 1, 3 and 5µM IBA produced 1 to 2 long adventitious roots with secondary and tertiary branching per shoot. Shoots that were cultured on half strength MS medium supplemented with 10µM IBA produced 2 to 3 long adventitious roots with secondary and tertiary branching per shoot.

Of the media with 71 to 82% rooting, half strength MS medium containing 0.3% AC and 10µM IBA with 82% rooting and 2 to 3 long adventitious roots per elongated shoot was adopted as the rooting medium in marula micropropagation.

4.1.7 Acclimatization of marula plantlets

Since *in vitro* grown plantlets are not well equipped to survive *ex vitro* conditions (Pierik, 1987; Preece and Sutter, 1991), marula plantlets were subjected to a hardening-off process (figure 8). Acclimatization took 4 to 6 weeks. Survival was 90%. Damping-off was observed on plantlets that did not survive.

Figure 8. Acclimatization of marula plantlets.

a and b (left). Plantlets on garden soil covered with transparent beakers. b (right) and c. Acclimatized plants on potted garden soil.

4.1.8 Rejuvenation of adult marula material

Rejuvenation is pursued as a path for the generation of source material for the propagation of trees of which mature tissues are often recalcitrant to propagation. Juvenile tissue possesses a high cell division potential and propagates easier (Pierik, 1987; Dixon, 1985; Franclet, 1991; Narayanaswamy, 1994). Sterilization of explant material from mature field-grown trees is often difficult due to high contamination rates. Success rates tend to be low in particular when not all parts of the tissue are readily accessed by the sterilizing agent. Rejuvenation of tree material involves the generation of new shoots and is often carried out under environmentally-controlled conditions such as a greenhouse or growth room. Such shoots provide a source of explant material with lower contamination rates, and aseptic cultures will be established easier and with greater success rates. Where field grown trees undergo a dormant phase such as marula which is dormant after fruit fall at the end of the rainy season, growth room conditions can be manipulated to allow for growth and access to explant material year round. Shooting *in vivo* prior to explant excision can add an additional multiplication step in a propagation procedure resulting in a higher number of plants per branch or node. In an effort to rejuvenate adult marula, truncheons were cut from adult trees towards the end of the rainy season and treated with SEDADIX and cytokinin. Only 8.5% of the truncheons formed new leaves 3 to 4 weeks after treatment (figure 9a), the leaves died and the truncheons did not form roots. Cytokinin treatment (4.8 µM BA and 2.4 µM KN) resulted in the formation of new leaves on 8% of the truncheons after 3 to 4 weeks (figure 9b). Similar to the SEDADIX treatment, truncheons did not root and dried out.
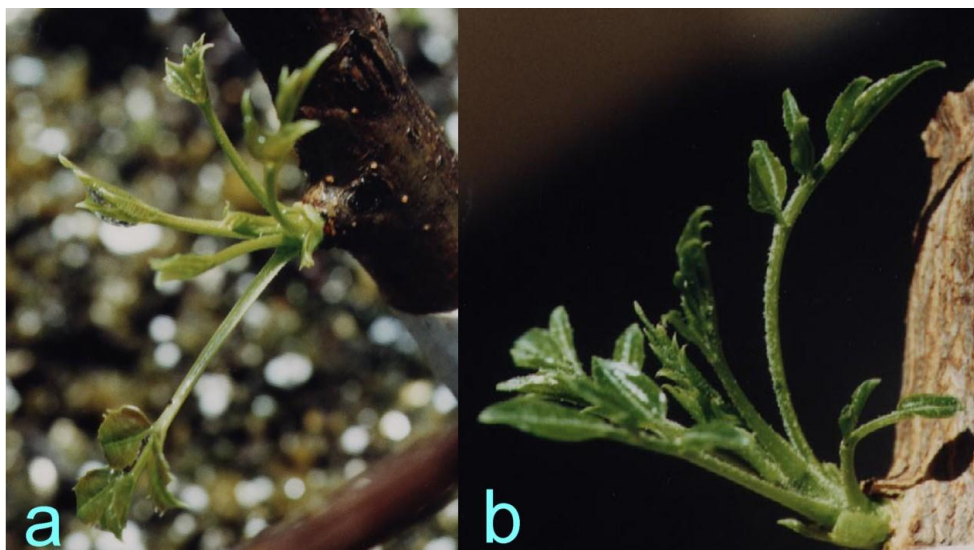
Figure 9. Rejuvenation of marula truncheons.

a. Sprouted shoot after IBA (SEDADIX) treatment. b. Sprouted shoot after cytokinin (4.8μM BA and 2.4μM KN) treatment.

4.2 Somaclonal variation

Marula leaves from source plants and their clones were harvested in duplicate and DNA was extracted. DNA at two different concentrations (5 and 10ng) from duplicate independent DNA extractions was subjected to PCR amplifications. A total of 7 primers Genosys were used (materials and methods). PCR products were size-fractioned by gel electrophoresis and visualized by UV illumination of ethidium bromide stained fragments (figure 10).
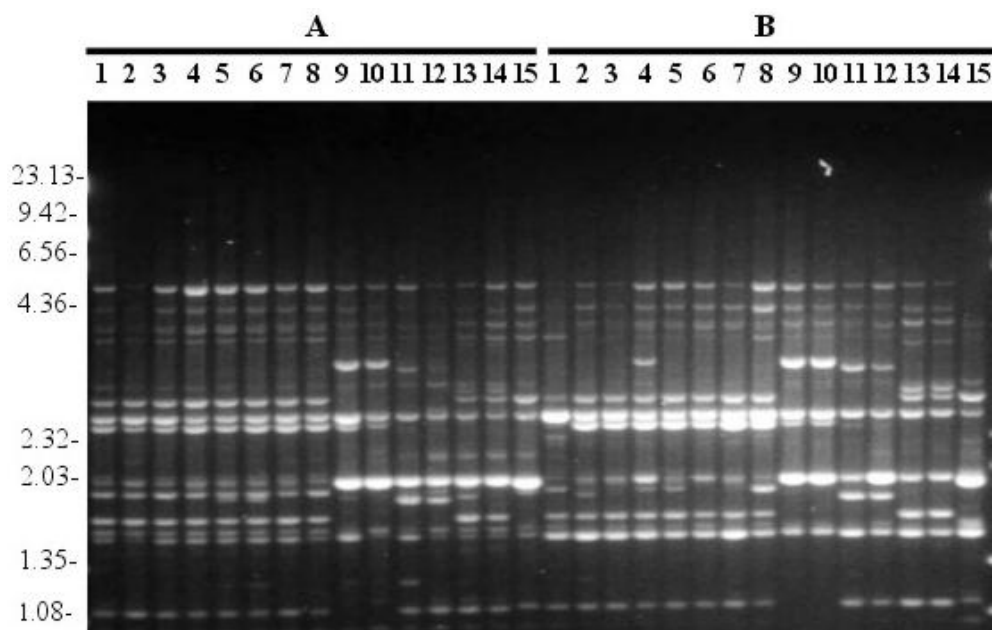
Figure 10. Representative RAPD.

DNA 5 (A) and 10ng (B) from independent isolations were subjected to RAPD analysis using decamer primer Genosys 60-8. Lanes 1, 9, 11, 13 and 15 represent DNA from seedling parents NS9, NS2, NS16, NS18 and NS6. Lanes 2-8, 10, 12 and 14 represent DNA of micropropagated plants from explant parents NS9, NS2, NS16 and NS18, respectively. DNA molecular weight is indicated in kbp on left.

Only bands, which were present as PCR products of both independent DNA extractions at both concentrations were scored. All RAPD gels were examined and scored by two independent researchers under the UV light to avoid potential misinterpretation due to limitations of UV photography. Special attention was given to the presence or absence of weaker bands which may not be visible after photography. A total of 1845 reproducible bands were scored in this manner for a total of 123 markers with an average of 17.6 markers per primer (table 21). Although aberrant bands were observed, they were not found in the independent replicate (figure 10, panel A versus panel B) and hence not scored. No reproducible differences were found. The RAPD results indicated that the

source plants were genetically identical to their clones (micropropagated plants) within the limits of the RAPD technology used here.

Table 21. RAPD analysis of micropropagated and parent plants

Number of markers	123
Number of polymorphic markers	60
Average markers per primer	17.6 ± 2.3
Average polymorphic markers per primer	8.6 ± 2.8

The RAPD primers used showed a high degree of polymorphism between source plants. On average 8.6 (49%) markers per primer were polymorphic.

4.3 *Agrobacterium*-mediated transformation of marula nodal explants

Sections 4.3.2 to 4.3.5 have been accepted for publication. The article entitled: "Preliminary examination of factors affecting *Agrobacterium tumefaciens*-mediated transformation of marula, *Sclerocarya birrea* subsp. *caffra* (*Anacardiaceae*)" was published in Plant Cell, Tissue and Organ Culture (2004) 79: 321-329 and is included as appendix two.

Given the time frame for tree tissue culture and optimization of *Agrobacterium*-mediated transformation procedure which normally involves a large number of factors (Yong-Woog *et al*., 1996; Hazel *et al*., 1998; Miguel and Oliveira, 1999; Kondo *et al*., 2000; Niu *et al*., 2000; Chateau *et al*., 2000; Mondal *et al*., 2001; Le *et al*., 2001; Bhatnagar and Khurana, 2003; Zambre *et al*., 2003), only three parameters (regeneration, kanamycin concentration for selection and cocultivation parameters) were evaluated.

4.3.1 Regeneration of marula

For *de novo* transformation, an efficient regeneration system is a prerequisite (Gheysen *et al*., 1997). Leaves (high bush blueberry, Callow *et al*., 1989; apple, Predieri and Malavasi, 1989; Maheswaran *et al*., 1992; spring glory, Rosati *et al*., 1996; plum, Yancheva *et al*., 2002; fig, Yancheva *et al*., 2004), nodes (eucalyptus, Herve *et al*., 2001) and petioles (kiwifruit, Gonzalez *et al*., 1995) have been used as source material for direct and indirect organogenesis of trees. Indirect regeneration of *Anacardiaceae* explants was achieved through somatic embryogenesis of nucellar tissue (cashew, Cardoza and D'Souza, 2002; Das *et al*., 1999; mango, Litz *et al*., 1984, 1998; Mathews *et al*., 1993). These thus offered possible research avenues for an efficient marula shoot generation method for *de novo Agrobacterium*-mediated transformation. Notwithstanding extensive efforts, sterilization of marula seed and nucellar tissue was not achieved and other explant sources were investigated for their regenerative potential. Preliminary regeneration experiments were performed to determine the PGR (cytokinin BA, KN and auxin IBA) requirements for different marula explants (leaf disks, stem and root sections). The explants were either obtained from growth room or tissue culture grown plantlets.

No direct regeneration was observed on the surface sterilized growth room explants (leaf disks and stem segments) inoculated on MS medium supplemented with different concentrations of BA (1, 1.5, 2, 2.5, 3μM), KN (1μM) and IBA (0.1μM). Callus formation was observed on 40 to 100% of the stem sections, whereas no response was observed on leaf disks (table 22). Stem sections, which were cultured on MS medium containing 1 to 2μM BA, 1μM KN and 0.1μM IBA resulted in a lower response

compared to those on MS medium supplemented with 2 to 3μM BA and 0.1μM IBA. Inclusion of 1μM KN in MS medium supplemented with 2μM BA and 0.1μM IBA decreased the response from 100% to 40%. Reproducibility and relevance need to be confirmed.

Different callus colors were observed from different PGR combinations (table 22). White and white and brown colored callus was produced on MS medium containing 1 to 2μM BA, 1μM KN and 0.1μM IBA. Stem sections on MS medium supplemented with 2 to 3μM BA, 0.1μM IBA produced green, white and green, brown and white and brown callus.

Table 22. Effect of BA, KN and IBA on callus formation on growth-room grown explants

PGR (μM)				
BA	KN	IBA	Response (%)	Callus color
1	1	0.1	60	W
1.5	1	0.1	60	W, WB
2	1	0.1	40	W, WB
2	0	0.1	100	G, WG, WB
2.5	0	0.1	80	B, WG,
3	0	0.1	100	WG, WB, GB

Results of 1 experiment of 10 explants per treatment (a total of 60 explants) are shown. W-white, WB-white and brown, G-green, WG-white and green, B-brown, GB-green and brown

After 4 weeks of culture, white brown callus (on MS medium with 3μM BA and 0.1μM IBA) which did not turn dark brown (20%), was split and transferred to the same medium composition or medium containing 5μM BA. Callus cultures continued to increase in size but no shoot formation was observed (figure 11a).
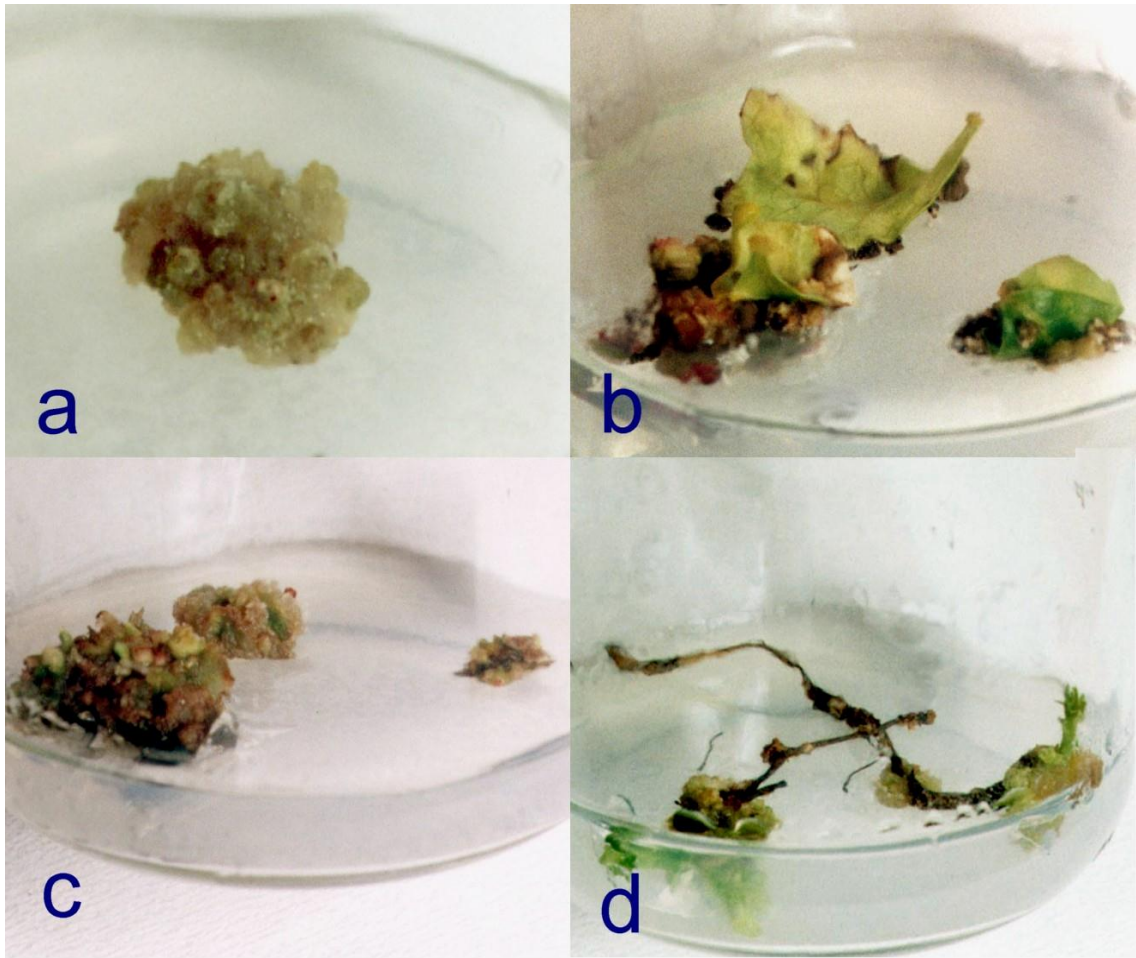
Figure 11. Callus formation on marula explants.

a. Callus formation on stem explant from the growth room inoculated on MS medium with 3µM BA and 0.1µM IBA. b. Purple structures on callus from tissue culture leaf explant cultured on MS medium containing 6µM BA and 0.1µM IBA. c. Purple structures on callus from tissue culture stem explant cultured on MS medium with 6µM BA and 0.1µM IBA. d. Green structures on callus from tissue culture root explant inoculated on MS medium containing 8µM BA and 0.1µM IBA.

In an effort to shift morphogenesis to shoot induction and to evaluate root sections as explants, the BA concentration was increased to 4, 6, 8 and 10µM at 0.1µM IBA and experiments were carried out on sterile rooted tissue culture propagated plantlets prior to

acclimatization since aseptic root explants could not readily be established from growth room grown seedlings.

Explant response varied for different concentrations of BA and IBA as well as explant type (table 23). Callus formation was observed on responding leaf disks, stem and root sections.

The percentage of responding leaf disks ranged from 14 to 86%. The lowest response (14%) was recorded on leaf explants inoculated on MS medium supplemented with 4µM BA and 0.1µM IBA. Fifty seven, 71 and 86% leaf explants formed callus on MS medium with 0.1µM IBA and 8, 10 and 6µM BA, respectively.

Table 23. Effect of BA and IBA on callus formation on tissue-cultured plantlets explants

Explant	BA (µM)	IBA (µM)	Response (%)	Callus color
Leaf	4	0.1	14	G
Leaf	6	0.1	86	G
Leaf	8	0.1	57	G
Leaf	10	0.1	71	G, WB, GB
Stem	4	0.1	43	WG
Stem	6	0.1	29	WG
Stem	8	0.1	100	W, WB, WG, GB
Stem	10	0.1	43	W, WG
Root	4	0.1	57	W, B
Root	6	0.1	29	WB, WG
Root	8	0.1	29	WB, WG
Root	10	0.1	14	G

Results of 1 experiment of 7 explants per treatment (a total of 84 explants) are shown. G-green, B-brown, W-white, WB-white and brown, GB-green and brown, WG-white and green

Callus generated from leaf disks on MS medium with 0.1μM IBA and 4 to 8μM BA was green. Green, white and brown and green and brown sectoral calli were observed on leaf disks on MS medium with 0.1μM IBA at the higher BA concentration (10μM).

The percentage of responding stem sections ranged from 29 to 100% (table 23). The lowest value (29%) was recorded on stem explants cultured on MS medium with 0.1μM IBA and 6μM BA. On MS medium with 0.1μM IBA and 6 and 10μM BA, 43% of the stem sections formed callus whereas all stem sections responded on MS containing 0.1μM IBA and 8μM BA. White and green sectored callus was mostly observed on all BA concentrations with the exception of 57% stem sections cultured on MS medium supplemented with 0.1μM IBA 8μM and 10μM BA that formed white, white and brown or green and brown sectored callus.

Root explants tended to respond to a lesser extent than other explants. Responding roots ranged from 14 to 57% (table 23). The lowest response (14%) was observed on root explants cultured on MS with 0.1μM IBA 10μM BA. Twenty nine percent root explants produced callus on MS containing 0.1μM IBA 6 and 8μM BA. The highest response (57%) of root explants was observed on MS with 0.1μM IBA 4μM BA. White or brown callus was observed on root explants cultured on MS supplemented with 0.1μM IBA 4μM BA, which gave the highest response. At higher BA concentrations, white and green or green callus was observed. Green callus survived, whereas brown callus died.

The callus observed on stem sections on MS medium with 0.1μM IBA 8μM BA, the medium with highest response, turned brown and died. Surviving calli of leaf, stem and root explants exhibited purplish and green structures, respectively (figures 11b-d). These

were transferred to the same MS medium or MS medium with a lower BA concentration (4μM) to achieve shoot formation.

