Evaluating the germination potential of *Pterocarpus angolensis* and *Strychnos cocculoides* with tissue culture techniques

By

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Declaration

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Date: March 2017

Abstract

The importance of indigenous tree species to local livelihoods can never be overstated. The species provide wood materials and non-woody resources, such as fruits and traditional medicines. *Pterocarpus angolensis* and *Strychnos cocculoides* are two important tree species found in a Namibian and adjacent countries' woodlands. Local people highly depend on the trees for wood (*P. angolensis*) and *fruits (S. cocculoides).* Due to over-exploitation of *P. angolensis* and *S. cocculoides*, the trees are on the verge of getting extinct. For instances; *P. angolensis* is mostly used for its ever-demanded wood products, thus become vulnerable to immense cutting down. Several attempts to propagate these two-tree species using traditional/conventional method i.e. nursery, are reported to have produced futile results. As such, there is need to explore other alternative germination methods for these trees. The current study evaluated germination potential of *P. angolensis* and *S. cocculoides* using the tissue culture germination method. The study objectives were: To compare the effect of tissue culture and nursery techniques on the germination success of *P. angolensis* and *S. cocculoides;* to develop a robust tissue culture protocol to optimise *in vitro* germination of *P. angolensis* and *S. cocculoides* and to evaluate the effect of different tissue culture aspects on the germination of these species. This was achieved by planting 60 seeds (30 each) of *P. angolensis* and *S. cocculoides* seed in the nursery and the performance of several *in vitro* tissue culture experiments under a controlled laboratory conditions. The influence of several aspects of tissue culture namely, the explant types (buds, seeds without seed coat, embryos, apical and axillary shoots); Agar media (Agar without hormone and Agar with hormones); pH (5.5 and 5.8); and surface sterilisation of explants on the germination success of *P. angolensis* and *S. cocculoides* were also investigated to identify optimal tissue culture protocol. Whereby, petri dishes with three treatments (sterilised, agar and explants) were placed randomly inside an SMC 1400 low-temperature incubator at 25°C with a 12-hour day and night photoperiod. Every third day, growth parameters such as germinated seedlings, plantlets length (roots and shoots), contamination and germination date were recorded up to 10 weeks after inoculation.

The results show that germination of *P. angolensis* and *S. cocculoides* can be promoted using tissue cultures as opposed to the nursery methods. For instance, up to seven plantlets (*P. angolensis* and *S. cocculoides*) can be produced in tissue culture methods within seven days. This however, cannot be reported from the nursery counterpart which only produces two and

seven seedlings from *P. angolensis* and S. *cocculoides* after 30 days respectively. From the tissue culture aspects, the results have shown that only seed (explants) without the seed coat and embryos yielded sufficient results for both species. For instance, there was no significant difference (*P = 0.12*) for germination percentage between the two types of explants (seeds without seed coat and embryo) for *P. angolensis* as contrasting to *S. cocculoides* explants (dry and fresh embryo) were a significantly different *(P* = 0.0010) *was* obtained. There was a significant difference in germination of explants between agar medium without hormones (A) and with hormones (A+H) in both *P. angolensis* (*P* = 0.0049) and *S. cocculoides* (*P* = 0.0001), with A producing high germination in all the species. *Pterocarpus angolensis* seed explants yielded high germination percentage at pH 5.8 while there was no significant difference in germination success between pH of 5.5 and 5.8 in *S. cocculoides* explants (fresh and dry embryos).

The study will be the first to demonstrate and develop a tissue culture protocol for *P. angolensis* and *S. cocculoides* of Namibia. It´s finding may contribute to the replanting of the two-tree species and eventually increase the tree stands reported depleting from the ecosystem. Therefore, the study recommends the use of tissue culture over the nursery germination method for *P. angolensis* and *S. cocculoides*. While suggesting for further investigation on aspects such as optimal temperature and light intensity required in tissue culture. Lastly, Improved germination of indigenous species has potential to contribute significantly to the conservation of these tree species which are under threat of extinction due to over-exploitation.

Opsomming

Die belang wat inheemse plant spesies het vir gemeenskappe kan nie oorbeklemtoon word nie. Hierdie spesies voorsien hout en nie-hout produkte soos vuurmaakhout, vrugte en tradisionele medisyne. *Pterocarpus angolensis* en *Strychnos cocculoides* is twee belangrike boom spesies wat in Namibië en aangrensende lande gevind word. Gemeenskappe is afhanklik van hierdie bome vir hout (*P. angolensis*) en vrugte *(S. cocculoides).* Weens oorontginning van beide *P. angolensis* and *S. cocculoides*, is hierdie bome op die brink van uitsterwing, veral *P. Angolensis* as gevolg van onbeheerde afkapping vir die toename in aanvraag van houtprodukte. Verskeie vergeefse pogings is al aangewend om hierdie twee boomspesie met behulp van traditionele kwekery metodes te vermeerder. Daar is dus ‘n groot behoefte om ander metodes van vermeerdering te ondersoek wat moontlik die ontkieming van *P. angolensis* and *S. cocculoides* kan verbeter, asook herplanting van woude. Daarom het hierdie studie die moontlikheid ondersoek om die ontkiemings persentasie van *P. angolensis* en *S. cocculoides* nie net met weefselkultuur te verbeter nie, maar ook met tradisionele kwekery metodes vergelyk.

Resultate het aangedui dat die ontkieming van *P. angolensis* en *S. cocculoides* verbeter kan word met weefselkultuur tegnieke in vergeleke met kwekery metodes. Byvoorbeeld, tot sewe plantjies (*P. angolensis* and *S. cocculoides*) kan binne sewed dae met weefselkultuur geproduseer word in vergelyking met twee (*P. angolensis*) en sewe saailinge (*S. cocculoides*) na 30 dae met die kwekery metode. Hierdie studie het verskeie aspekte ondersoek om die ontkieming te verbeter, byvoorbeeld: eksplante, agar media, pH en patogene beheer. Resultate het aangedui dat slegs saad (as eksplant), sonder die saadhuid, en embrios betekenisvol meer ontkieming tot gevolg gehad het vir beide spesies. Byvoorbeeld, daar was geen betekenisvolle verskil tussen die twee tipes eksplante (saad met saadhuid en embrio) vir *P. angolensis* in vergeleke met *S. cocculoides* eksplante (droog en vars embrios) wat wel betekenisvol verskil het. Verskillende agar media (A en A+H) het ook gelei tot betekenisvolle verskille in die ontkieming van eksplante in beide spesies, met agar medium A wat ‘n hoër ontkieming persentasie per spesies gehad het. *Pterocarpus angolensis* saad eksplante het ‘n hoër ontkiemings persentasie by beide pH’s gehad. Maar, geen betekenisvolle verskil was verkry by ‘n pH van 5.5 en 5.8 vir *S. cocculoides* vars en droë embrios. Die vlak van kontaminasie was aansienlik verminder met die gebruik van NaCIO en opwasmiddel.

In die algemeen, het resultate aangedui dat die weefselkultuur tegnieke die ontkieming van *P. angolensis* en *S. cocculoides* bevorder het, alhoewel aspekte soos optimale temperatuur, ligintensiteit nie ondersoek was nie. Hierdie aspekte moet egter in verdure studies ondersoek word. Ten slotte bevel hierdie studie aan dat daar ‘n behoefte is om ander inheemse spesies van Namibië te ondersoek en vas te stel of weefselkultuur op ‘n kommersiële skaal ingespankan word om die bewaring van hierdie spesies te bewerkstellig.

Dedication

This thesis is dedicated to my late mum Mee Eunike and Uncle Naftalie Haluodi, for always encouraging me to study. Although they could not witness this milestone, I am sure they are equally looking down on me. Thank you!

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Project rationale

1. Introduction

Indigenous tree species provide important services and goods to local communities. Wood is widely used for woodcraft, which is an important source of income (Jain and Häggman 2010), while indigenous fruit serves as an important source of cash income (Akinnifesi *et al.,* 2006) and nutrients as they are rich in vitamins and minerals (Mkonda *et al.,* 2002). However, the goods and services obtained from these species through cutting down the species for wood products, sometimes result in over-exploitation and extinction of these species (Botzat *et al*., 2015). As such, preservation measures targeting indigenous fruit and timber species can be essential for socio-economic development.

Indigenous tree preservation through germination is a less practised method as the process is mostly leave to nature. Most local people do not see the need to assist tree regeneration; this could be due to lack of understanding and or lack of resources. Although nursery and or conventional germination methods are sometimes used, they are not always producing good results (Kayofa, 2015). Historically, the conventional propagation methods of indigenous species yielded insufficient germination percentage, while the use of these species for indigenous products by the local communities has increased *(*Akinnifesi *et al.,* 2006). Additionally, due to lack of research, different germination aspects such as the low or poor growth of trees, low survival rates, low germination rates and sexual self-incompatibility need to be addressed in future studies (Giri *et al.,* 2004). On-going research needs to promote germination approaches for indigenous trees, by selection of plus trees and germplasm preservation. This could ensure and promote conservation of indigenous species and allow selection based on specific characteristics (Mkonda *et al.,* 2002). Henceforth, there is a need to explore alternative propagation/germination methods of tree species dissimilar from the conventional methods.

Plant tissue culture is an *in vitro* vegetative propagation method with biotechnology components (Hartmann *et al.,* 2014). It is known as a repeatable method of propagation for various forest and agriculture species around the world (Berlyn *et al.,* 1986; Balla *et al.,* 1997; Chisha-Kasumu *et al.,* 2006). Tissue culture can be based on different regeneration techniques such as micro-propagation; somatic embryogenesis, organogenesis, and axillary shoot production (Ahmed *et al.,* 2001). This technique is practised in a controlled and sterilized environment to promote propagation of plants in a short period by cultivating individual cells, tissues, and organs (stems, buds, seed or embryos) (Ahmed *et al.,* 2001). One of the main tissue culture technique benefits is that it produces large amounts of plants from limited propagation material and is a fast, repeatable and reliable technique. The method can also improve germination of plants that are not easily propagated (Benson, 1999). This study investigate germination of *Pterocarpus angolensis* (Kiaat) and *Strychnos cocculoides* (Monkey orange) by tissue culture.

Pterocarpus angolensis and *S. cocculoides* are socio-economical important species of southern Africa's forests that play a vital role in local livelihoods, such as Namibia and adjacent countries (e.g. Zambia and Malawi). Local communities largely depend on these species for fruit and wood products, which in return provide food consumption, income generation, and job creation. *Pterocarpus angolensis* is mainly utilised for the high-quality wood that is easy to work with, very durable and resistant to termites and woodborer insects, and several medicinal uses (i.e. ringworm) (Orwa *et al.,* 2009). *Strychnos cocculoides* is known as a source of fruit and can be processed into different products such as juices (Elago and Tjaveondja, 2015). One of the recognized juices from this tree is the Vigo juice, which is exported to South Africa and Angola. Previous studies indicated that the selling of *S. cocculoides* fruits and its processed products might contribute to the Namibian economy (Mendelsohn and Obeid, 2005; Elago and Tjaveondja, 2015).

However, *S. cocculoides* and *P. angolensis* are at risk of becoming extinct due to over-exploitation and unsustainable harvesting in Namibia (Directorate of Forestry, 2014). Natural disturbances such as prolonged drought, fire, pests and diseases also contribute to the gradual decline of these species (Kamminga, 2001). A study conducted in community forests of Tchaute in Kavango west regions of Namibia indicate that the regeneration of *P. angolensis* has declined drasticallyfrom 7% of total germination per year in 2003 to merely 2% in that specific area in 2014, while *S. cocculoides* declined from 12% in 2003 to 4% in 2014

(Directorate of Forestry, 2014). A decline in mature trees stand of *P. angolensis* was also noted in the north-eastern regions of the country by the local people (Kabajani, 2016). The decline of the species might be due to poor germination of seed, frequent fires, human impacts, livestock grazing and other abiotic factors such as storms (Kanime and Kakondo, 2003). Future studies and research need to explore alternative methods to germinate and protect the population of these species. Tissue culture might provide such a solution to conserve *P. angolensis* and *S. cocculoides* species, although limited genetic material might be available (Jaenicke, 1999; Barampuram *et al.*, 2014; Darius *et al.*, 2015). By contrast, tree species such as *Swartzia madagascariensis, Acacia mearnsii, Pterocarpus marsupium, Strychnos heignsii* and *Acacia auriculiformis* reported successfully propagated by tissue culture techniques (Giri *et al.*, 2004).

