HOGESCHOOL GENT
LID VAN DE ASSOCIATIE UNIVERSITEIT GENT

Geassocieerde Faculteit Toegepaste Bio-ingenieurswetenschappen

Academiejaar 2011 - 2012

Acclimatization of micropropagated hardwood *Melia volkensii*

Acclimatisatie van in vitro vermeerderend *Melia volkensii*

Masterproef voorgedragen door

Zjef Van Acker

tot het bekomen van de titel en de graad van

Master in de biowetenschappen: land- en tuinbouwkunde
Afstudeerrichting tuinbouwkunde

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Preface

This thesis was brought to a successful conclusion with the help of many people. First of all I would like to thank my promoters Prof. Stefaan Werbrouck and Ir. Jan Vandenabeele for all the support and guidance. I also would like to thank Better Globe Forestry Ltd. For the opportunity to do an internship during the summer of 2011, I had one of the best times of my life at the plantation in Kiambere. You are doing a great job on making this world a better place.

Next I would like to thank all the researchers at the Biotechnology laboratory of Ghent University College; Joris de Wilde, Hans Motte, Danny Vereecke and Pieter Verholle. Thank you for answering all my questions, advising me on my experiments and forgiving me for using a lot of working space. I also would like to thank the interns of the lab that helped me with my many experiments; Charline Bossuyt, Thomas Vervisch, Hans Santos and Gregory Daelman.

Finally I would like to pay tribute to all my friends and family for supporting me during this hard time in my life. My sister Jana Van Acker for being so awesome and understanding. My mother Magda Van Acker, Katleen Verhoest, Joery Neys, Silke Willaert and all my closest friends. This past year was very tough for me, but with your help, I managed to keep on going.

To end this preface, I would like to dedicate this work to my father and role model, Danny Decaestecker, who passed away last year.

Zjef Van Acker
Ghent, 25/05/2012

Abstract

Melia volkensii is a drought resistant, multipurpose hardwood tree, indigenous to East-Africa. Due to its difficult macropropagation, the technique of micropropagation is the solution to obtain a high rate of multiplication for superior trees. Although the in vitro multiplication goes very well, rooting of most of these shoots is still problematic. For this thesis, many experiments on in vitro multiplication, in vitro rooting and acclimatization were conducted. Meta topolin derivates (mTR and MemTR) added to the multiplication medium gave good multiplication, but moderate rooting results. Phenyl adenine, a cytokinin like substance, added to the micropropagation medium gave a delay in multiplication, but surpassed the meta topolin derivates on subsequent rooting. Many new insights on rooting and successful acclimatization have been obtained. Observations also confirmed that micropropagated Mukau shoots developed a fleshy tap root, an important asset of the trees drought resistant feature.

Abstract (dutch)

Melia volkensii is een Oost-Afrikaanse, droogteresistente hardhout boom die gebruikt wordt voor vele doeleinden. Conventionele vermeerderingstechnieken zijn zeer moeilijk, waardoor naar in vitro technieken moet gekeken worden voor het klonen van superieure bomen. Alhoewel de in vitro vermeerdering zeer succesvol is, blijft de beworteling voor de meeste van deze scheuten problematisch. Voor deze thesis werden meerdere experimenten op in vitro vermeerdering, in vitro beworteling en acclimatisatie uitgevoerd. Meta-topoline derivaten (mTR en MemTR), toegevoegd aan het vermeerderingsmedium, gaven een goede multiplicatie, maar matige bewortelings resultaten. Phenyl adenine, een cytokine achtige stof, toegevoegd aan het multiplicatie medium, gaf een vertraging tijdens de vermeerdering, maar oversteeg de resultaten van de meta-topoline derivaten tijdens de daaropvolgende bewortelingsfase. Vele nieuwe inzichten in verband met beworteling en succesvolle acclimatisatie werden verworven. Observaties bevestigden tevens dat de in vitro gekweekte Mukau scheuten een penwortel ontwikkelden, wat een belangrijk onderdeel is van de droogteresistentie van de boom.

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Used Abbreviations

IAA : indol-3-acetic acid
IBA : indol-3-butrytic acid
NAA : Naphthalene acetic acid
TIBA : 2,3,5-triiodobenzoic acid (polar auxin transport inhibitor)
NPA : N-1-naphthylphthalamic acid (polar auxin transport inhibitor)
2,4-D : 2,4-Dichlorophenoxyacetic acid
3,4-D : 3,4-Dichlorophenoxyacetic acid
CKX : cytokinin dehydrogenase
mT : meta-topolin
mTR : meta-topolin riboside
MemT : meta-methoxy topolin
MemTR : meta-methoxy topolin riboside
BA : benzylaminopurine
CEC : Cation Exchange Capacity
MS : Murashige and Skoog
BMM : Basic Medium *Melia*
WPM : Woody Plant Medium

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1. Introduction

In the semi arid areas of East Africa, the Mukau or *Melia volkensii* is a very coveted tree because of its multipurpose use. Due to the outstanding ability to withstand long periods of severe drought, it can be used as a weapon in the struggle against the expanding desertification that threatens these regions. Better Globe Forestry Ltd., has appropriated this mission and wants to create vast Mukau plantations in the drylands of Kenya. Though, many challenges have to be overcome.

Because of the fast growing human population in East Africa, Mukau has been overexploited, whereby the superior trees have disappeared and genetic diversity is very low. Another difficulty that Mukau growers have to deal with, is the fact that the extraction and germination of seeds is very hard, expensive and time-consuming. It is, thus, an extremely slow going activity to try and create immense plantations consisting of heterogeneous trees. Therefore, a new propagation method to obtain and grow only the best Mukau trees, was bound to be used; "micropropagation". With this technique it is possible to multiply the best Melia volkensii clones at an enormous high rate. But although protocols for the in vitro multiplication of this tree have been developed, the rooting and acclimatization of the micropropagated shoots is still a troublesome obstacle. A great deal of research has already been conducted on this subject, but up to the present, no conclusive method has yet been developed to root and acclimatize the majority of the these small and fragile shoots in order to develop into the big and strong trees that they are genetically predisposed to be. This thesis tried to combine all previous research on the micropropagation of *Melia volkensii* and complemented it with some new experiences and results on in vitro multiplication, in vitro rooting and ex vitro acclimatization. This purpose was successfully achieved, but many new questions were raised during this process. Plant science research is thereby a never ending story.

2. Literary study

2.1. *Melia volkensii*: Characteristics

Melia volkensii or Mukau is a fast growing, drought resistant, deciduous tree that can reach a height of 20m. It has a light, rounded or spreading crown and an important fleshly tap root. The bark is pale grey and rather smooth, its leaves are bright green and compound, they consist of lance shaped leaflets that grow up to 4cm long and have a slightly serrated margin (Tedd, Seabrook and Black 1997). The flowers are dense, white and worn on a branching head. The fruits are yellow and oval to 4cm long, visible on the tree (Maundu and Tengnäs 2005). All young shoots are densely haired and even in dry season the tree has a lot of green leaves (Tedd, Seabrook and Black 1997). Generally it grows good in well drained soils, sandy loam to sandy clays, but it's intolerant for heavy clays and black cotton soils (Stewart en Blomley 1994). It is visibly green throughout the year (Maundu and Tengnäs 2005), but shows major leaf cover reductions during the dry seasons. This and the fact that flushing starts before the rains begin, proves that *M. volkensii* has a very effective way of storing water in a hostile environment (Broadhead, Ong and Black 2002). Since *M.* volkensii has an effective mechanism for accumulating water in all tissues, it can withstand long periods of drought (Maundu and Tengnäs 2005).

Fig.1 : A seven year old Mukau tree during the dry season at Kiambere

Only the outer wood is affected by borers and termites, the hard inner wood of a mature tree is very hard, brown and resistant to all sorts of attacks (Tedd, Seabrook and Black 1997). The plant is also deeply rooted and performs well in resisting against attacks by common insects. Because it is a very drought resistant arbor, the Mukau is a valuable tree in the semi-arid areas of Ethiopia, Somalia, Tanzania and Kenya, from a geographical height of 400 to 1650m. The use of the tree is very versatile; it is applied as termite resistant timber, poles, bee hives, dry season fodder (fruits and leaves), mortar, fire wood, mulch against soil erosion and as maintainer of soil fertility, medicine (pain relief), carve wood, manure and as pesticide (Tedd, Seabrook and Black 1997), (Stewart en Blomley 1994), (Maundu and Tengnäs 2005). It seems that because of its multipurpose use and its high value, the Mukau has been overexploited (Runo, Muluvi and Odee 2004)**.** Therefore it can be stated that goats (Tedd, Seabrook and Black 1997) and poor management by the fast expanding human population in semi-arid areas, are the main threats for *M. volkensii*.

2.2. Propagation

2.2.1. Common Propagation

An universal prerequisite for plant propagation is to optimize the plant environment. Microclimatic conditions like light, temperature, humidity, gas exchange,… are altered in order to create the ideal circumstances for the development of the plant. Also edaphic (medium or soil, nutrition, water,…) and biotic (fungi, bacteria,…) factors can be used to optimize its growth. But in the process of its propagation, the plant has to be monitored very closely, because environmental conditions that are ideal for its propagation, are often very conductive for pests. (Hartmann, et al. 2002). A variety of structures, like greenhouses, cold frames, poly tunnels and hot beds are used to control the propagation environment (Klingaman 2008).

Hardening off is the stress adaptation process or acclimatization that occurs as a propagule, such as a cutting, is gradually weaned from a high to a low relative humidity environment during rooting. Over this process, the management of light and the temperature of the propagation medium can be critical for rooting the cuttings or shoot multiplication. Hartmann, et al. (2002) also claims that there is no factor more important than optimum temperature control for the propagation of plants.

High respiration rates occur during root formation at the base of a cutting. This consumes O_2 and produces CO_2. Therefore the medium must be sufficiently porous, but still dense enough to hold the cuttings. Firm, stable, easy to wet, free from pests, low salinity, high CEC, availability and economical are also some important parameters (Hartmann, et al. 2002). Pests are broadly defined as all biological organisms that interfere with plant production. Keeping stock-plants and propagules as clean and as pest-free as possible, and suppress pathogen organisms, is therefore one of the main goals in plant propagation (Hartmann, et al. 2002). Another very important factor with propagation is the selection of the cuttings; Health, age, growth stage, maturity of the stock plant, time of the year and type of cutting have to be taken into consideration (Osburn, Cheng and Trigiano 2008).

2.2.2. Common propagation of *Melia volkensii*

In most cases *Melia volkensii* is propagated with seedlings, but root cuttings, root suckers and wildlings are also commonly used (Maundu and Tengnäs 2005). This is because the epigeal sprouting seeds of the Mukau (P. Milimo 1989) are very hard to germinate. Even In well organized nursery's it is very difficult to achieve germination rates above 40% (Maundu and Tengnäs 2005). Placing the seeds under favorable conditions of moisture and temperature, is not enough. Maturation of seeds takes 11 to 13 months and lack seasonal patterns. Milimo (1986) witnessed that sprouting started after seeds with damaged integuments absorbed over 60% water. So they concluded that dormancy is imposed by the integuments because they don't allow water absorption and mechanically preventing the ridicule to emerge. Maundu and Tengnäs (2005) found that a good preparation of the seeds consist of removing the pulp from the fruits, drying the stones in the sun, cracking them to release the seeds and subsequently soak the seeds in warm water.

Better Globe Forestry Ltd., a company with multiple vast Mukau plantations in Kenya, presents following work instructions for the production of Mukau seedlings (Vandenabeele 2011).

1. Groomed collection and transport of the *M. volkensii* fruits. Ripeness, period of collection, location and quality of the parent trees are important.
2. Extraction and preparation of the Mukau seed.
    a. The cover of the fruit is removed by hitting it with a piece of wood
    b. The stones/nuts are washed and sun dried for 2 hours
    c. Only the best nuts are selected, the brown ones
    d. The nuts are cracked with a nutcracker or a knife (panga) hit softly by a piece of wood, the seed has to be carefully pulled out
    e. Darkbrown nuts are selected and put in a disinfected container
    f. The seeds are soaked overnight in a solution of salt-free water and Bavistin (1 g/l)
    g. Aat its small end, the seeds are nipped to break the coat and allow the entry of water
    h. The seeds are soaked in water with bavistin (1 g/l) for 12 hours
    i. Seeds are removed from the water and slit over its entire length by a razorblade, two slits on opposite sides of the seed
    j. Awaiting a large enough number to be sowed in the propagator, the seeds are put in water with bavistin.
3. The propagator is a wooden structure of 180 cm long, 60 cm wide, 20 cm high at the front and 25 cm at the back with a capacity of 1500 seeds. The whole structure

is covered in Polyethelene in a double layer, except the bottom which is perforated to allow drainage of water.

4. Coarse sand is used as medium, it is sterilized by sun drying or steaming.
5. 4cm of coarse sterilized sand is applied on the bottom of the propagator. Seeds are sown 1 cm apart from each other in rows that are 2 cm apart. Afterwards the seeds are covered with 1cm of coarse sand, and 0.5 cm of finer sand. After sowing, 6 l of water mixed with Bavistin (1 g/l) is used for watering one propagator.
6. When germinated seedlings reach 3-5 cm, they are transplanted into polybags and put under tunnels for a period of 7 days. 18 liters per 1000 seedlings every 3 days.
7. The seedlings are put under a plastic shelter for 3 weeks to harden of. 30 l of water per day/1000seedlings is applied.
8. The seedlings are hardened of without plastic shelter for a minimum of 6 weeks

Fig.2 : Slitting the seeds, a very precise and time-consuming practice

Fig.3 : Sowing of the seeds in the propagators.

Fig.4. : Young Mukau seedlings about one month old.

Fig.5 : A two year old plantation of Mukau trees during the dry season

Nowak (2010) Found that MAVUNO, a fertilizer commonly used in Kenya, had positive effects on the health and diameter of 1 year old Mukau seedlings. 50 g applied in the trial area when the trees were planted, gave the best results. The propagation of Mukau seedlings is very time consuming and costs a lot of money. Therefore other methods of multiplication can be used. Stewart en Blomley (1994) found that rejuvenation of mature *M. volkensii* is easiest by coppicing and root pruning, because the specie coppices good and the formation of root suckers is great when the roots are injured. But Indieka (2005) found that material obtained at the beginning and during the dry season is of bad quality, so the year round supply of planting material is uncertain. Mulatya, et al. (2002) also found that root architecture of *Melia volkensii* is influenced by the method of propagation. Roots of seedlings descend at greater angels into the soil then plants raised from cuttings. Therefore they hold a smaller fraction of their root system length at shallow depth. Because of this, they are more resistant to drought and are less competitive for nearby plants.

2.3. Root development

The requirements for the rooting of cuttings can be very species specific (Osburn, Cheng and Trigiano 2008). Some species root more easily with hardwood cuttings, others do better with softwood cuttings, semi-hardwood cuttings,… In most cases a cutting is treated with an auxin and stuck into a suitable rooting medium to start the production of adventitious roots (Osburn, Cheng and Trigiano 2008). If roots are generated, their morphology is regulated by many genes with small effects and highly influenced by the environment (Costa de Oliveira and Varshney 2011).