Only 2 shoots were observed on MS medium with 0.1μM IBA 8μM BA and MS medium containing 0.1μM IBA 4μM BA both transferred from leaf disk callus on MS medium with 0.1μM IBA 8μM BA. A total of 4 shoots were observed on MS medium with 0.1μM IBA 4μM BA transferred from stem callus on MS medium containing 0.1μM IBA 6μM BA. Transfer of callus from root explants on MS medium with 0.1μM IBA 8μM BA to the same MS medium composition and MS medium containing 0.1μM IBA 4μM BA resulted in 2 and 8 shoots, respectively. This set of experiments was repeated twice but could not be reproduced.

TDZ, used especially in woody plants, which mimics the effects of cytokinin (Bornman, 1998) and 2,4-D, suitable for callus and somatic embryo initiation (Gamborg and Phillips, 1995) at different concentrations (0.1, 0.25, 0.5, 0.75 and 1μM) on solid or in liquid medium were also tested but did not result in adventitious regeneration, callus or somatic embryo formation. Only swelling was observed on stem sections on MS medium supplemented with TDZ but no growth was observed. On MS medium supplemented with 2,4-D explants did not react, eventually turned brown and died.

In view of the low regeneration frequencies, which are inadequate for genetic transformation and non-reproducibility of experiments, lack of reliable access to a shaker to induce somatic embryogenesis and repeated problems with the growth room, this line of research was discontinued. The preliminary data hint that marula can be regenerated from leaf, stem or root explants as was reported for other trees (high bush blueberry,

Callow *et al.,* 1989; apple, Predieri and Malavasi, 1989; Maheswaran *et al.*, 1992; spring glory, Rosati *et al.*, 1996; plum, Yancheva *et al.*, 2002; fig, Yancheva *et al.,* 2004). However, a substantial amount of research will be needed to generate sufficient micropropagated explant material in order to optimize the PGR concentration and other conditions under which regeneration occurs reproducibly and efficiently. Given time constraints, an alternative strategy of transformation of existing meristems was employed to determine the feasibility of *Agrobacterium*-mediated transformation of marula. Such primary transformants will consist both of transformed and untransformed sectors (Knittel *et al.*, 1994; Park *et al.*, 1996; Bean *et al.*, 1997; Gheysen *et al.*, 1997; Gould and Magallanes-Cedeno, 1998; Han *et al.*, 2000).

4.3.2   Kanamycin concentration for selection

Since the vector used in marula transformation contained the *npt*II gene, transformants can be selected by inclusion of kanamycin in the medium. Kanamycin binds to the 30S subunit of the ribosome, blocking the formation of initiation complexes thus decreasing translation in the mitochondria and chloroplasts. The NPTII protein in transgenic plants deactivates the antibiotic by phosphorylation thus the binding of the antibiotic to the ribosome is prevented, allowing protein synthesis in the chloroplasts (Brasileiro and Aragao, 2001). Kanamycin can be used at a concentration at which the tissue is bleached but not killed (Knittel *et al.*, 1994; Gould and Magallanes-Cedeno, 1998; Mondal *et al.*, 2001).

Experiments to determine the effective kanamycin concentration that would bleach marula shoots were carried out in preparation for the transformation experiments. Marula

explants were inoculated in multiplication medium with different kanamycin concentrations (0, 10, 20, 30, 40, 50, 70, 100, 200, 250 and 300 mgl^{-1}). Plant growth was observed after 4 weeks in culture.

All marula shoots inoculated on MS multiplication medium without kanamycin were green (figure 12a). The presence of kanamycin in the multiplication medium did not kill marula explants because they attained bud break and shoot growth. However, shoots lacked chlorophyll and were bleached (figure 12b).
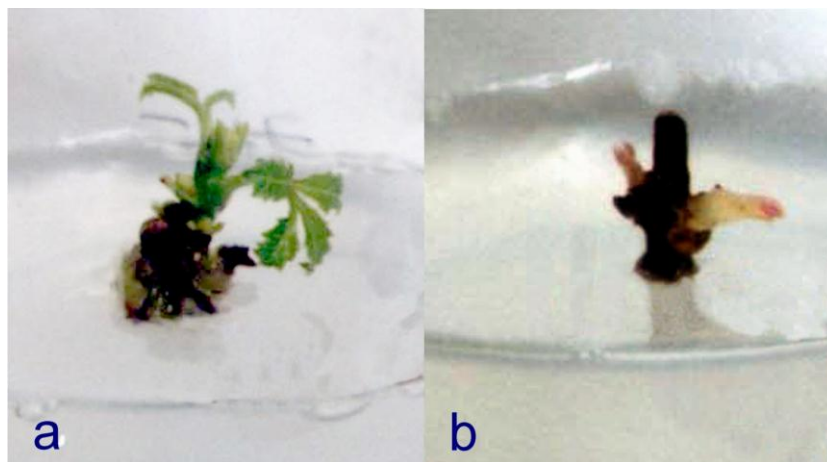
Figure 12. Marula shoots on selective medium.
a. Wild type shoot (green) on MS multiplication medium without kanamycin. b. Wild type shoot (bleached) on MS multiplication medium containing 300mgl^{-1} kanamycin.

The effect of kanamycin on the bleaching of marula shoots generally increased as the kanamycin concentration increased (table 24). Only at a concentration of 40 mgl^{-1} and above did kanamycin effect significant bleaching of emerging marula shoots (different from that observed on MS multiplication medium without kanamycin). No significant difference ($p < 0.05$) in the percentage of bleached shoots was observed between

kanamycin concentrations of 40 to 100 mgl^{-1}. Although 57% bleaching of emerging shoots cultured on MS multiplication medium containing 200mgl^{-1} kanamycin was not significantly different (p<0.05) from that observed on MS multiplication medium supplemented with 250mgl^{-1} kanamycin (63%), it significantly differed (p<0.05) from 90% bleaching of emerging shoots on MS medium supplemented with 300mgl^{-1} kanamycin.

Table 24. Effect of kanamycin on bleaching of marula shoots

Kanamycin (mgl^{-1})	Bleached shoots (%)
0	0±0a
10	17±14abc
20	17±14abc
30	13±10ab
40	30±5bcd
50	37±5bcde
70	43±5cde
100	37±5bcde
200	57±5de
250	63±5ef
300	90±5f

Different letters indicate significant difference at $p<0.05$ (one-way ANOVA posthoc Tukey HSD test). Data represent means ± standard deviation of 2 repeats of 15 explants each (a total of 330 explants)

The highest percentage of bleached shoots (90%) observed on MS multiplication medium supplemented with 300mgl^{-1} kanamycin was significantly different at $p<0.05$ from that observed on all other kanamycin concentrations tested except 250 mgl^{-1}. Since the selective agent would be used to select chimeric transformants, the kanamycin concentration was not further increased to achieve 100% bleaching. MS multiplication medium containing 300mgl^{-1} kanamycin was adopted as the selective medium for chimeric transformed marula shoots.

4.3.3 Cocultivation parameters of *Agrobacterium* and marula

In order to optimize *Agrobacterium* cocultivation, transient expression experiments were carried out using T-DNA harboring the GUS intron construct driven by the CaMV35S promoter. The effects of acetosyringone and wounding of marula explants were evaluated and reported as percentage number of GUS-positive explants and average number of blue stained zones per GUS-positive explant section.

4.3.3a Evolution of transient expression

Transient expression is characterized by an increase in transgene expression shortly after the transformation event followed by a decrease in transgene expression indicative of the frequency of transgene integration and stable transformation. Also in marula, GUS expression by plant cells was high on day 2 to 3 after the onset of cocultivation and was reduced to the low value observed on day 6 as observed both in the number of GUS-positive explants and the number of GUS-positive zones per explant section for all treatments (figure 13 and tables 27 and 30). A lag phase was observed in the evolution of the percentage of GUS-positive explants, reaching the highest value tested on day 3 for all treatments, significantly different ($p<0.05$) from that observed on other days (figure 13a and table 27). GUS-positive percentage of explants observed on day 1 did not differ significantly ($p<0.05$) from that observed on day 2, but both of these differed from those observed on day 0, 3 and 6 (table 25).

Table 25. Evolution of the number of GUS-positive explants

Time	GUS-positive explants (%)
Day0	0a
Day1	6.25b
Day2	6.88b
Day3	33.13c
Day6	1.25a

Different letters (a, b, c,…) indicate significant difference at $p<0.05$ (factorial ANOVA posthoc Tukey HSD test). Data on day 0 report the results from staining 30 control explants. Means of 120 GUS stained explants are reported for days 1, 2, 3 and 6.

No general trends for the evolution of GUS-positive zones per GUS-positive explant section were observed (figure 13b and table 30). Statistical homogenous grouping only

showed significant difference between the data of day 0 and those of all other days (table 26).

Table 26. Evolution of number of GUS-positive zones per GUS-positive explant section

Time		GUS-positive zones per GUS-positive explant section
Day0		0.00a
Day1		1.23b
Day2		1.45b
Day3		1.48b
Day6		1.00b
Time	**AS**	**GUS-positive zones per GUS-positive explant section**
Day0	+	0.00a
Day0	-	0.00a
Day1	+	1.36bc
Day1	-	1.16abc
Day2	+	1.67bc
Day2	-	1.33bc
Day3	+	1.69c
Day3	-	1.18b
Day6	+	1.50abc
Day6	-	0.50ab
Time	**WO**	**GUS-positive zones per GUS-positive explant section**
Day0	+	0.00a
Day0	-	0.00a
Day1	+	1.23ab
Day1	-	1.24b
Day2	+	1.53b
Day2	-	1.39b
Day3	+	1.46b
Day3	-	1.49b
Day6	+	0.33ab
Day6	-	1.40ab

Different letters indicate significant difference at $p<0.05$ (one-way ANOVA posthoc Tukey HSD test). Data on day 0 report GUS staining of 30 explants from 2 experiments. Daily means of 2 experiments each involving 480 explant sections from 120 explants and daily means for wounding or acetosyringone from 2 experiments each involving 240 explant sections of 120 explants are shown. AS-acetosyringone, WO-wounding, +-presence, --absence

However, when evaluating the evolution of the number of GUS-positive zones per GUS-positive explant section in further detail, those explants cocultivated in the presence of

acetosyringone exhibited a further increase on day 3 whereas those cocultivated in the absence of acetosyringone exhibited a maximum number of GUS-positive zones per GUS-positive explant section on day 2, followed by a decrease on day 3 (figure 13b and table 30). This was substantiated by the statistical analysis of results of day 2 and day 3 (table 26, F_{AS}=8.32, p=0.004). Wounding did not significantly impact the number of GUS-positive zones per explant section observed on day 2 and day 3 (table 26, F_{WO}=0.72, p=0.40).

a

b
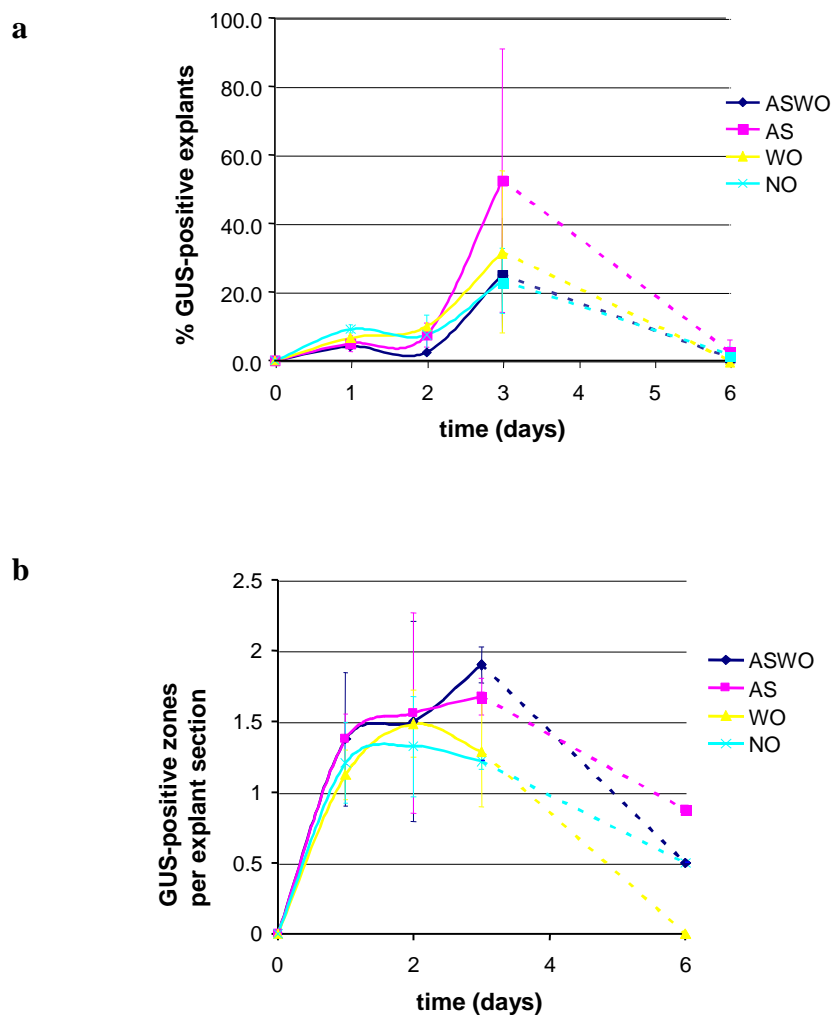
Figure 13. Evolution of transient expression in marula explants.

a. Percentage GUS-positive explants. b. Number of blue zones per explant section. Means of 2 experiments of 60 explant sections from 15 explants per treatment per day are shown. Vertical bars represent standard deviation. ASWO-cocultivation in the presence of acetosyringone and wounding of explants. AS-cocultivation in the presence of acetosyringone. WO-cocultivation of wounded explants. NO-cocultivation in the absence of acetosyringone and wounding.

4.3.3b Effect of acetosyringone and wounding on the percentage of GUS-positive explants

No significant differences ($p<0.05$) were observed among treatments on days 0, 1, 2 and 6 (table 27). On day 3, cocultivation in the presence of acetosyringone without wounding delivered the highest percentage of GUS-positive explants between 23.3 and 81.7%, 52.5% on average. This differed significantly ($p<0.05$) from other treatments on day 3 and all observations on days 1, 2, and 6. However, when explants were wounded and cocultivated in the presence of acetosyringone, the percentage of GUS-positive explants (25%) was lower than that when unwounded explants were cocultivated with acetosyringone (52.5%) and differed not significantly from that after cocultivation of wounded (31.7%) or unwounded (23%) explants in the absence of acetosyringone on day 3 (table 27). An antagonistic effect was thus observed between wounding and the application of acetosyringone during cocultivation on the percentage of GUS-positive explants on day 3 ($F_{ASWO}=18.36$, $p=0.00002$). The antagonistic effect of wounding and acetosyringone during cocultivation was further supported by factorial ANOVA and posthoc Tukey HSD tests when considering data for the total duration of experiment (table 28, $F_{ASWO}=15.11$, $p=0.0001$).

Table 27. Effect of acetosyringone and wounding on the percentage of GUS-positive explants

Treatment	Day 0	Day 1	Day 2	Day 3	Day 6
0	0.0a	0.0a	0.0a	0.0a	0.0a
ASWO	0.0a	4.2a	2.5a	25.0b	0.8a
AS	0.0a	5.0a	7.5a	52.5c	2.5a
WO	0.0a	6.7a	10.0a	31.7b	0.0a
NO	0.0a	9.2a	7.5a	23.3b	1.7a

Different letters indicate significant difference (factorial ANOVA, posthoc Tukey HSD test p<0.05). Data represent means of two experiments of 60 sections of 15 explants per treatment. Only 15 explants each were tested once for the day 0 and the no *Agrobacterium* control (0). ASWO-cocultivation in the presence of acetosyringone and wounding of explants, AS-cocultivation in the presence of acetosyringone, WO-cocultivation of wounded explants, NO-cocultivation in the absence of acetosyringone and wounding.

Table 28. Effect of wounding and acetosyringone during cocultivation on the percentage of GUS-positive explants

AS	WO	GUS-positive explants (%)
+	+	6.5a
-	-	8.3a
-	+	9.7ab
+	-	13.5b

Different letters indicate significant difference at p<0.05 (factorial ANOVA posthoc Tukey HSD test). Means of 2 experiments of 240 explant sections from 60 explants are shown. AS-acetosyringone, WO-wounding, +-presence, --absence

The percentage of GUS-positive explants observed on day 6, indicative of stable transformation correlated with the transient expression observed on day 3 for cocultivation of unwounded explants in the presence and absence of acetosyringone. Lowest putative stable GUS expression was observed when explants were wounded during cocultivation (table 27).

The percentage of GUS positive explants after cocultivation in presence of acetosyringone without wounding significantly differed (p<0.05) from that obtained after

cocultivation in the presence of acetosyringone plus wounding and cocultaivtion in the absence of both acetosyringone and wounding but not from that obtained after cocultivation of wounded explants without acetosyringone (table 28).

Explants that were not cocultivated with *Agrobacterium tumefaciens* were GUS-negative throughout the experiments in contrast to those cocultivated with *Agrobacterium tumefaciens* and the transgenic tobacco plant harboring the GUS transgene (figure 14).
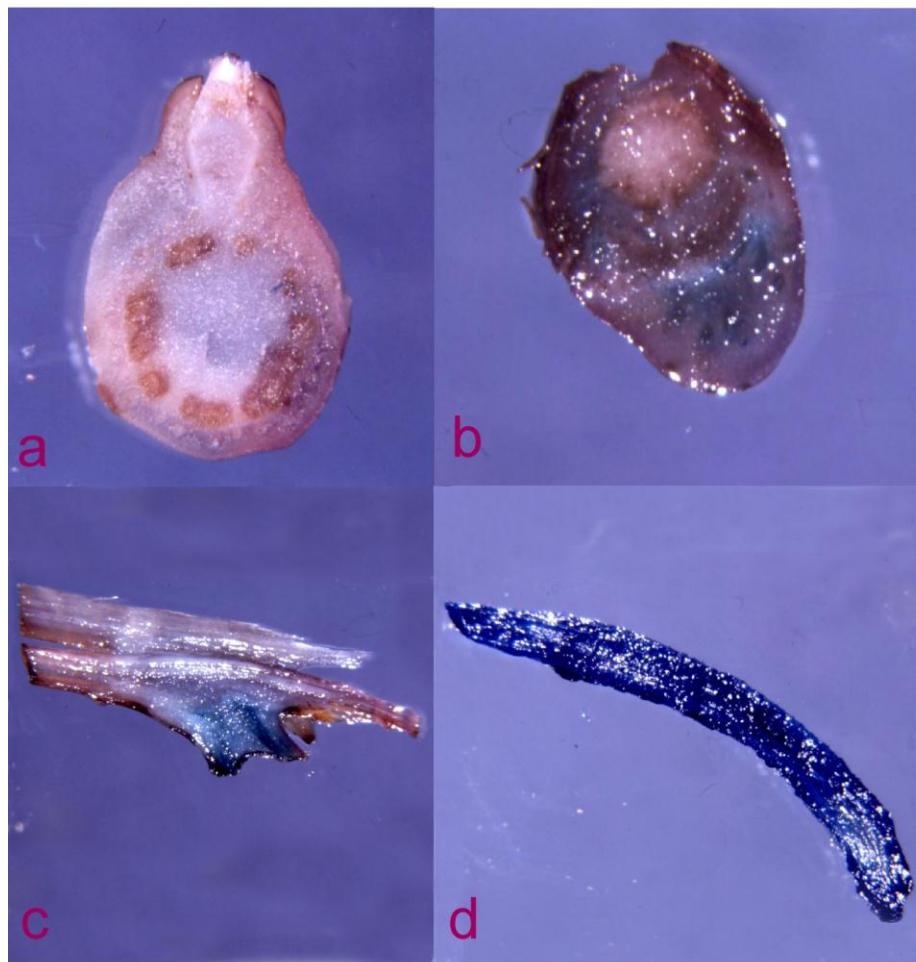

Figure 14. GUS expression of marula and tobacco explants.

a. Not cocultivated marula explant (transverse cut). b. Marula explant on day 3 after cocultivation in the presence of acetosyringone (transverse section). c. Longitudinal section of b. d. GUS-positive transgenic tobacco explant.