2 Problem statement

The over-exploitation of *P. angolensis* (timber and medicinal uses) and *S.* c*occuloides* (poor seedling establishment) has led to the diminishing of these species from wild populations (Chisha-Kasumu *et al.*, 2006). Previous studies aimed to improve the seed germination of the *P. angolensis* and *S.* c*occuloides*, reported unsatisfying results (Moses, 2012). Therefore, this study investigates the germination potential of *P. angolensis* and *S. cocculoides* with tissue culture techniques and develop a robust tissue culture protocol for optimum seed germination of each species.

The outcome of this study could promote germination that can contribute to reforestation of the two-tree species, preventing the depletion of these species from the ecosystem. The study was carried out over a 24 months period, aimed at comparing germination percentage between nursery and tissue culture (controlled laboratory conditions). To establish robust *P. angolensis* and *S. cocculoides* tissue culture techniques, the following factors were investigated: pH (5.5 and 5.8), explants (axially buds, seeds and embryo) and agar (pure agar and agar with growth regulator and hormones). The level of pathogens in the culture was also controlled.

3 Aims and objectives

The aim of the study was to compare the germination rate of *P. angolensis* and *S. cocculoides* between tissue culture and nursery experiments. Secondly, the study set out to develop a robust tissue culture protocol that is imitable to local communities. The specific objectives were:

- To compare the effect of tissue culture and nursery techniques on the germination success of *Pterocarpus angolensis* and *Strychnos cocculoides.*
- To develop a robust tissue culture protocol to optimise *in vitro* germination and growth of *Pterocarpus angolensis* and *Strychnos cocculoides* species.
- To evaluate the effect of different tissue culture factors (explant, agar, pH and contamination control) on the germination of *P. angolensis* and *S. cocculoides.*

4. Limitations

Given the narrow time frame and the limited resources during the study, limitations were not avoidable. The study was limited to two important tree species of Namibia (*Pterocarpus angolensis* and *Strychnos cocculoides*). Although the species are regarded as one of the important indigenous trees in the country, the plant materials used were imported from Namibia, the process found complex and costly to increase the number of tree species.

5. Research structure

The thesis is divided into six chapters. Chapter 2 provides a detailed literature review, while Chapter 3 explains the materials and methods used during this study. Chapter 4 presents the results, and Chapter 5 discusses the results. Chapter 6 highlights the main conclusions with a summary of recommendations for future studies, and Chapter 7 presents the appendixes.

Chapter 2:
Literature review

1. Introduction

Indigenous tree species are an integral part of the livelihoods of rural communities and the biodiversity of forests around the globe (Giri *et al.,* 2004). These trees are sources of food, fruits, fodder, timber, fuel and medicines (Akinnifesi *et al.*, 2007; Bijalwan and Dobriyal, 2015). However, the rapid population growth, high utilisation of trees, natural disasters and increase in land development has caused a drastic reduction in the cover of many indigenous species (Giri *et al.,* 2004). The current chapter reviews the literature on germination methods of two (*Pterocarpus angolensis* and *Strychnos cocculoides*) important indigenous tree species from Namibia, placing more emphasis on the importance of conserving these tree species. Propagation methods anticipated to conserve these indigenous tree species are equally described in detail.

In general, propagation aims to conserve species, especially scarce, endangered or socio-economic important species (Akinnifesi *et al*., 2006). A number of indigenous tree species are considered slow growing and unsuitable for propagation (Mkonda *et al.,* 2003). However, this might be due to a limited understanding of the natural variability, reproductive biology, propagation and lack of techniques to improve propagation and cultivation of these indigenous species. Currently, fruit trees are mostly retained and protected by the rural communities and farmers for socio-economic benefits (Mwamba, 2006). Therefore, these communities need scientific assistance in conserving their considered important trees.

Although conventional and or traditional (i.e. nursery) methods for propagation of indigenous tree species are well established, studies indicated that such methods produce limited success (Kayofa, 2015). Propagation of indigenous tree species can increase the number of trees in the wild (forests) and quantity of products from these species (Mungomba *et al.,* 2007). Not only can this provide food security, create job opportunities and generate income, but also conserve and prevent the extinction of these species. Historically, conventional or traditional methods have been used for propagation of indigenous species. In Namibia, nursery experiments with

indigenous woodland trees are done at a limited scale (Graz, 2004) and yielded variable results as the first steps towards establishing germination protocols (Moses, 2012; Van der Heyden, 2014). To improve the germination capacity of indigenous species, available technology and scientific assistance should be employed to assist and improve the natural redistribution and conservation of indigenous species (Akinnifesi *et al.*, 2007). Therefore, this study concentrated on two (*P. angolensis* and *S. cocculoides*) socio-economically important species to rural communities in the Kavango region of Namibia. The aim was to investigate tissue cultures as an alternative propagation method to improve the germination success of these species.

In contrast, perennial plants can be propagated by either sexual or vegetative methods (Abdullahi, 2013). During this study, both sexual (nursery germination) and vegetative propagation (tissue culture) for *P. angolensis* and *S. cocculoides* were evaluated. According to several document, tissue culture is a vegetative means of propagation (Abdullahi, 2013). It is a systematic procedure for establishment, stabilisation of shoots, shoot multiplication, root formation and acclimatisation after proliferation is observed. Although considered as one of the most important technologies for the production of high quality, disease-free and fast growing plants, the technique is not easily implemented (van der Riet *et al*., 1998; and Abdullahi 2013). As such, the following variables were investigated for both *P. angolensis* and *S. cocculoides* These were considered because, shoot and root development multiplication is the main criterion for successful tissue culture technique (Jaiswal *et al.*, 2015). Hence, the study believes they have a strong impact on the success of the technique.

2. Pterocarpus angolensis:

2.1 Distribution of *Pterocarpus angolensis*

Several reviews on the geographical distribution, taxonomy and ecology of *P. angolensis* (kiaat) has been published (Vermeulen, 1990; Mojeremane and Lumbile, 2013). This tree species is prominent in Southern African, with commercial importance (Chidumayo, 1994). *Pterocarpus angolensis* belongs to the Fabaceae family (Therrel *et al*., 2007; Mojeremane and Lumbile, 2013) and occurs naturally in the Miombo woodlands, particularly the Zambezian Miombo woodland (Mehl *et al.*, 2010). The Miombo woodland covers more than 1M ha in 11

African countries (Angola, Botswana, the Democratic Republic of the Congo, Malawi, Mozambique, Swaziland, Tanzania, Zambia, Zimbabwe, South African and Namibia) (Campbell, 1996; Kasumu *et al.*, 2006; Mojeremane and Lumbile, 2013). In Namibia, the species is found in Otjozondjupa, Kavango East and West, and the Zambezi (central and northeast) regions of the country. The species is known to adapt and survive under severe environmental conditions such as dry conditions, harsh temperatures and it can tolerate fire (Mehl *et al.*, 2010). The wood is very popular and a woodland without *P. angolensis* is considered less important by the rural communities (Caro *et al.*, 2005). However, *P. angolensis* can be propagated and re-introduced back into its natural environment from seeds and cutting (Mehl, 2010). A small-scale plantation for *P. angolensis* was usefully established from cutting method in Kenya and Mozambique (Takawira-Nyenya, 2005; Orwa *et al.*, 2009).

2.2 Current threats to *Pterocarpus angolensis*

The exploitation of *P. angolensis* have increased in Namibia over recent years (Moses, 2013) and can be attributed to the unique wood properties. Therefore, stricter regulations in terms of harvesting were introduced to conserve the species. It is considered an endangered species and protected by forestry legislation (Curtis and Mannheimer, 2015). Legislation indicates that harvesting of the species should be within a specified set of standards; for example, a harvesting permit must be issued from the line authority. The line authority can be the Ministry of Agriculture, Water and Forestry or community leaders with an official mandate. The permit instructs the logging conditions, such as the number of trees, size (diameter) and place of harvesting. *Pterocarpus angolensis* is also considered as a near threatened tree species by the IUCN Red List (IUCN, 1998).

More effort is still needed to protect *P. angolensis* in Namibia because regulations are currently being ignored, and as a result, illegal and unsustainable harvestings of *P. angolensis* are increasing on a daily basis (Mehl, 2010). Similar trend has been reported in Tanzania (Luoga *et al.*, 2002). As a source of concern, the annual demand may or is already exceeding the natural supply of the species, thus resulting in low seed germination and poor reforestation of the species (Moses *et al.*, 2013).

Another concern is that young trees are often being harvested to supply timber to the ever-expanding market of *P. angolensis* (Stahle *et al.*,1999; Chisha-Kasumu *et al.*, 2006). This will continue to cause major threats to the existing tree stands in coming years (Muhoka and Kamwi, 2013). Although *P. angolensis* is not sensitive to fire, the intensity or fire frequency can result in lower regeneration or lower number of seedlings (Desmet *et al*., 1996). Other threats include land clearing for agricultural purposes, browsing of newly regrowth by cattle and wildlife that possibly will inhibit the coppicing growth of tree and infrastructure development (Mehl *et al.*, 2010).

While natural regeneration is known to rescue species from becoming extinct, the current natural regeneration of *P. angolensis* is unsatisfactory, because of poor survival of seedlings during the developmental stage (Mojeremane and Lumbile, 2013). This is likely to be caused by too frequent forest fires, harsh climatic conditions, animal browsing, recurrent yearly dieback of seedlings, and competition from other plants for resources, and delayed seed production. Therefore, collaborative research is required to find ways of improving *P. angolensis* seed germination (Mojeremane and Lumbee, 2013).

Previous studies have attempted to germinate *P. angolensis* seed with standard nursery methods with limited success (Hengari, 2004). This might be attributed to a delayed seed production, dishusking of the seeds coats without damaging the inner seeds and hard seeds coats that makes it difficult for water absorption. There is, however, a successful small-scale seedling establishment of *P. angolensis* in the warmer areas of Mozambique (Mojeremane and Lumbile, 2013). Germination techniques to improve the seed production of *P. angolensis* need further investigation.

2.3 Germination of *Pterocarpus angolensis*

In Nature, fire is the main germination facilitator of *P. angolensis* seed as it breaks down the woody pod to facilitate sprouting (Banda *et al.*, 2006). However, previous studies indicate that the reproduction of *P. angolensis* by seed increases with the degree of openness of the stand as the seed is de-husked from the fruit during the rainy season. After germination, a tap root and shoots develop (Banda *et al.*, 2006). The developed shoot, however, dies back during the following dry season, a cycle that is repeated for several seasons until the root system is fully

developed. The root system then continues to grow until seedlings can survive the dry season (Mehl, 2010). On average, the growth of seedlings is slow, only growing by to 15cm per growing season (Mehl, 2010).

Pterocarpus angolensis can be propagated from seed and cuttings (Shackleton 2002). Previous studies indicate that *P. angolensis* seeds have a low germination success in nature (Muhoka and Kamwi, 2013) and under controlled laboratory conditions (Von Breitenbach, 1973). However, there is some evidence of propagation success under controlled laboratory conditions (Chisha-Kasumu *et al.*, 2006), thus indicating that the species has a potential of being propagated through *in vitro* propagation techniques. In contrast, stem cuttings have a low survival rate (Vermeulen, 1990). Studies also indicate that untreated *P. angolensis* seeds may yield lower germination success than seeds that are treated (Shackleton, 2002). This means, pre-treatment of the seeds prior to sowing may promote germination of *P.angolensis.* Mature trees of *P. angolensis* can also be coppiced to improve regeneration (Caro *et al.,* 2005).