According to Hartmann, et al. (2002) the stages of novo adventitious root formation are; (1) Dedifferentiation of specific differentiated cells, (2) formation of root initials from certain cells near vascular bundles or vascular tissue, which have become meristimatic by dedifferentiation, (3) subsequent development of root initials into organized root primordial and (4) growth and emergence of the root primordia outward through other stem tissue, plus the formation of vascular tissue between the root primordia and the vascular tissue of the cutting. The development of the right root system architecture is very important, it allows a plant to survive periods which it is meant to overcome. The number, the direction of growth of each root and its placement are highly variable, even when plants have identical genomes. This development plasticity is driven by the environment. The plant possesses many undifferentiated cells that are able to change their purpose to benefit the growth, though there are still genetic constraints. Drought resistant varieties of rice have deeper roots that are more branched in comparison to less drought resistant ones (Price, Tomos and Virk 1997). There are two categories of mechanisms that that influence the root architecture; (1) pathways that are essential for organogenesis and growth, (2) pathways that determine how plants respond to external signals (J. Malamy 2005). Malamy en Benfey (1997) found that genetic markers with *A. thaliana* showed similar patterns of expression in primary and lateral roots. Although the primary root is the only one that develops during embryogenesis, adventitious, lateral and primary roots are essentially identical in structure (J. Malamy 2005). However, this does not mean they are the same; with Maize, adventitious roots and lateral roots are genetically different. Adventitious roots originate from the dedifferentiation of parenchyma cells associated with vascular tissues, whilst lateral roots originate from pericycle cells of a root from a higher branching order (Srivastava 2002). It can be concluded that unrooted macro- and micro-cuttings develop adventitious roots.

Auxins are Planth Growth Regulators (PGR's) that induce rooting, though they inhibit root growth (Osburn, Cheng and Trigiano 2008). For root growth with *Arabidopsis thaliana*, it has been found that IAA, NAA and TIBA slow it down primarily through reducing the

magnitude of the plants growth zone. Auxins like 2,4-D and NPA inhibit root growth by reducing the cell production rate (Rahman, et al. 2007). Reed et al. (1998) found that lateral root initiation at a root junction is inhibited by blocking the polar auxin transport. Overproduction of endogenous or application of exogenous auxin leads to more initiation events (J. Malamy 2005) (Boerjan, et al. 1995). Auxin/cytokinin ratios are implicated in many development processes (J. Malamy 2005) and many sources report that high endogenous IAA content and low cytokinin content is needed for good rooting. Malamy (2005) found that *Arabidopsis thaliana* plants, engineered to have lower cytokinin levels, have a larger number of lateral roots. Other hormones have also been known to affect root initiation, but merely because of their effect on auxins. Brassinosteroids, Abscisic acid and nitric oxide have been known to influence either the transport of auxin, or it's signalling (J. Malamy 2005). A high ratio of sucrose to nitrogen in the plant medium, inhibits lateral root initiation with *A. thaliana* (Malamy and Ryan 2001), and so does a high sulphate content. Sulphate deprivation triggers a response pathway that leads to increased formation of IAA (Kutz, et al. 2002) (J. Malamy 2005). It seems logical, but also water stress inhibits lateral root initiation with *A. thaliana* (van der Weele, et al. 2000). Indieka (2005) observed that callus forms at the base of *M. volkensii* cuttings. Callus is an irregular mass of cells in different stages of lignifications that develops at the basal end of a cutting that is placed under environmental conditions favorable for rooting. In some species, callus is a precursor for root formation. (Hartmann, et al. 2002)

Etiolation consists of growing a plant in the total exclusion of light, Banding or partial etiolation comprises of applying materials to exclude light from localized portions of the stems of plants (Bachman 2008). Some sources suggest that anatomical and physiological changes, that enhance rooting, can occur in etiolated stem tissue. Firstly, it changes the stem's sensitivity to exogenous applied and endogenous auxin (Bachman 2008) (Gillman 2008). Secondly, these cuttings may develop chemicals in them, that can act as cofactor to the rooting hormone (Gillman 2008). Thirdly, the lower chlorophyll content may have an impact on root formation (Bachman 2008). The fourth change in favor of rooting, is that etiolated cuttings have less lignin in them, a lower concentration of this structural compound may allow roots to penetrate more easily trough tissues that normally inhibit their extension (Gillman 2008). Normal stems have a high number of Sclerenchyma cells, which give the stem strength and rigidity. Etiolated stems have a much lower quantity of sclerenchyma cells and an increase in relatively simple parenchyma cells, which present less of a mechanical barrier for new, developing roots (Bachman 2008). Lake and Slack (1961) also suggest that in some plant species, light of appropriate spectral composition will slow down root extension and elicit a gravitropic response. That is, if it reaches the root apex. With peas, an increase of roots formed was observed when the part of the stem, that would act as the base of the cutting, was etiolated before propagation. If the pea-plant was fully etiolated, the endogenious auxin

level was higher, but the number of roots formed did not increase (Koukourikou-Petridou 1998).

2.4. Micropropagation

Micropropagation is defined as aseptic asexual plant propagation on a defined culture medium in culture vessels under controlled conditions of light and temperature. The term in vitro propagation is also commonly used as to refer to micropropagation (Kane, Kauth and Stewart 2008).

Genetics, the surrounding environment and the components of the culture medium, these factors influence the growth and development of an in vitro plantlet. Therefore the objective determines the type of explants and/or culture (George, Hall and De Klerk 2008). The most frequently used method of micropropagation is shoot cultures, it relies on enhanced axillary shoot proliferation from cultured meristems. It provides the most genetic stability and is more readily attainable for many plant species (Kane, Kauth and Stewart 2008). A culture mediums consist of 95% water, Plant Growth Regulators (PGRs), vitamins, sugar, a culture support element (like agar) and sometimes various other organic materials (Beyl 2008). With in vitro propagation, closed vessels are used to prevent microbial contamination, this decreases air turbulence, which increases leaf boundary layer and limits the inflow of CO_2 and outflow of gaseous plant products like ethylene. All these special conditions result in abnormal morphology, anatomy and physiology of the small plantlet that has to correct all these effects when it is planted ex vitro (Baroja-Fernández, et al. 2002).

2.4.1. Plant Growth Regulators

Plant Growth Regulators (PGRs) can have large effects in small concentration, they regulate the initiation and development of the explants in in vitro cultures. Every plant creates their own endogenous PGRs, but applied exogenous PGRs can manipulate the growth for a specific purpose. The effect of growth regulators are not absolute and specific, therefore the responses of the organisms in vitro, can vary according to the in culture conditions, type of explants and the plant genotype (George 1993). Endogenous hormonal levels change over the course of plant development, these changes determine intrinsic patterns of growth and development. They are also key components of response pathways, as hormone levels are modulated in response to environmental signals (J. Malamy 2005). Because of this, exogenous applied PGRs can change the normal course of plant development to serve a specific goal in micropropagation. In tissue cultures many different classes of hormones can be used, but the most important ones are the auxins and cytokinins (George 1993).

Auxins

Auxins are commonly used in micropropagation to promote the growth of callus or organs, and to regulate morphogenesis. They are also responsible for the maintenance of apical dominance in plants and treatments for rhizogenesis are usually with auxins alone (George 1993). The uptake by the plants is more rapid in tissue cultures with a pH less than 5-6. Auxin is transported in a polar fashion to the cells.

IAA or indole-3-acetic acid (molecular weight = 175,18 g/mol) is the most widely distributed, naturally occurring auxin in vascular plants (George 1993). It is involved in almost every aspect of plant growth and development (Srivastava 2002). The synthesis of IAA occurs in young plant tissue, such as shoot tips or young buds. This happens through many pathways, most of which use tryptophan as precursor, though there is some evidence that IAA can also be formed without this amino acid (Srivastava 2002). Synthetically prepared IAA, used in tissue culture media, denaturates quickly or is rapidly metabolized within the plant tissues (George 1993). The rate in which IAA degrades is even more rapid in light, in presence of MS salts and heat (Dunlap, Kresovich and McGee 1986). Stasinopoulos et al. (1990) Found that IAA is susceptible to photolysis from blue and TL-light, but that yellow light prolongs the lifetime of IAA in culture media.

IBA or indol-3-butrytic acid (molecular weight = 203,24 g/mol) is one of the many chemical analogues of IAA, but it is more stable (George 1993). IBA can be derived from IAA through chain elongation. IAA is converted to IBA by an enzyme termed IBA-synthetase (Kaldorf and Ludwig-Muller 2000). This process also occurs in a reversed manner, thence IBA may be a part of the system that maintains IAA homeostasis (Srivastava 2002).

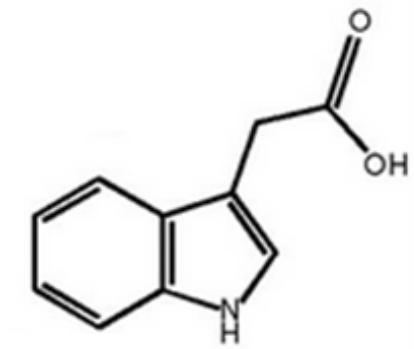

Fig.6 : IAA or indol-3-acetic acid (Won, et al. 2011)

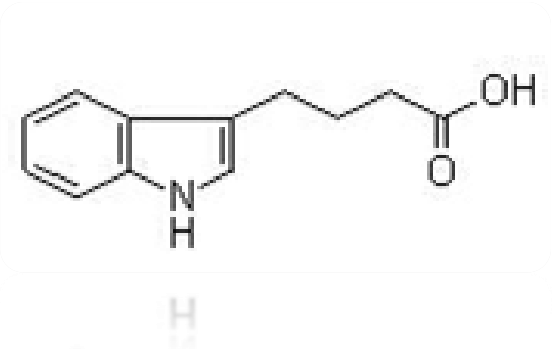

Fig.7 : IBA or indol-3-butrytic acid (Kaldorf and Ludwig-Muller 2000)

Cytokinins

Cytokinins (CKs) are used in tissue cultures because they stimulate cell division and control morphogenesis, furthermore they break the dormancy of lateral buds by overcoming apical dominance. On the other hand, CKs are inhibitory to rhizogenesis and leaf senescence (George 1993). The synthesis of CKs happens in organs where cell divisions occur at a high rate, such as root apices, cambial tissue, developing seeds,… (Srivastava 2002). The Roots are the main site of CK biosynthesis. Therefore, many isolated, small organs are unable to synthesize sufficient amounts of this plant hormone to sustain growth (George 1993).

Meta-topolin riboside (mTR) is an naturally occurring aromatic cytokinin derived from mT through hydroxylation (Tarkowska, et al. 2003). According to some, mT and mTR have the same biological activity as trans zeatin, the most active natural CK. mTR gives good multiplication rates, but inhibits root development with bananas (Bairu, et al. 2008). Though mT caused good multiplication and in vitro rooting with *Spathiphyllum floribundum* (Werbrouck, et al. 1996). Also Mutuia et al. (2012) observed better rooting with *Pelargonium x Hortorum* after an in vitro treatment with mT. With potatoes, the in vitro multiplication medium supplemented with mTR improved its ex vitro survival rate and rooting, this is in contrast with other CKs that generally inhibit root formation (Baroja-Fernández, et al. 2002).

MemTR or meta-methoxytopolin riboside is a naturally occurring aromatic CK derived from MemT; a methoxy derivate from mT (Tarkowska, et al. 2003). Not only is the uptake of ribosides by plants better, it is suggested that mT and MemT are supplied with a riboside-group after they have entered the plant system. In vitro shoot tip explants of *Barleria greenii* produced most adventitious shoots on a medium with MemTR (Amoo, et al. 2011), but with *Petunia* Chimeras it showed a low multiplication rate, though very good anti-senescing effects with roses (Werbrouck, et al. 2006).

Phenyl adenin (Phe-Ade; molecular weight = 223,26 g/mol) is a synthetic anilinopurin. It has a structural similarity to aromatic cytokinins but it does not get any response from cytokinin receptors, it also does not inhibit cytokinin dehydrogenase (CKX) (Zatloukal, et al. 2008).

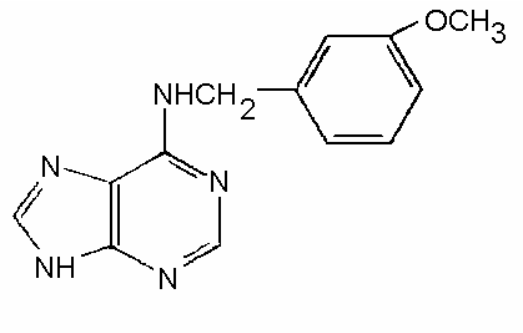

Fig.8 : MemT (Werbrouck, et al. 2006)

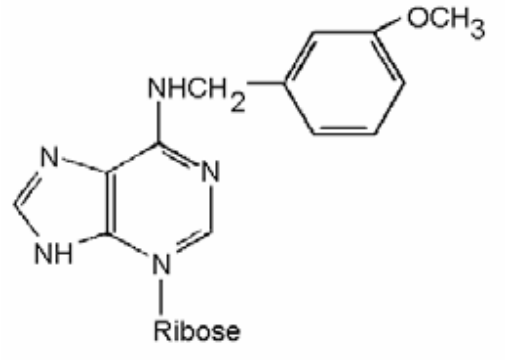

Fig.9 : MemTR (Werbrouck, et al. 2006)

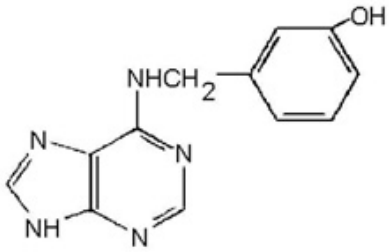

Fig10. : mT (Werbrouck, et al. 2006)

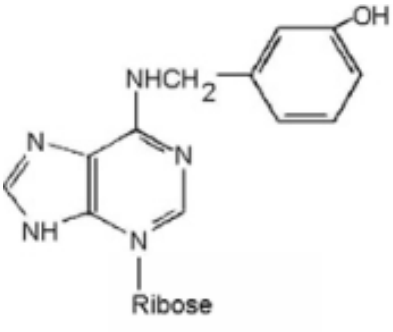

Fig.11 : mTR

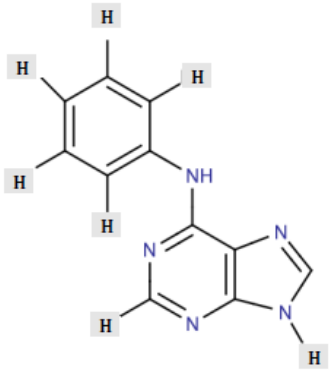

Fig12. : Phenyl Adenin
(Zatloukal, et al. 2008)

Other regulators

Activated charcoal (AC) is not a growth regulator, but it has large effects on the culture medium it is used in. It absorbs compounds present in the agar, it prevents unwanted callus growth, it promotes morphogenesis and is beneficial to root formation (partially due to light exclusion) (George 1993). AC can also absorb gasses, like ethylene, that are released from the tissue culture (Ernst 1974). The presence of charcoal can also cause a rise in pH.