4.3.3c Effect of acetosyringone and wounding on the number of blue stained zones per explant

Cocultivation in the presence of acetosyringone showed a significant positive effect on the number of GUS-positive zones per GUS-positive explant section (table 29, F_{AS}=5.51, p=0.02), whereas wounding did not show an effect (table 29, F_{WO}=0.46, p=0.50). No interaction between acetosyringone and wounding was observed (table 29, F_{ASWO}=0.073, p=0.79).

Table 29. Effect of wounding and acetosyringone during cocultivation on the number of GUS-positive zones per GUS-positive explant

AS	WO	GUS-positive zones per GUS-positive explant
-	+	1.13a
-	-	1.15a
+	-	1.55b
+	+	1.68b

Different letters indicate significant difference at p<0.05 (factorial ANOVA posthoc Tukey HSD test). Means of 4 samplings of 15 explants each per treatment in 2 experiments are shown. AS-acetosyringone, WO-wounding, +-presence, --absence

Little significant difference among the individual treatments at days 1, 2, 3 and 6, resulting in 1.1 to 1.9 GUS-positive zones per GUS-positive explant section was observed (table 30). Only the number of GUS-positive zones per GUS-positive explant section after cocultivation of wounded explants in the presence of acetosyringone observed on day 3 (1.9) differed significantly (p<0.05) from that observed on day 3 after cocultivation of wounded explants in the absence of acetosyringone (1.1). None of the other observations differed significantly.

Table 30. Effect of wounding and acetosyringone on the number of GUS-positive zones per GUS-positive explant

Treatment	Day 0	Day 1	Day 2	Day 3	Day 6
0	-	-	-	-	-
ASWO	-	1.4ab	1.7ab	1.9b	1.0ab
AS	-	1.3ab	1.7ab	1.6ab	1.7ab
WO	-	1.1ab	1.5ab	1.1a	-
NO	-	1.2ab	1.1ab	1.3ab	1.0ab

Different letters indicate significant difference (factorial ANOVA, posthoc Tukey HSD test $p < 0.05$). Data represent means of two experiments of 60 sections of 15 explants per treatment. ASWO-cocultivation in the presence of acetosyringone and wounding of explants, AS-cocultivation in the presence of acetosyringone, WO-cocultivation of wounded explants, NO-cocultivation in the absence of acetosyringone and wounding, -- samples without GUS-positive explants, zones could not be reported

Therefore the addition of acetosyringone without explant wounding was proposed as the cocultivation conditions for marula and *Agrobacterium tumefaciens*.

4.3.4 Location of GUS stain

In cocultivated marula nodal explants, the GUS-positive zones were observed in parenchyma cells and vascular bundles that lead to the leaf stalk (figure 14).

4.3.5 Preliminary data on putative chimeric transformants

After 4 weeks on selection medium (MS multiplication medium supplemented with 300mgl^{-1} kanamycin and 500mgl^{-1} cefotaxime), 130 shoots emerged from a total of 104 explants from two experiments, a multiplication rate of 1.25 shoots per explant, half of that observed during micropropagation (table 15). One hundred and two (78%) emerging shoots were bleached, 25 shoots (19%) exhibited both bleached and green sectors and 3 shoots (2%) were completely green. After 6 weeks on elongation medium supplemented

with 300mgl^{-1} kanamycin, only two of the green shoots (1.5%) survived and remained

green. One of the subcultured green shoot and all the subcultured partially green shoots

died or bleached substantially (figure 15a and b). GUS staining of putative chimeric

transgenic marula shoots revealed high level of GUS activity in virtually all tissues of one

shoot whereas a low level of and zonal GUS activity was observed in the second shoot

(figure 15c). No molecular analysis was conducted to determine the presence of the *npt*II

and *uid*A transgenes or the number of integration events in the putative chimeric

transformed marula shoots.

Figure 15. Chimeric marula shoots.

a. Cocultivated shoots with both green and bleached sections on MS elongation medium supplemented with 300mgl^{-1} kanamycin. b. Chimeric green shoot on MS elongation medium supplemented with 300mgl^{-1} kanamycin. c. Chimeric GUS-positive explants (top-deep blue and bottom-light blue) of putative transformed shoots.

CHAPTER 5. DISCUSSION AND CONCLUSION

5.1 Micropropagation

5.1.1   Surface sterilization of explants from growth room versus field plants

Establishment of aseptic cultures is a first step towards any *in vitro* plant multiplication (Narayanaswamy, 1994; Gamborg and Phillips, 1995). Often, establishment of aseptic cultures is easier of explant materials obtained from a contained environment compared to field collected materials due to their heavy contamination with dust and both endophytic and surface microorganisms (Cassells, 1991; George, 1993; Narayanaswamy, 1994; Gamborg and Phillips, 1995). This was also observed in this work.

Marula nodal explants harvested from the growth room were effectively surface sterilized with 70% ethanol for 1 minute, 30% (v/v) bleach containing 0.1% (v/v) Tween 20 for 10 minutes, resulted in routine 100% aseptic cultures (table 12). Field marula nodal explants collected in the October-November season decontaminated with the same solutions for longer exposure times of 2 minutes in 70% ethanol and 20 minutes in 30% (v/v) bleach containing 0.1% (v/v) Tween 20 resulted in only 13% microbial free cultures. Additional procedures (soaking in fungicide followed by wash in antibacterial dishwashing detergent) prior to surface sterilization of field explants had to be included to achieve 73% aseptic cultures of nodal explants harvested from the field (table 14). Although *Anacardiaceae* explants harvested from the field and green house were mostly decontaminated with $HgCl_2$ (0.05 to 0.1%) and some with NaOCl (1.05 to 8%) the aseptic culture establishment was lower (3 to 74%) (table 3) compared to results obtained

in this work. In marula, acceptable success rates (73 to 100%) for aseptic culture establishment were achieved using the gentler and safer NaOCl (1.5%) immersion. Lievens *et al*. (1989), Das *et al*. (1996) and Thomas and Ravindra (1997) included fungicide soak pretreatment steps prior to surface sterilization of cashew and mango explants. Lievens *et al*. (1989) obtained 74% healthy cultures of cashew from green house grown seedlings after a fungicide soak for 10 minutes followed by 9% $Ca(OCl)_2$ solution for 25 minutes. Only Das *et al*. (1996) obtained limited success (3 and 21%) when establishing aseptic cultures of explants from mature cashew trees. Other authors reported on the *in vitro* germination of field harvested seeds or nuts (Barghchi and Alderson, 1983; Litz *et al*., 1984; Philip, 1984; Leva and Falcone, 1990; Abousalim and Mantell, 1992; D'Silva and D'Souza, 1992; Das *et* al., 1996; Anathakrishnan *et al*., 2002; Cardoza and D'Souza, 2002).

Both $HgCl_2$ and NaOCl immersion with or without pretreatment were reported for surface sterilization of the non-*Anacardiaceae* (Mhatre *et al*., 2000; Rama and Pontikis, 1990; Naik *et al*., 1999). In pomegranate, 95% cultures of field origin were free of the microbial contaminants after a 3-hour detergent soak prior to 0.1% $HgCl_2$ decontamination procedure (Naik *et al*., 1999). Percentage aseptic cultures were not reported for other field-harvested explants.

Eradication of fungal or bacterial contaminants was a major problem in mature pistachio, mango and cashew explants (Barghchi and Alderson, 1983; Abousalim and Mantell, 1992; Thomas and Ravindra, 1997; Das *et al*., 1996; 1999). In pistachio, one measure to decrease contamination was to reduce the size of explants cultured; this was unsuccessful

(Onay, 1996). Onay (2000) sprouted tree truncheons of pistachio in a controlled environment before *in vitro* establishment. In mango, explants collected from field-grown trees remained aseptic for only 2 to 3 weeks (Thomas and Ravindra, 1997). Cashew shoot tips and nodes from the field only exhibited a 3 and 21% survival rate after decontamination but tissue cultures were not established (Das *et al*., 1996). In contrast, all aseptic cultures of marula explants (73%) harvested from field-grown trees in October-November exhibited growth.

5.1.1a Explant types

Of the field harvested marula explants, shoot tips were more difficult to decontaminate than stem and leaf explants (table 13). An underlying reason may be found in the structure of the shoot tip. Marula shoot tips harbor numerous leaves in close-set scales, which prevent penetration of a decontaminating solution. Thomas and Ravindra (1997) experienced a similar problem when surface sterilizing mango shoot tip explants. The authors reported that additional decontamination treatments delayed appearance of contamination to 4 weeks (Thomas and Ravindra, 1997).

Surface sterilization methods, which proved efficient to decontaminate other marula explant types, did not establish aseptic cultures from root explants. Roots are underground organs which grow in close proximity with vermiculite or soil and may have a high microbial load in contrast to above ground plant parts (leaves, shoot tips, nodal sections). Narayanaswamy (1994) suggested that surface sterilization of heavily contaminated explants materials such as underground explants can be achieved by using

HgCl$_2$. HgCl$_2$ however has been implicated in growth inhibition (Narayanaswamy, 1994) and was not evaluated in this study.

5.1.1b Seasonal effect

Apart from the general health of the parent tree, seasonal changes impact the load of microorganisms and influence the attainment of aseptic cultures (Cassells, 1991; Narayanaswamy, 1994; Gamborg and Phillips, 1995). In marula, nodal explants collected from the field during the rainy season (October-November) were heavily contaminated compared to those collected at the end of the rainy season (February-March) as evidenced by percentages of aseptic cultures obtained (13 and 80%, respectively) using 2 minutes in 70% ethanol, 20 minutes in 30% bleach containing 0.1% Tween 20 surface sterilization method. The difference in percentage of aseptic marula nodal cultures between different collection periods could be due to the abundance of microorganisms in the field as influenced by weather conditions. February-March is characterized by dryer weather whereas October-November is the rainy season characterized by high humidity. The latter condition is conducive for the flourishing of microbial organisms. The implied higher microbial load of explants collected in October-November than those collected in February-March was not directly investigated.

Seasonal effects on microbial contamination of explant materials were also reported in mango (Thomas and Ravindra, 1997), papaya (Litz and Conover, 1981) and capsicum (Mythili and Thomas, 1995). Thomas and Ravindra (1997) reported that mango shoot tips collected during the June-August season (spring-summer season in Asia) exhibited minimum microbial contamination in comparison to shoot tips collected in other months.

5.1.2 Polyphenols

Freshly explanted tissues of woody plants often secrete polyphenols into the medium in response to wounding (Pierik, 1987; Debergh and Read, 1991; Nayaranaswamy, 1994). Generally explants taken from older trees tend to produce more phenolics (Thorpe *et al*., 1991). Phenolic compounds released into the medium inhibit growth and can kill the explant (Pierik, 1987; Debergh and Read, 1991; Nayaranaswamy, 1994). In preliminary experiments, browning of any type of marula explants and media was excessive, which resulted in explant death. Similar effects of polyphenols were reported in pistachio (Barghchi and Alderson, 1983; 1985; Martinell, 1988), cashew (Das *et al*., 1996; 1999), mango (Thomas and Ravindra, 1997), pomegranate (Naik *et al*., 1999; 2000) and olive (Rugini, 1984). In contrast, Boggetti *et al*. (1999) reported that browning of cashew nodal explants was not inhibitory to the survival of more than 80% of explants.

In order to overcome the browning problem, antioxidants or adsorbents are supplemented in the tissue culture medium (Pierik, 1987; Debergh and Read, 1991; Narayanaswamy, 1994). In marula, the adsorbent PVP (0.1%) was supplemented to the multiplication medium. This and the bi-daily transfer of field explants to fresh media during the first week in culture alleviated the problem of browning and premature death of marula explants. A similar approach of using an adsorbent AC (0.5%) was used in cashew (Das *et al*., 1996) and mango (Thomas and Ravindra, 1997). In cashew, frequent transfers and dark incubation for the first week of culture supplemented AC in overcoming the polyphenol problem (Das *et al*., 1996). Two consecutive transfers to fresh medium solved the problem of browning in pomegranate explants (Naik *et al*., 1999). Lievens *et al*.

(1989) reported that addition of an antioxidant (ascorbic acid) in the medium alleviated the polyphenol problem in cashew.

Presence of adsorbent in the medium can be beneficial or detrimental to growth *in vitro* (Boggetti *et al*., 1999). In marula, 0.1% PVP was useful in alleviating the polyphenol and premature explant death problem in shoot multiplication and elongation. In contrast, 0.08% PVP in the rooting medium resulted in stubby adventitious roots, which did not elongate. Whereas inclusion of AC (0.3%) in the rooting medium, which also reduced light penetration in the medium resulted in long adventitious roots. In cashew, presence of AC in the medium and cultivation in darkness for the first week of culture benefited shoot elongation but suppressed bud sprouting (Boggetti *et al*., 1999). Conditioning of shoots in AC containing medium was beneficial for sour sop rooting (Lemos and Blake, 1996).

5.1.3 PGR for *in vitro* shoot proliferation

PGR concentrations and combinations optimal for bud break, shoot multiplication, elongation and root induction are plant species and often variety specific (Pierik, 1987; Litz and Jaiswal, 1991; Narayanaswamy, 1994) and are thus determined empirically for each plant variety. As far as known this is the first report on micropropagation of marula. For new plant species, often protocols developed for closely related species serve as a starting point for experimentation and development of protocols.

5.1.3a Bud break

Bud break rates of 72 and 100% were recorded from seedling and field-grown tree marula nodal explants cultured on MS medium containing 4.8μM BA and 2.4μM KN, respectively. Similar bud break rates of 70 and 80% on cashew nodal explants cultured on MS medium supplemented with 1μM 2iP and 8.9μM BA were reported by Boggetti *et al*. (1999) and Lievens *et al*. (1989), respectively. In pistachio, a bud break rate of 82% was observed on MS medium containing 17.75μM BA (Barghchi and Alderson, 1985). Macadamia nodal explants cultured on MS medium supplemented with 8.87μM BA and 2.89μM GA_3 resulted in 100% bud break (Mulwa and Bhalla, 2000). Naik *et al*. (1999) reported 85% bud break of nodal explants of pomegranate cultured on MS medium supplemented with 5.69μM ZR. In guava, 77% bud break was recorded on nodal explants cultured on MS multiplication medium supplemented with 4.5μM BA (Amin and Jaiswal, 1987). In marula, bud break at a lower rate of 30% was also observed in the absence of exogenous PGR, a response indicative of the presence of endogenous PGR in marula nodal explants. The authors above did not comment on the response of respective explants on media without PGR.

5.1.3b PGR for microshoot proliferation

Juvenile growing tissues with preformed meristems (nodal explants with axillary buds) were used in the study. These explants have an advantage of easy response *in vitro* (Vasil, 1980; Yeoman, 1986; Stafford and Warren, 1991) and show least somaclonal variation among propagated plants (Gamborg and Phillips, 1995; Bornman, 1998). MS medium supplemented with 4.8μM BA and 2.4μM KN was optimal for *in vitro*

proliferation of marula nodal explants obtained from 2 to 24 month old seedlings. An average of 2.5 shoots per responding node or 1.8 shoots per sterile explant were recorded. Das *et al*. (1996) recorded a maximum of 12, 4 and 2 buds for cashew cotyledonary nodes, leaf axils and shoot tips respectively from 1 month old seedlings on the very similar MS medium supplemented with 4.4µM BA, 2.32µM KN and 9.12µM ZN. The maximum of 4 buds per cashew leaf axil compared to the 2 to 4 shoots observed per responding marula nodal section, which is referred to as leaf axil by Das *et al*. (1996). In pistachio, MS medium supplemented with BA alone (8.8µM, Onay, 2000; 17.75µM, Barghchi and Alderson, 1985; 3.1, 4.44, 8.87µM Martinelli, 1988) was sufficient for both shoot proliferation and elongation. Whereas MS medium containing 17.75µM BA and 1.34 µM NAA resulted in 5.5 shoots per pistachio explant and the same medium was used for shoot elongation (Barghchi and Alderson, 1983). Onay (2000) and Barghchi and Alderson (1985) reported that pistachio shoots and nodal explants produced 3.7 and 0.89 to 2 shoots per explant, respectively. In other woody species, cytokinins, especially BA alone or in combination with auxin or gibberellins, were useful for shoot amplification (Mhatre *et al*., 2000; Rama and Pontikis, 1990; Amin an Jaiswal, 1987; Purohit and Dave, 1996; Lemos and Blakes, 1996; Gavidia *et al*., 1996; Mulwa and Bhalla, 2000). In marula, BA concentration was a significant factor in bud break in contrast to KN concentration whereas both BA and KN concentrations impacted on shoot number (table 16 and 17). In cashew, 2iP favored proliferation of cashew nodal explants (Boggetti *et al*., 1999). In contrast KN and TDZ inhibited sprouting and branching of nodal explants from the glasshouse, whereas BA (5 to 20µM) promoted branching of subcultured branches of cashew (Boggetti *et al*., 1999).

5.1.3c Callus formation

Callus formation on marula explants was excessive on multiplication media containing total cytokinin concentrations above 8.4μM. Callus started at the base of explants and at higher cytokinin concentrations spread to growing shoots. Boggetti *et al.* (1999) and Barghchi and Alderson (1983, 1985) reported similar observations on cashew explants cultured on multiplication media supplemented with 20μM of BA, ZN and KN and pistachio explants on media containing more than 4μM BA, respectively. In pistachio explants, callus growth was followed by senescence at the base of explants, which according to Barghchi and Alderson (1983) slowed down nutrient uptake by the shoots. Callusing of shoots will increase somaclonal variation, reduce the efficiency of generating phenotypically normal shoots and impact nutrient and water transport when occurring at the shoot-root interface. In marula multiplication on MS medium supplemented with 4.8μM BA and 2.4μM KN callus remained near the bottom of the shoots and was removed before transfer of shoots to elongation MS medium supplemented with 1.2μM BA and 1.0μM KN. Callus was never observed on elongation medium and rooting medium and thus did not affect the vascular connection between shoot and root.

5.1.3d PGR for microshoot elongation

Marula microshoots were elongated on MS medium containing a low concentration of cytokinin (1.2μM BA and 1.0μM KN). Lowering cytokinin concentration in the tissue culture media was used to achieve elongation in cashew (D'Silva and D'Souza, 1992), pomegranate (Naik *et al.*, 1999) and guava (Amin and Jaiswal, 1987). D'Silva and

D'Souza (1992) lowered cytokinin concentration from 22.2µM to 4.4µM to elongate cashew shoots. Naik $et\ al$. (1999) reduced ZR concentration from 5.69µM to 2.85µM to achieve elongation of pomegranate shoots. In guava elongation, BA concentration was lowered from 4.5µM to 0.5µM. Das $et\ al$. (1996) and Onay (2000) achieved both proliferation and elongation of cashew and pistachio microshoots on medium containing the same concentration of cytokinin (4.4µM BA, 2.32µM KN, 9.12µM ZN and 8.8µM BA), respectively. In contrast, prolonged incubation of marula shoots on a similar high cytokinin (4.8µM BA and 2.4µM KN) medium resulted in excessive callus.

GA_3 (1.5µM, Lievens $et\ al$., 1989; 20µM, Boggetti $et\ al$., 1999; 2.89µM, Mulwa and Bhalla, 2000) has been applied successfully in tissue cultures to promote shoot growth and elongation. In marula, however GA_3 incorporation in the media (1, 5µM) did inhibit microshoot elongation. Similarly, Sriskandarajah $et\ al$. (1990) reported a negative effect of GA_3 on apple microshoot elongation, which remained short. In addition, GA_3 yielded pale shoots compared to those on BA and KN media. Yang and Lüdders (1993) also reported that GA_3 did not improve shoot growth of mango. In contrast, Lievens $et\ al$. (1989), Boggetti $et\ al$. (1999) and Leva and Falcone (1990) reported that GA_3 aided the bud break, sprouting and elongation of cashew. Also in macadamia, microshoots elongation was achieved on culture media supplemented with 8.87µM BA and 2.89µM GA_3 (Mulwa and Bhalla, 2000).