2.4 *Pterocarpus angolensis* uses

The wood of *P. angolensis* is primarily used for furniture and firewood, while the tree's phloem sap has numerous traditional and medicinal uses (Vermeulen, 1990; Van der Riet, 1998; Graz, 2004; Orwa *et al.*, 2009). Roots, stems, branches, bark, sap and leaves are used in traditional remedies (Takawira-Nyena, 2005). *Pterocarpus angolensis* is also a good pollen source for honeybees (Orwa *et al.*, 2009). As a leguminous tree species (Graz, 2004), it can play an important role as nitrogen-fixing species and promote soil minerals (Mendelson, 2005).

3. Strychnos cocculoides:

3.1 Distribution of *Strychnos cocculoides*

Strychnos cocculoides (monkey orange or Maguni) is another socio-economically important species found in the Namibian woodland towards the northern regions. The evergreen tree which can grow up to 8 m tall, belongs to the Loganiaceous family (Mwamba, 2006). It is commonly grow in the Kavango West and Eastern regions, on sandy plains and dunes and in

areas with mixed woodland and riverine (Curtis and Mannheimer, 2015). Rural communities depend on the tree for fruits, wood and traditional medicines and in many cases protect the trees(Akinnifesi *et al*., 2006). *Strychnos cocculoides* is one of the top five-miombo fruit tree species selected for domestication by farmers in southern Africa (Mkonda *et al.,* 2002).

3.2 Propagation of *Strychnos cocculoides*

Strychnos cocculoides can be propagated from seed and other different parts of the plant through vegetative propagation such as air layering and grafting (Akinnifesi *et al*., 2006). According to earlier findings, grafting of *S. cocculoides* can have a success rate of up to 49% depending on skill of a person performing it (Akinnifesi *et al*., 2006). Seed propagation is reported to be challenging, but germination success can significantly increase with the correct pre-treatment application prior to sowing (Mkonda *et al.,* 2002; Mateke, 2000a; Mateke, 2003b). Despite these findings, propagation efforts of *S. cocculoides* are hindered by poor seed germination and slow growth (Mkonda *et al.;* 2002).

3.3 Current *Strychnos cocculoides* threats

Unlike *P. angolensis*, *S. cocculoides* is not threatened by unsustainable harvesting in Namibia. The tree is mostly sought after for the fruit that results in non-destructive harvesting. However, poor seedling establishment gives rise to lower reforestation levels of the species (Elago and Tjaveondja, 2015). The available tree stands are becoming extinct because of natural disasters such as pest and diseases, drought as well as ageing (Mkonda *et al.;* 2002).

3.4 *Strychnos cocculoides* uses

The fruits of *S. cocculoides* are edible and are an important cash crop for rural communities. Different value-added products of *S. cocculoides* include jams, juices and cakes (Bille *et al.*, 2013). If made available on a large scale, this industry would create jobs and generate income for rural communities (Elago and Tjaveondja, 2015). The fruit is also eaten and sold around the country, making it one of the most important indigenous products in Namibia. The roots

and green fruits are mostly used in traditional medicine to cure coughs and wounds, while wood is used for crafts such as utensil handles.

4. Propagation methods

Generally, there are two propagation methods in plants, namely sexual (seed) and asexually (vegetative) (Hartmann *et al.*, 2014). Sexual reproduction is the most common and cost-effective propagation method as opposed to asexual. During this study, the two methods were assessed, the nursery germination method as a sexual reproduction and tissue culture method as an asexual method. Below are two examples of each germination method.

4.1 Seed germination

Seed germination of indigenous tree species can be a valuable forest management tool for species with slow growth and on the verge of extinction (De Cauwer and Younan, 2015). The process can allow selection of the desired tree qualities, such as drought resistance, good timber and fruit quality. In Namibia, several studies show that seed germination of these tree species is under-represented (Kanime and Laamanen, 2002; Kanime and Kakondo, 2003; De Cauwer and Younan, 2015). Various seed germination studies have been undertaken to improve the germination success and re-introduce tree species into forests or woodland as part of rehabilitation programs (De Cauwer *et al.*, 2015). However, more effort is still needed to investigate other processes of increasing indigenous tree species such as enrichment planting in the forest, agroforestry and intercropping, to meet socio-economic needs (De Cauwer *et al*., 2015).

4.2 Tissue culture

Tissue culture is one of the leading universal agro-technologies (George 1993), defined as a vegetative propagation of plants *in vitro* to ensure rapid multiplication of plant production material on a defined solid or liquid medium under aseptic conditions. Through tissue culture, plants can be regenerated from small parts, such as cells and tissues (Murashige and Skoog,

1962; Hartmann *et al.*, 2002; Hartmann *et al.*, 2014). The methods can potentially increase seed germination of species known to be difficult to propagate from seed in the natural environment (Ahmed *et al.*, 2001). Furthermore, it can be used for rapid production of high-quality materials within a limited time and space, producing plants irrespective of the season and weather (George and Manuel, 2013).

Tissue culture success is largely dependent on various pathways and stages that produce true to type plants in multiple numbers (Ahloowalia *et al.*, 2002). The following four developmental stages are important (Hartmann *et al.*, 2014):

- **Establishment:** the tissue is placed into culture and initiates micro shoots. Micro shoots are initiated by successfully placing a plant part (explants) into aseptic culture (growing medium) while avoiding any contamination but providing a conducive *in vitro* environment.
- **Shoot multiplication**: maintaining the culture by promoting and multiplying the micro shoots through different nutrient supplements.
- **Root formation**: promote rooting of the explants and prepare the micro cuttings for transplanting.
- **Hardening off (acclimatisation**): transferring the plantlets (culture micro shoots) to a natural environment.

Tissue culture is mostly practised in the agriculture sector as a micro propagative tool in plant production and as an integral part of breeding programs (Gatti *et al.*, 2016). Apart from the advantage, that tissue culture is applicable to species that are difficult to propagate, the technique can offer economic benefits (Berlyn *et al.*, 1986; Chisha-Kasumu *et al.*, 2006). However, lack of skilled personnel and poor infrastructure can limit success (Abdullahi, 2013). Nonetheless, the abilities for tissue culture to improve the propagation potential of indigenous species by providing sufficient quality and quantity materials to produce rooted plantlets (Pijut *et al.*, 2012) outweighs the shortcomings. Hence, investing in such technique will be worth the resources. Although the technique is not really a preferred method of propagation in forestry or in the field of indigenous forests, forestry sector is at the starting point of introducing tissue cultures as an operational practice (Sedjo, 2016). This is because, the technique can assist with re-introducing species (rare and endangered) that are difficult to propagate from seed (Wochok,1981; Rathore *et al.*, 2004; Foden and Potter 2005; Lobine *et al.*, 2015). In addition,

many natural germination rates of forest tree species are low and tissue culture is strongly proposed as an alternative for mass-propagation of such species. Therefore, the tissue culture technique can increase the commercial exploitation of more forest species (Bonga and Durzan, 1987), and it can be extensively applied in the propagation and the management of botanical collections (Lobine *et al.,* 2015).

5. Importance of plant tissue culture in forests trees

Tissue culture can lead to mass production of forests trees (George and Manuel, 2013) and creates new and challenging opportunities in the global trading for producers and nursery owners, leading to improvement of countries' economies (FAO, 2000b in George and Manuel, 2013). It can establish forests and forest plantations to meet the ever-increasing demand for tree products, which has been a long-standing tradition, especially in the tropics (Evans, 1999; Kumar *et al*., 2015). Apart from alleviating the pressure on the valuable primary forests, tissue culture in forestry can provide continuous production of tree materials through intensive management practices. Although traditional propagation (e.g. nursery method) of forest trees have been used for the past decades, only a few successful outcomes have been reported (Chisha-Kasumu *et al.,* 2006). Therefore, tissue culture can potentially produce trees in a short time with limited space and can reduce seedling mortality rate while promoting a strong primary stage during the earlier stages of the plants. Similarly, the method will improve the potential move for making faster gains and will offer numerous possibilities for advancement in forest protection, regeneration and improvement (Sedjo, 2016). This is because of its ability to produce a high number of plants from a single tissue, organs and plant cells. Henceforward, the use of tissue culture holds the greatest promise for forest improvement around the globe (Bonga and Durzan, 1987).

6. Low-cost options for tissue culture costs

The benefits of tissue culture technology are mainly in the production of good quality plants which can be multiplied any time of the year under a disease-free environment irrespective of the climate (IAEA, 2002). However, the technology is reported to be capital and energy intensive (George and Manuel, 2013) as equipment and skilled personnel are not always available. Also, electricity and clean water are important factors that might not always be available especially in African countries (IAEA, 2002). Therefore, it is important to investigate low-cost alternatives with a high success rate.

The most expensive components of the tissue culture are the equipment and laboratory structures, thus, careful planning is essential to increase cost-effectiveness (George and Manuel, 2013). For example, artificial lighting inside the growth room can be the most expensive and most ineffective method (George and Manuel, 2013). Therefore, an effective alternative to reduce the costs of lighting without compromising the quality of the plant can be essential to plant breeders. One strategy to limit the costs of electricity is changing from artificial (electricity) to natural light (sunlight). Most laboratories maintain the temperature in the growing chambers with air conditioners; this however, can increase production costs due to high electricity consumption. A suggested better way would be to allow *in vitro* growing at various temperatures so that plantlets are able to easily adapt to the field environments (Ahloowalia *et al.*, 2002). Seedlings can also be hardened off under shade netting with natural light, replacing the ventilated air rooms with artificial lighting which are mostly applied. Sucrose, which is a carbon source, can perhaps be replaced with table sugar or molasses, which is less expensive and more freely available. Careful planning of growth medium and laboratory containers i.e. reusable glass jars can be also applied to further lower the costs. Preparation of the growth medium in bulk can also lower the labour costs (George and Manuel, 2013). A practical example of cost-effective practices using the aforementioned techniques was applied during a production of sweet potatoes, thus decreasing the production costs by 96% (Ogero *et al.*, 2012). Similar findings were obtained in the production of banana (George and Manuel, 2013). However, careful consideration to avoid substituting all the techniques with low costs is needed as this may result in lower production (George and Manuel 2013).

7. Important tissue culture aspects

There are number of complex factors that determine the *in vitro* growth and development of plants (Pierik, 1997a). Pathogens on the explants and from non-sterile equipment are major constraints (Abdullahi 2013 and Kumar *et al.,* 2015). A well-mixed medium is needed to initiate the growth of the plants and should contain mineral salts, carbon source (usually sucrose), vitamins, growth regulators, amino acids and plant extracts. The poorly developed culture medium may lead to poor survival of the plants (Ahloowalia *et al.,* 2002). The culture environment (light intensity, pH and temperature) needs to be adjusted based on the plant's needs (Hartmann *et al.,* 2014). Both growing medium, light and temperature adjustments can be mishandled due to poor skills and lack of human resources in tissue culture laboratories (Chisha-Kasumu *et al.,* 2006).

7.1 Culture medium

Tissue culture media can contain different growth regulators (Hartmann *et al.,* 2002), also known as plant hormones. These are chemical substances that influence the growth and cell differentiation (George *et al.,* 2008). Hormones such as auxins (IAA), Gibberellins (GA), Cytokinin's, and Abscisic acid (ABA) can be used. These growth regulators work together to promote plant growth (George *et al.,* 2008). The use of these hormones and growth regulators can be costly and if used incorrectly, it can limit the success of tissue culture (Ahmed *et al.,* 2001). For example, the ratio of auxin and cytokinins needs to be balanced well as this determines shooting and rooting in plant. A wrong measurement or application of these regulator may result in one feature abundonment. However, different species have different hormone or growth regulator requirements.