2.4.2. Initiation of tissue cultures

The initiation is started with pieces of complete plants or in vitro sown seedlings. It is Important that these explants are free of micro-organisms and pests, because the conditions favorable for the growth of in vitro plants, are often beneficial for the multiplication of these unwanted organisms (George, Hall and De Klerk 2008). According to George et al. (2008), several elements are important for the initiation of tissue cultures; the type of explants, the isolation and incubation, the cultural environment and the solidified or liquid culture medium with vitamins, amino acids, sucrose (energy),…

2.4.3. Multiplication

The multiplication of micropropagated shoots can be achieved by generating axillary shoots on the plantlets. Usually a culture medium with a high cytokinin/auxin ratio is prepared to acquire the most side shoots. Another method is growing the shoot while keeping the apical dominance, afterwards the plant is cut into pieces that each can be inoculated and grown again. With these methods it is possible to obtain homogeneous shoots very easily and very fast (Swamy, Ganguli and Puri 2004). The success of the multiplication depends on the species, explants type and culture conditions (Yeoman 1986).

2.4.4. Rooting

Micropropagated plantlets are very weak, it is therefore advised that shoots that have already developed roots, are used for acclimatization. They are more capable of withstanding pathogens, dry atmospheres, fluctuating temperatures and high irradiance light. During the acclimatization to the outdoor environment and rooting of the shoots, there is a shift from a heterotrophic to an autotrophic condition. By gradually reducing the humidity or removing the cap of the in vitro culture, prior to transplanting, the cuttings can be hardened off milder (Hartmann, et al. 2002). Most treatments for multiplication do not induce root formation, so a

different procedure must be applied. The shoots can be rooted as small conventional cuttings out of culture, or they may be rooted in vitro during a separate stage (George 1993), (Hartmann, et al. 2002). Roots that have developed in vitro, are never fully effective when they are transferred into the soil, they frequently lack root hairs and vascular connections. They also have a high risk of getting damaged when they are removed from the culture medium, this increases the chance of infection. The vascular connection can be insufficient because there is callus formation at the base of the shoot, prior to root formation. Callus formation should therefore be kept to a minimum (George 1993). A shoot that does not possess roots, is obligated to dedifferentiate stem cells, so they can redifferentiate and form root initials. Wysokinska (1993) saw that microcuttings of *Penstemon serrulatus* formed long thin roots after adding IBA or IAA to the medium, and short fat roots with secondary rooting after NAA was added.

2.5. Micropropagation of *Melia volkensii*

Melia volkensii trees that have been grown from cuttings, develop shallow roots and the germination of seeds is very low, moreover it results in very heterogeneous planting material. Therefore the micropropagation of *M. volkensii* can be an important tool for its the mass-propagation and the distribution of superior clones that develop an efficient root system.

2.5.1. Sowing in vitro

Vermeir (2008) disinfected the seeds with seedcoat for 20 minutes in a 10% javel-solution, after that they were washed three times with sterile water. Subsequently the seedcoat was removed and the seeds were decontaminated again for 15 minutes in a 10% javel-solution. Before inoculation on a culture medium (4,4 g/l MS, 30 g/l sucrose, 7 g/l agar and 0,1 mg/l kinetin) with a pH of 5,7; the seeds were again rinsed three times in aseptic water. She concluded that unpeeled seeds of Mukau don't germinate in vitro, unless the seedcoat is removed, then they sprout very fast, though the embryo must stay connected to the endosperm (Vermeir 2008)

Fig.13 : A peeled seed germinating in vitro

2.5.2. Somatic embryogenesis

Indieka et al. (2007) cultured mature zygotic embryos and cotyledon explants on MS medium supplemented with BAP, NAA and 2,4-D (0,5; 1,0 and 2,0 mg/l) alone, and with BAP ((0,5; 1,0; 2,0 and 4,0 mg/l) in combination with 2,4-D or NAA (0,2 and 0,5 mg/l). After four weeks they observed the initiation of direct somatic embryos on 60% of the cotyledonary explants from seeds stored for less than three months. This was on the MS medium completed with BAP (0,5-4,0 mg/l) in combination with 2,4-D (0,2 mg/l). Only 20% of Cotyledonary explants form seeds stored for over 12 months, produced somatic embryos. The mature zygotic embryos on the other hand, failed to produce any somatic embryos (Indieka, et al. 2007).

Verhaeghe (2009) did many experiments to induce somatic embryogenesis on *Melia volkensii* leaves, mature seeds and immature fruits. Firstly callus was generated on the explants inoculated on a basic medium with MS (4,4 g/l) or WPM (2,46 g/l), sucrose (30 g/l), agar (7 g/l) supplemented with 2,4-D (0,2 mg/l), it had a pH of 5,7. After a couple of weeks they were transplanted on to a with MS medium with sucrose (30 g/l) and agar (7 g/l), the pH was 5,7. The basic medium was supplemented with combinations of BA (0; 0,5; 4 mg/l), 2,4-D (0,2 or 0,5 mg/l), 3,4-D (0,2 mg/l), $AgNO_3$ (5 mg/l), NaFeEDTA (36,7 mg/l), mT (0,5 mg/l) and MemTR (0,85 mg/l). Only on the mature cotyledons, longitudinally cut in half and placed on the medium with its outer shell, produced somatic embryos. This happened on the Basic MS medium with 2,4-D (2 mg/l) supplemented with BA (0,5 and 4 mg/l), though when this trial was repeated, the mature seeds generated no callus. There was also generation of somatic embryos on mature cotyledons inoculated on basic WPM medium supplemented with $AgNO_3$ (5 mg/l), NaFeEDTA (36,7 mg/l), BA (0,5 mg/l) and 2,4D or 3,4-D (0,2 mg/l). In another experiment somatic embryos were created on mature cotyledons inoculated on basic MS medium with BA (0,5 mg/l), MemTR (0,85 mg/l) alone and combined with $AgNO_3$ (5 mg/l) and NaFeEDTA (36,7 mg/l). Verhaeghe (2009) concluded that adition of $AgNO_3$ and NaFeEDTA is not neccesairy for the generation of zygotic embryos on mature cotyledons. The adittion of BA and 2,4-D to the medium gives the most creation of zygotic embryos. Whether the development of zygotic embryos was the best with a light or a darkness treatment could not be concluded. All the produced somatic embryos were transferred on to a new medium. Only one embryo developed in a normal manner (shoot + root), this happened on the basic MS medium. On the other media, the somatic embryos just developed into shoots that could be used for multiplication. These shoots were generated on basic MS-medium supplemented with BA (0,25 mg/l), GA (0,17 mg/l) or 2,4-D (0,2 mg/l). Secundary embryos, some shoots and fasciation were the results of somatic embryos transplanted to a basic MS-medium supplemented with BA (0,2 mg/l), GA3 (0,01 mg/l) and Putrescine (161,1 mg/l).

Embryos had to deal with hyperhydricity on a basic MS medium with BA (1 mg/l, Casein hydrosylate (200 mg/l), putrescine (161,1 mg/l) and Adenin (40 mg/l).

Lamberigts (2010) tried to create somatic embryos from leaf explants from one parent tree. Callus was formed, but after transplanting them on to hormone-free medium, there was no formation of embryos. Vermeir (2008) also got a lot of callus formation on leaf explants put on BMM supplemented with BA (0; 0,5; 1 and 5 mg/l) and 2,4-D. When the explants were put on 2,4-D-free medium, there was no initiation of somatic embryos. Braem (2011) did neither acquire somatic embryos from seeds whose epicotyls and radicula was removed, using BMM augmented with 2,4-D (0; 0,1 or 0,2 µM) and TDZ, BA or Incyde (0, 2 or 4 µM). Vermeir (2008) inoculated Root pieces on BMM with caseïnehydrosylate, nicotin acid, myo-inositol, Ca-panthothenoate, pyridoxine, thiamine and 2,4-D. When callus appeared they were transplanted on to the same medium, but without 2,4-D, there was no formation of zygotic embryo's.

2.5.3. Shoot induction

On leaves

Braem (2011) tried to find out if it was possible to regenerate shoots from leaves of *M. volkensii* on BMM supplemented with Zeatin or 2-ip in combination with Incyde-Cl, Incyde-F, Thidiazuron or 4-CCPu. After 50 days good results were obtained with both Incyde variants. The combination of the Incydes with or without 2-ip or Zeatin showed no significant differences. The adventitious shoots were not grown into full plants.

On roots

Braem (2011) also tested if root pieces of *M. volkensii* could generate shoots if placed on BMM culture medium completed with Incyde or BA (0,25; 0,5; 1 or 2 µM). The treatments with BA had significant better results than those with Incyde. BMM supplemented with 2µM BA gave the most shoots.

2.5.4. Multiplication

Indieka (2005) did six experiments on the multiplication rate of *Melia volkensii* shoots. The culture medium used for his experiments consisted of MS combined with sucrose (30 g/l) and agar (0,8%), supplemented with different PGRs or combinations of PGRs. He tested the use of zeatin, BAP, kinetin, IAA and NAA (see table 1) . The best multiplication rate (x 5,4),

after 30 days of subculture time was achieved with BAP (0,5 mg/l) combined with IAA (0,2 mg/l).

Table 1: Multiplication rates of treatments from Indieka (2005)

PGRs used	Best Multiplication rate (30 days of subculture)
zeatin/BAP/kinetin (0,25; 0,5; 1 or 2 mg/l)	2
BAP (0,5; 1; 2 or 3 mg/l) NAA (0,1; 0,2 or 0,3 mg/l)	1,4
kinetin (0,25; 0,5; 1 or 2 mg/l) IAA (0,05; 0,1 or 0,2 mg/l)	1,3
zeatin (0,25; 0,5; 1 or 2 mg/l) IAA (0,05; 0,1 or 0,2 mg/l)	1,7
BAP (**0,5**; 1; 2 or 3 mg/l) IAA (0,1; **0,2** or 0,3 mg/l)	5,4
kinetin (0,1; 0,2; 0,4; 1 or 3 mg/l) BAP (0,1 or 0,2 mg/l)	1,8

Vermeir (2008) used Woody Plant Medium (WPM) with 20 g/l sucrose, 8 g/l agar, IAA (0,1 mg/l) and BA (0,1 mg/l), kinetin (0,5 mg/l) or mT (0,25 mg/l). After eight weeks mT gave a multiplication rate of 6,1 and BA a multiplication rate of 4,52. Vermeir (2008) also used charcoal in the Basic Medium for *Melia* (BMM) and found that the shoots grew up to be larger and nicer than the ones on normal medium, though the number of shoots didn't increase much.

Lamberigts (2010) tested the influence of different types of cytokinins (CKs) on the multiplication rate and the growth of the explants. Basic Medium Melia (BMM) supplemented with IAA (1,14 µM) and mFT, diFT, mFTR, BA, MemTR or mT (2,22 µM) was used. mT gave the most shoots per explants (x 3,24), the complete shoots had an average length of 23,0 mm. Though, there was no significant difference from results on the other meta-topolin-derivates, MemTR and MemT. Verhaeghe (2009) did the same experiment with BA, MemTR and mT. the conclusion was that the medium with MemTR gave the longest and the finest shoots. Lamberigts (2010) also found that the optimum pH value of the multiplication-medium for *Melia volkensii* is 4,6. This causes a more fluid medium, so the addition of extra agar is advised.

2.5.5. Rooting

Indieka (2005) used second generation shoots inoculated in full (4,4 g/l) and half strenght MS, supplemented with sucrose (30 g/l), agar (0,8%) and different concentrations of IAA, NAA or IBA (0,5; 1; 2 and 3 mg/l). There was no root formation with the shoots on IAA and NAA medium. The full strenght MS medium suplemented with IBA (2 mg/l or 9,84 µM) gave 40% rooting, with IBA (1 and 3 mg/l) there was a 20% rooting percentage. In the medium with half strenght MS, 30% succes was achieved with IBA (3 mg/l) and 20% with IBA (1 and 2 mg/l). Full strenght MS supplemented with IBA (2 mg/l) gave the best root quality and the highest number of roots, but S. Indieka (2005) did not find a significant difference between all the IBA-results after conducting an ANOVA.

Vermeir (2008) put shoots on Woody Plant Medium (WPM) completed with agar (7 g/l), sucrose (20 g/l) and a supplement. It was found that with charcoal (2 g/l) as supplement, the shoots grew up very nicely without the formation of callus, but they didn't develop roots. A treatment with sequestrene (50 mg/l), IBA (2 mg/l) and riboflavin (0; 2,5; 5 and 10 mg/l) neither gave roots. The riboflavin inhibited callus formation, but at 10 mg/l, it decreased the growth of the shoots. Another experiment with mT (0,5 mg/l) in combination with IBA or NAA (10 and 20 µM) and sequestrene (50 mg/l), showed that the NAA-treatment killed all the shoots. The IBA-treatment gave no roots, but a lot of callus.

Verhaeghe (2009) put micropropagated *M. volkensii* shoots on basic MS medium under red, far red or blue LED-lights. After 6 weeks there was no root development on none of the shoots. In another experiment, basic WMP medium and basic MS medium were used, with or without charcoal (2 g/l). After 44 day only the shoots on the media without charcoal had generated roots (10% for MS and 9,8% for WPM, no significant difference). In a subsequent experiment, the same media (without charcoal) were used, but other amounts of MS (4.4; 2,2 and 1,1 g/l) and WPM (2,46; 1,23 and 0,615 mg/l) were applied. Where with the previous experiment, the full MS treatment induced only 10% rooting with micropropagated *M. volkensii* shoots, it now reached 40% rooting. The half MS treatment (2,2 mg/l) even caused 62% rooting. Unfortunatly, when the half MS and half WPM treatment was repeated again later on, Verhaeghe (2009) only achieved 12% rooting. These very variable results (10%-40% and 12%-62%) show that in vitro root development with *Melia volkensii* depends on many unknown factors. But nevertheless, it can be concluded that the half MS treatment gives better results then the full MS treatment.

Vermeir (2008) tried some pulse-treatments to initiate rooting with in vitro *M. volkensii* shoots. For 1, 2 or 4 hours the shoots were put in an medium completed with IBA (200 mg/l

or 984 µM) with or without mT (0,5 mg/l). After the pulse, they were put on hormone free V-WPM-medium supplemented with charcoal (2 g/l), where they grew for 6 weeks. The experiment was repeated twice and gave very different results. The best rooting percentage was 33,3%. This was obtained through a 4h pulse without mT, though the repetition only gave 13,4%.