5.1.3e   PGR for rooting microshoots

Auxin, especially IBA is most often used for rooting microshoots (table 6). Marula microshoots were successfully rooted by continuous incubation for 6 to 8 weeks in MS

medium at half strength salts supplemented with 10μM IBA and 0.3% AC. A rooting efficiency of 82% and 2 to 3 long adventitious roots per microshoot were recorded. Similarly, MS at half salt strength supplemented with different concentrations of IBA (10, 0.53, 2.5, 4.92, 1.5, 9.8μM) and continuous incubation of microshoots in rooting media was used in strawberry (Mereti *et al*., 2002), grape (Heilor *et al*., 1997; Mhatre *et al*., 2000), peach (Dimassi-Theriou, 1995), pear (Baraldi *et al*., 1995) and pistachio (Onay, 2000), respectively. Amin and Jaiswal (1987) rooted guava microshoots on half strength salts MS medium supplemented with 1μM of both NAA and IBA.

Alternatively, treatment of shoots with high concentration of auxin for several hours to days followed by transfer of shoots to auxin free media for further elongation have been reported (table 6). Cashew (Das *et al*., 1996; Boggetti *et al*., 1999), pomegranate (Naik *et al*., 1999), macadamia (Mulwa and Bhalla, 2000) and kadaya gum (Purohit and Dave, 1996) shoots were subjected to auxin (2460, 100, 4.92, 14.92, 2460.6μM, respectively) treatment prior to transfer to auxin free media.

The latter approach to rooting is mostly applied when no or low rooting is observed on low auxin media and callus forms upon prolonged incubation on auxin containing media. No callus was observed at the shoot/root zone transition although marula shoots were rooted continuously in IBA supplemented MS medium. This may imply that plantlets developed a well-connected vascular system. In contrast, incubation of pistachio rooted shoots beyond 4 weeks in IBA (9.8μM) containing medium led to excessive callus formation at the base of the shoots and deformation of the roots (Onay, 2000). Barghchi and Alderson (1983, 1985) also reported that culture of pistachio microshoots on rooting

media beyond 15 days resulted on callus formation and darkening of the roots. Although, guava microshoots were successfully rooted on 1μM IBA and 1μM NAA supplemented media, root growth was arrested after a few millimeter extension and root tips became blunt and blackened (Amin and Jaiswal, 1987). In marula, stubby adventitious roots were formed on microshoots when PVP (0.08%) was included in the rooting medium supplemented with IBA (1, 3, 5, 10μM), which even with prolonged culture period did not elongate.

Reasons for callus formation upon transfer from a cytokinin medium to an auxin medium could be found in the carry-over effect of PGR from one medium to the other. A recovery period is important to prevent carry-over of PGR from one treatment to the next (Stafford and Warren, 1991). Without a recovery period between high cytokinin containing multiplication and elongation media and high auxin containing rooting media, the newly transferred microshoots are reacting to the presence of both cytokinin and auxin at concentration, which may induce callus formation. In marula, recovery from cytokinin effect prior to rooting was achieved on MS medium free of PGR. This procedure was also used for sour sop microshoots (Lemos and Blake, 1996). A well-developed root-shoot vascular connection and root system underlie successful acclimatization, growth and production of micropropagated trees in the field.

5.1.4   Acclimatization

Gradual reduction in humidity, gradual shift from heterotrophic to autotrophic and sterility are key to high acclimatization success (Pierik, 1987; Preece and Sutter, 1991). Marula plantlets were acclimatized gradually in sterile vermiculite watered with half

strength MS salts followed by transfer to sterile garden soil. Ninety percent plantlets survived weaning. A comparable survival rate of 70 to 80% was achieved by Barghchi and Alderson (1985) who hardened-off pistachio plantlets, in a peat and compost mixture. In contrast, Onay (2000) recorded a survival rate of 50% only for pistachio plantlets originated from rejuvenated mature branches. In cashew, Boggetti *et al*. (1999) and Das *et al*. (1996) obtained survival rates of 40 to 50% and 28% on vermiculite and peat mixtures, respectively. These rates are low compared to this work and that of guava (Amin and Jaiswal, 1987) and may have among others have resulted from poor vascular root-shoot connectivity and a poorly developed root system.

In strawberry (Mereti *et al*., 2002), pomegranate (Naik *et al*., 1999), apple (Bolar *et al*., 1998), olive (Rama and Pontikis, 1990) and guava (Amin and Jaiswal, 1987) survival rates of 60%, 80%, 70 to 100%, 50%, and 90% respectively were recorded. Plantlets were acclimatized either on vermiculite, peat and vermiculite mixture, vermiculite and sand mixture or garden soil respectively.

Marula plantlets, which did not survive weaning, died of damping-off, a fungal infection. In order to further increase acclimatization rates, fungicide spraying could be included in marula hardening. Spraying plantlets with a fungicide (Thirame) solution and biological protectant prevented fungal infection in cashew (Lievens *et al*., 1989) and apple (Bolar *et al*., 1998) plantlets, respectively. Pierik (1987) and Preece and Sutter (1991) recommended prevention of any pathogens and insects from the plantlets by inclusion of fungicide and pesticide application in routine hardening procedures.

Apart from plant protectants, apple plants were fertilized during acclimatization (Bolar *et al*., 1998). Fertilization was also applied to olive plants during hardening-off (Rugini, 1984). Marula plants were watered with half strength MS salts during initial phases of hardening. Good root-shoot vascular connection as implied by the absence of callus on rooting media and the high rooting efficiency, fertilization, the sterilization of soil, extensive washing to remove media from roots and the gradual decrease in humidity started before the shift of plantlets from the heterotrophic MS medium to the autotrophic soil may underlie the efficient marula plantlet acclimatization.

5.1.5 Recycling of proliferated marula microshoots

Cycling of newly generated shoots onto a high cytokinin medium can dramatically increase the shoot proliferation rate from one explant. Prolonged incubation of shoots on proliferation medium increased the amount of callus dramatically. Lowering of sucrose concentration to 0.3%, application of B5 medium with MS vitamins or half strength MS medium did not alleviate the callus problem. Pistachio (Onay, 2000), guava (Amin and Jaiswal, 1987) and grape (Heilor *et al*., 1997; Mhatre *et al*., 2000) microshoots however were successfully cycled using the same high cytokinin media composition or lowered cytokinin concentration media, respectively. Marula research on cycling has not been exhaustive and incorporation of a recovery medium for a prolonged period may enable cycling of marula to achieve higher proliferation rates from fewer explants.

Differences observed in marula tissue culture behavior could be linked to subtle differences in endogenous PGR. For example, although elongation was readily achieved at high cytokinin concentration in cashew (Das *et al*., 1996) and pistachio (Onay, 2000)

in marula, elongation efficiency is presently the bottleneck to marula micropropagation efficiency.

5.1.6   Propagation of adult tree material

5.1.6a   Rejuvenation

Due to difficulties of *in vitro* establishment of explants derived from adult trees, rejuvenation is often recommended (Pierik, 1987; Franclet, 1991; Narayanaswamy, 1994). Attempts to rejuvenate marula by imbibition in 4.8μM BA and 2.4μM KN, and SEDADIX (IBA rooting powder) application resulted in sprouting in 8 and 8.5% of tested truncheons, respectively. Shoots did not continue to grow and with time they withered. Frequencies were considered too low for eventual commercial application and sprouts were not transferred to tissue culture. Onay (2000) obtained a similar response rate of 10% by rejuvenating adult pistachio branches in 44μM BA. Upon introduction of the shoots *in vitro* he attained 3.7 shoots per explant and 20 shoots per explant after subcultivation. However, in cases where explant material is limited, the micropropagation rate per amount of source material must be considered.

Harvesting of explants from zones, which are still juvenile (Pierik, 1987) formed a viable alternative to rejuvenation of adult tree material in marula. An average of 1 shoot per nodal explant, a juvenile stem cutting of around 1.5 cm in length, was recorded.

Due to the difficulty of rejuvenation of mature trees, much research concentrates on explants of younger materials such as somatic embryos from nucellar tissue and cotyledonary explants (Litz *et al*., 1984; Philip, 1984; Ananthakrishnan *et al*., 2002;

Cardoza and D'Souza, 2002) and adventitious shoot formation from seedling explants (Litz *et al*., 1984; Barghchi and Alderson, 1983, 1985; Martinelli, 1988; Leva and Falcone, 1990; D'Silva and D'Souza, 1992; Boggetti *et al*., 1999). However both of these explants do not provide good starting materials for commercial propagation of a superior tree (Gamborg and Philip, 1995). Offspring of heterozygous parent trees are neither genetically homogenous nor identical to their parents.

5.1.6b   Age of explant source

The developed protocol for *in vitro* multiplication of marula seedling explants was adopted for adult field materials. During the October-November season juvenile explants were harvested directly from field-grown trees and were successfully established in tissue culture. In cashew, materials from mature trees did not survive *in vitro* due to inefficiencies of sterilization and browning in culture (Das *et al*., 1996). These problems were adequately overcome in the establishment of marula tissue culture from timely harvested new sprouts from field-grown adult trees. After 2 weeks in culture marula nodal explants obtained from seedlings and field-grown adult trees resulted in a bud break rate of 72 and 100%, respectively. Proliferation on tree nodal explants however was limited to 1 shoot per node even at apparent high exogenous cytokinin levels, which achieved 2 to 4 shoots on 72% of the seedling nodal explant. These observations may be attributed to the larger size of tree explants as well as the ratio of endogenous to exogenous PGR, which drove the growth of one shoot for each tree nodal explant harvested from the field at the beginning of the rainy season. Thicker explants have more food reserves and endogenous PGR than thinner explants (Pierik, 1987). Other factors

may include the physiological state and developmental program of the marula tree versus seedling explant. The results however may thus hint towards the necessity to optimize media compositions for tree versus seedling explants, which is contrary to present accepted practice.

In marula the lower bud break response (72%) of young seedling material versus older tree material (100%) however contrasts other reports. Das *et al.* (1996) recorded 90% response of cashew seedling explants compared to only 3% of field materials. The latter low response may however be due to other factors including insufficient medium optimization since with time all tree explants turned brown and died. Age of explant also had an effect on *in vitro* establishment and multiplication as exemplified by 1-month-old cashew material, which performed better than 1-year old material (Boggetti *et al.*, 1999). In marula, the impact of age of seedlings (2 to 24 months) on *in vitro* establishment and development was not evaluated. The unpredictable cycles of elevated temperatures in the growth room may have affected the physiological state of certain seedling axillary meristems resulting in the lower bud break for seedling than tree explants. In addition, the effect of cut back and removal of apically dominant shoots, which may have impacted endogenous PGR in seedling, cannot be excluded.

As in marula, greater proliferation of shoots on young materials (6.1 shoots per explant) than old material (5 shoots per explant) was reported in laurel (Gavidia *et al.*, 1996). Boggetti *et al.* (1999) also reported that young seedling explants responded better than older seedling explants. In contrast, Mulwa and Bhalla (2000) recorded a better *in vitro* response for 2-year-old macadamia trees (10.7 shoots per explant) than 1-month old

seedlings (6 shoots per explant). In marula, comparison of *in vitro* response of seedling explants from seedlings of different ages (2 to 24 months) was not evaluated. However, a relatively large variation of *in vitro* response of marula was observed indicated by the large relative standard deviations. This may be due to an explant age effect, differences in physiological states of the preformed apical meristems as well as the heterogeneous genetic background of the explant source evidenced from the RAPD analysis (table 21). Barghchi and Alderson (1985) also reported a high variation in the response of pistachio explants to culture conditions.

5.1.6c Effect of season

The season at which explants are harvested affects *in vitro* establishment (Pierik, 1987). In marula, explants harvested in February-March when winter dormancy may have set in did not show any growth, whereas those collected in October-November in the beginning of the rainy season, responded *in vitro* with a response of 100% bud break and 1 shoot per responding explant. Similarly, seasonal effect on *in vitro* establishment was reported in guava (Amin and Jaiswal, 1987), mango (Yang and Lüdders, 1993; Thomas and Ravindra, 1997), apple (Hutchinson, 1984), papaya (Litz and Conover, 1981), red raspberry (Anderson, 1980) and sweet gum (Sutter and Barker, 1985). In guava, explants harvested in April-June (Asia, Amin and Jaiswal, 1987) established well, whereas in mango, June-August (Asia, Thomas and Ravindra, 1997) and May-June (Europe, Yang and Lüdders, 1993) were the best for explanting compared to other harvesting months. In Asia and the northern hemisphere, April-August and May-June are the growing seasons during which young sprouts are on the trees, respectively.

5.1.7 Somaclonal variation

The variation observed between parent plants and their clones produced *in vitro* could either be genetic or epigenetic (Larkin and Snowcroft, 1981; Evans, 1989; Peschke and Phillips, 1992; Skirvin *et al*., 1994). In marula, clones propagated *in vitro* through axillary bud proliferation did not exhibit genetic variation to their source plants as tested by RAPD analysis. Out of the 123 markers scored, none were polymorphic within the cloned plantlet sets. For example, the seven clones NS9-1 to NS9-7 and parent plant NS9, 85 markers were monomorphic suggesting the absence of intraclonal polymorphism within the limits of the RAPD technique. Similar observations were made for *in vitro* axillary bud proliferated kiwifruit plants and their source plants (Rugini *et al*., 2000). No intraclonal variation was observed between oak source plants and their clones that originated from nodal explants (Barrett *et al*., 1997). But even in cases where propagation procedures involved dedifferentiation stages, no variation was detected within the limits of RAPD analysis between source plants and their clones (table 8). Somatic embryo derived plants of spruce (Isabel *et al*., 1993), Norway spruce (Rani *et al*., 1995; Fourre *et al*., 1997), ginseng (Shoyama *et al*., 1997), oak (Wilhem, 2000) and asparagus (Raimondi *et al*., 2001) were identical to their source plants as were Japanese black pine (Goto *et al*., 1998) and begonia plants (Bouman and De Klerk, 2001) derived from leaf explants. In contrast, wheat (Brown *et al*., 1993), beet (Munthali *et al*., 1996), oil palm (Rival *et al*., 1998), rye (Linacero *et al*., 2000) and tea (Devarumath *et al*., 2002) clones micropropagated from protoplasts, leaf explants, somatic embryos, immature embryos and nodal segments with axillary buds, respectively were genetically different from their parent plants as determined by RAPD analysis.

5.1.7a   Reproducibility and limitations of RAPD

Reproducibility of results is often a problem associated with RAPD (Muenier and Grimont, 1993; Buscher *et al*., 1993; Micheli *et al*., 1994). In marula RAPD, mostly faint bands that were near the limit of detection in an ethidium bromide stained gel were not reproducible (figure 10). Faint bands may result from amplification of single alleles or poorly amplifying sites compared to repeat sequences and easily amplified sites. Aberrant bands occurred occasionally that were not reproduced when using DNA of an independent DNA extraction at another concentration from the same plant. These prominent bands could be due to contamination during the extraction and handling. Variations in band intensities were observed. These could have resulted from subtle differences in DNA extracts and PCR conditions, hidden mutations among alleles since RAPD are mostly expressed in a dominant fashion (Williams *et al*., 1990; Isabel *et al*., 1993) and differences in ploidy. Since the RAPD procedure in this study involved 45 PCR cycles, amplification was not quantitative and differences in ploidy levels, which have been reported in somaclonal variation, could not be detected.

5.1.7b   Combined analyses for screening somaclonal variation

Although RAPD analyses do have limitations, it was useful in marula to detect the monomorphism of parent seedlings and their clones despite the repeated excessive growth room temperatures. The same method was able to detect polymorphisms between seedling plants. Despite RAPD being a useful method in the detection of somaclonal variation, Fourre *et al*. (1997) reported that sometimes the method is not sufficient because somaclonal variation is a complex problem that requires the use of several

approaches to be correctly appreciated. The absence of intraclonal RAPD polymorphism cannot ensure genetic stability because morphological, hidden mutations such as ploidy and point mutations or subtle deletions, insertions and mutations within amplified fragments can be missed (Isabel *et al*., 1993; Raimondi *et al*., 2001). The technique is not exhaustive. Also complete genome coverage by RAPD, as a method per se is questionable and certainly here in the marula study. A total of 123 markers scored in marula, using 7 primers with 60 to 70% GC content roughly examined up to 500kb of the unknown genome size of marula. This coverage is by far too small even when considering the smallest plant genome (*Arabidopsis thaliana*, 125Mb, The *Arabidopsis* Genome Initiative, 2000). Similarly other authors investigated 5 to 259 markers (table 8) using 29 to 71 primers, an examination of 20 to 1036kb.

Bouman and De Klerk (2001) used morphological analyses (qualitative and quantitative) in begonia, of which only the quantitative analysis detected variations between plants. In asparagus (Raimondi *et al*., 2001) and spruce (Fourre *et al*., 1997) cytogenetic analyses detected mixoploidy, trisomic and tetraploid cells in buds and roots of dwarf plants and aneuploidy in plants, which were phenotypically normal, in roots, buds, embryogenic mass and somatic seedlings. Wilhem (2000) reported that DNA-content measurement by flow cytometry detected tetraploidy in some cell lines. Long-term field evaluation of micropropagated apples trees revealed interclonal variation in terms of vegetative vigor, flowering age and fruit yield, but trees did not display intraclonal variation (Zimmerman and Steffens, 1996). Even though a combined approach may give more information about somaclonal variation, interpretation will still depend on the combined limitations of all techniques used. The choice of which method to use probably will depend on various

aspects such as time, cost, equipment and the ultimate end product of micropropagation. In marula the ultimate goal of micropropagation is the multiplication of a selected superior tree. DNA polymorphisms have been documented using RAPD analyses in propagated plants cloned from tea nodal explants (Devarumath *et al*., 2002). The RAPD technique can be applied as a first screen of clones that would be used as source materials in a chain of *in vitro* multiplication. Eventual analysis will however be field performance.

5.1.7c   Occurrence of somaclonal variation

Although somaclonal variation is sometimes considered a problem in breeding and mass propagation, mutation during mitosis is a natural process. In tomato, flow cytometric analysis detected changes in ploidy levels in plants originated from hypocotyl explants but not from leaf and cotyledon explants (Van den Bulk *et al*., 1990). In senescing cork tissue, which is subject to much oxidative stress, one mutation in 1520 nucleotides was found after sequencing nuclear DNA of old and young tissue (Pla *et al*., 2000). No difference was detected between the epicormic and crown shoots of a 150 year old oak by microsatellite and RAPD analyses (Barret *et al*., 1997). Polanco and Ruiz (2002) reported that the value of nucleotide diversity estimated by AFLP analysis for each group of regenerated *Arabidopsis* plants and that can be ascribed to somaclonal variation were 2 to 3 orders of magnitudes smaller than natural variation described for natural ecotypes of *Arabidopsis*. At what frequency ploidy and phenotypic differences naturally exist and/or are generated during the tissue culture of marula needs further investigation and study over several years, which is outside the scope of this PhD thesis.

5.1.7d   Polymorphism among source plants

In marula, interclonal variation was observed between seedling source plants confirming their different genetic background. Forty nine percent of the markers were polymorphic among seedlings, which is greater than 2% observed in oak (Barrett *et al*., 1997) and 19% in oil palm (Rival *et al*., 1998). Polymorphism between source plants was expected because the seeds that were used to germinate source plants were collected from different marula trees. The greater degree of polymorphism observed in marula compared to oil palm (Rival *et al*., 1998) and oak (Barrett *et al*., 1997) could be due to absence of breeding of marula as well as its dioecious nature.

5.2 Genetic modification of marula

Due to the difficulty in the development of an efficient regeneration procedure, transformation of existing meristems offered an alternative route to evaluate the feasibility of marula transformation. The advantage of this method is the avoidance of somaclonal variation as the *de novo* regeneration procedures are bypassed (Gamborg and Phillips, 1995; Bornman, 1998). The transgenic shoots obtained can be multiplied *in vitro* through axillary bud proliferation.