According to Hartmann *et al.,* (2014) explants are small parts of the plant that are used in tissue culture. They are sometimes referred to as building block of tissue culture. They are extracted from mature trees as well as from young seedlings called donor or mother plants. Explants can be a cell, tissue and/or organs extracted from root tips, leaves, buds and apical meristems. Juvenile explants are mostly preferred as compared to the mature explants because they are more responsive to growth regulators such as cytokinin's, gibberellins, auxins and other inhibitors.

The selection of the explant type requires a systematic eliminating process as it can directly influence the tissue culture success rate (Hartmann *et al.*, 2014). For instance, some explants may produce seedlings faster than others; some may not require any hormones to initiate plant parts like roots, while others may require growth hormones to initiate roots and shoots. The most popular explants are shoot tips (apical meristems), seeds, buds, embryos, leave blade pieces, flower and root tissue (Hartmann *et al.*, 2014). In this study, seeds, buds and embryos were investigated as possible explants. During the study, explants were selected based on their accessibility from the species, for instance, the study could obtain dry seeds (harvested over six months) for P. *angolensis* seeds and fresh and dry seeds for *S. cocculoides.* Similar procedure could not be done with *S. cocculoides* seeds. This is because they do not portray the same feature as the thin outer-coat is attached to the cotyledon making it hard to manually remove.

7.2 Embryo

Embryos are the most preferred explant in tissue culture, because they are believed to have a high potential of forming plantlets that are not duplicate copies of the mother plants as opposed to other explants such as buds and shoot tips. According to a recent study, this is because embryo comes from a zygotic embryo that has already gone through a sexual recombination (Hartmann and Davies, 2014). Clones are sometimes considered not good, as they are vulnerable to uniform conditions such as pest and diseases (Kagithoju *et al.*, 2013).

7.3 Shoot tips or apical meristems

The shoots sprout from a small cluster of cells known as shoot apical meristem (Ahloowalia, *et al.*, 2002). They are mostly used to eliminate systemic virus, fungi and bacteria (pathogens) from the plantlets. The shoot tip explants sizes can vary, but should not be too big because the bigger the explants, the higher the chances of infection by pathogens. The suggested size of a shoot explant is 100µm in diameter and 250µm in length (Ahloowalia *et al.*, 2002).

8. General control conditions

8.1 Explant Disinfestation

Hartmann *et al.* (2014) defines disinfestation "as a process of removing contaminants from the surface of the organ rather than from within the organ." This is simply a removal of possible contamination on the surface of the explants (also referred to as surface sterilization). A typical procedure of explant surface sterilisation is repeatedly washing with sterile water and sometimes with added chemicals.

8.2 Temperature

The germination and growth of a plant can only occur between its maximum and minimum temperature requirements (Pierik, 1977a). Extreme high or low temperatures can be detrimental to plants in the culture. Different plants survive at different temperatures; therefore, the culture need to be adjusted at a specific plant required temperature (FAO, 1989). Even though some woody plants have a cold requirement for root formation, adventitious root and shoot formation are generally promoted at a high temperature of about 23 to 27ºC (Pierik, 1997a).

8.3 Light

The presence and absence of light in the *in vitro* culture generally have negative and positive effects on root and shoot formation of the explants (Edwin, 1993). Pierik (1997b) reported that plants which have been grown in the dark might root more easily than light-grown plants. Therefore, light intensity requirements of plants being cultured must be known.

8.4 pH

There is limited information about the influence of the pH on a nutrient medium of *in vitro* growth (Pierik, 1987). A pH range from 5.0 to 6.5 is normally suitable for many species *in vitro* growth (Pierik, 1987; Kifle *et al.*, 2014; Hartmann *et al.*, 2014). Low (less than 4.5) or

high pH (more than 7.0) generally stops the growth and development of *in-vitro* culture. Low pH also complicates nutrients such as auxin (e.g. IAA) and gibberellic acid to become less stable, sloppy agar and precipitation of salts. Different species may have different pH requirements, thus, background information on the species pH requirement is essential in tissue culture.

8.5 Orientation of inoculation

The inoculation position of the explants plays an important role in shoot and root inducing of plants. The apolar inoculation (up-side down) promotes and polar inoculation (natural orientation, base down) inhibits regeneration (Pierik, 1997b). Studies have advised the use of apolar inoculation because it allows for better oxygen supply above the medium hence encourage regeneration.

Chapter 3:
Materials and Methods

1 Introduction

Plant materials (seed and buds) were collected from mature trees of *P. angolensis* and *S. cocculoides* at Hamoye state forest, Kavango West region, north-eastern Namibia (Figure 3.1). *In vitro* tissue culture experiments were performed under controlled conditions in a plant propagation lab at the Department of Forest and Wood Science, Stellenbosch University, South Africa.

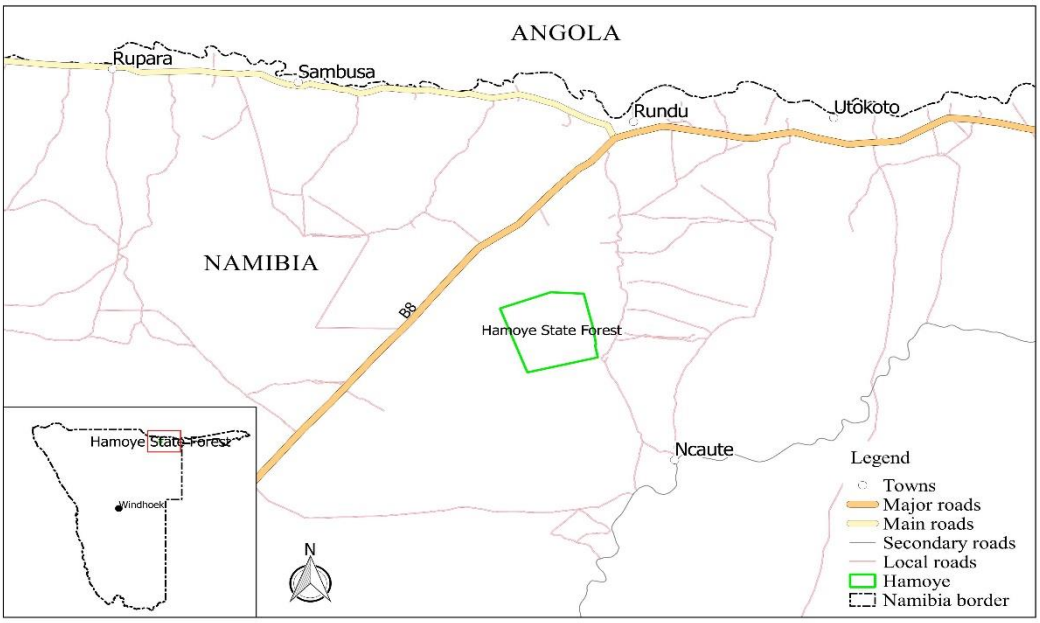

Figure 3. 1: Location of Hamoye State Forest, Kavango West in northeastern Namibia

Seeds were collected from phenotypic superior trees as they had a good tree shape, straight and long branch free stem with few knots, and undamaged seed pods. The tree stands found in the state forest have grown under the same agro-climatic conditions in terms of the geography and ecology of the two species. The forest locations of the mother trees (seed donors) were marked and recorded for future references. After collection, *P. angolensis* pods were burned on a small fire to remove bristles that can be spiky and cause harm while extracting the seeds. The seeds

were then de-husked from their hard coats using tweezers, secateurs or scissors to extract the seed. After the extraction process from the hard coats, the *S. cocculoides* seeds went through several washes with tap water to remove any pulp. The seeds were then dried with limited exposure to direct sunlight pending sowing. For transportation, the seeds of both species were packed in polythene bags together with phytosanitary certificates (Appendix A) from the Ministry of Environment in Namibia, and the Department of Agriculture Forestry and Fisheries in South Africa. The seeds were harvested and prepared in August and September 2016, transported during October 2016 and used in experiments shortly thereafter. Figure 3.2 illustrates the schematic outline (distinct steps) followed during this study.

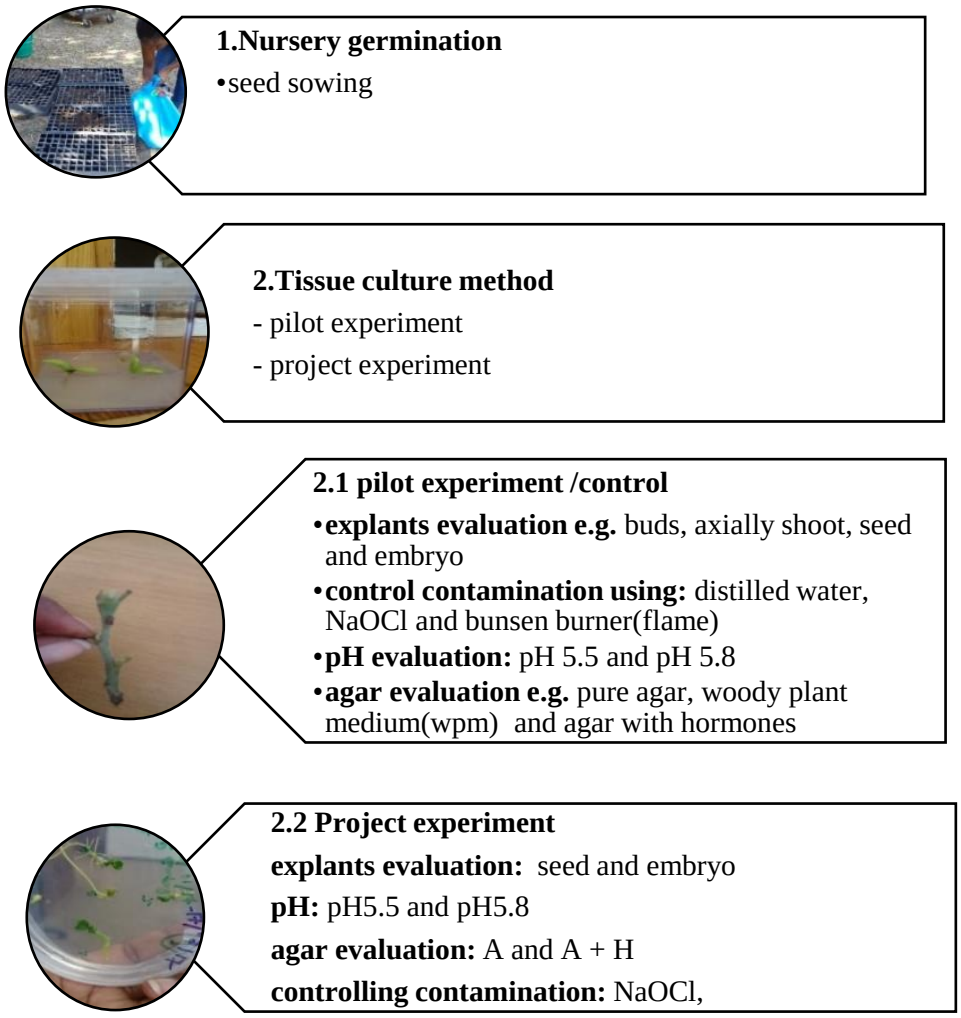

Figure 3.2: The distinct steps embarked on during the two-year period of this study

2 Nursery experiments

Pterocarpus angolensis and *Strychnos cocculoides* seeds were sown in the nursery at the Stellenbosch University within six months of collection and preparation as juvenile seed have a higher germination potential (Hartmann *et al.*, 2014). This was done to compare traditional nursery germination with tissue culture experiments. Seeds were sown in a Reliance potting soil mix that resembles the natural soil requirements needed for the two species. The reliance potting mix is a basic growing soil medium, weed-free enriched with organic compost and with a required optimum pH of plant growth Orwa *et al.,* (2009). Takawira-Nyena (2005) shown that *S. cocculoides* seedlings prefer slightly heavier soils found in dry riverbeds. Previous studies on *P. angolensis* show that the species are typically found in well-drained, medium-to-light soils with moderate fertility and a pH between 5.5 and 7 (Banda *et al.*, 2006). This make both species suitable in the Reliance potting soil mix.