Verhaeghe (2009) also conducted some pulse treatments on micropropagated *Melia volkensii* shoots. For the first experiment a pulse of 0, 2 or 4h was given with a pulse-medium (pH = 5,7) that consisted of IBA (800 mg/l) and agar (7 g/l) suplemented with or without mT (0,5 mg/l). A rooting percentage of 56,67% was obtained after 1 month with a 4 hour pulse. But compared to the roots generated from non treated shoots, they were fat and soft (See Fig. 14). The longer the pulse, the more roots developed, mT did not have any additional effects. In another trial, a 24h pulse treatment was given with a IBA (200 mg/l), afterwards the plantlets were transferred to a basic WPM-medium. After 27 days 78% rooting was obtained, however only 6,7% of the rooted shoots survived the acclimatization. The same experiment was repeated with a 0, 2, 4, 18 and 25 hour pulse. After 1 month, 100% of the shoots who were subjected to an 18h pulse had developed roots, though only 38% survived in vitro. 46% of the shoots who received a 2 h pulse gave good in vivo plants. Another repetition with a 5, 6, 18 and 20h pulse gave 84% rooting (20 h), these shoots were not acclimatized. These pulse trials show that longer IBA pulses generate more roots and in a shorter time period. Unfortunaltly these roots, developed on micropropagated *Melia volkensii* shoots, are of poor quality (Verhaeghe 2009).

Fig.14-15 : Roots developed after pulse with 800 mg/l IBA (left) and without a pulse (right) (Verhaeghe 2009)

Lamberigts (2010) tried to root micropropagated *M. volkensi* shoots on a BMM-medium supplemented with IAA (2,0 mg/l). After 8 weeks, only 13,7% of the shoots had formed roots which were very fragile and didn't have a direct connection with the vascular bundle of the stem.

Table 2: Overview of all in vitro rooting experiments

Basic medium	Treatment	Best in vitro rooting	Reference	Extra
Basic WPM medium	IBA (200 mg/l) pulse 5, 6, 18, 20h	**84%** (20 h)	Verhaeghe (2009)	Not tested for survival after acclimatization
Basic MS/WPM medium	Variable MS/WPM concentrations	**62%** (MS 2,2 mg/l)	Verhaeghe (2009)	Repetition only gave 12%
MS (4,4 g/l)	IBA, IAA or NAA	**40%** (IBA 2 mg/l)	S. Indieka (2005)	No significant difference for IBA results
V-WPM with charcoal	Pulse-treatment IBA + mT 1, 2 or 4 hours	**33,3%** (4h without mT)	Vermeir (2008)	Much callus; Repetition was different
BMM	IAA	**13,7%**	Lamberigts (2010)	Much callus
Basic MS/WPM medium	charcoal	**10%** (MS without charcoal)	Verhaeghe (2009)	
V-WPM	Charcoal	**0%**	Vermeir (2008)	Nice looking shoots
V-WPM	Sequestrene IBA Riboflavin	**0%**	Vermeir (2008)	Riboflavin inhibited callus
V-WPM	mT IBA or NAA Sequestrene	**0%**	Vermeir (2008)	Much callus

2.5.6. Whitening of shoots with *M. volkensii*

Lamberigts (2010) observed whitening of *M.volkensii* shoots after a couple of weeks of in vitro culture. This discoloration started at the top, after a while the complete shoot died. Oxidative stress may have caused this whitening. Though, use of Basic Medium melia (BMM) enriched with IAA (1,14 µM, MemTR (2,22 µM) and four different concentrations of the anti-oxidant, Sodium nitroprusside (0; 25; 50; 100 µM SNP), didn't show any improvement.

The addition of sulphates (30 µM adenine sulphate, 250 mg/l ammonium sulphate and 100 mg/l Potassium sulfate) to BMM enriched with IAA (1,14 µM) and MemTR (2,22 µM) or BA (2,22 µM), decreased the amount of whitening significantly, but it also diminished the growth potential of the shoots (Lamberigts 2010).

Lamberigts (2010) also measured an evolution of pH during the culture period. In the first three weeks of the culture period of *Melia volkensii* shoots, the pH dropped very fast from 5,7-5,9 to 5,1. After five weeks the pH of the culture medium started to slowly rise again, three weeks later it had increased 0,1-0,3 units. MES (1 g/l), an commercial available Buffer was added to BMM complemented with IAA (1,14 µM) and MemTR (2,22 µM) or BA (2,22 µM). After 8 weeks there was a slight decrease in whitening. In de treatment with BMM, IAA and MemTR, the addition of MES even gave a higher amount of shoots. Though adding MES to the multiplication medium gave good results, it couldn't buffer pH completely.

3. Overall materials and methods

3.1. Plant material

The shoots used for all experiments originated from the subcultures of Braem (2011). The two clones used, from now on referred to as “65” and “08”, originated from in vitro sown *Melia volkensii* seeds that were selected for their good in vitro growth and multiplication.

3.1.1. Lab material

Plastic Zeshine (top diameter=9,5cm; base diameter = 8 cm; height = 8,5 cm) or glass (top diameter = 7,5 cm; base diameter = 8,5 cm; height = 8,5 cm) containers, filled with 100 ml culture medium, were used. To enable aseptic working procedures, a laminar sterile airflow was used as working bench. It was equipped with a glass bead sterilizer (at 220°C), three tweezers and three scalpels, sterile paper sheets and a sprayer filled with 70% ethanol.

3.1.2. Inoculation of shoots in vitro

Before inoculation, the containers and the medium inside, were put in the autoclave. An autoclave sterilizes everything for 15 minutes at a temperature of 120°Celcius, under and elevated pressure of 2 atm. After this treatment, everything was left to cool down to room temperature. The working bench and the operators hands were cleaned by spraying 70% ethanol upon them, afterwards they were dried with paper towels. The tweezers and scalpels were sterilized by putting them in the heated glass bead sterilizer for a minimum of 5 minutes. Sterile paper was placed as deep as possible into laminar airflow, organized in a manner that enabled efficient working procedures.

Plant cells may die when they make contact with tools at high temperature, therefore all materials had to be at room temperature before proceeding with the inoculation. The tweezers and scalpels were sterilized after every operation, hence the sets were used in a rotating manner to allow cooling down before usage without loss of working time. After dead leafs and callus were removed the shoot was cut into its desirable size (depends on purpose). Each new shoot was then inoculated on the medium of the container that was opened in the laminar airflow. It was gently punctured into the medium until its base reached a depth of 2-5 mm. After inoculation the containers with the plantlets were put in a phytotron with a temperature of 26°C and a daylight management of 16 hours light.

3.1.3. Culture medium

Unless it is mentioned otherwise, a basic medium was used for each in vitro experiment. This basic medium consisted of Murashige and skoog (MS) medium (4,4 g/l, Duchufa), sucrose (20 g/l) and plant agar (6 g/l). Before autoclavation, the pH was set to 5,4. This basic medium was named Z-7300. In function of a particular experiment, some other components were added to this culture medium; PGRs like IBA, IAA, MemTr, mTR, phe-ade or charcoal. This is always described very clearly in the specific Materials and methods of each experiment.

3.1.4. Acclimatization procedures

For each acclimatization experiment, a specific soil mixture was prepared and put in the propagation trays. Before planting the medium was moisted, not wetted. Small plant holes were made with a pencil or tweezer. Then the shoots were carefully removed from the culture medium and submerged in an fungicide solution for ten minutes. Afterwards the length of the shoots was measured and they were lowered into their plant holes, the soil was gently pushed around the stem to keep the plantlet steady and make good contact with the medium. Each plantlet was planted in one cell (diameter = 2 cm, depth = 5 cm) of a plastic plate (45 cm on 35 cm) with 51 cells. Before planting, the shoots were measured and sometimes the base of the shoot was dipped in rooting powder (Rhizopon A with IAA as active ingredient). After planting, mist or water was sprayed over the clones. The propagator was filled with 2 cm of water to maintain a relative humidity (RH) of 100% and the plate was placed inside the propagator above the water level (except for “Cutting trial IV”).

Fig.16 : A top view of *Melia volkensii* shoots during acclimatization

Fig.17 : Type of propagator used for most cutting trials

The small shoots were very weak, so some guidelines were used to obtain the best results. The environment was kept as clean and hygienic as possible. Everything that came in contact with the shoots (operators hands, soil, ruler,…) had to be free of pathogens so the risk of infection could be kept to a minimum. When the shoots were handled, they were grasped by one leave, not the stem. If because of bad working practices the tissue was damaged, it was a less vital part of the plantlet. Excluding "Cutting trial IV and V", the micro cuttings were put in an phytotron with a constant temperature of 26°C, a relative humidity of 80% and a daylight management of 16hours. After planting, the shoots were watered every three days with a water sprayer.

Table 3 : Overview of Fungicides used during acclimatization trials

Trial	Fungicide used	Active ingredient	Protection results
Cutting trial II and Cutting trial VIII	Topsin® M 70 WG (1 g/33cl)	Thiofanaat-methyl	Good protection for Cutting trial VIII, though not enough for Cutting trial II
Cutting trial III	Bavistin® FL (1 ml/l)	Carbendazim	Good protection, though phytotoxic damage to plants
	Euparen M (1 g/l)	Tolylfluanide	
	Rovral® (1 ml/l)	Iprodione	
Cutting trial IV	BAVISTIN (1 g/l)	Carbendazim	Not good enough
Cutting trial V	Aliette® WG (1 g/l)	Fosethyl	Good protection
	Rovral SC (1 g/l)	Iprodione	
Cutting trial VI and Cutting trial VII	FENOMENAL (1 g/l)	Fenamidone and Fosethyl	Not good enough

4. Experiments

4.1. Overview

The experiments can generally be subdivided into 3 main groups; Multiplication, in vitro rooting and acclimatization trials. Some research was conducted across the boundaries of these divisions or combined them, so they are described separately.

4.1.1. Multiplication

Purpose

To find out the effect of the different components in the culture medium on the multiplication-rate of in vitro grown *Melia volkensii*. Multiplication was done for the purpose of obtaining a lot of shoots to conduct rooting and acclimatization experiments.

Materials and method

With every inoculation the shoots were removed from their old container, cut into pieces of 15 mm and punctured into the new culture medium of a sterile container. Two different clones were used, the plant density varied and the subculture time also wasn't similar. The culture medium used for multiplication was Z-7300 supplemented with MemTR (2 µM).

Table 4 : Overview multiplication trials

	Clone (08 or 65)	Subculture time	Plant density (shoots per container)			Total amount of shoots		
Counts1	08	38	6			18		
Counts2	65	38	6			18		
Counts3	65	46	6			30		
Counts4	08	70	3	5	6	3	20	18

Fig.18 :Small shoots, ready to be inoculated

Fig.19 : Glass containers, used for multiplication

Results and discussion

In table 5 the number of shoots counted and the multiplication rates after the culture period are displayed. A two sample t-test was performed on the data obtained from the shoots that had a subculture time of 38 days. The multiplication rate of the shoots of clone 65 (x 3,83) is significantly higher than the multiplication rate of clone 08 shoots (x 3.44). This indicates that the multiplication rate of *Melia volkensii* shoots inoculated on Z-7300 culture medium supplemented with MemTR (2 µM) depends on the genotype of the plant.

Table 5 : Results multiplication trials

	Plant density			Amount of shoots (start)			Amount of shoots (end)			Multiplication rate		
Counts1	6			18			62			3,44		
Counts2	6			18			69			3,83		
Counts3	6			30			149			5,00		
Counts4	3	5	6	3	20	18	15	134	65	5,00	6,70	3,61

Using the data from 70 days subculture time with clone 08, a one way ANOVA showed that the multiplication rates with different plant densities are significantly different. Both "Bonferroni t test" and "Tuckey's studentized range test" indicated that the multiplication rate

for the plant densities (3, 5 or 6) are significantly different. Five plants per container gave the best multiplication rates (x 6,70) for the shoots, inoculated on Z-7300 medium supplemented with MemTR (2 µM).

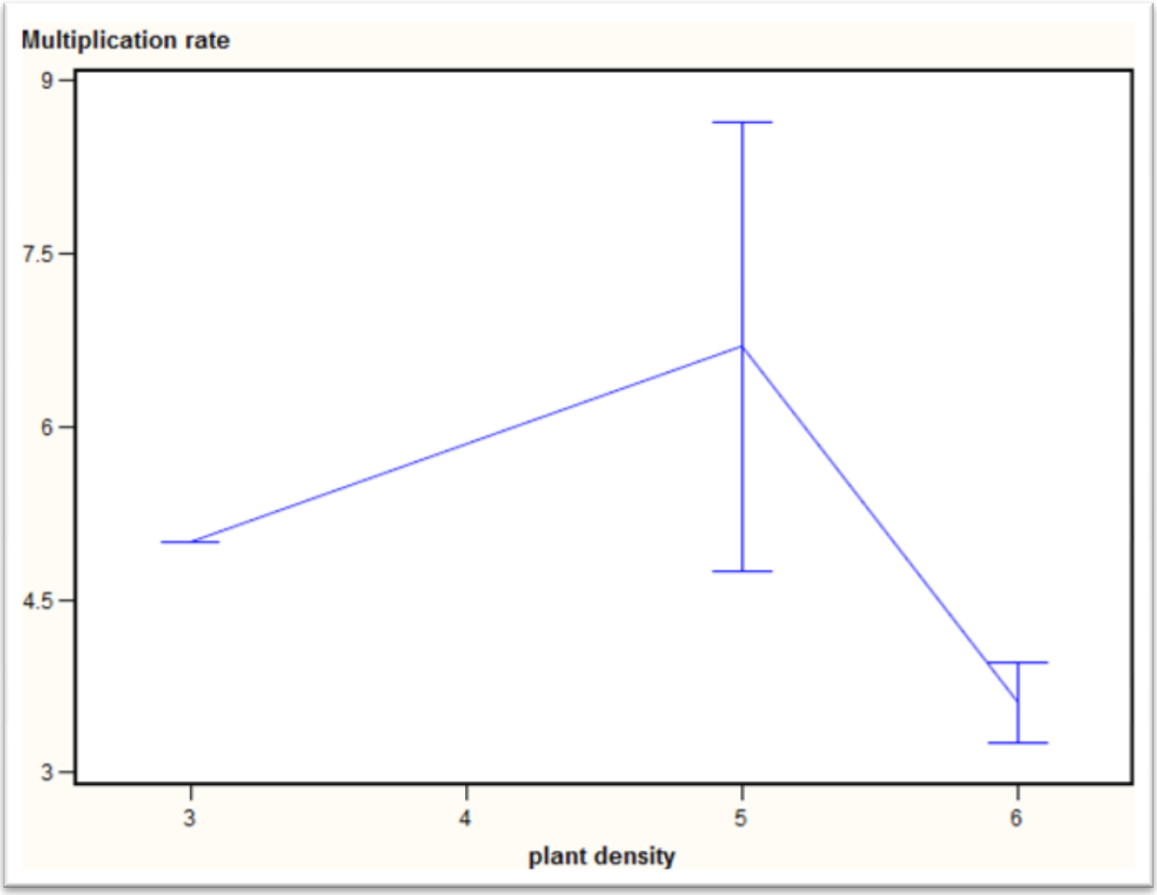

Fig.20 : Means plot; multiplications rates of plant densities of Clone 08,
(70 days of subculture time)

For clone 8 a two sample t-test was performed on the multiplication rates data from 70 days (x 3,61) and 38 days of subculture time (x 3,44). There is no significant difference in the multiplication rate of 08 shoots, that have been growing with a plant density equal to 6, for 38 or 70 days on a Z-7300 culture medium supplemented with MemTR (2 µM). For shoots of clone 65 with a plant density of 6, a similar experiment was conducted. After 46 days the average multiplication rate of the shoots was 5,00. This was significantly higher than the multiplication rate after 38 days (x 3.83). These results showed that the genotype of the *Melia volkensii* shoots had a large impact on the in vitro multiplication on Z-7300 culture medium supplemented with MemTR (2 µM). Every clone responded differently to the same treatment and had its own optimum growing period for the largest multiplication rate.