The optimization of a procedure for transfer of T-DNA to plant genome involves a large number of factors such as explant precultivation and physiological condition (Hazel *et al*., 1998; Bhatnagar and Khurana, 2003), cocultivation period (Kondo *et al*., 2000), temperature (Kondo *et al*., 2000), light intensity (Zambre *et al*., 2003), medium composition including supplements (Niu *et al*., 2000), PGR (Chateau *et al*., 2000), *vir*

gene inducers (Yong-Woog *et al*., 1996), inoculation density (Mondal *et al*., 2001), *Agrobacterium* strain and virulence plasmid (Miguel and Oliviera, 1999), wounding and concentration and timing of selective agent (Le *et al*., 2001). Of these, *Agrobacterium* strain and virulence plasmid, wounding and inclusion of *vir* gene inducers are most often reported to impact significantly. In this study only explant wounding and acetosyringone on *Agrobacterium*-mediated transformation of marula nodal explants were evaluated.

5.2.1   Concentration of selective agent

Kanamycin can be used at sublethal concentration to select for chimeras. Sublethal concentration bleaches the non-inoculated tissue but does not affect organogenesis (Knittel *et al*., 1994; Gould and Magallanes-Cedeno, 1998; Mondal *et al*., 2001). Kanamycin at 300mgl^{-1} supplemented in MS multiplication medium bleached 90% of wild type marula nodal explants. The bleached nodal explants were not killed as they exhibited bud break and shoot growth. The same kanamycin concentration (300mgl^{-1}) in MS multiplication and elongation medium resulted in 1.5% green chimeric putatively transformed marula shoots, respectively after *Agrobacterium*-mediated gene transfer. Kanamycin as a selective agent for putatively transformed meristems was also reported in sunflower (Knittel *et al*., 1994), guayule (Pan *et al*., 1996) and cotton (Gould *et al*., 1998) with transformation efficiencies of 4% for cotton and less than 1% for sunflower and guayule.

Putatively untransformed marula shoots turned yellowish (bleached) on selection medium compared to green (potentially chimeric) putatively transformed shoots indicative of deactivation of aminoglycoside antibiotics by the NPTII protein present in the cells of

putative transgenic marula shoots. Bean *et al*. (1997) observed that untransformed pea shoots turned brown compared to green shoots of putatively transgenic peas. Enrichment of transformed tissue at each subculture was accomplished by removal of bleached tissues and the transfer of only green materials (Knittel *et al*., 1994) a procedure that was not done in marula.

Putatively transformed shoots originated from *de novo* regeneration were also selected on media containing kanamycin (table 10). In mango, transformed somatic embryos were selected on medium containing kanamycin 400mgl^{-1} (Mathews *et al*., 1993). Kanamycin inclusion in the media at 100mgl^{-1} was adequate for recovery of putatively transformed kiwifruit, apple, black poplar and lavender (Janssen and Gardner, 1993; Sriskandarajah *et al*., 1994; Confalonieri *et al*., 1995; Dronne *et al*., 1999).

Kanamycin did not appear to negatively impact shoot multiplication and elongation in marula. In lavender, kanamycin resistant callus was recovered at rates between 3 and 89%, however presence of kanamycin in the media inhibited organogenesis (Dronne *et al*., 1999). In plum (Yancheva *et al*., 2002), kanamycin at 50mgl^{-1} inhibited regeneration whereas a lower kanamycin concentration (25mgl^{-1}) resulted in escapes. In contrast, Confalonieri *et al*. (1995) recorded approximately 6 to 29% kanamycin resistant and organogenic callus in poplar. In marula, 90% bleaching of emerging shoots and 10% escapes were obtained at a kanamycin concentration of 300mgl^{-1}. The concentration of kanamycin used for selecting chimeric shoots was not tested in *de novo* regeneration.

5.2.2 Level and timing of transient expression

Transient expression of the *uid*A reporter gene is used to test the efficiency of T-DNA transfer to plant cells (Vancanneyt *et al*., 1990; Confalonieri *et al*., 1995). In marula, the highest level of transient expression of 52.5% GUS-positive explants with an average of 1.6 blue stained zones per GUS-positive explant section was measured for acetosyringone without wounding treatment on day 3 followed by a much lower expression level, a first indication of stable transformation frequency, on day 6. The transient expression is due to the fact that not all T-DNA that is transferred to a nucleus becomes integrated and unintegrated T-DNA is eventually lost (de la Riva *et al*., 1998; de Villiers, 1998). In marula this was demonstrated by the significant decrease in percentage of GUS-positive explants between day 3 and day 6 (figure 13a). Similar transient expression frequencies of 51.7% in wheat (Amoah *et al*., 2001), 50% in kiwifruit (Janssen and Gardner, 1993) and 50% in sunflower (Rao and Rohini, 1999) have been reported. In contrast, Atkinson and Gardner (1993), Knittel *et al*. (1994) and Dronne *et al*. (1999) reported lower than 50% transient expression efficiencies in tamarillo (35%), in sunflower (41%) and in different lavender cultivars (1 to 40%), respectively. In wheat (Khanna and Daggard, 2002) and different *Arabidopsis thaliana* genotypes (Chateau *et al*., 2000) transient expression efficiencies of between 35 and 63% and, 14 and 69% have been reported, respectively. In pine and spruce, Wenck *et al.* (1999) obtained transient expression efficiencies of 60% and 90%, respectively. Dronne *et al*. (1999) observed 3 to 6 GUS-positive spots per lavender explant whereas, Chateau *et al*. (2000) observed 0.5 to 10.1 spots per *Arabidopsis thaliana* explant. GUS staining of cocultivated marula explant sections revealed a similar number of 1 to 5 blue zones per positive explant section, 1.7

blue zones on average per explant section or 6.8 per explant (4 sections per explant). In black poplar (Confalonieri *et al*., 1995) and wheat (Amoah *et al*., 2001) a much higher number of 15.6 and 33.8 GUS-positive spots per explant were recorded, respectively.

5.2.3 Duration of cocultivation

The cocultivation period of explants and *Agrobacterium* is important as it allows the bacterium to be in close contact with explants and thus its colonization (de la Riva *et al*., 1998). Although no optimization of cocultivation period was undertaken, a cocultivation period of 3 days was effective for *Agrobacterium* to transfer T-DNA into the nucleus of marula which resulted in optimal transient expression levels on day 3 of between 23.3 and 81.7%, 52.5% on average, with 1 to 5 blue stained zones per GUS-positive explant section, 1.7 on average. Various cocultivation periods of 2 days in Norway spruce and tamarillo (Wenck *et al*., 1999; Atkinson and Gardner, 1993, respectively), 3 days in guayule (Pan *et al*., 1996), 2 and 4 days in apple cultivars (Bolar *et al*., 1999, Sriskandarajah *et al*., 1994), 4 to 5 days in kiwifruit (Janssen and Gardner, 1993) and 6 days in lavender (Dronne *et al*., 1999) were reported. Although a longer period has been prerequisite to achieve transformation in white spruce (Le *et al.,* 2001), cocultivation period is limited by the minimal period of 2 hours required by *Agrobacterium* to produce and transfer the T-strand and a maximum period that is determined by the extent of bacterial overgrowth (Narasimhulu *et al.,* 1996).

5.2.4 Choice of strain

A commonly used octopine strain LBA4404 of *Agrobacterium tumefaciens* harboring the p35SGUSintron plasmid was used in marula transformation as this strain was readily available for experimentation. The strain was effective as evidenced by the transient and

stable GUS expression and the putative transgenic chimeric plantlets. The percentage of GUS-positive explants was within the range reported by other authors on other plants where the same strain of *Agrobacterium* was used (wheat, Khanna and Daggard, 2002; sunflower, Rao and Rohini, 1999) and where other strains of *Agrobacterium* were used (wheat, Amoah *et al*., 2001; *Arabidopsis thaliana*, Chateau *et al*., 2000). Supervirulent strains which harbor multiple copies of certain virulence genes, in particular *vir*A and G, have enabled and increased the efficiency of transformation of many recalcitrant species, of monocots including maize (Ishida *et al*., 1996) and rice (Liu and Gelvin, 1992), cassava (Li *et al*., 1996) and conifers such as spruce (Wenck *et a*l., 1999; Le *et al*., 2001) white pine (Levée *et al*., 1999) and larch (Levée *et al*., 1997). The cocultivation of marula with such strains is expected to positively impact transformation efficiencies beyond the present observed 1.5%.

5.2.5   Effect of acetosyringone and wounding on transformation

Addition of acetosyringone and wounding of explants before cocultivation have been demonstrated to increase transformation frequencies (Park *et al*., 1996; Gheysen *et al*., 1997; de Villiers, 1998). Acetosyringone binds to the cell wall VirA-ChvE complex, the generated signal is transduced via VirG which in turn induces expression of the virulence genes. The virulence gene products then produce the T-strand, effect transfer of the T-strand complex to the plant nucleus and potentially mediate integration of the T-DNA into the plant genome. Wounding may increase the chances of *Agrobacterium* to find plant cells by chemotaxis, induce the production of polyphenols for *vir* gene induction as well as improve the competence of plant cells for transformation (Park *et al*., 1996; Gheysen *et al*., 1997; de Villiers, 1998).

Addition of acetosyringone to the cocultivation medium significantly increased transient transformation of marula. This treatment resulted on average in 52.5% GUS-positive explants with an average of 1.6 blue zones per GUS-positive explant section on day 3 and 2.5% GUS-positive explants with an average of 1.7 blue zones per GUS-positive explant section on day 6. Addition of acetosyringone to the bacterial suspension and during cocultivation enhanced transformation efficiency in tamarillo (Atkinson and Gardner, 1993), black popular (Confalonieri *et al*., 1995), guayule (Pan *et al*., 1996) and tea (Matsumoto and Fukui, 2002). Matsumoto and Fukui (2002) reported that the presence of acetosyringone during cocultivation was essential for the formation of putative transgenic callus in tea. Incorporation of acetosyringone in the cocultivation medium was also essential for poplar transformation (Confalonieri *et al*., 1995) and to achieve a high transformation efficiency of Norway spruce. In Norway spruce however, an acetosyringone concentration of 100μM and above exhibited a phytotoxic effect (Wenck *et al*., 1999). In marula, 100μM acetosyringone contributed to a significantly augmented transient transformation efficiency, which may possibly translate into a higher stable transformation rate as hinted by the not significantly higher percentage of GUS-positive explants observed on day 6. This needs to be confirmed since the correlation between transient and stable transformation efficiencies does not always hold (Maximova *et al*., 1998; Weber *et al.,* 2003). Understanding of a possible correlation between the observed significant increase in the number of GUS-positive zones per GUS-positive explant section from day 2 to day 3 when acetosyringone was included versus omitted from the cocultivation medium and stable transformation rates may determine the potential

usefulness of the study of the evolution of transient expression as an indicator of stable transformation rates.

Wounding by particle bombardment of meristematic cells of rice shoot apices (Park *et al.*, 1996), by syringe of the axillary buds of guayule (Pan *et al.*, 1996), by macerating enzyme treatment and sonication of shoot apices of sunflower (Weber *et al.*, 2003) and by sonication of immature cotyledon explants of soybean (Santarém *et al.*, 1998) and zygotic embryos of loblolly pine (Tang, 2001) enhanced *Agrobacterium*-mediated transformation. In contrast, wounding of marula nodal explants prior to cocultivation did not significantly impact the transient transformation efficiency (number of GUS-positive explants). Moreover, on day 6 which may be indicative of stable transformation, a not significantly lower number of GUS-positive explants and a lower number of blue zones per GUS-positive explant section were observed when explants were wounded prior to cocultivation than when explants were not wounded. Furthermore when wounded explants were cocultivated in the presence of acetosyringone a significant antagonistic effect between wounding and acetosyringone was observed on day 3 as well as when the data for the full duration of the experiment were considered. Mondal *et al*. (2001) reported that both acetosyringone and wounding did not enhance but reduced the efficiency of transformation in tea (10% compared to 40% for control). The authors ascribed this effect to the high levels of phenolics and excessive tissue browning in tea explants which may be inhibitory to transformation or phytotoxic. Also marula tissue culture is characterized by substantial production of polyphenols, which prevented explant survival in preliminary micropropagation experiments (Mollel and Goyvaerts, submitted) where phenolic compounds and tissue browning of explants were observed

after 2 days in culture. This problem was alleviated by incorporation of PVP in the multiplication medium. During transformation however PVP was omitted from the medium as it may adsorb the acetosyringone added to induce *vir* gene expression. The large amounts of phenolics produced upon wounding of the axillary bud area in marula explants may have prevented binding by the acetosyringone to the VirA-ChvE cell wall protein complex (Gheysen *et al*., 1997) and thus reduced *vir* gene induction to a level similar to that in the absence of acetosyringone. Alternatively, wounding may have lead to localized cell necrosis. Joubert *et al*. (2002) reported that certain phenolic compounds inhibited T-DNA transfer and related this to the general toxicity of phenolics. Further experimentation is required to elucidate the molecular mechanisms of the impact of wounding on marula nodal explant transformation.

5.2.6 Patterns of transient expression

Blue stained zones rather than spots were observed in marula GUS-positive explants. Similarly, in fig large blue sectors characterized GUS-positive areas (Yancheva *et al*., 2004) whereas in kiwifruit and petunia, small zones of blue stained cells and large blue zones, respectively characterized GUS-positive areas (Janssen and Gardner, 1993). In addition, blue stained individual cells, which were not observed in marula, were also observed in kiwifruit (Janssen and Gardner, 1993). The failure to observe individual blue stained cells in marula may be due to characteristics of the *Agrobacterium*-mediated transformation of marula or a procedural artifact. Small blue spots may have remained undetected in the bluish green marula tissue at the lighting and magnification used (625X) or small blue precipitates may have been washed out during extensive 70% ethanol incubation. In marula, blue precipitate was observed in parenchyma cells and

vascular bundles that lead to the leaf stalk. This co-localized with the wounded sites during explanting, implying that the *Agrobacterium* got access to the marula cells through the cut surfaces. Similarly, in *Arabidopsis* cotyledons, blue stained cells were observed at the cut surface and in the neighborhood of vascular tissue (Vancanneyt *et al*., 1990). Uematsu *et al*. (1991) also observed blue precipitates in guard and mesophyll cells of transgenic kiwifruit explants. In contrast, the stained zones in kiwifruit and petunia were found near veins rather than the basal end (Janssen and Gardner, 1993). The site of transformation is impacted by *Agrobacterium* access and the competence of transformation of a particular plant cell. High transformation success is achieved when the transformed cells coincide with those that are regenerated in *de novo* regeneration and those meristematic cells that give rise to the chimeric shoots in *in planta* transformation.

5.2.7 Stable transformation

Stable transformation is characterized by stable integration of the transgene in the genome. In marula, 2.3 and 19% emerging shoots from cocultivated explants remained green and white green on selection medium, respectively and may have been putative and/or chimeric transformants. Two green shoots (1.5%) survived after six weeks on elongation medium supplemented with 300mgl^{-1} kanamycin whereas all white green and one green shoots bleached, browned and eventually died. Dead shoots may have been untransformed escapes, chimera with smaller sections of transformed tissue unable to sustain survival under the relatively high selective pressure or transformants exhibiting insufficient expression levels of the NPTII enzyme to confer kanamycin resistance. The percentage of putative and/or chimeric marula transformants was within the range reported by other authors (wheat, 1.2 to 3.9%, Khanna and Daggard, 2002; plum, 2.7%,

Yancheva *et al*., 2002). GUS staining of the green shoots revealed high level of GUS activity in virtually all tissues of one shoot whereas a low level of and localized GUS activity was observed in the second shoot. It is rather surprising that the second shoot that remained green under prolonged selection expressed GUS at an apparent low level and in only few tissues. Although possible, it is not very plausible that the substrate did not permeate to a similar extent in both explant sections which were of similar thickness and preparation, and thus gave rise to artifacts. More likely, the observed differences may have been due to the genetic background of the particular explant which enabled its escape or to the specific transformed blue tissues which NPTII expression effectively supported the survival of other non transformed tissues. Alternatively the GUS gene may have been disrupted in independently transformed tissues of the chimeric shoot and its expression in those tissues may not have correlated with NPTII expression. Further, the marula shoot with low level GUS activity may have been subject to *uid*A gene silencing possibly caused by the presence of more than one transgene copy which were highly transcribed and in turn triggered a regulated mechanism of mRNA degradation resulting in low levels of translation product. This phenomenon has been reported in transgenic citrus (Cervera *et al*., 2000) and aspen (Tuominen *et al*., 2000), high transgene number and post translational gene silencing caused low GUS activity. Partial methylation of either *GUS* gene or its promoter could have translated in low level of GUS activity. In transgenic sorghum methylation-based silencing of the *uid*A gene inhibited expression of the reporter gene (Emani *et al*., 2002). Lastly, expression of *uid*A and *npt*II genes which are driven by two different promoters, may not be co-regulated and *GUS* is subjected to down regulation in particular tissues when integrated near particular regulatory elements.

However, no DNA work was carried out to confirm the integration or the number of copies and loci of *npt*II and *uid*A genes in the marula genome. Since *in planta* transformants are often reported to be chimera, Knittel *et al*. (1994), Park *et al*. (1996), Bean *et al*. (1997), Gould and Magallanes-Cedeno (1998) and Han *et al*. (2000) used continued selection of putative chimeric plants at sublethal concentration in order to continuously kill non transformed cells whereas transformed surviving cells regroup and form the bud resulting in a non-chimeric transgenic shoot. Such an approach will eventually result in a plant consisting of all transformed cells that may be resulting from one or more transformation events. Notwithstanding, even when using a *de novo* transformation approach, chimeric transgenics consisting of transformed and non-transformed cells have been reported (Domínguez *et al*., 2004). The interaction between the particular explant and genotype during and after cocultivation, the particular nature of the selectable marker, the selection regime and tissue culture protocol must be considered in evaluating and optimizing a new transformation protocol and its resulting transgenics.

5.3 Conclusions

This is the first report on the micropropagation and genetic transformation of marula, *Sclerocarya birrea* subsp. *caffra*, a wild fruit tree with great commercial potential for Africa and a member of the mango family. From the findings of the study the following conclusions were reached:

5.3.1 Marula micropropagation

1) Surface sterilization of marula seedling explants harvested from the growth room was achieved routinely at 100% aseptic rates using 70% ethanol for 1 minute followed by

30% (v/v) bleach solution containing 0.1% (v/v) Tween 20 for 10 minutes. Field harvested materials required additional treatments prior to decontamination, which included a soak in fungicide solution and washing in antibacterial dishwashing detergent, followed by immersions in 70% ethanol for 2 minutes and in 30% (v/v) bleach solution containing 0.1% (v/v) Tween 20 for 20 minutes. This lead to routine 73% aseptic culture establishment of adult tree explants harvest at the onset of the new growth season.

2) 0.1% PVP and bi-daily transfers of field-grown tree explants alleviated the problem of polyphenols and premature death of explants in culture and enabled routine tissue culture establishment of seedling and field-grown explants.

3) MS medium supplemented with 4.8μM BA and 2.4μM KN was effective in microshoot proliferation at 72 and 100% bud break and an average of 2.5 and 1 shoots per responding explant for seedling and field-grown trees, respectively.

4) A reduction in cytokinin concentration to 1.2μM BA and 1.0μM KN was prerequisite for microshoot elongation (42%) from growth room seedlings.

5) After a 2-week recovery period on MS medium without PGR, MS medium at half strength salts containing 10μM IBA and 0.3% AC resulted in root formation (2 to 3 long adventitious roots) on seedling-propagated microshoots at 82%.

5) Acclimatization of plantlets at rates of 90% was achieved by gradual reduction of humidity and fertilization.

6) No somaclonal variation was detected between clones and parent plants within the limits of the RAPD technique despite the recurring extreme temperatures while clones were in tissue culture.

These results deliver an opportunity for commercial amplification of selected elite marula trees *in vitro* followed by grafting or rooting. Conventionally, marula is vegetatively propagated by cuttings/truncheons of about 10cm in length (Mbuya *et al*., 1994). In our method a stem nodal explant of around 1.5cm in length is used to generate 1 plant and tree material is thus used more efficiently.