Before sowing, a seed sample was drawn to test for viability with the water soak test (Ham *et al.*, 2017). Seeds were soaked for 24 hours in tap water, and the sinking seed was considered viable. Fifteen P. *angolensis* and *S. cocculoides* seeds (15 each) were tested for viability, of which 90% were viable in overall. Thereafter, seeds were subjected to a pre-treatment to improve the germination success by softening the seed coat and breaking dormancy (Heita and Ham 2015). Pre-treatment was done by soaking the seeds in warm water (45˚C) overnight, before sowing in black Unigro 92 seedling trays which were filled with the potting mix and placed under standard green net nursery treatments (sun protection, irrigation and weeding). The seed were let in trays for up to 10 weeks in anticipation of increasing germination over time. Irrigation was scheduled daily for 60 minutes at 12:00 for 21 weeks (from September 2016 to January 2017). The following data were collected weekly for a period of 10 weeks in the nursery: many germinated seeds; shoot length; the number of leaves; and germination dates. The seeds were considered germinated when the embryonic plant begins to grow and the seed coat breaks open above the substrate (Figure. 3.3) (Hartmann *et al*., 2014).

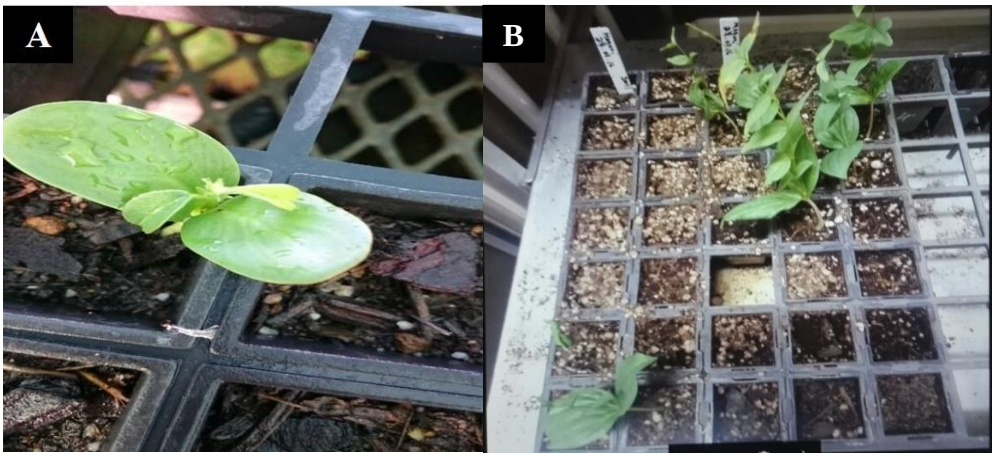

Figure 3.3: Germinated seedlings of Pterocarpus angolensis (A) and Strychnos cocculoides *(B) grown in the Reliance potting mix in the nursery*

3 Tissue culture

To initiate a tissue culture recipe, different aspects or factors that affect the culture success (germination) must be taken into consideration (Hartmann and Davies, 2014). This can be the external and internal environments of the culture, for example, temperature, light intensity, agar, explants and growth vessels (Hartmann *et al.*, 2014). This study employed five different stages to develop a tissue culture recipe for successful propagation of *P. angolensis* and *S. cocculoides*. These stages were a pilot experiment and consisted of: surface sterilisation of explants, pH (5.5 and 5.8), type of explants, and agar medium (with or without growth hormones. The pilot experiment specifically tested four types of explant surface sterilisation (distilled water, NaOCl, ethanol and flame), pH (5.5 and 5.8), type of explant (buds, axially shoots, apical shoots, fresh seed, dry seed and embryo), and the agar medium (agar with or without growth hormones). Results from the pilot experiments were then used in establishing the protocol (recipe) for the main tissue culture experiments. The tissue culture experiments partially repeated the pilot experiment tests, however with few changes as specified in **Fig** 3.2 -2.2. For instance, the explants tests changed from buds, axially shoot, seed and embryo to embryo and seeds only. Unlike nursery method with only once off sowing, tissue culture germination was repeated each week, which means, 30 explants were inoculated in the agar for germination each week.

3.1 Surface sterilisation of explants

As aforementioned in the previous sections, surface sterilisation was conducted in reference to removal of contaminants from the surface of the organ rather than from within the organ (Hartmann *et al*., 2014). In this study, elimination of any possible contamination before placing the explants on the agar media was essential. Explants were repeatedly washed in distilled water. Instruments like scalpels, forceps, needles, and tweezers which were used during the inoculation were sterilised by placing them in a glass steriliser (Bacti-Cinerator 250) at 250˚C for at least 10 minutes to eliminate pathogens.

In addition to instruments sterilization, three different sterilisation treatments were tested on the explants: (1) washing with sterile water; (2) ethanol and heat treatment (flame); and (3) NaOCl with dishwashing soap. For treatment 1, distilled water was autoclaved for 20min, at 120°C. Approximately five explants from each group (buds and/or seeds) were washed with autoclaved water and rinsed five times under running distilled water. Explants were then air-dried in a sterilised laminar flow prior to inoculation onto the agar medium inside plastic 65mm Petri dishes. For the second treatment, another five explants were dipped into 70% ethanol (v/v) for 2s and heated on a flame for one second inside a laminar flow before inoculated onto the agar media within 65mm Petri dishes. For treatment 3, explants were dipped into a solution of diluted NaOCl (0.75ml/l) and 1ml dishwashing soap. Thereafter, explants were incubated for 3 to 5min in a shaker (80rpm) taking note of any colour change which may implies decay. Shaking by hand can replace the shaker if necessary. Explants were rinsed three times with distilled water until no foam was visible and air-dried in a laminar flow. Explants were then transplanted onto the agar medium inside 65mm Petri dishes. All the Petri dishes were closed with Para film to further limit contamination.

To avoid further contamination, in particularly in the internal (within organ), juvenile explants (shoots from seedlings, seed, and embryos) were considered. Several studies show that young explants can have low contamination level, which often results into improved *in vitro* germination when compared to the matured explants (George 1993; Yu Xiaoling and Reed, 1995). Embryos are less exposed and have less external contamination as opposed to seed with a seed coat (Hartmann *et al*., 2014). The contamination in petri dishes were then rated into three categories, of which the highest contamination level is rated as number three (more than

70%), two was medium (30 to 70%) and one was a low contamination (less than 30%). The levels of contamination were determined through daily observation. Any kinds of foreign outgrowth in the petri dish were considered contaminants. Contaminants were mainly mould and fungal hypha. The levels were classified as follow: a highly infected petri dish with no germination potential explants was considered as level 3; Level 2 could be a petri dish with medium contamination; whereas level 1, is a few contaminations of which some explants can still survive.

3.2 Agar media

For easy assessment during data collection, a semi-clear agar was used, as it allow clear observation of plantlets i.e. roots (Scholten and Pierik, 1998). To simulate *in vivo* growing conditions of *P. angolensis* and *S. cocculoides*, the data sheets of the FAO were referred to (FAO, 2017). Also, two different growing media were investigated during the study; agar without added hormones (A) and agar with added hormones (A+H). Two 0.7% (v/v) agar media (Sigma – Aldrich A1296; Appendix A) were prepared. The initial agar solutions were divided into two equal parts. The pH of one solution was adjusted to 5.5 and the second to a pH of 5.8 with KOH (0.1M) and HCl (0.1M). Thereafter the initial agar solution was divided into two equal parts for both pH solutions. This represents the following agar media: A pH 5.5; A pH 5.8; A+H pH 5.5; and A+H pH 5.8. To complete the A+H solution, auxin-IBA (0.7g/1000ml) and cytokinins- kinetin (0.7g/1000ml) were added as recommended by Chisha-Kasumu *et al.* (2006). All four-agar media were autoclaved for 60min at 120°C, cooled down and poured into 65mm plastic Petri dishes in a laminar flow.

3.3 Determination of explants

Explants from seedling shoots in the nursery (seed germinated during the first segment of the study, Figure 3.3) as well as shoots and seeds from the plus trees of *P. angolensis* and *S. cocculoides* were used. One internode with an enclosed bud in the axil of a leaf was collected from the seedlings. Pieces were approximately 1 to 3cm in length (Figure 3.4A). The leaves were carefully removed and care was taken to not damage the buds. Seeds with the intact seed

coat were soaked overnight in warm water to soften the seed coat and remove the embryos with ease (Figure 3.4B, C). As *P. angolensis* has a softer seed coat than *S. cocculoides,* care was taken to not over soak the seeds. The embryos were carefully extracted from the seeds using sterile tweezers, before inoculation onto the agar media. For each explant (approximately 10 for each treatment), four mini-experiments were conducted as follows: A pH 5.5; A pH 5.8; A+H pH 5.5; and A+H pH 5.8.

Petri dishes for each treatment (sterilised, agar and explants) were placed randomly inside an SMC 1400 low-temperature incubator at 25°C with a 12 hour day and night photoperiod. Every three days, growth parameters such as shoot and root length, number of visible leaves and microbial contamination were recorded over a period of 10 weeks after inoculation. Contaminated Petri dishes were removed and discarded as soon as contamination was visible.

After data collection, plantlets (seedlings) that were too tall for the Petri dishes were transferred to a 0.7% (v/v) Murashinge and Skoog Woody Plant Medium (WPM) in sterilised glass jars (250ml). Jars were sterilised with an autoclave for 60min at 120°C. Germination rates were calculated by investigating the type of explants (buds, seeds or embryos), agar type (An or A+H), pH (5.5 or 5.8) and contamination. The axial shoot proliferation from each species was counted when the shoot and roots were visible.

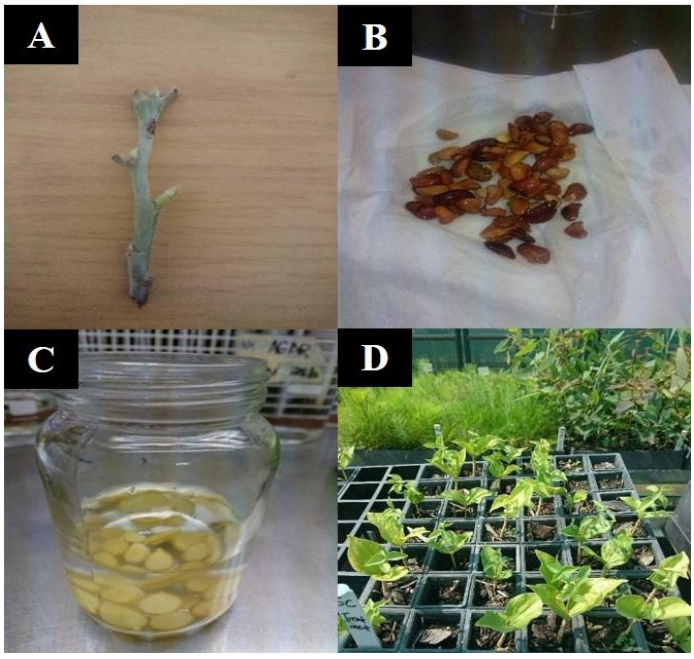

Figure 3. 4: Different explants of Pterocarpus angolensis and Strychnos cocculoides *sampled from plus trees (A=buds and B= seeds(Pterocarpus angolensis) C= Seeds and D=nursery seedling (Strychnos cocculoides).*

3.4 Tissue culture general control conditions

Embryo and seed explants were placed on top of the agar media with the tip slightly pushed in the agar (Figure 3.5). This was done to prevent oxygen deficiency that may occur when an embryo is pushed too deep into the agar media (Pierik, 1997a). While buds were pushed approximately half-way into the agar, care was taken that the shoot tips were still visible.