Conclusion

The multiplication rate of *Melia volkensii* shoots inoculated on Z-7300 medium supplemented with MemTR (2 µM) depended on the genotype of the plant and the amount of plants per container (or plant density). Statistical analyses also showed that the period of growth to obtain the highest multiplication rate, was different for every clone. Repetitions have to be conducted to validate these findings. The experiments can be expanded to find out if the influence of the plant density and composition of the culture medium on the multiplication rate, is also genotype dependant.

4.1.2. Rooting in vitro

Purpose

To find out the effect of different components in the culture medium and the influence of the environment, on the amount of rooting of in vitro propagated *Melia volkensii*.

Materials and method

Darkness-induction

The shoots were inoculated in glass containers with Z-7300 culture medium supplemented with IBA (5, 10 and 20 µM), the plant density was high; 15 shoots per jar. The first 7 days they were put in complete darkness, the 7 days later, they were put in in an in vitro growth room with a 12h light/ 12h night regime. After the first 14 days, the plantlets were transferred on a hormone free medium (Z-7300). 35 days later, the amount of rooting was assessed. The control plants (without IBA) were not transferred after 14 days.

Pulse-trial 1

Before the shoots were inoculated on a hormone-free Z-7300 medium, a pulse of 4 hours on an identical medium supplemented with a high concentration of IBA (400 and 800 mg/l) was given. Per treatment, four containers with each 17 shoots were used. After 30 days, the number of rooted shoots was counted.

Pulse-trial 2

For the second Pulse-trial, two different concentrations of IBA were used (100 and 200 µM = 20 and 40 mg/l). Also the time of the pulse varied; 5 seconds, 1, 2 or 4 hours, afterwards they were rinsed in sterile water. The plantlets that were only dipped in the medium for 5seconds, were not. After every pulse, the shoots were transferred to hormone free Z-7300 medium. The control treatment was inoculated without any pulse. This resulted in nine different treatments with each 50 shoots divided over 5 jars (plant density per jar was 10). After 55 days this pulse-trial was assessed.

Results and discussion

Darkness-induction

After the first 14 days, all the treatments showed a lot of callus development at the base of the shoots, though the shoots inoculated on the medium with 20µM IBA seemed to have formed the least. On the root-assessment day, the treatments of 5 and 10µM had 0% rooting, while 11 of the 20µM plantlets had generated roots (7,3% rooting). 8 of those 11 rooted shoots had developed in only one of the ten containers used for the 20µM-treatement. The

variation within treatments is therefore very high. The control treatment showed a rooting percentage of 1,3%. IBA did not seem to be a good auxin to induce rooting in micropropagated *Melia volkensii* shoots. Although the treatment with 20 µM showed less formation of callus and produced an outlier jar with 53,3% rooting, the overall results were bad. These results didn't match with the results from Indieka (2005), he achieved good rooting (40%) with IBA (2 mg/l or 9.84µM). The difference might have been caused by the darkness-treatment, but like with the multiplication rates, each clone might respond differently to the same treatment.

Pulse-trial 1

Thirty days after the pulse was given, no roots had developed. The plants looked very bad and the growth of callus was immense, even the part of the shoots above the surface was overgrown with callus. Verhaeghe (2009) obtained 56,67% rooting with an 4h IBA pulse (800 mg/l), but on a medium without MS. Vermeir (2008) gave a 4h pulse-treatment with 200 mg/l IBA and obtained 33,3% rooting. It is not mensioned if Vermeir (2008) used MS in the pulse-medium, but MS might be inhibitory for the development of roots with micropropagated *Melia volkensii* shoots.

Pulse-trial 2

50 days after the pulse treatment was given, not many roots had developed. The plants from the control treatment and from the 4 hours 100 µM IBA pulse developed 6% rooting. A 4hour IBA pulse treatment at 100 µM could not induce more roots then the hormone free control treatment. Compared with the results from the pulse trial from Vermeir (2008) and Verhaeghe (2009), our low rooting percentage can be attributed to a suboptimal concentration of IBA. It is also possible that the IBA was washed away and therefore the pulse lost its effect when the shoots were rinsed in water.

Table 6 : Results Pulse trial 2

Treatment	Rooting per container	Percentage rooting
200µM IBA - 4h	1 - 0 - 0 - 0 - 0	2%
100µM IBA - 4h	1 - 0 - 1 - 1 - 0	6%
200µM IBA - 2h	0 - 0 - 0 - 0 - 1	2%
100µM IBA - 2h	1 - 0 - 0 - 0 - 0	2%
200µM IBA - 1h	0 - 0 - 2 - 0 - 0	4%
100µM IBA - 1h	1 - 0 - 0 - 0 - 0	2%
200µM IBA - 5sec	0 - 0 - 0 - 1 - 0	2%
100µM IBA - 5sec	0 - 0 - 0 - 0 - 0	0%
control	2 - 1 - 0 - 0 - 0	6%

Conclusion

These three in vitro rooting experiments showed no good results. This confirms that micropropagated *Melia volkensii* shoots are very difficult plants to induce rooting upon. In all three experiments IBA was used as PGR to initiate rooting. If it were not for the moderate rooting results of Indieka (2005), Vermeir (2008) and Verhaeghe (2009), the conclusion would be that IBA was not a good auxin to induce rooting on micropropagated *M. volkensii* shoots. The addition of IBA to the culture medium used for rooting must be further investigated to pass final judgment upon the influence of this PGR on micropropagated *M. volkensii*.

Allthough Indieka (2005) found no significant difference between the rooting of shoots on half and full strength MS, it is very possible that MS in too high concentrations inhibited root development. The highest in vitro rooting percentage (84%) was obtained with a pulse by Verhaeghe (2009), who didn't even use MS in the pulse-media. The influence of MS was not yet conclusive, therefore it is necessary to conduct more experiments on this matter. Other factors like genotype, light treatment, plant length,… that can influence the in vitro root initiation, should be investigated too.

4.1.3. Acclimatization

Because the in vitro rooting experiments didn't give a very high rooting percentage, unrooted shoots from the darkness-induction trial were taken and planted in vivo. In its early stage, this first acclimatization trial was merely orientating. But when most of the plantlets survived and some even generated roots, a new kind of rooting experiments was suggested.

Fig.21 : Cutting trial I

Fig.22 : A close up of Cutting trial I

Cutting trial II and III

Purpose

To find out the survival and rooting rate during acclimatization of micropropagated *Melia volkensii* shoots.

Materials and method

For Cutting trial II, 34 shoots (clone 65) were taken from their Z-7300 medium supplemented with MemTR (2 µM) and put in a fungicide solution of Topsin® M 70 WG (1 g/33 cl). Before they were planted in the plastic trays, the base of the shoots was dipped in rooting powder (Rhizopon A). The soil composition used was unfertilized peat mixed with cocos fiber (3:2). After 42 days the number of survivors and rooted shoots was assessed.

26 plantlets (clone 65) were taken from their Z-7300 culture medium supplemented with MemTR (2 µM) for Cutting trial III. They were put in a fungicide solution of Bavistin® FL (1ml/l), Euparen M (1 g/l) and Rovral® (1ml/l). No rooting powder was used and they were planted in a soil mixture of unfertilized peat combined with cocos fiber (3:2). The trial was assessed after 27 days.

Results and Discussion

In the first week after the start of Cutting trial II, the leaves of many shoots were infected by fungus (*Botrytis*). This infection didn't seem to kill the plantlets, only weaken them. The shoots also endured some water stress because of excessive irrigation, followed by drought stress. After 42 days of acclimatization 75% of the shoots had survived and 26% had developed roots. The shoots from Cutting trial III did not endure fungal infection during acclimatization, but many showed phytotoxic symptoms after only 3 days; young leaves got a yellowish color. Though this trial was assessed to soon (27 days), 19% of the 61% survivors had already developed roots. Because no rooting powder was used, these roots seemed to show more secondary rooting.

Fig.23 : Rooted shoot from Cutting trial II

Fig.24 : Rooted shoot from Cutting trial III

Conclusion

Shoots that did not undergo an in vitro root initiation step, could survive and generate roots in vivo. Treating the weak shoots with fungicides was necessary for their protection, too much, however, harmed the plant more than a microbiological attack. The use of rooting powder (Rhizopon A) before planting seemed to induce less secondary rooting. If placed under conditions of high humidity, micropropagated *Melia volkensii* shoots could survive for a very long time without roots, 75% of the shoots of Cutting trial II had survived fungal infection and were still green after 42 days.

Cutting trial IV and V

These trials were conducted at Kiambere (Kenya), in the nursery of Better Globe Forestry.

Purpose

To find out if it is possible to acclimatize micropropagated *Melia* volkensii shoots in the semi-arid area of Kiambere. The use of locally available soil mixtures and the differences between clones 08 and 65 is also taken into consideration.

Materials and method

For transportation from the biotechnology laboratory in Ghent to the Kiambere Site, the shoots were placed in small plastic tubes (12,5 ml per tube; Z-7300 medium with 0,1 µM mT (0,024 mg/l), 7 shoots per tube). The plants travelled for 10 days and endured a lot of different temperatures (See Fig.25). For these trials, two different clones were used (08 and 65). Before planting, the base was dipped in rooting powder (Rhizopon A). The propagators (pr's) that were used for these trials, are the same that are used by Better Globe Forestry for germinating the seedlings. The propagators are coffin shaped, have a length of 180 cm, are 65 cm wide and have a height of 30 cm. For Cutting trial IV the pr's were aligned with pr1 positioned closest to the north and pr6 closest to the south, they were also shielded from direct sunlight. For Cutting trial V only one propagator was used, it took the position of pr3 from Cutting trial IV. The Temperatures the shoots had to withstand during acclimatization varied greatly because of weather conditions throughout the day (see table 7 and Fig.28). The propagators buffered the biggest variations (especially at night), but this did not prevent the exposure of these weak shoots to extreme temperatures.

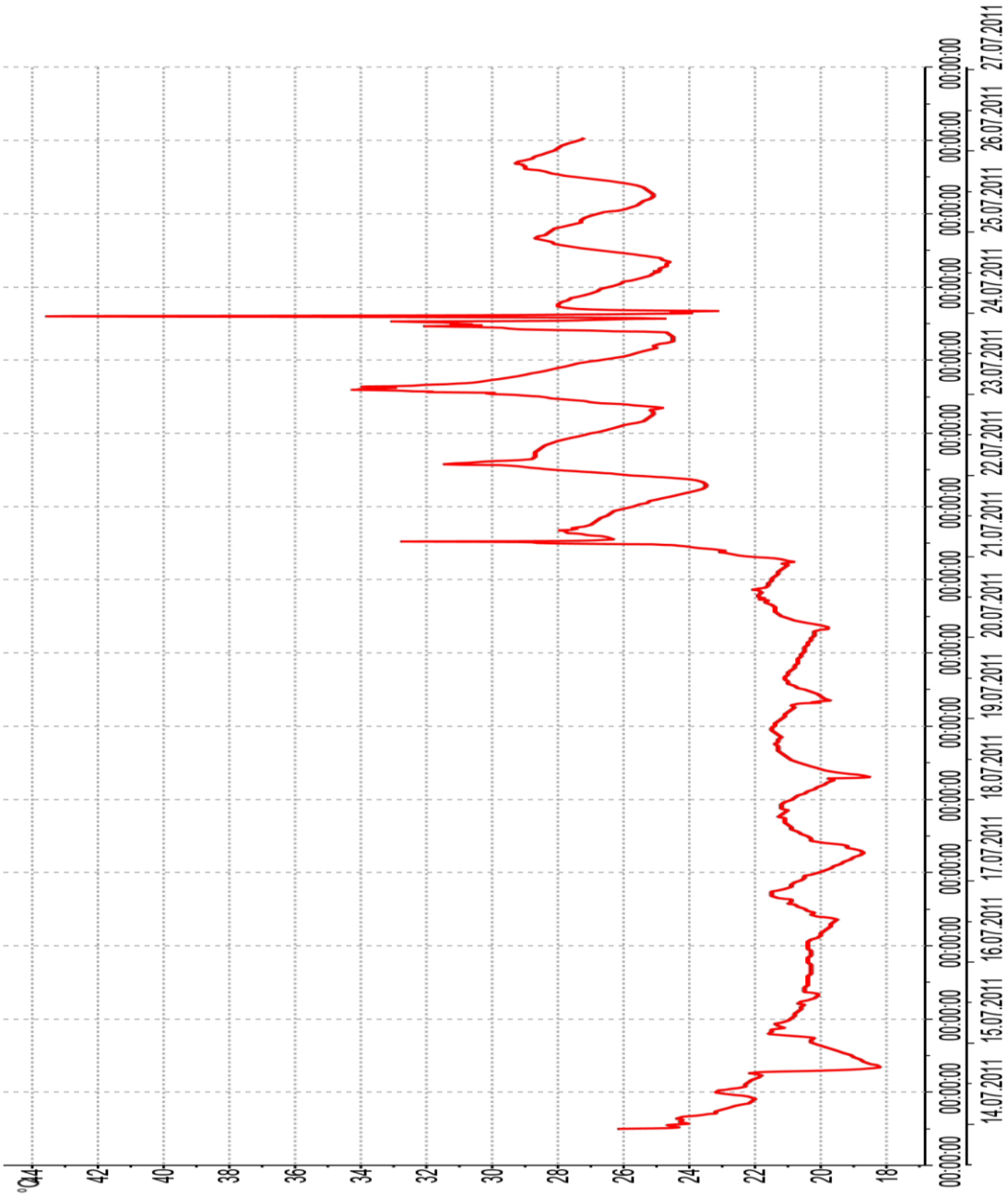

Fig.25 : logger data on the temperature variation during the journey of the shoots from the biotechnology lab in Ghent until the start of Cutting trial IV in Kiambere (Kenya). 14/07; arrival at Nairobi airport - 21/07; arrival at Kiambere - 24/07; start Cutting trial IV

Fig.26 : transport method of the *Melia volkensii* shoots.