5.3.2 *Agrobacterium*-mediated transformation of marula

1) *Agrobacterium*-mediated transformation of the marula tree is feasible as evidenced by the observed T-DNA transient expression of 52.5% GUS-positive explants with 1.6 blue stained zones per GUS-positive explant section in marula explants on day 3 which persisted at the much lower level of 2.5% GUS-positive explants and 1.7 stained zones per GUS-positive explant on day 6, suggesting T-DNA integration and stable transformation.

2) Presence of acetosyringone without wounding of the nodal bud area was an important cocultivation parameter for *Agrobacterium tumefaciens*-mediated transformation of marula nodal explants.

3) MS multiplication and elongation media supplemented with 300mgl^{-1} kanamycin were effective in selecting 1.5% putatively chimeric transgenic shoots.

5.4 Recommendations

5.4.1 Micropropagation

Although much effort in micropropagation of important cash crops of the *Anacardiaceae* family such as mango, cashew and pistachio is evidenced from the literature, only few reports show success with explants from adult tree material, which is a prerequisite to *in vitro* propagate genetically uniform material of a dioecious tree. Therefore the following opportunities could be pursued to further improve the applicability of marula *in vitro* propagation procedure.

-Further optimization of the microshoot elongation procedure, which at 42% is a bottleneck for commercial application.

-Further improvements of the marula micropropagation procedure for adult tree material must target the proliferation rate. Since marula tree explants showed behavior in tissue culture that may hint towards different endogenous/exogenous PGR ratios from seedling explants, the PGR type, combination and concentration may require optimization. Although rooting was not attained from tree explants due to recurring heating of the growth room, since tree explants were readily established in culture, and rooting was attained at 82% for seedling explants, a similar rooting response may be expected from marula tree explants. Alternatively shoots of superior tree material may be grafted on suitable rootstocks for commercial exploitation.

-Opportunities still exist for the development of cycling parameters for proliferated shoots and increasing the multiplication rate *in vitro*.

5.4.2   Genetic transformation

-Opportunities exist to further optimize cocultivation parameters, especially the *Agrobacterium* strain, timing and concentration of selective agent, recovery of chimera.

-The stable transformation rates of *Agrobacterium*-mediated transformation of marula need to be confirmed.

-The commercial applicability of chimeric and transgenic marula derived from enrichment of transgenic tissue must be determined.

-Optimization of an efficient regeneration system is a prerequisite for regeneration independent transformation.

-Optimization of cocultivation and selection conditions for *de novo* transformation.

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APPENDICES

Appendix 1: Micropropagation of marula, *Sclerocarya birrea* subsp. *Caffra (Anarcadiaceae)* by axillary bud prolifaration and RADP analysis of plantlets

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Abstract The availability of a rapid vegetative amplification procedure of mass-selected

trees with superior characteristics greatly accelerates the development of a new tree

species as a crop. A protocol for routine micropropagation of marula, *Sclerocarya birrea*

subsp. *caffra (Anacardiaceae)* has been developed. Marula plants were established in soil

in the growth room at an average rate of 0.57 per axillary bud of a nodal stem section

explant. Application of this new procedure readily established plantlets in tissue culture

from nodal stem explants derived from adult field grown trees. Randomly amplified

polymorphic DNA (RAPD) analysis scoring 1845 markers showed intraclonal genetic

stability between explant parent and micropropagated plants.

Keywords Anacardiaceae . *Axillary bud proliferation . Marula . RAPD . Somaclonal variation*

Abbreviations *AC:* Activated charcoal . *AFLP*: amplified fragment length

polymorphism . *BA:* N^6-Benzyladenine . *GA$_3$:* Gibberellin A3 . *IBA:* Indole-3-butyric

acid . *IPA:* 2-Isopentenyladenine . *KN:* kinetin . *MS:* Murashige and Skoog . *PVP:*

polyvinylpyrrolidone . *RAPD:* randomly amplified polymorphic DNA . *ZN:* zeatin

Introduction

The marula tree, *Sclerocarya birrea* subsp. *caffra*, is an indigenous, drought tolerant multipurpose tree of the *Anacardiaceae* and is widely distributed in Africa (Palmer and Pitman 1973, Mbuya et al. 1994, Simute et al. 1998). The marula tree has many indigenous uses as food, oil, dye, wood timber and medicine. The tree is also important to a variety of animals for shade, feed and shelter. The high nutritional value of the fruit in terms of vitamin C (0.68 mg/g) and protein content (60%) presents a good source of nutrients and vitamins for people and animals (Venter and Venter 1996). The high quality stable oil, about 56% of the nut, offers additional exploitation opportunities (Shone 1979). In view of the novel flavor of the fruit, the highly stable oil, the derivation of many commercial products and the potential for rural job creation, *Sclerocarya birrea* subsp. *caffra* has been earmarked for crop development.

The marula tree can be propagated by seed or vegetatively. However, propagation by seed is unsatisfactory since a very heterogeneous progeny is obtained as a result of heterozygosity. Vegetative propagation plays an important role in agriculture because vegetatively propagated trees are homogeneous and thus ensure uniform quality and production, enabling standardization of growth, harvesting and processing. *In vitro* propagation offers a larger number of cloned materials to be produced in a shorter time starting from fewer plant materials. This is important especially for the rapid amplification of improved tree material.

Genetic stability of tissue culture material depends on the stability of donor material (D'Amato 1986) and the propagation method (Saieed et al. 1994). Tissue culture

procedures, especially when callus is observed and when culture stages are long, are reported to introduce somaclonal variation (Evans 1989). Somaclonal variation is traditionally determined by chromosome staining, DNA measurement, cytogenetic and phenotypic evaluation (Freytag et al. 1989, Linacero and Vazquez 1992, Leitch et al. 1993, Vuylsteke and Ortiz 1996). Recently, amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD) techniques have been used successfully to determine somaclonal variation in among others, poplar (Rani et al. 1995), oak (Barrett et al. 1997), spruce (Fourre et al. 1997), asparagus (Raimondi et al. 2001), pecan (Vendrame et al. 1999) and oil palm (Rival et al. 1998).

Here, we report a micropropagation protocol for marula seedling explants *(Sclerocarya birrea* subsp. *caffra*) with preliminary evaluations on adult tree explants. The latter forms a first report where a species of the *Anacardiaceae* family could be readily established in tissue culture from adult tree explants without a rejuvenation stage. The protocol may offer a commercial propagation method for superior tree material. We further used RAPD analysis to determine somaclonal variation of micropropagated plants.

Materials and methods

Plant material

Marula seeds were germinated in sterile vermiculite. Seedlings over 2 months old were used as a source of explants. Alternatively, young shoots from healthy adult trees were harvested in the field and either kept in water or fungicide (Funginex™: active ingredient Triforine 190 mg/l, Sentrachem) during transfer to the laboratory. Shoots from both sources were defoliated before surface sterilization.

Surface sterilization

Explants were washed in running tap water prior to surface sterilization. Surface sterilization of seedlings was by immersion for 1 minute in 70% ethanol followed by 10 minutes in 30% bleach containing 0.1% (v:v) Tween 20. Explants were then rinsed five times in sterile distilled water.

Adult tree material was surface-sterilized by submersion for 2 minutes in 70% ethanol followed by 20 minutes in 30% bleach containing 0.1% (v:v) Tween 20. Additional steps were evaluated: soaking in antibacterial dish washing liquid for 10 minutes followed by rinsing under running tap water before surface sterilization; soaking in fungicide for 2 h (Funginex™: active ingredient Triforine 190mg/l, Sentrachem), rinsing prior to 10 minutes soaking in antibacterial dishwashing liquid, rinsing and surface sterilization; or dipping in sterile fungicide solution after surface sterilization before inoculation. Explants were cut into 1.5 cm sections before cultivation. Contamination was assessed 7 and 14 days after culture initiation. Live explants that were sterile at both 7 and 14 days were scored as the percentage of the total surface sterilized explants.

Culture conditions

The basal media contained Murashige and Skoog (MS, 1962) nutrients at full or half-strength salts, 3% sucrose, 0.6% agar and supplements of 0.08 or 0.1% PVP and 0.3% AC. The pH of the medium was adjusted to 5.8 prior to autoclaving. PGR were added to the media before autoclaving (20 minutes at 121°C). Cultures were incubated in the growth room at 25 ± 1°C with a 16-hour photoperiod of 81 μmol m^{-2} s^{-1}.

Optimization of culture media for propagation, elongation and rooting

Surface sterilized nodal explants were placed on MS medium containing different concentrations of cytokinins. Explant browning and subsequent death was observed in preliminary experiments. Incorporation of PVP (0.1%) in the medium with and without bi-daily transfers to fresh media during the first week of cultivation prevented browning and premature death of field grown adult tree and growth room grown seedling explants, respectively and enabled establishment in tissue culture. For shoot induction various concentrations of the cytokinins, kinetin (KN) at 1.2-6.0 μM and benzyladenine (BA) at 1.2-6.0 μM were evaluated. Initial duplicate experiments included all cytokinin combinations after which only those with above 40% budbreak and with more than one shoot per node were evaluated in additional repeat duplicate experiments. Data of all duplicate and repeat experiments were combined for statistical analysis. In addition to BA and KN, gibberellin A3 (GA_3) at 1 and 5 μM was evaluated for shoot elongation. For root induction, half-strength MS supplemented with 0 to 10 μM of the auxin IBA and either 0.3% AC or 0.08% PVP and 0.08% PVP with the bottom wrapped in aluminium foil for 7 days were evaluated.

Acclimatization of plantlets

Rooted plantlets were transferred into baby food jars containing sterile vermiculite supplemented with half-strength MS. The caps were gradually opened to reduce relative humidity in the jars. After 2 weeks plantlets were potted in garden soil and covered with beakers that were gradually lifted over a period of 4 weeks. Acclimatized plantlets were transferred to potting bags in the plastic house and reported as a percentage of the total.

Micropropagation rate was calculated as the multiplication of aseptic, bud break, shoot

proliferation, elongation, rooting and acclimatization rates of the adopted

micropropagation protocol.

Statistical analysis

One-way and factorial ANOVA were conducted on results of duplicate and repeat

experiments using STATISTICA version 6 software from Softstats[TM]. Percent values of

budbreak and elongation were log-transformed before analysis. Rooting percent values

were normally distributed (Chi-Square = 0.92; P = 0.33) and mean percent values were

compared between treatments using factorial ANOVA. Multiple comparison of treatment

means was carried out using Tukey HSD post-hoc test (Winer et al. 1991) to determine

significant differences between treatments at an error level of <5% (p<0.05).

RAPD analysis

Leaves from five seedlings and ten micropropagated plantlets were harvested twice. DNA

was extracted from both leaf sets using the cetyl triammonium bromide (CTAB) method

according to a modification by Doyle and Doyle (1987) of the method of Saghai-Maroof

et al. (1984) and Rogers and Bendich (1985). The DNA was re-precipitated, dissolved in

1 mM TRIS and 0.1 mM EDTA and its concentration estimated by comparison with a

known concentration of lambda *Hind*III molecular weight marker after gel

electrophoresis and ethidium bromide visualization. Polymerase chain reactions were

carried out in 20 μl volumes containing 5 and 10 ng template DNA of the two

independent extractions, 0.2 μM dNTP, 0.3 μM primers Genosys 60-4, 60-6, 60-8, 60-9,

60-10, 70-6, and 70-9, 1x *TaKaRa Ex-Taq™* buffer and 2.5 units *TaKaRa Ex-Taq™*

polymerase. Denaturation was at 94°C for 5 minutes, followed by 45 cycles of [94°C for

45 seconds, 36°C for 1 minute, 72°C for 2 minutes] and a final extension at 72°C of 7

minutes, in a Perkin Elmer 9700 PCR machine. PCR products were size-fractionated by

1.5 % agarose gel electrophoresis and visualized by UV-illumination of ethidium

bromide stained fragments. Only reproducible bands observed among the RAPD products

obtained with 5 and 10 ng template DNA from two independent DNA extraction

procedures were scored.

Results

Surface sterilization

Surface sterilization of nodal explants grown in the growth room routinely resulted in

100% aseptic culture establishment. Immersion for 2 minutes in 70% ethanol followed by

20 minutes in 30% bleach supplemented with 0.1% (v:v) Tween 20 was sufficient to

decontaminate field-grown adult tree material harvested at the end of the rainy season.

However, in October-November at the beginning of the rainy season when new shoots

sprout, this sterilization method resulted in all contaminated cultures. Among the

modifications tested, a 2-hour fungicide soak, followed by 10 minutes in antibacterial

dishwashing detergent proved significantly more effective than others, although it

remained significantly lower than the aseptic rate obtained for growth-room-grown

seedling nodal explants. On average, an aseptic rate of 73% was obtained for field-grown

adult tree nodal explants from young twigs.

Bud break and shoot proliferation

Axillary bud break was observed within 14 days of culture on medium with as well as without cytokinins in 20 to 72% of the explants (Table 1). Bud break of 72% was observed on MS media containing 4.8 μM BA plus 2.4 μM or 3.6 μM BA plus 3.6 μM KN. More than 50% bud break was observed on media containing an intermediate cytokinin concentration, MS 2.4 μM BA plus 6.0 μM KN, MS 3.6 μM BA plus 2.4 or 3.6 μM KN, MS 4.8 μM BA plus 1.2 to 4.8 μM KN. Lower and higher cytokinin concentrations resulted in lower bud break percentage rates.

The highest proliferation rate was observed on MS medium supplemented with 4.8 μM BA and 2.4 μM KN (Table 1 and Fig. 1). Two to four shoots, on average 2.5 shoots per responding bud or 1.8 shoots per sterile cultured explant, was significantly different (Tukey HSD post-hoc test, $p < 0.05$) from all but three other cytokinin concentrations tested. This proliferation rate was not significantly different at $p < 0.05$ from that observed on MS 6.0 μM BA plus 1.2, 3.6 or 6 μM KN when shoots per responding bud and from that observed on MS 4.8 μM BA plus 3.6 μM KN and 6.0 μM BA plus 3.6 or 4.8 μM KN when shoots per explant were compared. However, on the latter media much callus was observed and MS 4.8 μM BA plus 2.4 μM KN was chosen as the medium with the highest bud break and proliferation rate. Absence of cytokinin and low cytokinin concentrations generally resulted on average in only one shoot per responding bud.

Higher cytokinin concentrations resulted on average in more than one but less than 2.5 shoots per responding bud. On high cytokinin media callus proliferation was excessive. The combination and concentration of 4.8 μM BA and 2.4 μM KN was adopted for nodal explants from field grown adult trees. A bud break of 100% and one shoot per responding

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bud or a rate of 0.73 shoots per cultured explant when loss due to contamination was considered was observed for adult tree material (Fig. 2). This was significantly different from that observed for seedling explants (Table 1, Tukey HSD post-hoc test, p<0.05).

Shoot elongation

After 4 weeks on shoot proliferation medium, elongated shoots with two nodes and measuring around 2 cm were transferred from MS supplemented with 4.8 µM BA and 2.4 µM KN to MS medium supplemented with zero or lower cytokinin concentration (1.2 µM BA plus 1.0 µM KN) or 1 and 5 µM GA_3 (Table 2). Inclusion of 1 and 5 µM GA_3 in the medium resulted in low elongation rates of 10 and 4% of the transferred microshoots, respectively. Transfer to zero or low cytokinin medium resulted in elongation of 34 and 42% of the transferred microshoots, respectively; a response that was significantly different from the tested GA_3 containing media. MS medium supplemented with 1.2 µM BA plus 1.0 µM KN was adopted as the shoot elongation medium for marula microshoots (Fig. 3).

Adult explant microshoots placed on elongation medium behaved similarly to seedling explant derived microshoots. Results of these experiments are not tabulated as most tissue culture material died as a result of repeated overheating of the tissue culture growth rooms during the December-January summer holidays.

Rooting of marula shoots

After 4 weeks on the adopted shoot elongation medium, shoots were transferred to MS medium without PGR for 2 weeks before transfer to root induction medium (Table 3).

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Microshoots in media supplemented with PVP or PVP wrapped with aluminum foil rooted poorly, significantly differing from those on the AC supplemented media (Tukey HSD post-hoc test p<0.05). Plantlets on half-strength MS media supplemented with PVP exhibited stubby and short adventitious roots, which did not elongate upon prolonged incubation. Although IBA did not have significant impacts on rooting per se, rooted plantlets on half-strength MS medium containing 0.3% AC plus 0-5 μM IBA produced one to two long adventitious roots and those on half-strength MS medium containing 0.3% AC plus 10 μM IBA two to three long adventitious roots (Fig. 3). Maximum rooting of 82% was observed on half-strength MS supplemented with 0.3% AC and 10 μM IBA. This medium was adopted in routine micropropagation. No callus was observed on microshoots for any of the tested rooting media.

Some elongated shoots from field grown adult tree explants were transferred to rooting medium. However, due to repeated problems with the temperature control of the growth room, tissue culture plants did not survive the December-January summer holidays. Collection of explants from adult trees at the end of the rainy season in January-March did not result in the establishment of tissue culture. Soaking of tree branches in PGR as a rejuvenation step prior to tissue culture resulted only in few shoots.

Acclimatization and field transfer

Rooted plantlets were acclimatized by gradually lowering the humidity (Fig. 5 and 6). Acclimatization of plantlets was achieved with a survival rate of 90%. Potted plants were transferred to planting bags and kept in plastic houses for several months up to 1 year before planting in the field at the beginning of the rainy season.

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Analysis of somaclonal variation by RAPD

Figure 7 shows a representative agarose gel of the RAPD products obtained with primer

Genosys 60-8. Only bands reproduced at both DNA concentrations and extractions were

scored. On average 17.6 reproducible markers were scored per decamer primer. Of the

1845 bands scored, 900 (49%) were polymorphic among the seedling plants (Table 4).

No reproducible polymorphic bands were observed between micropropagated seedlings

and their respective explant parents.

Discussion

This is the first report on the micropropagation of marula, *Sclerocarya birrea* subsp.

caffra, a wild fruit tree with great commercial potential for Africa. Similarities and

differences were observed between procedures developed for marula and those reported

for other members of the *Anacardiaceae*, pistachio, mango and cashew (refer to the

supplementary material for an in-depth discussion). One nodal explant from a growth

room grown seedling resulted on average in a success rate of 0.57 potted hardened

marula plants in a period of 22 to 26 weeks i.e. 5 to 6 months. Das et al. (1996) achieved

a micropropagation rate of 0.5 to one potted cashew plant per leaf axil from one-month

old seedling grown cashew in a calculated 15 weeks. Onay (2000) attained a

micropropagation rate of 16 potted pistachio plants in 22 weeks per shoot tip induced on

10% of imbibed stems. However, when the amount of superior plant material is limited

propagation rates per tree node must be considered when comparing protocols. We were

unsuccessful in attaining cycling of marula explants as excessive callusing on newly

formed shoots was observed after repeat incubation on high cytokinin medium even when transferred from a low cytokinin concentration.

The relatively large variation in tissue culture response may be due to the heterogeneous nature of the starting material. Growth room grown marula seedlings were of different genetic background as evidenced by the interclonal RAPD polymorphism as well as different ages (2 to 24 months).

Much effort in propagation for the important cash crops, mango, cashew and pistachio is evidenced from reports on somatic embryogenesis from cotyledon explants (Litz et al. 1984, Philip 1984, Anathakrishnan et al. 2002, Cardoza and D'Souza 2002) and adventitious shoot formation from seedling explants (Barghchi and Alderson 1983, 1985, Martinelli 1988, Lievens et al. 1989, Leva and Falcone 1990, D'Silva and D'Souza 1991, Bogetti et al. 1999). These explants however form inappropriate starting materials for commercial propagation, as offspring of heterozygous parent trees are not genetically homogeneous nor identical to their selected superior parent. In our experience with marula, once the major bottlenecks of contamination and browning were overcome, the time of harvest proved most important to establish explants from adult trees. Bogetti et al. (1999) achieved 14% bud break of nodes from 5-year old glass house grown cashew trees but did not report on further progression of explants from adult trees through tissue culture. Shoot tip necrosis was observed in in vitro generated pistachio plantlets (Barghchi and Alderson 1983, 1985, 1996). For marula, bud break of field-grown adult tree explants was observed at a rate of 100% in contrast to 72% from seedling (two to 24 months old) derived explants. Only 1 shoot per node was formed whereas seedling-

derived material which had a much smaller mass than tree-derived material, formed 2 to 4 shoots per responding node on the same medium. Opportunities for the optimization of the marula micropropagation procedure for adult trees may exist in the further optimization of PGR concentrations for tree explants specifically.