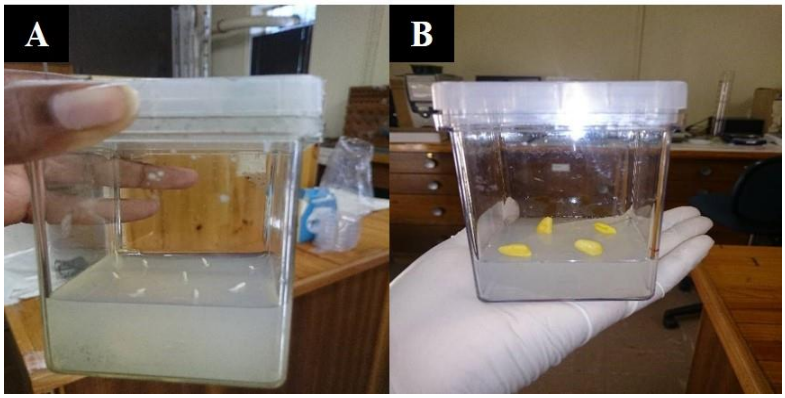

Figure 3.5: Embryo of Strychnos cocculoides (A) and seed explants of Pterocarpus angolensis *(B) inoculated into the agar media*

After 10 weeks of data collection in a tissue culture environment, plantlets (referred to now as seedlings) (Figure 3.6 A) were transferred to the soil (pit bricks supplemented with urea (Figure 3.6B) for natural growth

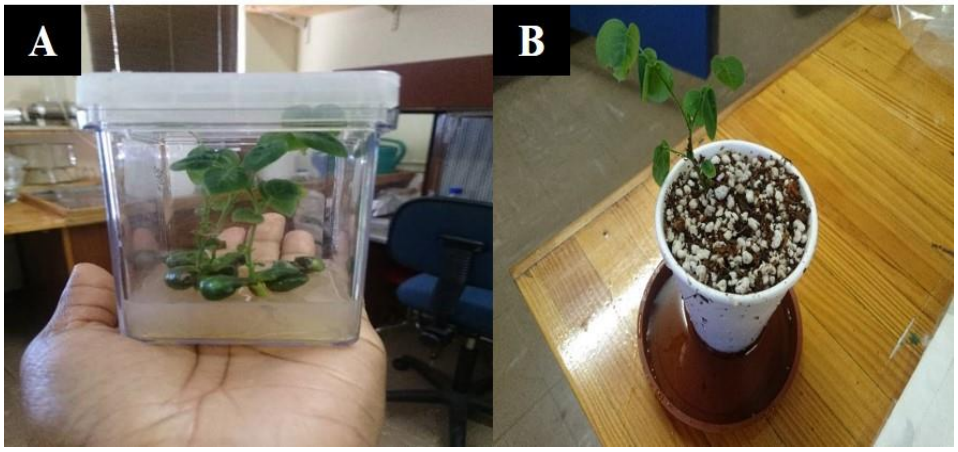

Figure 3.6: Germinated seedlings of Pterocarpus angolensis *in tissue culture (A) and transferred seedling (B)*

4 Data Analysis

This study was initiated to develop a robust tissue culture recipe for the successful germination of *P. angolensis* and *S. cocculoides* seed. Variables which were analysed were explants (seed and embryo), pH (5.5 and 5.8) and agar (with and without hormones). These variables were tested one by one on different batches of explants (seed and embryo), eliminating any possible interaction between variables. Growth parameters, such as a number of roots and shoots, plantlets length, percentage contamination, type of agar, and germination date were recorded every third day for 10 weeks. Data were analysed with SAS/STAT® software (version 7.1, 64 bit, System for Windows 7) and XLStat. For all disinfestation experiments, Surface sterilisation of explants was ranked and the contamination presence data were recorded and rated from 1 to 3 as counts of high contaminated (3), medium contamination (2) and low contamination (1). A Chi-square test was performed to identify significant differences ($P<0.05$).

All data were tested for normality and homogeneity of variance (Shapiro and Wilk of more than 0.85 for all tests) and then subjected to an analysis of variance, multiple comparison tests (using one-way ANOVA). To test for normality assumptions, each treatment's observations such as pH, Agar etc., were tested separately. For instance, data on the effect of the two agar (A and A+H) on the total germination and or the effect of pH on the total germination. Example, in one-way ANOVA, the response (dependent variable), was the germination rate while the treatments (independent variable) were the agar types, explants and or pH level separately. Significance was calculated at $P=0.05$(95%) confident interval and least significant difference (LSD) was used for comparison of treatment means. The statistical model was:

$$\gamma = \mu + \alpha i + \epsilon$$

Where γ is the response measured on the experimental plot (for example germination rate of explants); μ the overall mean; α^j the mean effect of the j^th treatments and ε the unexplained error.

Chapter 4:

Results

1 Introduction

Germination success of *P. angolensis* and *S. cocculoides* in the nursery (control) and through tissue culture methods were investigated. In the control germination experiments, seeds were sown under standard nursery conditions. In tissue culture, different explants (e.g. seeds and embryos) were inoculated in agar media. This was to compare germination success from the nursery with that of the tissue culture. The following section presents the results from this study. This section also narrates specific results obtained from different variables of tissue culture (i.e. such as explants, agar media and pH) reported to influence germination.

2 Nursery experiments

Only, one *P. angolensis* (week 3) and two *S. cocculoides* (week 2) seeds germinated during the nursery experiments (Figure 4.1). After 10 weeks, only two (6%) *P. angolensis* and seven (23%) *S. cocculoides* seed germinated and had grown into seedlings. The method extended germination

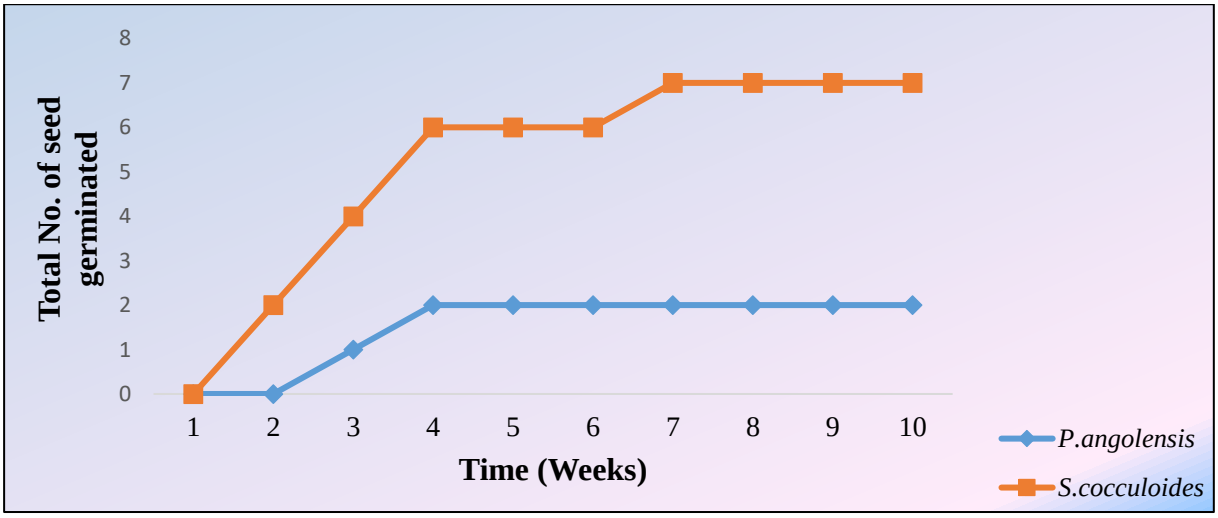

Figure 4.1: Total Number of germinated seed recorded per week during the nursery experiment for Pterocarpus angolensis and Strychnos cocculoides

3 Tissue culture experiments

3.1 Pilot experiments

The pilot experiments were used to pre-screen the optimal type of explants, the method for surface sterilisation, determine pH and type of agar medium. Buds, seeds, embryos, apical and axillary shoots were tested as possible explants (Figure 4.2). Only seed without the seed coat and embryos yielded sufficient results for both species. For *P. angolensis* no plantlets were obtained from buds and shoots, while less than 20% of the buds resulted in plantlets and none for shoots with *S. cocculoides*. Due to high contamination observed, surface sterilisation by means of distilled water was also considered not appropriate (Figure 4.3). Therefore, tissue culture experiments were conducted with two types of explants (seed without seed coat and embryos); surface sterilisation of NaClO; pH of 5.5 and 5.8; and two types of agar media (A and A+H).

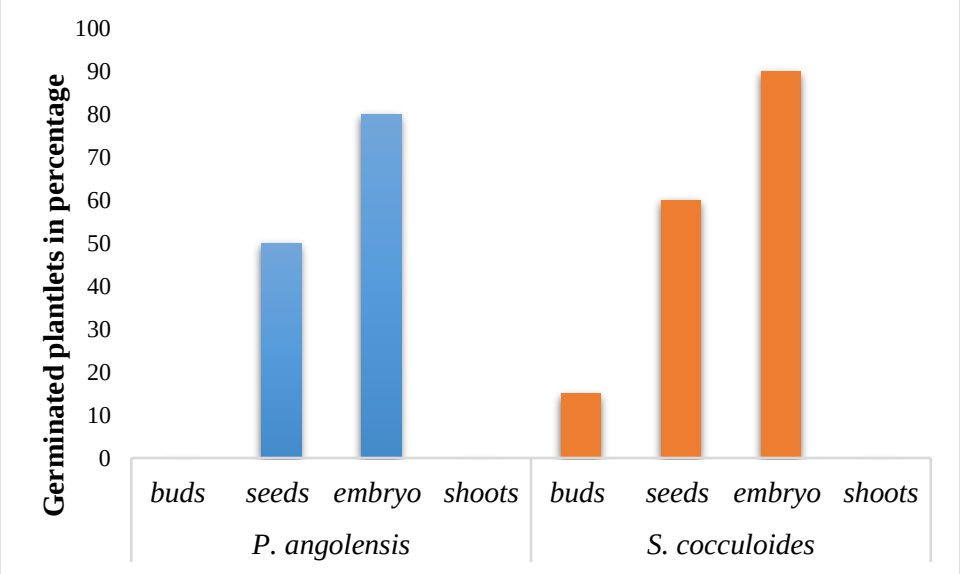

Figure 4.2: Germination and/or generation percentage recorded per 10 number of explants, per type of explant per species tested during the pilot experiments

Figure 4.3: An example of pathogens observed on the explants after surface sterilization with only distilled water

3.2 Testing of variables and treatments: Project experiment

3.2.1 Effects of explant type on germination

There was no significant difference ($P = 0.12$, $R^2 = 0.28$) for germination success between the two types of explants (seeds without seed coat and embryo) for *P. angolensis* over the 10-week period. Recorded germination distribution for embryo was between 30 and 73%, while 70 to 75% for seed over the 10-week period (Figure 4.4).