Fig.27 : The pr's used for Cutting trial IV

Table 7 : Temperature varations at the Kiambere plantation

date	hour	T in pr1 (°C)	T in pr3 (°C)	T in pr6 (°C)	Weather conditions	outside T (°C)
29/07/2011	10h	**31**	**36**	**32**	clouds/sun (3:1)	33
29/07/2011	11h	**35**	**36**	**33**	clouds/sun (3:1)	35
29/07/2011	12h	**36**	**36**	**33**	clouds/sun (3:1)	35
29/07/2011	13h	**34**	**33**	**32**	very cloudy	34
29/07/2011	14h	**35**	**35**	**32**	clouds/sun (3:1)	35
29/07/2011	15h	**36**	**36**	**33**	clouds/sun (3:1)	35
29/07/2011	16h30	**35**	**34**	**31**	clouds/sun (1:1)	33
29/07/2011	17h30	**33**	**33**	**30**	clouds/sun (2:1)	30
29/07/2011	20h30	**28**	**27**	**26**	clear night	25
30/07/2011	01h	**25**	**25**	**24**	clear night	19
30/07/2011	06h	**23**	**23**	**22**	clear sky/dawn	15
30/07/2011	07h	**23**	**23**	**22**	clear sky/dawn	16
30/07/2011	08h	**26**	**26**	**25**	clear sky	25
30/07/2011	09h	**36**	**36**	**31**	clear sky	33
30/07/2011	10h	**39**	**39**	**34**	clear sky	37
30/07/2011	12h	**39**	**39**	**34**	clear sky	39
30/07/2011	14h	**39**	**39**	**35**	clear sky	39

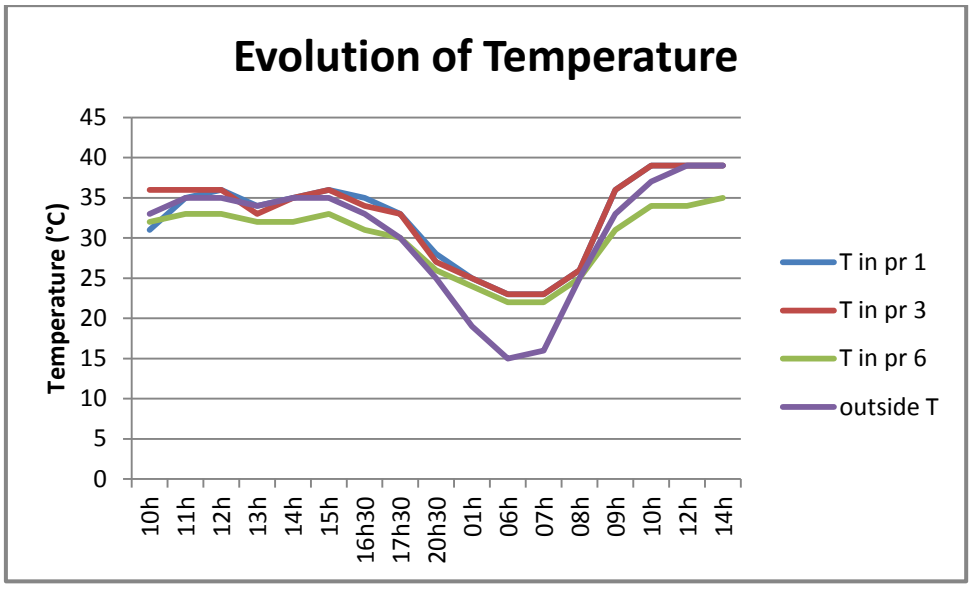

Fig.28 : Evolution of temperatures the plantlets had to endure during acclimatization at Kiambere

Cutting trial IV

Before planting the shoots were disinfected by rinsing them in a BAVISTIN-solution (1 g/l). Four different soil mixtures were used;

- **Mixture A:** cocos fiber + subsoil (ratio 1:2)
- **Mixture B:** cocos fiber + subsoil (ratio 2:1)
- **Mixture C:** cocos fiber + coarse sand (ratio 1:2)
- **Mixture D:** cocos fiber + coarse sand (ratio 2:1)

The cocos fiber was washed in disinfected lake water. By stirring the mixture with the hands very thoroughly, it was desalinated. This was done three times. The subsoil was dug up at the Kiambere site, it was a mixture of sandy-loam with some clay particles. The first 40 cm of the top soil were not used. Before application, the subsoil was sieved to remove the stones. The coarse sand that was used, was river sand, it was supplied by a local company. The relative humidity (RH) inside the propagator was maintained by two buckets filled with lake water, they had a combined evaporation-surface of 12 dm^2. The RH was measured with the difference between the dry- and the wet-bulb temperature, it varied from 75 to 85%. Irrigation was done with a sprayer filled with lake water mixed with BAVISTIN (1 g/l).

Cutting trial V

Before planting the shoots were submerged in an fungicide solution of Aliette (1 g/l) combined with Rovral (1 g/l), thereafter the base was dipped in rooting powder. Only two different soil mixtures were prepared;

- **Mixture E:** 100% cocos fibre
- **Mixture F:** 50% Peat + 30% Cocos fibre + 20% Perlite

Because of many fungal infections with cutting trial IV, the cocos fiber and the peat were cooked for 10minutes and subsequently oven dried for another 10minutes. Also the relative humidity was raised to 95-100% by increasing the evaporation surface inside the propagator to 83,25 dm^2. Watering was done with a sprayer filled with clean water obtained from the local water company (Kiambere–Mwingi water and sewerage company).

Results and Discussion

69,1% of the shoots used in Cutting trial IV suffered from a fatal fungal infection. Stem rot was the main evildoer, this indicates that the soil-mixtures used were not disinfected sufficiently, and that Bavistin (1 g/l) is not effective enough. Also a lot of shoots died very fast because of dehydration. The plantlets, coming from a very protective environment, were not able to withstand the very hostile environment. After 26 days only 5% had survived and 0% had developed roots. After 72 days only 0,5% of all the shoots had survived and 0,25% had generated roots. All survivors and rooting happened with the shoots that were planted in the soil mixture of subsoil + cocos fiber (2:1). So for soil mixture A, there was a survival rate of 2% and a rooting percentage of 1%. A very remarkable fact is that after 72 days without roots, a plantlet still survived in these harsh conditions.

Due to malpractices with Cutting trial IV, many lessons were learned and much better results were obtained with Cutting trial V. Soil mixture F (50% peat, 30% cocos fiber and 20% perlite) induced the best survival percentages; 75% for clone 65 shoots and 57% for clone 08 shoots. Also for rooting, soil mixture F caused the best results; 29% of the plantlets from clone 08 developed roots.

Table 8 : Survival of shoots during Cutting trial V

Survival %	Clone 65	Clone 08
Soil Mixture E	35%	29%
Soil Mixture F	75%	57%

Table 9 : Rooting of shoots during Cutting trial V

Rooting %	Clone 65	Clone 08
Soil Mixture E	6%	0%
Soil Mixture F	13%	29%

There is a significant difference in rooting between the clones and between the soil mixtures used. Cocos fiber alone is definitely not the most ideal medium for survival and rooting. The mixture of peat, cocos fiber and perlite gives much better results, this is probably because the mixture is more stable due to its higher water retention. There is also a significant difference between the two clones, clone 65 seems to have the best genes to survive acclimatization, though clone 08 has a higher rooting percentage.

While analyzing the data, some other interesting insights were obtained. The rooted shoots were significantly larger when they were planted, also the survival rate was higher with shoots that were larger from the beginning. The number of leaves that the shoots had when they were planted did not influence the survival or rooting percentage. It seems that the loss by evaporation is eliminated by the extra photosynthetic production of sugar that the plants need to protect themselves and generate roots.

Conclusion

The very weak plantlets were an easy prey for microbiological predators. Their size and overall quality had to improve seriously to obtain a better survival rate during acclimatization. Cutting trial IV showed that it was very important to use proper working practices and thoroughly disinfected materials. Though cutting trial IV was an absolute failure, many lessons were learned on how to properly acclimatize micropropagated *Melia volkensii* shoots in a Semi Arid area like Kiambere. This knowledge was used to obtain 29% rooting with Cutting trial V. Important improvements were the more sterile environment (soil, water for irrigation, propagator,..), cleaner and more careful working practices, better soil composition, higher humidity and a better fungicide solution. It can be concluded that the genotype has an important effect on the rooting and the survival rate of the plantlets. It also seemed that shoots with greater length had a better chance of surviving and rooting.

Cutting trial VI

Purpose

To find out if taller and thicker shoots had a better survival and rooting percentage during acclimatization.

Materials and Method

Shoots (clone 65) of different sizes were used for this rooting experiment. To obtain this variation in shoots, this trial was conducted with shoots coming from two different glass containers, 4 small ones and 4 large ones. The small jars (height = 8,5 cm, diameter = 8,5 cm) are the same ones as the ones used for the overall multiplication of *M.* volkensii shoots, unless it is indicated differently (Zeshine trials). The small containers were filled with 100 ml Z-7300 medium supplemented with MemTR (2 µM). The large jars (height = 12.5 cm, diameter = 8,5 cm) were filled with 250 ml of the same medium. Shoots of 15 mm were inoculated on the medium in the small jars, plantlets with a length of at least 40 mm were transplanted into the large jars. After 36 days of growing in vitro the shoots were measured, treated with fungicide, dipped in self made rooting powder (50 g talc powder mixed with 0,25 g IAA) and subsequently planted in a soil mixture of cocos fiber and perlite (ratio 2:1) for acclimatization. The callus overgrown zone of some plantlets was cut off and after 44 days of acclimatization the shoots were assessed.

Fig.29 : The two different containers

Fig.30 : A large shoot with a lot of callus

Results and discussion

The shoots from the large containers did not grow as good as expected. They started developing more callus than usual and they also generated a whitish type of callus on the parts of the plantlet that were not submerged in the culture medium (see Fig. 30). Because of this, they also didn't grow as large and thick as foreseen. It can also be said that the shoots from the large containers were of lower quality. This might be because the volume of culture

medium was too high and the plantlets were punctured in too deep. Nevertheless; average length of the shoots before planting is displayed in Table 10. There were also a lot of infections observed during acclimatization.

Table 10 : Average shoot length of shoots from cutting trial VI

Average shoot Length	Small containers (A)	Large containers (B)
Callus	52mm	78mm
Callus removed	47mm	64mm

The overall survival rate was 33%. The shoots from which the callus was removed had a higher survival rate (44%) than the shoots which were planted without the removal of callus (22%). This was the same within the groups (A and B). Strangely the shoots that were grown in the large containers (B), on double medium volume, had a lower survival rate (31%) than the ones grown in the small containers (35%). The highest survival rate (50%) was obtained with shoots from the small containers without callus (see Table 11).

Table 11 : Survival% of Cutting Trial VI

Survival %	Small containers (A)	Large containers (B)
Callus	20%	25%
Callus removed	50%	38%

Table 12 : Rooting% of Cutting trial VI

Rooting %	Small containers (A)	Large containers (B)
Callus	0%	12,5%
Callus removed	40%	0%

The highest rooting percentage (40%) during acclimatization was obtained with shoots that were grown in the small containers with removed callus (see Table 12). Shoots from the large containers without callus didn't develop roots at all. Though, when the data was analyzed while only observing the length of the shoots and not taking the containers from which the shoots originate into consideration, there seemed to be an optimum length for survival and rooting. Shoots that had a length between 45 mm and 75 mm seem to have considerably better survival and rooting rate than smaller or larger shoots (see Fig.31 and 32). This might indicate that micropropagated *M. volkensii* shoots have an optimum size for acclimatization. Because the experimental design was not adapted to this, and the larger shoots were of poorer quality, the influence of shoot size itself, not the container which they

originated from, should be investigated further. The fact that the shoots ,of which the callus was not removed, had a higher mortality rate, is probably because of accumulation of cytokinin metabolites which can inhibit rooting (Werbrouck et al. 1996). Moreover the soft callus rots away and thereby affects the base of the shoot in a negative manner. Another interesting observation was that the shoots, of which the callus was removed, developed a hard semi spherical type of whitish callus at the base of the shoot.

Some of the rooted shoots of Cutting trial VI were transferred into larger pots so they could continue to grow with less restrictions. After variable periods of time, the shoots were taken out and pictures were taken of their root development (see Fig 33, 34 and 35). The pictures show that the clones develop fleshy tap roots. If the plantlets had not been transplanted, the roots would even look nicer. All of the clones show side branching at the height they had reached before transplantation, this is probably a stress response of the plant as a result of transplantation, it also might be an expression of genotype because the clones were all the same.

Conclusion

Unrooted micropropagated shoots of *Melia volkensii* survived and rooted better during acclimatization, when callus was removed from their base. There also seemed to exist an optimum shoot length for acclimatization, though this should be further investigated. The shoots from the larger containers did not grow ideally, this might have occurred because of the high amount of medium in the large jars and because the shoots were punctured in too deep. Rooted shoots that were transferred into largerr containers develop fleshy tap roots.