Rani et al. (1995) reported that RAPD markers are useful tools in genetic identification of micropropagated plants. Any of the seven decamer primers used was able to distinguish between the five different seedlings (Table 4). The high interclonal polymorphism rate of 49% in marula compared to 20% in oak (Barrett et al. 1997) and 19% in oil palm (Rival et al. 1998) may be in part due to the lack of cultivation and breeding of marula and its dioecious nature.

The data suggested that all micropropagated plantlets although subject to extreme temperatures while in tissue culture, were genetically uniform within the limits of the RAPD method. Out of the 1845 bands of 123 markers scored, none were polymorphic within the cloned plantlet sets. For the seven clones NS9-1 to 7 and explant parent plant NS9, 85 markers were monomorphic suggesting an intraclonal polymorphism frequency below 0.1%.

Although RAPD and AFLP have been successfully used to determine somaclonal variation in *Triticum* (Brown et al. 1993), beet (Munthali et al. 1996) and *Arabidopsis* (Polanco and Ruiz 2002), in other cases these molecular techniques failed to detect genetic differences underlying obvious phenotypic changes due to somaclonal variation (Goto et al. 1998, Rival et al. 1998). Fourre et al. (1997), Wilhelm (2000) and Bouman and De Klerk (2001) observed differences in ploidy levels between clones and their

explant parents that remained undetected when screening a large number of RAPD and AFLP markers. Although somaclonal variation is considered a problem in breeding and mass propagation, mutation during mitosis is a natural process. In senescing cork tissue, one mutation in 1520 nucleotides was found after sequencing nuclear DNA of old and young cork tissues (Pla et al. 2000). AFLP analysis of *Arabidopsis* somaclones showed that the values of nucleotide diversity estimated of each group of regenerated plants were two to three orders of magnitudes smaller than natural variation described for natural ecotypes of *Arabidopsis* (Polanco and Ruiz 2002). At what frequency phenotypic differences are generated during marula micropropagation needs further examination of traits in the field over several years.

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Table 1. Effect of BA and KN concentrations on bud break and shoot proliferation of marula explants.

Origin of nodal explants	BA , KN (μM)	Bud break (%)	Mean number of shoots per responding bud	Mean number of shoots per sterile explant
Growth room grown seedlings	0.0 , 0.0	30 (14)[ab]	1.0 (0.0)[a]	0.3 (0.1)[a]
	1.2 ,1.2	40 (14)[ab]	1.5 (0.0)[a]	0.4 (0.0)[a]
	1.2 , 2.4	30 (14)[ab]	1.0 (0.7)[a]	0.3 (0.1)[a]
	1.2 , 3.6	30 (14)[ab]	1.0 (0.0)[a]	0.4 (0.0)[a]
	1.2 , 4.8	30 (14)[ab]	1.0 (0.0)[a]	0.3 (0.1)[a]
	1.2 , 6.0	20 (0)[a]	1.0 (0.0)[a]	0.2 (0.0)[a]
	2.4 , 1.2	40 (0)[ab]	1.0 (0.0)[a]	0.4 (0.0.)[a]
	2.4 , 2.4	40 (0)[ab]	1.0 (0.0)[a]	0.4 (0.0)[a]
	2.4 , 3.6	30 (14)[ab]	1.0 (0.0)[a]	0.3 (0.1)[a]
	2.4 , 4.8	40 (0)[ab]	1.3 (0.4)[a]	0.5 (0.1)[a]
	2.4 , 6.0*	61 (14)[b]	1.2 (0.2)[a]	0.7 (0.2)[a]
	3.6 , 1.2	40 (0)[ab]	1.3 (0.4)[a]	0.5 (0.1)[a]
	3.6 , 2.4	64 (17)[b]	1.3 (0.2)[a]	0.8 (0.2)[a]
	3.6 , 3.6	72 (12)[b]	1.2 (0.2)[a]	0.9 (0.2)[a]
	3.6 , 4.8*	30 (14)[ab]	1.0 (0.0)[a]	0.3 (0.1)[a]
	3.6 , 6.0*	40 (0)[ab]	1.3 (0.4)[a]	0.5 (0.1)[a]
	4.8 , 1.2	66 (21)[b]	1.4 (0.3)[a]	0.9 (0.3)[a]
	4.8 , 2.4	72 (27)[bx]	2.5 (0.4)[bx]	1.8 (0.9)[bx]
	4.8 , 3.6*	69 (20)[b]	1.6 (0.4)[a]	1.1 (0.4)[ab]
	4.8 , 4.8*	53 (12)[ab]	1.4 (0.1)[a]	0.7 (0.1)[a]
	4.8 , 6.0*	47 (12)[ab]	1.4 (0.3)[a]	0.6 (0.3)[a]
	6.0 , 1.2	40 (0)[ab]	1.8 (0.4)[ab]	0.7 (0.1)[a]
	6.0 , 2.4*	50 (14)[ab]	1.3 (0.4)[a]	0.6 (0)[a]
	6.0 , 3.6*	50 (14)[ab]	1.8 (0.2)[ab]	0.9 (0.1)[ab]
	6.0 , 4.8*	35 (14)[ab]	1.6 (0.1)[a]	0.8 (0.3)[ab]
	6.0 , 6.0*	40 (0)[ab]	1.8 (0.4)[ab]	0.7 (0.1)[a]
Field grown adult trees	4.8 , 2.4	100 (0)[y]	1.0 (0.0)[y]	1.0 (0.0)[y]

Surface sterilized nodal explants were placed on MS medium containing different concentrations and ratios of BA and KN in one to four duplicate experiments of five to eight explants per treatment (a total of 465 growth room grown seedling and 71 field grown adult tree explants). Bud break is shown as the percentage of responding nodes of the total number of nodes placed on the respective medium. The mean number of shoots per responding node was calculated. Standard deviations are shown between parentheses. * indicates excessive callus proliferation. Different letters (a, b, c,...) in superscript along the column indicate significant difference (Factorial ANOVA, Tukey HSD post-hoc test, df=55, p<0.05). Different letters (x,y,z,…) in superscript along the column indicate significant difference between the explant source i.e., seedling and field explants at the same PGR treatment (One-way ANOVA, Tukey HSD post-hoc test, df=12, p<0.05)

Table 2. Effect of BA, KN and GA3 on shoot elongation.

PGR (µM)	Elongated shoots (%)
0.0	34 (3)a
BA (1.2), KN (1.0)	42 (3)a
GA3 (1.0)	10 (3)b
GA3 (5.0)	4 (0)b

After bud break and shoot multiplication, microshoots were transferred from MS supplemented with 4.8 □M BA and 2.4 □M KN to MS medium without PGR or supplemented either with low concentrations of BA and KN or GA3 for shoot elongation. Percentage shoot elongation was calculated as the number of shoots elongated out of the total number of shoots transferred. Results of two experiments of 25 explants per

treatment are shown (a total of 200 microshoots). Standard deviations are shown in parentheses. Different letters (a, b, c,...) in superscript indicate significant difference (One-way ANOVA, Tukey HSD post-hoc test, df=4, $p < 0.05$).

Table 3. Effect of IBA concentration, AC, PVP and wrapping of bottles on rooting (%) of elongated shoots.

IBA (µM)	AC	PVP	PVPdark	Average
0	79 (10)	50 (10)	36 (10)	55 (21)^x
1	71 (20)	54 (15)	57 (20)	61 (17)^x
3	61 (5)	43 (10)	46 (15)	50 (12)^x
5	61 (15)	68 (15)	43 (10)	57 (16)^x
10	82 (5)	61 (15)	57 (10)	67 (15)^x
Average	71 (13)^a	55 (13)^b	48 (13)^b	

Elongated shoots were transferred from MS supplemented with 1.2 µM BA and 1.0 µM KN to half-strength MS medium without PGR for 2 weeks before transfer to half-strength MS medium containing different concentrations of IBA, AC (0.3%) and PVP (0.08%). PVP bottles were also wrapped in aluminium foil for seven days (PVPdark). Rooting was scored as the percentage of transferred shoots that produced roots. Results of two experiments of 14 explants per treatment are shown (a total of 420 elongated shoots). Standard deviations are shown in parentheses. Different letters (a, b, c,… and x, y, z,…) in superscript indicate significant difference among the treatments and IBA concentrations, respectively (Factorial ANOVA, Tukey HSD post-hoc test, df=15, $p < 0.05$).

Table 4. RAPD analysis of micropropagated and explant parent plants.

Number of decamer primers	7
Number of markers	123
Number of polymorphic markers	60
Average markers per primer	17.6 (2.3)
Average polymorphic marker per primer	8.6 (2.8)

Standard deviations are shown in parentheses.

Figure 1 to 4. Micropropagation of marula explants. 1 and 2. Propagation of marula explants on MS medium supplemented with 4.8 ☐M BA and 2.4 ☐M KN. 1. Seedling material. 2. Adult tree material. 3. Elongation of shoots from seedling explants on MS medium supplemented with 1.2 ☐M BA and 1.0 ☐M KN. 4. Rooting of elongated shoots derived from marula seedling explants on half strength MS supplemented with 10 ☐M IBA and 0.3% AC.

Figures 5 and 6. Acclimatization of micropropagated marula plants. 5. Hardening off. 6. Ready for transfer to the plastic house.

Figure 7. Agarose gel electrophoresis of a representative RAPD of parent and micropropagated marula plants. DNA 5 (A) and 10 ng (B) from independent isolations were subjected to RAPD analysis using decamer primer Genosys 60-8. Lanes 1, 9, 11, 13 and 15 represent DNA from seedling parents NS9, 2, 16, 18 and 6. Lanes 2-8, 10, 12 and 14 represent DNA of micropropagated plantlets from explant parents NS9, 2, 16 and 18, respectively. DNA molecular weight is indicated on the left in kbp.
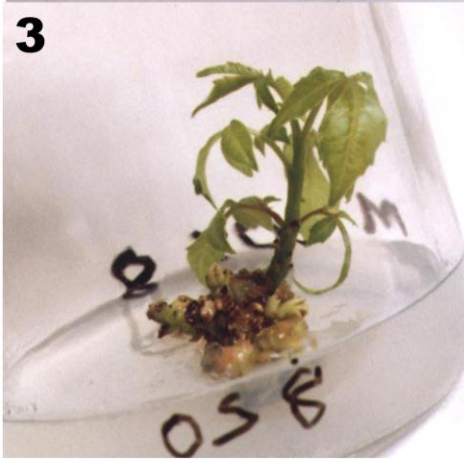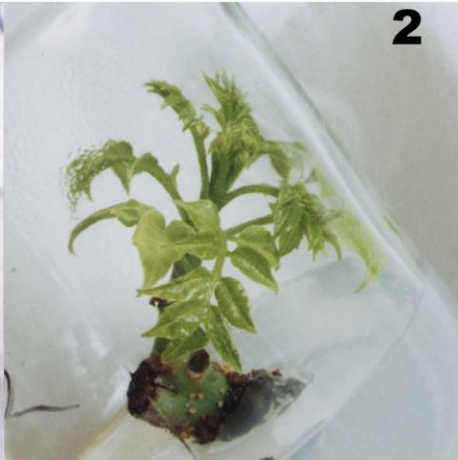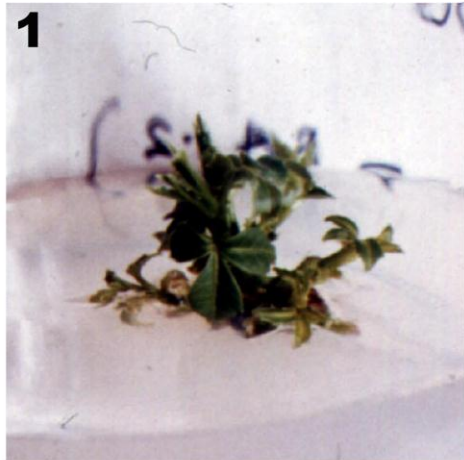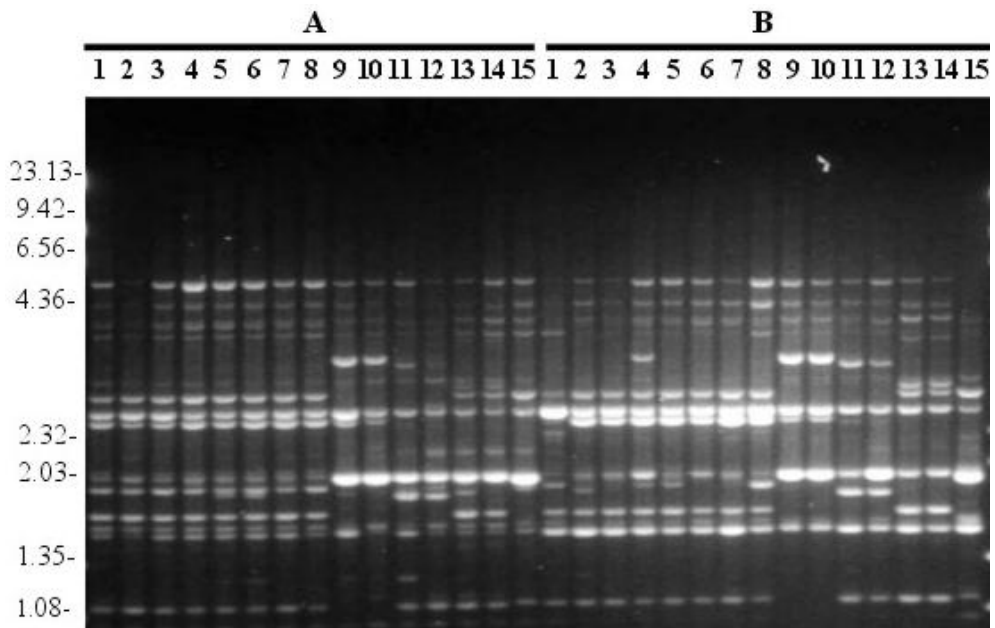
Appendix 2: Preliminary examination of factors affecting *Agrobacterium tumefaciens* mediated transformation of marula, *Sclerocarya birrea subsp. Caffra (Anarcadiaceae)*

1 Preliminary examination of factors affecting
2 *Agrobacterium tumefaciens*-mediated transformation of marula,
3 *Sclerocarya birrea* subsp. *caffra* (*Anacardiaceae*)

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10 **Abstract**

11 In planta *Agrobacterium tumefaciens*-mediated transformation of marula, *Sclerocarrya birrea* subsp. *caffra*
12 (*Anacardiaceae*) resulted in chimeric transgenic *in vitro* shoots at a rate of 0.8–1.5%. Average transgene
13 expression rates of 33.1 and 1.3% GUS-positive explants were observed on days 3 and 6 after agroinfection,
14 respectively. One to 4 GUS-positive zones were observed per GUS-positive explant section. Addition of
15 acetosyringone (100 μM) during co-cultivation significantly improved transient transformation efficiency as
16 determined by the percentage of GUS-positive explants on day 3 ($p < 0.05$) whereas wounding did not
17 show a significant effect. However, wounding and acetosyringone acted antagonistically reducing transient
18 transformation rates.

19 *Abbreviations:* BA – N[6]-benzyladenine; GA3 – gibberellin A3; GUS – β-glucuronidase; KN – kinetin; MS –
20 Murashige and Skoog (1962); X-Gluc–5-bromo-4-chloro-3-indolyl glucuronide

22 **Introduction**

23 The marula tree, *Sclerocarya birrea* subsp. *caffra* is
24 an indigenous, drought tolerant multipurpose tree
25 of the Anacardiaceae. It is widely distributed from
26 SubSaharan west and central Africa in the north
27 to the northern parts of South Africa in the south
28 (Pitman and Palmer, 1973; Mbuya et al., 1994;
29 Simute et al., 1998). Its many indigenous uses, high
30 nutritional value of the fruit in terms of vitamin C
31 (0.68 mg g^{-1}) and protein content (60%) (Venter
32 and Venter, 1996), the novel flavor of its fruit and
33 the high quality stable oil, about 56% of the nut
34 (Shone, 1979), underpin the derivation of many
35 commercial products and the potential for rural
36 job creation. *Sclerocarya birrea* subsp. *caffra*, has
37 hence been earmarked for crop development.

38 The long regeneration cycle and the hetero-
39 zygous nature of marula make conventional
40 breeding a slow and difficult process. Genetic
41 engineering followed by vegetative propagation
42 offers the opportunity of single and multiple gene
43 improvement of a selected superior genotype.
44 Genetic transformation has been reported for
45 other fruit-bearing woody species such as apple
46 (James et al., 1989), citrus (Hidaka et al., 1990),
47 grape (Mullins et al., 1994), peach (Hammerschlag
48 et al., 1989) and apricot (Laimer de Camara
49 Machado et al., 1992). Generation of transgenic
50 plants depends on the transformation of cells that
51 can be efficiently regenerated. Only aberrant
52 regeneration was achieved from few marula leaf,
53 stem, shoot and root explants, which was insuffi-
54 cient to support the development of a de novo

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transformation procedure (Mollel and Goyvaerts, unpublished data). An alternative approach may be found by transforming preexisting meristems. From the resulting chimeric plants, transgenic tissues can be excised and re-cultured until a homogeneous transgenic plant is attained. Shoot apex transformation has been applied successfully in petunia (Ulian et al., 1988), sunflower (Knittel et al., 1994), maize (Gould et al., 1991), rice (Park et al., 1996) and cotton (Gould and Magallanes-Cedeno, 1998). The optimization of transformation procedures is a lengthy process. Monitoring transient expression enables early results of experiments, optimization of procedures to target specific cells that are easily regenerated and assaying of gene transfer efficiencies separately from regeneration. Transient β-glucuronidase (GUS) transgene expression has thus been used successfully to optimize transformation procedures and provides a good indication of the rate of stable transformation (Janssen and Gardner, 1993).

Here, we set out to evaluate the feasibility and factors affecting *Agrobacterium*-mediated transformation of preformed axillary meristems of marula as assayed by transient expression analysis. Putative chimeric transformed shoots were obtained.

Materials and methods

Plant material

Marula seeds were harvested from wild-grown trees of different regions and randomly germinated in vermiculite in the growth room. Eight centimeter long stem sections from seedlings between 2 and 24 months old were harvested, exfoliated and surface-sterilized by immersion for 1 min in 70% (v/v) ethanol followed by 20 min in 30% bleach containing 0.1% (v/v) Tween 20. Surface-sterilized stem sections containing one or two axillary buds were cut in 1.5 cm (Mollel and Goyvaerts, submitted) and used as nodal explants in transformation experiments.

Agrobacterium *strain and cultivation*

For cocultivation the disarmed octopine Ach5 *Agrobacterium* strain LBA4404 (Hoekema et al.,

1983) harboring the p35SGUSintron (Vancanneyt et al., 1990) plasmid, a derivative of the binary vector pBIN19 (Bevan, 1984) was used. p35SGU-Sintron possesses the nopaline synthase promoter – *npt*II – nopaline synthase terminator polyA cassette and the GUS reporter gene with a portable plant intron (*gus*-int) under the regulatory control of the CaMV35S promoter and CaMV35S-polyA terminator sequence at the left border. Bacterial cultures were grown from frozen stocks on solid YEB supplemented with 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin for 3 days at 25 °C. A single colony was grown overnight in 10 ml YEB supplemented with 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin at 25 °C. The bacteria were precipitated at 2500 rpm for 2 min and the pellet washed twice with 10 ml 10 mM MgSO$_4$ before resuspension in 10 ml Murashige and Skoog (MS, 1962) medium (Janssen and Gardner, 1993; Wenck et al., 1999). The *Agrobacterium* suspension was supplemented with or without 100 μM acetosyringone for cocultivation.