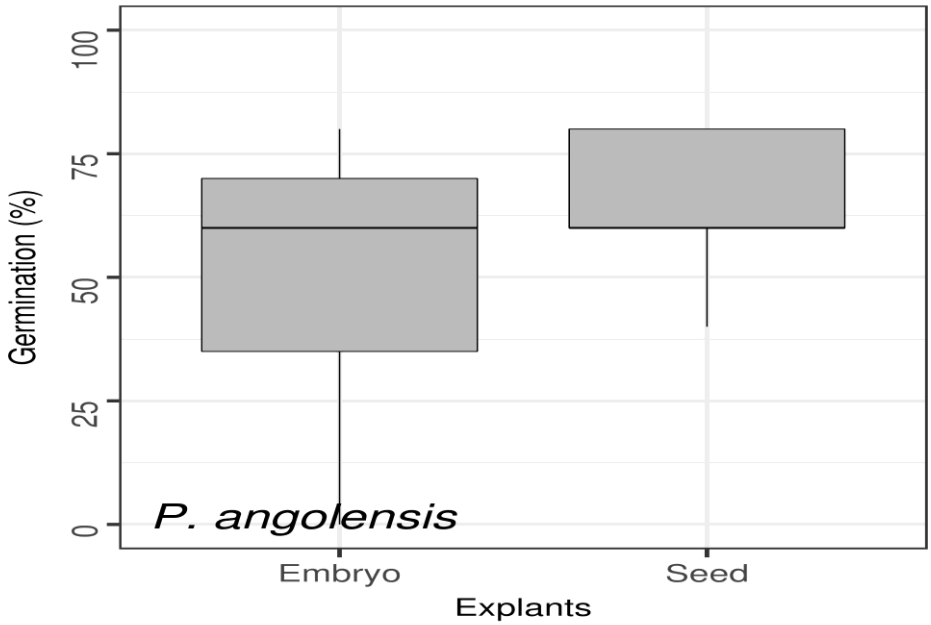

Figure 4.4: Germination percentage distribution for embryo and seed explants of Pterocarpus angolensis

Halfway through the study, fresh seed explants was obtained for *S. cocculoides*. Therefore, the germination potential of the embryos obtained from fresh seed was compared to that of one-year-old seed (dry embryo). A significant difference (P = 0.0010, R^2 = 0, 25) between the germination rates over a ten-week period were obtained, indicating a higher germination potential in the embryos of the dry seed (Figure 4.5). The germination success was 92% and 67% in the dry embryo and fresh embryo respectively for *S. cocculoides* over the 10-week period. Lastly, the coefficient of determination (R^2) was low, indicating that the applied model explains only some (which was explants) of the variability of the response data around its mean.

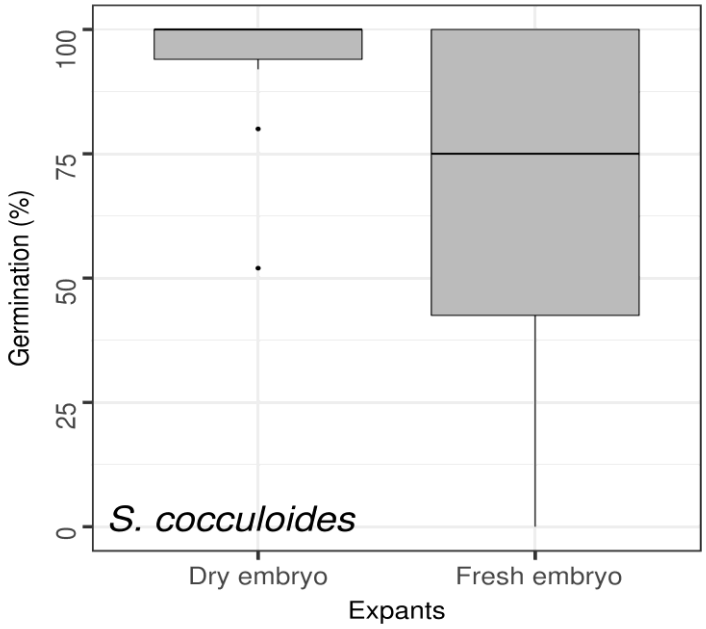

Figure 4.5: Germination percentage distribution for dry and fresh embryos in Strychnos cocculoides

3.2.2 Effect of agar type on explants germination

Two agar media were investigated: agar without hormones (A) which served as a control, and agar with hormone (A+H). There was a significant difference in germination of explants between A and A+H in both *P. angolensis* (P = 0.0049, R^2 = 0.13) and *S. cocculoides* (P = 0.0001, R^2 = 0.25). Agar medium A produced a higher germination in all the species (Figure 4.6). *Pterocarpus angolensis* embryos and seed explants which were grown in agar medium A also had a higher mean total length (5 to 7cm) than those in A+H (1.5 to 3.5cm). For *S. cocculoides*, agar medium A performed better, with a mean total length of 3.7cm. The mean total length for *S. cocculoides* in agar medium A+H was 1.9 cm.

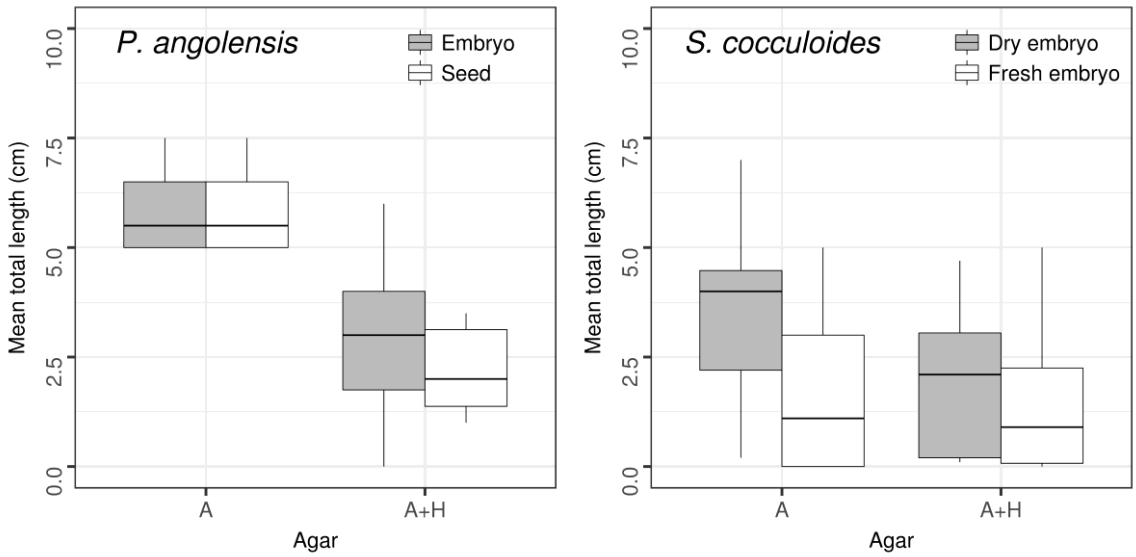

Figure 4.6: Mean total length of plantlets (cm) for agar without hormones (A) and agar with hormones (A+H) for Pterocarpus angolensis and Strychnos cocculoides

The effect of agar type on shoot length, root length, (shoot length + root length = total length) and a number of visible leaves were recorded (Figure 4.7). In *P. angolensis*, there were highly significant differences in mean length for shoot (P = 0.0019, R^2 = 0.15) and root length (P = 0.0145, R^2 = 0.09) at both A and A+H agar media. Medium A had longer roots and shoots, which eventually resulted in a higher mean total length than agar medium A+H in for each species. Also, *P. angolensis* produced a higher average number of leaves in both agar media (2.1cm for A and 1.8cm for A+H). Similar findings were observed in *S. cocculoides*, with highly significant differences for shoot length (P = 0.0001, R^2 = 0.20), root length (P = 0.0001, R^2 = 0.20) and average number of visible leaves (P = 0.0001, R^2 = 0.13) in both agar media. In addition, medium A promoted shoot and root length, as well as the average number of visible leaves in this species, more than medium A+H (Figure 4.7).

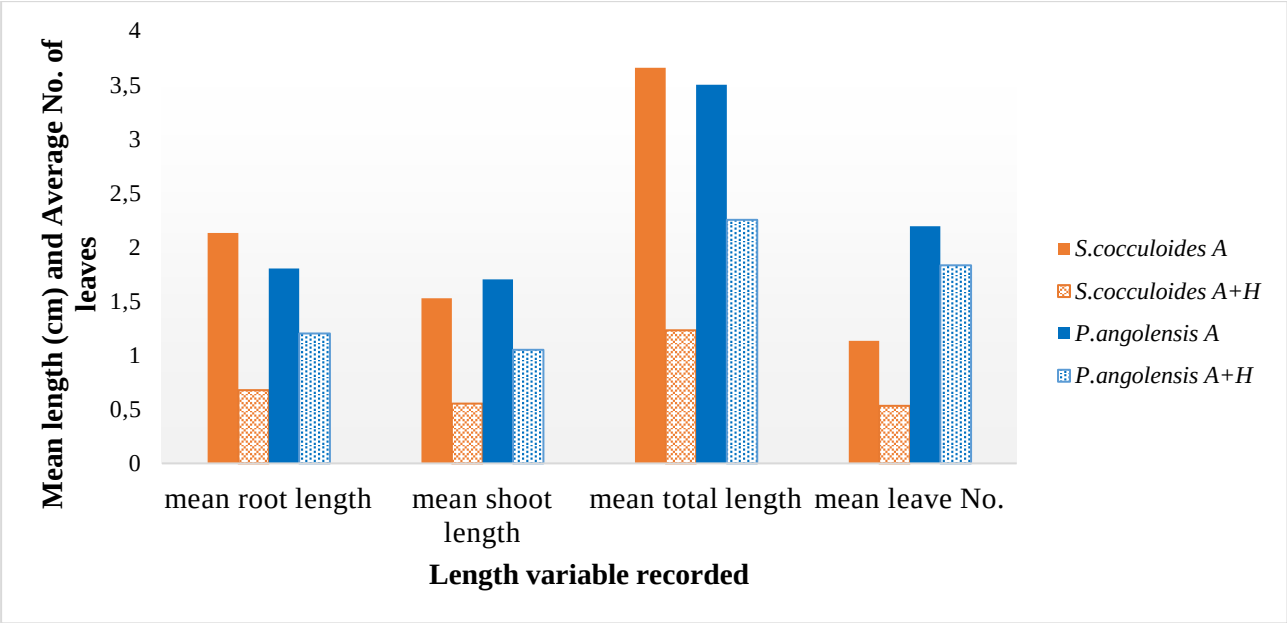

Figure 4.7: Mean differences of vegetative variables recorded (shoot length, root length (cm) and average number of leaves) for Agar without hormones and Agar with Hormones in Pterocarpus angolensis *and* Strychnos cocculoides

3.2.3 The effect of An agar medium, pH on explants germination success

There was a significant difference ($P = 0.001$, $R^2 = 0.31$) for germination success at pH 5.5 and pH 5.8 for *P. angolensis* seed and embryo explants (Figure 4.8). At pH 5.8, higher germination success for seed and embryo's explants was recorded than at pH 5.5 in *P. angolensis.* In general, *P. angolensis* seed explants showed higher germination success at each pH levels than embryo explants.

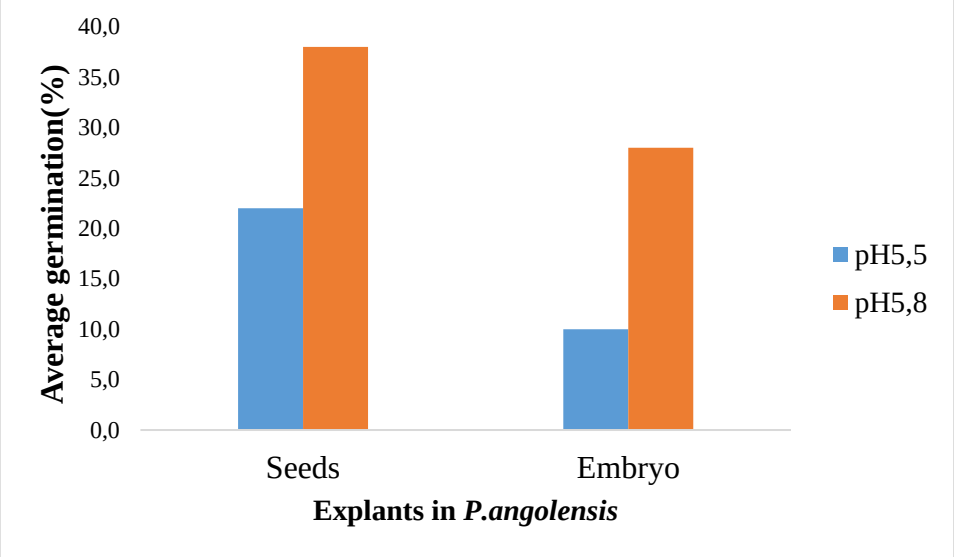

Figure 4.8: The comparisons of pH levels mean germination for P. angolensis

No significance difference (P = 0.21 and R^2 = 0.40) at pH of 5.5 and 5.8 was evident for *S. cocculoides* fresh and dry embryos. At pH 5.5 the fresh embryo (28%) performed slightly lower than at pH 5.8 (29%). However, for the dry embryo, both pH 5.5 and 5.8 had a value of 29 and 30% respectively.