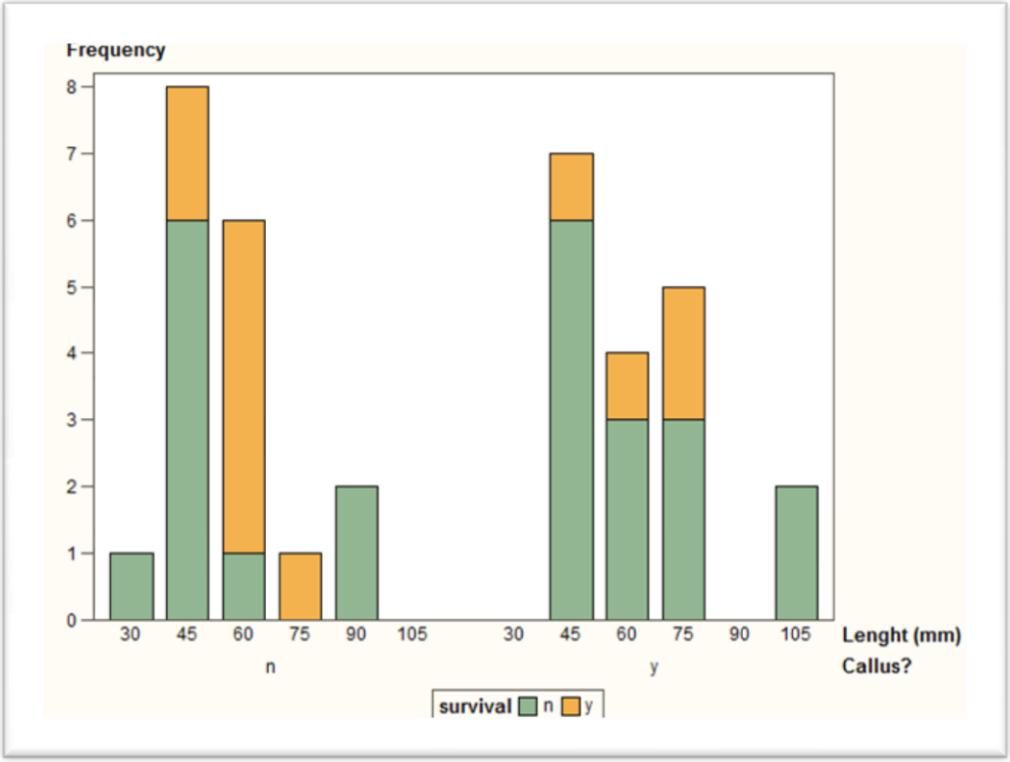

Fig.31 : Survival frequency of shoots with (y) or without (n) callus by shoot size

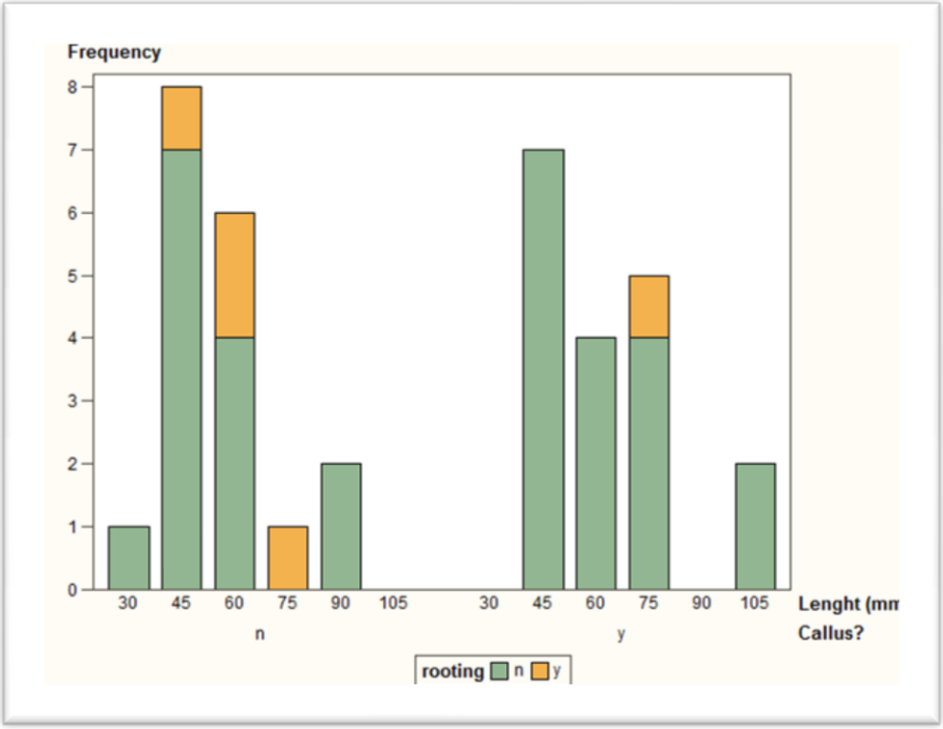

Fig.32 : Rooting frequency of shoots with (y) or without (n) callus by shoot size

Fig.33 : the development of a rooted shoot: Top left corner; rooted shoot one month after the start of Cutting Trial VI. Top right corner; same plant, two months after acclimatization. Lower left corner, again the same plant, three months after acclimatization. In the right lower corned; a more detailed picture of the root.

Fig.34 : Roots compared; The left root is the tap root from a normal germinated seedling (four months old). The two roots on the right are from two identical clones (65), rooted during Cutting trial VI, (three months after acclimatization)

Fig.35 : A root system of a shoot (clone 65) that rooted during Cutting trial VI. It developed multiple Fleshy tap roots, though it seems that there is one dominant.

4.1.4. Zeshine trials

Purpose

To find out if the type of container used for micropropagation has any effect on in vitro multiplication-rate and the in vitro rooting.

Materials and method

The model of the Zeshine container that was used is TC- R500 (See Fig. 36). The TC-R500 is a vented tissue culture container, specifically designed for plant cell/tissue culture applications. It is made of highly purified clear polypropylene which provides good light transmission (81%) and is autoclavable at 121℃. The vent on the cap is sealed by a PTFE filter membrane (pore size: 0.2µm) which allows good gas exchange but eliminates germs (Shanghai Zeshine 2009).

Fig.36 : the TC-R500 Zeshine container (Shanghai Zeshine 2009)

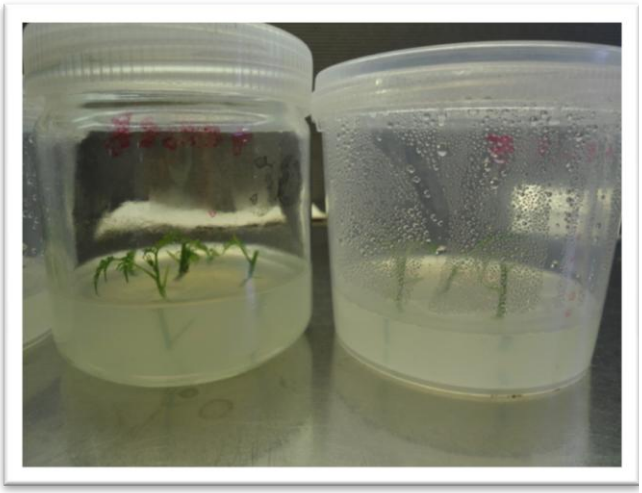

Fig.37 : On the left a glass container compared to a Zeshine container (right)

Five Zeshine and Five glass containers with each 100 ml Z-7300 culture medium supplemented with mTR (2 µM) were used. Each jar was inoculated with five shoots (clone 65) of equal size, this trial was repeated once. The same setup was used to find out if the type of container has an impact on rooting percentage, only the medium that was used for this rooting experiment was different (Z-7300 without mTR). The assessment happened 39 days after inoculation.

Results and discussion

For the first trial, the Zeshine containers gave an average multiplication rate of 4,5 and the glass jars a multiplication rate of 4,2. For the second trial it was respectively 3,4 and 3,6. With a two sample t-test was found that the first trial had significantly higher results ($p>0,02$) than the second one. So for each trial 2 sample t tests were conducted. Trial one gave no significant difference between the multiplication rate of the shoots grown in Zeshine or Glass containers ($p>0,70$) . Also the second trial did not indicate any significant differences ($p>0,65$). This indicates that Zeshine and glass containers generate the same multiplication rates for in vitro *Melia volkensii*. Though, something that has to be noted, is that of the two out of ten glass jars suffered from microbiological contamination, while none of the Zeshine jars was infected. More important is that the quality of the plants was better in the Zeshine containers, which could have an effect on the acclimatization success

For the rooting experiment, both treatments gave 8% rooting (2 of 25). Thus it can be concluded that the use of the Zeshine containers, compared to the glass ones, has no positive or negative effect on the in vitro rooting of *M. volkensii.*

The shoots that have been micropropagated in Zeshine containers had better developed leaves and looked firmer than those from glass containers, they probably would be more resistant to fungi in acclimatization trials better. Though, this has not been tested yet. Because Lamberigts (2010) reported that because of the growth of callus, the roots from the in vitro shoots had no direct contact with the vascular bundles. To find out if this was also the case with shoots that had developed roots in the Zehsine trial, the shoot was longitudinally cut in two (see Fig.38). This was compared with a random rooted shoot from Cutting trial VI, that also was cut in half (see Fig.39). There was a clear connection between the vascular bundle of the shoots and their roots. The root of the in vitro shoot developed straight through the soft callus, while the root of the acclimitized shoot could not penetrate the hard callus at the base, and had to grow throught the side. Though, as seen with the 2 months older shoots, there is no long term effect and the tap root will develop nicely.

Fig.38 : A rooted shoot from the Zeshine trial, longitudinally cut in half

Fig.39 : A rooted shoot from cutting trial VI longitudinally cut in half

Conclusion

The Zeshine containers did not improve rooting or multiplication with micropropagated *Melia volkensii* shoots. But the shoot quality in the Zeshine containers was better, which might indicate that they could have survive acclimatization better. This should be tested in the future

4.1.5. Light trials

purpose

To find out if the use of charcoal and blue, red or TL light has an effect on the in vitro rooting and acclimatization of *Melia volkensii shoots*.

Materials and method

Thirty Zeshine containers with each 100ml if Z-7300 medium were used, of these, fifteen were supplemented with charcoal (1 g/l) (see Fig. 41). Five shoot-tips per container were inoculated, subsequently the containers were exposed to their light treatment (see Fig. 40). For each treatment (red, blue or TL) ten containers were used; five with charcoal, and five without. After 35 days the in vitro rooting was assessed and Cutting trial VII was initiated. The 150 shoots were rinsed in a solution with FENOMENAL (1 g/l) and planted randomly, all together without the use of rooting powder in a soil mixture of cocos fiber with perlite (2:1). After 45 days, this acclimatization trial was assessed.

Fig.40 : The growth chamber with the different light treatments

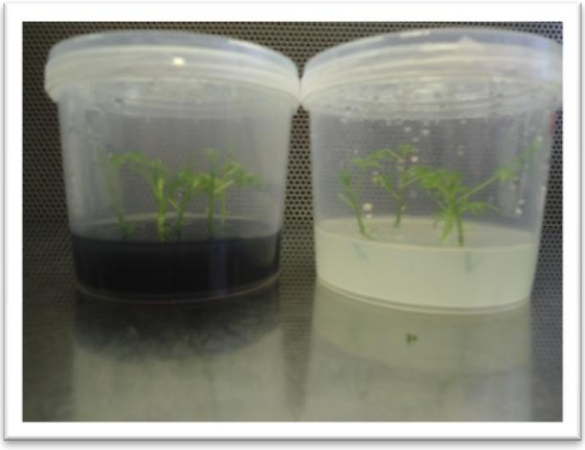

Fig.41 : Zeshine containers containing mediu with (left) or without charcoal (right)

Results and discussion

Shortly After the shoots were subjected to their light treatment, it was clear that the this had a direct effect on the size of the shoots. The average shoot length after the treatment was not equal (one way ANOVA; p>0,0091). The shoots that had grown under blue (40,4mm) and TL light (39,6mm) with Charcoal were significantly larger than all the other treatments. This proves that the shoots inoculated in a medium supplemented with charcoal grow larger.

Though, if the shoots are subjected to only red light, they remain behind in growth. Rooted shoots did not have a larger size than unrooted shoots. Also, in line with the findings from Cutting trial VI, the surviving shoots were significantly taller than the shoots that hadn't survived acclimatization (there were no shoots that were larger than 70mm).

The in vitro rooting percentage was highest (30%) for the shoots inoculated on a medium without charcoal and subjected to a blue light treatment. In general, rooting is better on media without charcoal (see table 13). Not all the shoots that rooted survived the acclimatization. The shoots that developed roots under the red light treatment suffered al lot. The plantlets from blue and TL without charcoal had a high survival rate (86% and 83%, see table 14). Of all the shoots that were transplanted for acclimatization only the ones with the TL light without charcoal treatment obtained a high survival rate (76%). This is because the survival % of the not in vitro rooted shoots was also very high (74%) compared to the survival rate of the Blue light without charcoal treatment (38%). It can be concluded that blue or red light treatments and charcoal during the in vitro phase didn't improve rooting.

Table 13 : Rooting% and average lenght after light treatment. Blue, red ot TL-light treatment with or without charcoal (+ or - C).

Treatment	Length (mm) after treatment	In vitro rooting %
Blue – C	32,6	30%
Blue + C	40,4	0%
Red – C	31,2	16%
Red + C	33,2	12%
TL – C	36,4	24%
TL + C	39,6	0%

Table 14 : Survival and rooting% of Cutting trial VII

Treatment	Total Ex vitro Survival %	not in vitro rooted Survival %	in vitro rooted Survival %
Blue – C	52%	38%	**86%**
Blue + C	36%	36%	-
Red – C	32%	29%	50%
Red + C	24%	27%	0%
TL – C	**76%**	**74%**	**83%**
TL + C	36%	36%	-

The ex vitro rooting percentages of the shoots that had not developed roots in vitro, was very low (11% for TL-C). The shoots were grown on an PGR free medium. This might have caused a low endogenous auxin content. The plantlets hadn't been able to initiate rooting in

vitro, so why would they develop them easier ex vitro? Also rooting powder was not used to try and initiate rooting during acclimatization. The low ex vitro rooting rate caused an low total rooting percentage (see table15). The highest rooting percentage was obtained with plantlets grown in a medium without charcoal, under TL light condition (TL-C : 28%) The shoots grown under blue light in a medium without charcoal had a non significantly different rooting percentage (Blue-C : 26%), but their ex vitro survival was much lower (52%) than the survival of the shoots from the TL-C treatment.

Table 15 : Rooting summary of the Light trial

Treatment	not in vitro rooted Rooting %	Total rooting % (surviving shoots)	Total Ex vitro survival %
Blue – C	0%	**26%**	52%
Blue + C	8%	8%	36%
Red – C	10%	16%	32%
Red + C	5%	4%	24%
TL – C	**11%**	**28%**	**76%**
TL + C	0%	0%	36%

Conclusion

Micropropagated *Melia volkensii* shoots, grown on a hormone free Z-7300 medium without charcoal, rooted best in vitro when they were subjected to blue light. Though, during acclimatization they had a lower survival rate than plants from the TL-C treatment. At the end, the treatment with classical TL light and without charcoal, yielded better rooting and survival rates.

Charcoal, added to the hormone free medium inhibited rooting and induced lower survival and rooting rates during acclimatization.

4.1.6. Micropropagation trials

Purpose

To find out if the use of different types of cytokinins (CK's) in the culture medium has an effect on multiplication-rate and the rooting

Materials and method

Three different CK's were used in addition to the Z-7300 medium; MemTr (2 µM), mTR (2 µM) and Phenyl Adenine (2 µM). For each medium a stock was made (five shoots per container) and after every subculture (two times) the multiplication-rate was assessed by counting the shoots in Five randomly taken containers. All the shoots that were used to initiate the stocks originated from a Z-7300 medium supplemented with MemTR (2 µM). After the second subculture (2^{nd} generation), the first micropropagation trial was conducted with shoots that had been grown on Z-7300 medium supplemented with MemTR and mTR. They were inoculated on Z-7300 medium with or without IAA (1,75 mg/l). For this, Zeshine containers were used and a density of five plants per container was applied. After 41 days rooting was assessed and some of the shoots were transplanted in vivo for rooting and acclimatization (cutting trial VIII). For this experiment the largest of the non rooted shoots were taken (six for each treatment), the rooted ones were taken at random. After a 10 min bath in a TOPSIN (1 g/l) solution the shoots were planted in a soil mixture of cocos fiber and perlite (2:1). No rooting powder was applied. The callus of the non-rooted shoots was removed because Cutting trial VI showed that the survival rate was lower and less roots had developed on the shoots with attached callus. The plantlets which had formed roots, were transplanted without removal of callus, because the roots had formed in the callus-overgrown-zone. After 35 days of acclimatization, Cutting trial VIII was assessed.