Cocultivation and selection of putative transgenic chimera

Surface-sterilized marula nodal explants were incubated in the *Agrobacterium* suspension with or without 100 μM acetosyringone for 10 min and with or without wounding the axillary bud area with a sterile needle. Explants were blotted dry on sterile 3 mm filter paper and inoculated on cocultivation medium for three days at 25 °C and a 16-h photoperiod at 81 μmol m^{-2} s^{-1}. Cocultivation medium was multiplication medium [MS medium containing 3% sucrose, 0.6% agar and 4.8 μM benzyladenine (BA) and 2.4 μM kinetin (KN); Mollel and Goyvaerts, submitted] supplemented with or without 100 μM acetosyringone. Incorporation of kanamycin at a concentration of 300 mg l^{-1} in the shoot multiplication medium resulted in 90% bleaching of emerging wild-type shoots and was chosen as the concentration for selection of putative chimeric transformants. After cocultivation explants were transferred to shoot multiplication medium supplemented with 300 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. Few explants that were not assayed for transient expression, were kept on multiplication medium supplemented with 300 mg l^{-1} kana-

149 mycin and 500 mg l^{-1} cefotaxime. Shoots
150 emerged after 2 weeks. After 4 weeks, green
151 putative chimeric transformed shoots were
152 transferred to elongation medium [MS medium
153 supplemented with 1.2 μM BA and 1.0 μM KN;
154 Mollel and Goyvaerts, submitted] supplemented
155 with 300 mg l^{-1} kanamycin.

156 *Transient expression assays and GUS staining*

157 On days 1, 2, 3 and 6 after the onset of cocculti-
158 vation for each treatment 15 explants were re-
159 moved from the medium, cut longitudinally in four
160 and assayed for GUS activity by incubation in
161 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc)
162 for 24 h at 37 °C (480 explants in two experiments
163 and 15 explants each for the no *Agrobacterium* and
164 day 0 controls) according to Jefferson et al. (1987).
165 Stem sections were soaked in 70% ethanol for 48 h
166 to remove chlorophyll prior to enumeration of
167 blue zones per explant section.

Statistical analysis 168

Means of GUS-positive explants and the mean 169
number of GUS-positive zones per GUS-positive 170
explant section were calculated. Factorial ANO- 171
VA was conducted using STATISTICA version 6 172
software from Statsoft™. For comparison of 173
treatment means the posthoc Tukey HSD test was 174
used to determine significant effects at an error 175
rate of 5% ($p < 0.05$). 176

Results 177

Agrobacterium tumefaciens is able to transfer its 178
T-DNA harboring the GUS gene transiently and 179
stably to marula (Figure 1). Explants that were 180
not cocultivated with *Agrobacterium tumefaciens* 181
were GUS-negative throughout the experiments in 182
contrast to those cocultivated with *Agrobacterium* 183
tumefaciens and a transgenic tobacco plant har- *184*
boring the GUS transgene (Figure 1a–c). 185
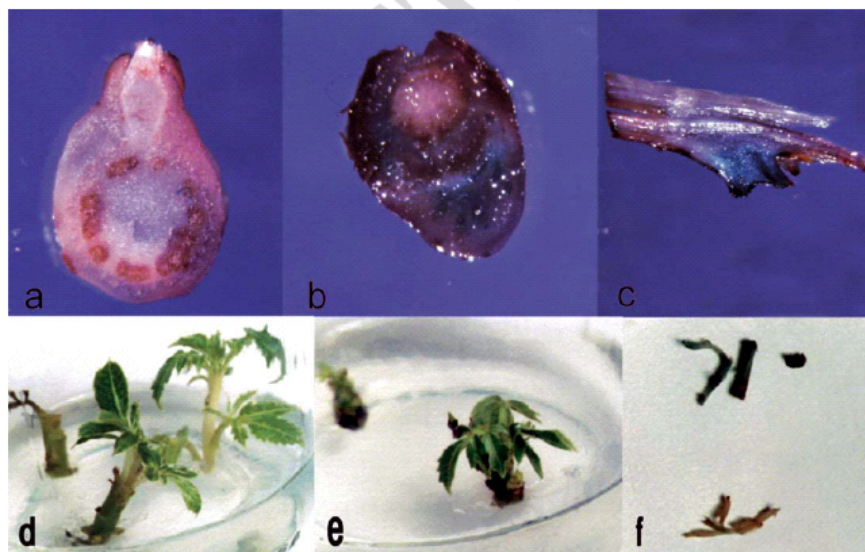
Figure 1. (a)–(c) Transient expression of cocultivated marula explants. Explants were cut and stained for GUS activity, (a) Transverse section of no *Agrobacterium* marula explant control; (b) transverse section of marula stem explant on day 3 after agroinfection; (c) longitudinal stem section on day 3 after agroinfection. (d)–(f) Putative chimeric transgenic marula shoots; (d) bleached and white green shoots on selective elongation medium; (e) green putative transgenic chimeric shoots on selective elongation medium; (f) GUS staining of two surviving putative chimeric transgenic shoots.

4

Evolution of transient expression 186

Figure 2 shows the evolution of transient GUS 187
expression of explants cocultivated with and 188
without wounding in the presence and absence of 189
acetosyringone. Factorial ANOVA indicated a 190
significant impact of the factor time on the per- 191
centage of GUS-positive explants ($F_{time} = 127.76$, 192
$p < 0.000001$). A lag phase was observed in the 193
evolution of the percentage of GUS-positive 194
explants (Figure 2a). The highest percentage of 195
GUS-positive explants among the days tested was 196
observed on day 3 for all treatments, significantly 197
different ($p < 0.05$) from that observed on other 198
days. The percentage of GUS-positive explants 199
observed on day 1 did not differ significantly 200
($p < 0.05$) from that observed on day 2, but both 201
of these differed from those observed on days 0, 3 202
and 6. The percentage of GUS-positive explants 203
was reduced on day 6 for all treatments. 204

No general trends for the evolution of GUS- 205
positive zones per GUS-positive explant section 206
were observed (Figure 2b; $F_{time} = 1.02$, $p = 0.36$). 207
However, when evaluating the evolution of the 208
number of GUS-positive zones per GUS-positive 209
explant section in further detail, those explants 210
cocultivated in the presence of acetosyringone 211
exhibited a further increase on day 3 whereas those 212
cocultivated in the absence of acetosyringone 213
exhibited a maximum number of GUS-positive 214
zones per GUS-positive explant section on day 2, 215
followed by a decrease on day 3 (Figure 2b). This 216
was substantiated by the statistical analysis of re- 217
sults of day 2 and day 3 ($F_{AC} = 8.32$, $p = 0.004$). 218

Wounding did not significantly impact the number 219
of GUS-positive zones per explant section ob- 220
served on day 2 and day 3 ($F_{WO} = 0.72$, $p = 0.40$). 221

Effect of acetosyringone and wounding on the 222
percentage of GUS-positive explants 223

No significant differences ($p < 0.05$) were ob- 224
served among treatments on days 0, 1, 2 and 6 225
(Table 1). On day 3, cocultivation in the presence 226
of acetosyringone without wounding delivered the 227
highest percentage of GUS-positive explants be- 228
tween 81.7 and 23.3%, 52.5% on average. This 229
differed significantly from other treatments on day 230
3 and all treatments on days 1, 2 and 6. However, 231
when explants were wounded and cocultivated in 232
the presence of acetosyringone, the percentage of 233
GUS-positive explants (25.0%) was lower than 234
that when unwounded explants were cocultivated 235
with acetosyringone (52.5%) and differed not sig- 236
nificantly from that after cocultivation of wounded 237
(31.7%) or unwounded (23.0%) explants in the 238
absence of acetosyringone on day 3 (Table 1). An 239
antagonistic effect was thus observed between 240
wounding and the application of acetosyringone 241
during cocultivation on the percentage of GUS- 242
positive explants on day 3 ($F_{AC*WO} = 18.36$, 243
$p = 0.00002$). The antagonistic effect of wounding 244
and acetosyringone during cocultivation was also 245
supported by factorial ANOVA and posthoc Tu- 246
key HSD tests when considering data for the total 247
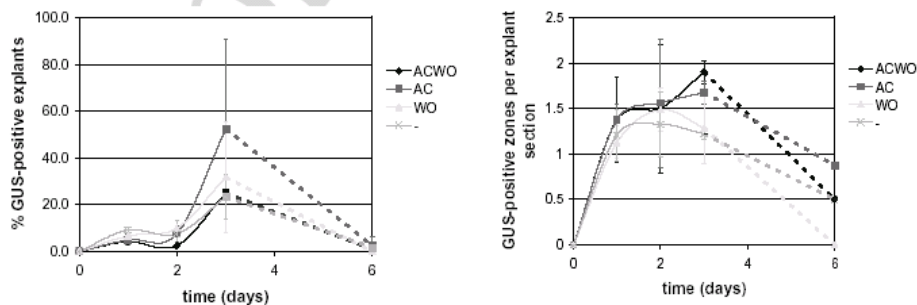duration of the experiment ($F_{AC*WO} = 15.11$, 248
$p = 0.0001$). 249

Figure 2. Evolution of transient expression, (a) Percentage of GUS-positive explants; (b) number of GUS-positive zones per explant section. AC, cocultivation in the presence of acetosyringone; WO, cocultivation of wounded explants; –, cocultivation in the absence of wounding and acetosyringone. Vertical bars indicate standard deviation.

Table 1. Effect of acetosyringone and wounding on the percentage of GUS-positive explants

AC	WO	Day 0	Day 1	Day 2	Day 3	Day 6
0	0	0.0	0.0	0.0	0.0	0.0
+	+	0.0 a	4.2 a	2.5 a	25.0 b	0.8 a
+	−	0.0 a	5.0 a	7.5 a	52.5 c	2.5 a
−	+	0.0 a	6.7 a	10.0 a	31.7 b	0.0 a
−	−	0.0 a	9.2 a	7.5 a	23.3 b	1.7 a

Different letters (a, b, c, …) indicate significant difference (factorial ANOVA, posthoc Tukey HSD test $p < 0.05$). Data represent means of two experiments of 60 sections of 15 explants per treatment. Only 15 explants each were tested once for the day 0 and the no *Agrobacterium* controls (0). Treatment factors were wounding of explants prior to agroinfection (WO) and cocultivation in the presence of acetosyringone (AC).

Effect of acetosyringone and wounding on the number of blue stained zones per GUS-positive explant section

Cocultivation in the presence of acetosyringone showed a significant positive effect on the number of GUS-positive zones per GUS-positive explant section ($F_{AC} = 5.51$, $p = 0.02$) whereas wounding did not show an effect ($F_{WO} = 0.46$, $p = 0.50$). No interaction between acetosyringone and wounding was observed ($F_{AC*WO} = 0.073$, $p = 0.79$). Little significant difference among the individual treatments at days 1, 2, 3 and 6, resulting in 1.1 to 1.9 GUS-positive zones per GUS-positive explant section, was observed (Table 2). Only the number of GUS-positive zones per GUS-positive explant section after cocultivation of wounded explants in the presence of acetosyringone observed on day 3 (1.9) differed significantly from that observed on day 3 after the cocultivation of unwounded explants in the absence of acetosyringone (1.1).

Putative chimeric transformants

After 4 weeks on selective multiplication medium, 130 shoots emerged from a total of 104 explants from two experiments, a multiplication rate of 1.25 shoots per explant, half of that observed during micropropagation (Mollel and Goyvaerts, submitted). One hundred and two (78%) emerging shoots were bleached, 25 shoots (19%) exhibited both bleached and green sectors and 3 shoots (2%) were completely green. After 6 weeks on selective elongation medium, only two of the green shoots (1.5%) survived and remained green whereas subcultured partially green and 1 green shoot died or bleached substantially (Figure 1d and e). GUS staining of putative chimeric transgenic marula shoots revealed a high level of GUS activity in virtually all tissues of one shoot whereas a low level of and zonal GUS activity was observed in the second shoot (Figure 1f). No DNA analysis was conducted to determine the presence of the *npt*II and *uid*A transgenes or the number of

Table 2. Effect of wounding and acetosyringone during cocultivation on the number of GUS-positive zones per GUS-positive explant section

AC	WO	Day 0	Day 1	Day 2	Day 3	Day 6
0	0	−	−	−	−	−
+	+	−	1.4 ab	1.7 ab	1.9 b	1.0 ab
+	−	−	1.3 ab	1.7 ab	1.6 ab	1.7 ab
−	+	−	1.1 ab	1.5 ab	1.1 a	−
−	−	−	1.2 ab	1.1 ab	1.3 ab	1.0 ab

Different letters (a, b, c, …) indicate significant difference (factorial ANOVA, posthoc Tukey HSD test $p < 0.05$). Data represent means of two experiments of 60 sections of 15 explants per treatment. Treatment factors were wounding of explants prior to agroinfection (WO) and cocultivation in the presence of acetosyringone (AC). The control was cocultivation in the absence of *Agrobacterium* (0) and for samples without GUS-positive explants (−) zones could not be reported.

6

292 integration events in the putative chimeric trans-
293 formed marula shoots.

294 **Discussion**

295 *Evolution of transient expression*

296 Transient expression of the *uid*A reporter gene has
297 been used successfully to study and optimize the
298 efficiency of T-DNA transfer to plant cells in
299 *Agrobacterium*-mediated transformation (Nara-
300 simhulu et al., 1996; Egnin et al., 1998; Wenck
301 et al., 1999; Amoah et al., 2001; Chakrabarty
302 et al., 2002). In marula, after the lag phase ob-
303 served on day 1 and 2 GUS expression increased
304 on day 3 and was reduced on day 6 after agro-
305 infection (Figure 1 and Table 1). Transient
306 expression occurs due to the transfer of the T-
307 DNA to the plant nucleus followed by transcrip-
308 tion, splicing, mRNA export, translation and
309 expression of the GUS enzyme. A number of po-
310 tential factors including a higher transcription le-
311 vel from an episomal than from a chromosomal
312 gene and a higher T-DNA copy number per cell
313 before than after integration could contribute to
314 the observed initial high expression followed by a
315 reduction. But the transient effect is mainly as-
316 cribed to the fact that not all T-DNA that is
317 transferred to a nucleus becomes integrated and
318 unintegrated T-DNA is eventually lost (De Vil-
319 liers, 1998). The observed lag phase in percentage
320 of GUS-positive explants concurred with the
321 change in environment requiring metabolic
322 reprogramming and switch-on of the transforma-
323 tion machinery by *Agrobacterium*. Optimization of
324 *Agrobacterium*-mediated transformation normally
325 involves a large number of factors of which,
326 *Agrobacterium* strain and virulence plasmid
327 (Miguel and Oliveira, 1999), wounding (Le et al.,
328 2001) and inclusion of *vir* gene inducers (Khan,
329 2003) are most often reported to impact signifi-
330 cantly. In this preliminary study only the effects of
331 explant wounding and acetosyringone on *Agro-
332 bacterium*-mediated transformation of marula no-
333 dal explants were evaluated by measuring transient
334 GUS expression. Generally addition of the *vir* gene
335 inducer acetosyringone and wounding of explants
336 before co-cultivation increase transformation fre-
337 quencies (de Villiers, 1998; Park et al., 1996;
338 Gheysen et al., 1998). Presence of acetosyringone

339 without wounding of explants was effective in
340 T-DNA transfer to marula. Addition of acetosy-
341 ringone to the bacterial suspension also increased
342 transformation efficiencies in tamarillo (Atkinson
343 and Gardner, 1993), Norway spruce (Wenck et al.,
344 1999) and guayule (Pan et al., 1996) and was
345 essential for putative transgenic callus differentia-
346 tion in black poplar (Confalonieri et al., 1995) and
347 tea (Matsumoto and Fukui, 2002). Wounding has
348 been reported to promote *Agrobacterium*-mediated
349 transformation (Pan et al., 1996; Park et al., 1996;
350 Santarém et al., 1998; Tang, 2002; Weber et al.,
351 2003). Wounding may enhance the accessibility of
352 plant cells for *Agrobacterium*, stimulate the pro-
353 duction of *vir* gene inducers and enhance the plant
354 cell competence for transformation. However, in
355 marula wounding *per se* did not significantly in-
356 crease transient expression. Moreover in combi-
357 nation with acetosyringone, wounding exhibited a
358 significant antagonistic effect (Table 1). Mondal et
359 al. (2001) reported that both acetosyringone and
360 wounding reduced the efficiency of transient
361 transformation in tea (10% compared to 40% for
362 control). In tea, survival of explants was affected
363 due to the inherent prevalence of high amounts of
364 phenolics upon wounding in woody plant tissues
365 and the high rate of tissue browning. Explant
366 browning upon initiation in tissue culture was also
367 observed for marula and during routine micro-
368 propagation polyvinylpyrrolidone at a concentra-
369 tion of 1% was included in the medium (Mollel
370 and Goyvaerts, submitted). During transforma-
371 tion however it was omitted from the medium as it
372 may adsorb the acetosyringone added to induce *vir*
373 gene expression. Browning of the medium and
374 explants was effectively prevented by transfer to
375 fresh medium and the use of explants from growth
376 room grown seedlings. Phenolics produced upon
377 wounding of the axillary bud area in marula ex-
378 plants may have prevented binding by the aceto-
379 syringone to the VirA-ChvE cell wall protein
380 complex (Gheysen et al., 1998) and thus reduced
381 *vir* gene induction to a level similar to that in the
382 absence of acetosyringone. Alternatively, wound-
383 ing may have lead to localized cell necrosis. Jou-
384 bert et al. (2002) reported that certain phenolic
385 compounds inhibited T-DNA transfer and also
386 related this to the general toxicity of phenolics.

387 At 23.3–81.7%, 52.5% on average, we obtained
388 transient GUS expression in marula explants at
389 rates similar to those reported by others. Atkinson

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and Gardner (1993) reported transient expression frequencies of 35% in tamarillo, Janssen and Gardner (1993) 50% in kiwifruit, Dronne et al. (1999) 1 to 40% dependent on cultivar in lavandin, Knittel et al. (1994) 41% in sunflower, and Wenck et al. (1999) 60% in pine and 90% in spruce. Blue stained zones along the vascular bundles rather than spots were observed in marula GUS-positive explants (Jansen and Gardner, 1989, 1993; Vancanneyt et al., 1990). The rate of 1–4 GUS zones per GUS-positive stem section (four sections per explant) compared to three to six spots at the edges of lavandin explants (Dronne et al., 1999) but was lower than the average rate of 15.6 GUS spots per leaf explant of black poplar (Confalonieri et al., 1995).

Of those elongated in the presence of kanamycin, only two out of three green shoots survived whereas white and white green shoots died. White green shoots may have been escapes or chimera with smaller sections of transformed tissue unable to sustain survival under the relatively high selective pressure. Two green shoots elongated in the presence of 300 mg l^{-1} kanamycin of which one showed GUS activity in virtually all tissues more than 10 weeks after co-cultivation, a putative transformation rate of 0.8–1.5% for all treatments combined. It is rather surprising that the second shoot that remained green under prolonged selection expressed GUS at an apparent low level and in only few tissues. This may have been due to the genetic background of the particular explant which enabled its partial escape. This or alternative explanations would need to have been confirmed by DNA work. The percentage of putative chimeric marula transformants was close to the range reported by other authors (1.2–3.9% for wheat, Khanna and Daggard, 2002; 2.7% for plum, Yancheva et al., 2002).

In conclusion, this is the first report on the genetic transformation of marula. We used an approach of *Agrobacterium*-mediated transformation of pre-existing shoot apices and high selective pressure which lead to the recovery of 0.8–1.5% of putative transgenic chimera. Transgenic chimera exhibiting desired novel traits may offer an opportunity to generate commercial superior marula trees and to carry out basic research on, among others, signal transduction in trees. Much scope for further optimization of transformation efficiency by using supervirulent *Agrobacterium* strains and by optimization of the recovery of transgenic chimera as well as non-chimeric transgenic plants through repeat subculture of transgenic tissues followed by rooting or grafting exists.

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