For the second micropropagation trial, shoots of the third subculture were used. The plantlets were inoculated on Z-7300 medium supplemented with IAA (1,75 mg/l). The influence of Charcoal (1 g/l) and blue or TL lights on the rooting of the in vitro *Melia volkensii* shoots was assessed. Zeshine containers (plant density = 4) were used and after 29 days, the rooting was assessed

Multiplication trial

In every subculture, the shoots on a Z-7300 medium supplemented with phenyl-adenin remained small and formed a lot of callus in the first two weeks after inoculation. If a plantlet was punctured to deep into the medium it was overgrown with callus and did not form any new shoots. After these first two weeks, a lot of new shoots developed (see Fig.44). Shoots on the other two mediums did not suffer from abnormal callus growth or this delay in shoot-development (see Fig.42). The shoots on Z-7300 medium with mTR generated a lot of thick sprouts, usually equal in height, but with small leaves and many sprouts showed necrosis at the top (see Fig.45). Shoots on medium with MemTR developed long, thin shoots with large leaves and few side branches (see Fig.43).

Fig.42 : containers with MemTR/Phe-ade/mTR (20 days of subculture)

Fig.43 : shoot from MemTR stock (35 days of subculture)

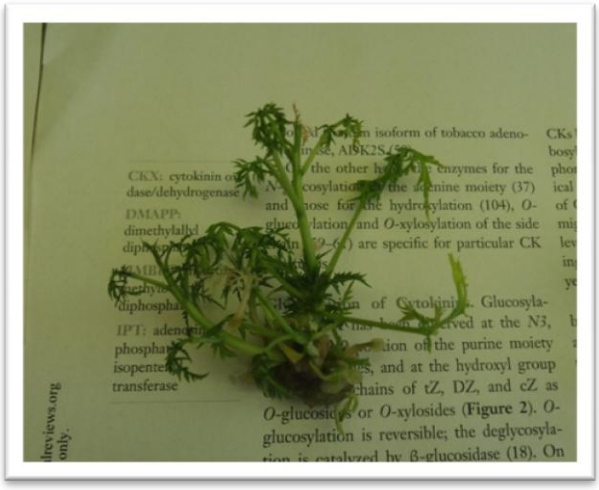

Fig.44 : shoot from Phe-Ade stock (35 days of subculture)

Fig.45 : shoot from mTR stock (35 days of subculture)

Table 16 : Multiplication results of micropropagation trials. Multiplication rates with the same letter (a or b) belong in the same significance group

	Amount of shoots (start)	Amount of shoots (end 1st generation) 35 days subculture	Amount of shoots (end 2nd generation) 40 days subculture	Multiplication rate	
MemTR	25	67	71	2,68[a]	2,84[a]
mTR	25	105	102	4,20[b]	4.08[b]
Phe-Ade	25	69	80	2,76[a]	3,2[a]

The multiplication rates of the 1st generation are not significantly different from the multiplication rates of the 2nd generation. This means that prior growth history of the shoots (in this case on a Z-7300 medium supplemented with MemTR) doesn't affect the multiplication rates.

A separate one way ANOVA was performed for each generation. And for both generations, the Bonferoni t-test showed that mTR had a significant higher multiplication rate than the two other CKs. This is because MemTR doesn't generate a lot of side branches, and phe-ade has a growth delay of two weeks.

Micropropagation trial I

The highest in vitro rooting rate (29%) was obtained with *Melia volkensii* shoots from the MemTR multiplication stock who were inoculated on an Z-7300 medium for 41 days (see Table 17). Shoots that had been put on hormone free Z-7300 medium did not root well. As described in material and methods, the largest of these shoots were transplanted in vivo for acclimatization and further rooting.

Table 17 : in vitro rooting% of micropropagation trial I

Treatment	In vitro Rooting %
Z-7300 + IAA (from mTR-stock)	17%
Z-7300 + IAA (from MemTR-stock)	29%
Z-7300 (from mTR-stock)	6%
Z-7300 (from MemTR-stock)	0%

The ex vitro survival rate on all treatments was 100% (see Fig.46) . There were no infections, but this might also be because all the shoots were very healthy and had an optimum length, as described in Cutting trial VI. Most of the shoots that had been planted for acclimatization also developed a hard hemispherical white callus at the bottom (see Fig. 48 and 49). Even the ones that did not originate from the medium supplemented with IAA developed this structure. This is result from cutting of the base of the shoot to remove the soft

callus that develops in vitro. This hard callus seems to protect the base of the shoots, the part that is very susceptible to decay. Root development occurs just above this callus.

After 35 days of acclimatization, none of the shoots showed actual withering. Also, most of the ex vitro roots that were observed, found themselves in a very early stage of their development, they were still very short (see Fig.48). Therefore it can be concluded that the shoots were assessed too early and that ex vitro rooting rate might still increase.

Fig.46 : 100% survival with Cutting trial VIII

Fig.47 : A rooted shoot taken out of its cell

Fig.48 : The spherical shaped callus at the base of a shoot. The growth of a root is initiated just above this structure.

Fig.49 : An unrooted shoot from Cutting trial VIII. After 35 days of acclimatization this shoot still looks lively

Table 18 : Rooting% of Cutting trial VIII

Treatment	ex vitro Rooting %
Z-7300 + IAA (from mTR-stock)	14%
Z-7300 + IAA (from MemTR-stock)	17%
Z-7300 (from mTR-stock)	33%
Z-7300 (from MemTR-stock)	17%

Micropropagation trial II

After 29 days a lot of callus had developed around the base of the shoots, as a result of the IAA treatment. Though, the shoots inoculated on medium supplemented with charcoal showed less development of callus. The plantlets that were micropropagated on Z-7300 medium supplemented with phe-ade gave the most rooting (38%). If the results from micropropagation trial I are compared to the rooting percentages obtained for the same experiments in this trial, big differences were observed. Where the plants from the MemTR-stock showed a rooting percentage of 29% in the first trial, they only achieved a rooting percentage of 15% with the same treatment, in this trial. The shoots from the mTR stock gained 0% rooting in this trial and 17% in micropropagation trial I. These differences can be the result of the fact that the subculture period is different for both trials (29 days compared to 41 days). The length of the subcuture needed for optimal root development should be investigated to draw permanent conclusions on this theory. The other difference between the two trials is the plant density. Micropropagation trial I is performed with a plant density of 5, trial II with a plant density of 4. Though it seems highly unlikely that this would induce such big differences.

As observed in the light trials, charcoal added to the medium inhibits callus growth, but also the development of roots. Together with the results of Verhaeghe (2009), it can be concluded that adding charcoal to the rooting medium of micropropagetd *Melia volkensii* shoots, is not beneficial.

During the light trials, blue light induced the highest amount of rooting with shoots on Z-7300 medium without supplements. But it seems that when IAA is added to the same medium, rooting is much lower. IAA is degraded by blue and TL light, An explanation might be that IAA is degraded fasted by TL light because it has a wider spectral composition. Therefore, root growth will be earlier with shoots exposed to TL light.

Table 19 : Rooting% micropropagation trial II. Treatments are TL or blue light, with or without charcoal (+ or - Ch); The CK between parentheses indicates the original stock of the shoot.

Treatment	Rooting %	Treatment	Rooting %
TL + ch (phe-ade)	0%	Blue + ch (MemTR)	0%
TL - ch (phe-ade)	38%	Blue - ch (MemTR)	0%
TL + ch (MemTR)	0%	Blue + ch (mTR)	0%
TL - ch (MemTR)	20%	Blue - ch (mTR)	5%
TL + ch (mTR)	0%		
TL - ch (mTR)	0%		

Conclusion

The best multiplication rates for micropropagated *Melia volkensii* shoots are obtained when mTR is added to the Z-7300 medium. Z-7300 culture medium supplemented with MemTR gives long unbranched healthy shoots. Phe-ade also induces many side branches but the plants were subjected to a delay of two weeks in which a lot of callus developed.

The cytokonin added to the multiplication medium had a direct impact on the in vitro root development afterwards. With the results from the first micropropagation trial, it could be concluded that MemTR inhibited root development less than mTR. The second trial proved the same, but the positive effect of phe-ade on rooting exceeded that of MemTR by far. Between MemTr, mTR and phe-ade, phe-ade had the best combined in vitro multiplication and rooting properties. If a shoot had not developed roots during its in vitro rooting process, it was better to remove the callused part of the plant before acclimatization.

4.2. Overall conclusion and discussion

4.2.1. Multiplication

In vitro multiplication of *Melia volkensii* was very easy. But it was still a goal to find the best preparation for rooting and acclimatization. Lamberigts (2010), Verhaeghe (2009) combined with this research confirm on the fact that meta topolin derivates added to the culture medium produced the healthiest shoots and the best multiplication rates. Though, they still inhibited the subsequent root initiation. Phenyl Adenin, a cytokinin-like substance, had an average multiplication rate due to the two week growth delay it induced on the micropropagated shoots. But phe-ade had very little inhibitory impact on the subsequent root development.

Another conclusion is that the number of days the plants spend in subculture had an optimum. This means that there was an amount of days a shoot could spend growing on a specific medium to develop a maximal amount of shoots in a minimum amount of time. Though, this depended on the genotype of the plant. There was also an optimum plant density for the same purpose. The Zeshine trials showed that the type of container used for micropropagation did not necessarily have a direct impact on the multiplication rate of *Melia volkensii* shoots. But it did demonstrate that it has an impact on the health of the plantlets and therefore their subsequent survival during acclimatization.

4.2.2. In vitro rooting

The in vitro rooting of micropropageted *Melia volkensii* shoots was very unpredictable. Many factors influencing this process were known, though, most of them are yet to be unraveled.

In contrast to the findings of Indieka (2005), Vermeir (2008) and Verhaeghe (2009), IBA didn't seem to be a good PGR for the initiation of roots. IAA added to the rooting medium gave better results, this occured because IAA is less stable than IBA in the culture medium. Therefore, root growth was inhibited for a shorter period of time. An extensive experiment that compares all root inducing hormones, is a designated next step in this research. It should be combined with the results from the micropropagation trials that clearly indicated that the use of a specific CK added to the multiplication medium has a direct impact on the subsequent rooting percentage. Phenyl-adenin, a CK-like substance, inhibited root initiation less than the meta topolin derivates mTR and MemTR. Phe-ade was not recognized by the

CK-receptors of the plant and had therefore no immediate inhibitory effect on root initiation. The endogenous auxin:CK ratio of the plant was much higher.

The addition of MS to the rooting medium was also an inhibiting factor for root development. Although this was not tested during the experiments for this thesis, the trials of Verhaeghe (2009) clearly demonstrated this. Supplementing the rooting medium with charcoal generated a lower amount of callus, though it definitely inhibited root formation and induced lower survival rates with the subsequent acclimatization of the shoots. The spectral composition of the light that the shoots were exposed to, during the in vitro rooting step, had also an impact. Red light was inhibitory to plant growth and rooting, while blue light and TL-light induced normal shoot development and rooting on hormone free medium. If IAA was added to the medium, shoots exposed to TL light grew roots earlier. Fast rooting is important because the roots have to penetrate the callus that develops around the base of the shoot. Observations demonstrated that root initiation and growth started from the parenchyma cells of the vascular bundles near the base of the shoot itself, not from the callus that had formed around it.

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4.2.3. Acclimatization

Research on the acclimatization of micropropagated *Melia volkensii* shoots was scarce and undetailed. Indieka (2005) and Verhaeghe (2009) did not obtain good survival rates during acclimatization of in vitro rooted plantlets. To obtain good survival rates, the shoots had to be protected from possible threats. Removal of the soft callus that developed at the base of the shoot during the last in vitro step, was a first critical practice. All materials that were used for acclimatization were disinfected thoroughly; it was also important to treat the shoots with a good systemic fungicide. The soil composition used for acclimatizing the shoots should have a good water retention capacity. Peat added to the ex vitro medium induced the best results, cocos fiber mixed with perlite dried out more easily, but gave good results too. The trials with the best results were conducted with a relative humidity that was close to 100%, evaporation by the plantlets was kept to a minimum.

The preceding multiplication treatment of the shoots had a big impact on rooting and survival. If shoots were misshaped, like with mTR added to the medium, they had to correct this by using a lot of energy. The container used for micropropagation needed to have good gas exchange capacities so the shoots could develop stronger and healthier before they were acclimatized. Micropropagated Mukau-shoots also had an optimum length to obtain the highest survival and rooting rates during acclimatization. Shoots that were too small or too large didn’t acclimatize as well as shoots that had a length between 45 and 75 mm. Small shoots were weaker, larger shoots dried out more easily and had a suboptimal endogenous distribution of assimilated auxins. Though, this needs to confirmed with further research.

Charcoal added to the culture medium, used prior to acclimatization, induced low survival rates due to the weakening effect it had on the base of the shoot. The partial etiolation even had an inhibitory effect on the root initiation during acclimatization. When the spectral composition of the light, used to grow the shoots prior to and during acclimatization, was similar, the survival rate of the shoots increased. Changing the irradiance induced extra stress on the shoot.

Micropropagated *Melia volkensii* shoots developed fleshy tap roots that allowed them to withstand severe drought. If transplanted, the tap root showed a higher amount of branching.

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6. Appendix

6.1. List of all media

name	content	extra	reference
IND-01	MS (4,4g/l) Sucrose (30g/l) agar (0,8%)	pH= 5,8	(S. Indieka 2005)
BMM	MS (4,4g/l) Scurose (2%) agar (0,7%)	pH =5,7 (can vary)	(Lamberigts 2010) (Braem 2011)
V-WPM	WPM sucrose (20g/l) agar (7g/l)	pH=5,8	(Vermeir 2008)
Z-7300	MS (4,4g/l) Sucrose (20g/l) agar (6g/l)	pH= 5,4	
basic MS medium	MS (4,4g/l) Sucrose (30g/l) Agar (7g/l)	pH= 5,7	Verhaeghe (2009)
basic WPM medium	WPM (2,46g/l) Sucrose (30g/l) Agar (7g/l)	pH= 5,7	Verhaeghe (2009)

6.2. Data of Micropropagation trials after Thesis Due date

Table extra 1 : Rooting% of micropropagation trial I (71 days after acclimatization)

Treatment	In vitro Rooting %	Ex vitro rooting % (unrooted shoots)	Total Rooting % (in vitro + ex vitro)
Z-7300 + IAA (from mTR-stock)	17%	57%	64%
Z-7300 + IAA (from MemTR-stock)	29%	17%	41%
Z-7300 (from mTR-stock)	6%	50%	53%
Z-7300 (from MemTR-stock)	0%	50%	50%

Table extra 2 : Rooting% micropropagation trial II (65 days of culture time). Treatments are TL or blue light, with or without charcoal (+ or - Ch); The CK between parentheses indicates the original stock of the shoot.

Treatment	Rooting %	Treatment	Rooting %
TL + ch (phe-ade)	0%	Blue + ch (MemTR)	0%
TL - ch (phe-ade)	50%	Blue - ch (MemTR)	15%
TL + ch (MemTR)	0%	Blue + ch (mTR)	0%
TL - ch (MemTR)	25%	Blue - ch (mTR)	30%
TL + ch (mTR)	0%		
TL - ch (mTR)	5%		