3.2.4 The level of contamination during experiments

Throughout the 10 weeks, the level of contamination was rated between one and three according to the total area covered by contamination (Figure. 4.9). Contamination level of three indicated a high presence of contamination (more than 70%), two was medium (30 to 70%) and one was a low contamination (less than 30%). During week one (first experiments), contamination was high,but descended as the weeks passed and surface sterilisation methods were improved.

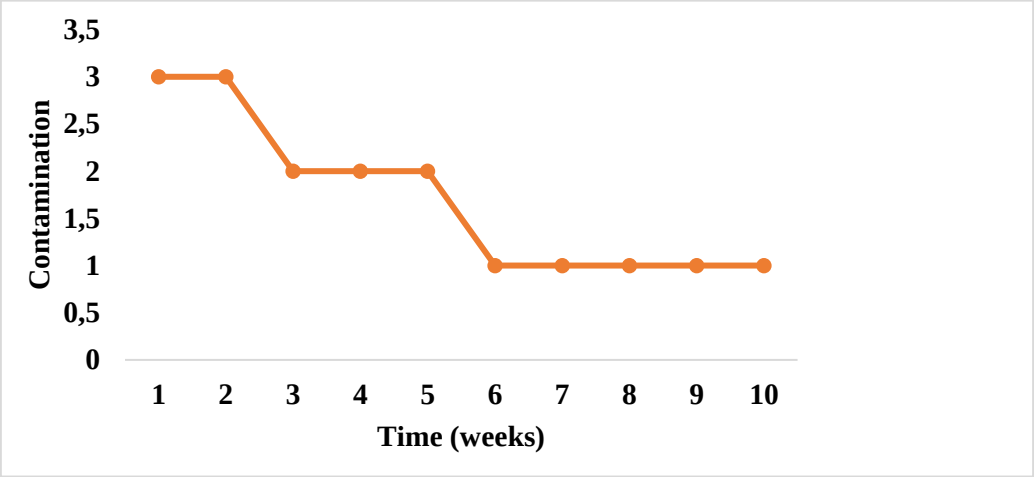

Figure 4.9: The level of contamination during the tissue culture experiments for the 10-week period

4 Recipes:

Both species can germinate in the tissue culture within few days (approximately six days) after inoculation. After five weeks in the culture, the plantlets grew fully into seedlings (approximately 7cm in height) and can be transferred to a soil media as seedlings (Figure 4.10). After exploring and observing different explants, the results show that using seeds explant yielded the best germination success of *P. angolensis* and *S. cocculoides* in tissue culture. In *P. angolensis* seeds with outer coat removed and the embryo can yielded high germination success, but seeds without outercoat yielded best results. For *S. cocculoides*, dry seeds (stored

for approximately one year) yielded a higher germination rate than fresh seeds. The comparisons between the agar medium (A and A+H) indicated that the agar media A yielded the highest germination success for both *P. angolensis* and *S. cocculoides*. Lastly, the pH of the agar medium can range from 5.5 to 5.8 with better germination at a pH 5.8 for both *P. angolensis* and *S. cocculoides*. The results simply illustrate the important of control the level of contamination in the culture. Thus, NaCIO with dishwashing detergent (e.g. sunlight dish wash) can sufficiently control contamination.

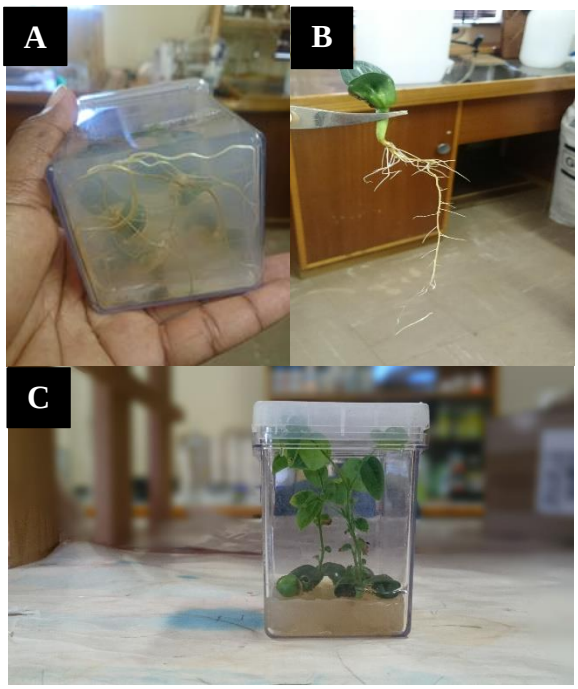

Figure 4.10: Three weeks old plantlets of Pterocarpus angolensis (A and C) and Strychnos cocculoides (B) grown in tissue culture technique

5 Comparison of germination rate between nursery and tissue culture

During the nursery, 30 seeds were sown in potting soil medium for 10 weeks, while in tissue culture, 30 seeds per week, were inoculated in agar media for both species. Over a 10-week period, germination success from the two methods for both species differed significantly (P = 0.0001; R^2 = 0.59) between the experiments.

The *P. angolensis* nursery experiment yielded only two seedlings, while the tissue culture experiments resulted in up to 20 seedlings per week (Figure 4.11A). *Strychnos cocculoides* yielded seven seedlings during the nursery experiment, while the tissue culture experiments had a weekly total germination of 15 seedlings (Figure 4.11B). In general, the tissue culture

experiments not only improved the germination of seed significantly but also the number of days until germination (Figure 4.13).

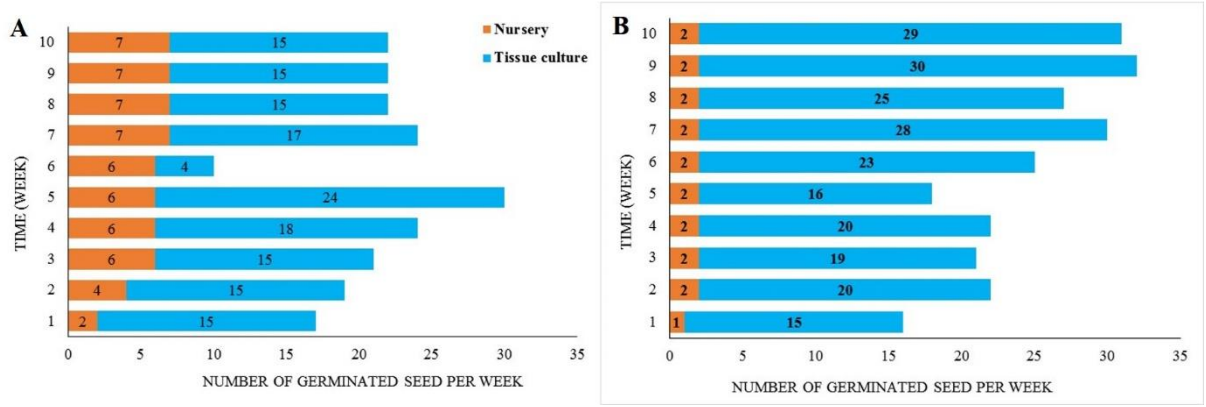

Figure 4.11: Comparison of germinated seedlings/plantlet per week over a 10-week period between the nursery and tissue culture(TC) experiments for Strychnos cocculoides *(A)* and Pterocarpus angolensis *(B)*

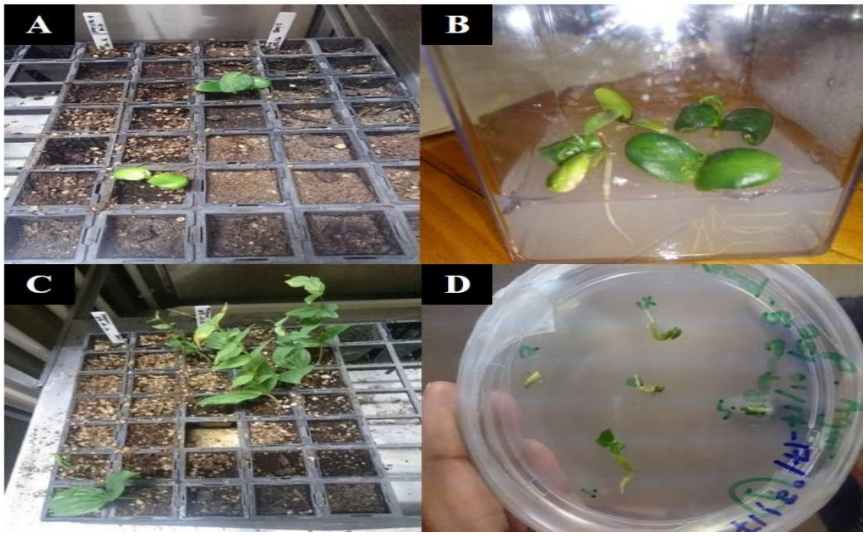

Figure 4.12: Nursery germination at week 10 (A and C) compared to tissue culture germination at week 1 (B and D) for Pterocarpus angolensis (A and B) and Strychnos cocculoides *(C and D) respectively.*

Chapter 5:
Discussions

This study investigated and compared the germination of two important tree species (*P. angolensis* and *S. cocculoides*) from Namibia using the nursery and tissue culture (TC) methods. The species were chosen based on their essential roles they play in many local communities around the country.

Based on the results, there were differences between the two methods (Figure 4.11). For instance, germination of *Pterocarpus angolensis* and *Strychnos cocculoides* was higher in tissue culture method than in the nursery.

In addition, nursery method extended germination (Figure 4.1); for example, the first germination of *P. angolensis* occurred after 3 weeks of sowing, and S. *cocculoides* took two weeks to germinate. Only two *P. angolensis* and seven *S. cocculoides* seeds germinated and grew into seedlings after 10 weeks of the nursery experiment. Similar research studies (see Moses, 2012; De Cauwer *et al.;* 2015) indicated that *P. angolensis* seed can take up to three weeks to germinate. These studies also reported low nursery germination success for both *P. angolensis* and *S. cocculoides.* Rising concern about the time of planting and temperature differences to may possibly be the reason for such low germination success (Moses, 2012). In comparisons, tissue culture increased the germination success in both *P. angolensis* and *S. cocculoides* when compared to nursery experiments. For *P. angolensis*, tissue culture had up to 80% germination success weekly, much higher when compared to 7% for nursery experiments. The *S. cocculoides* had 80% germination success weekly in tissue culture, which was also far higher than the 23% germination success in the nursery experiments over a 10-week period. In addition, up to 15 *S. cocculoides* and *P. angolensis* plantlets were produced per week with the newly developed protocol of the present study. By contrast, the environmental conditions subjected to the two methods could cause the differences in germination, for instance, the controlling of pathogens as well as light and temperature constant set-up in the tissue culture. This, however, could not be a case in the nursery methods. The study therefore, suggests nursery germination with other means of assistance such as scarification pre-treatment as an option to promote high germination.

Higher germination success from tissue culture recorded in this study is consistent with findings reported from previous studies (Chisha-Kasumu *et al.*, 2006). Based on an earlier study, tissue culture propagation has numerous advantages over nursery plant propagation techniques (Ahmed *et al.,* 2001). For example, planting materials that can withstand environmental conditions such as drought, temperature and low fertility (Opabode, JT 2005). The technique has also improved some indigenous trees by providing sufficient quality and quantity materials (Kitch *et al.,* 2002). It also helps in multiplying plants, which produces few or no seeds, or plants that can be hard to propagate from seed. In addition, the technique provides clean plant materials that are free from diseases and pests.

The influence of several aspects of tissue culture namely, the explant types, Agar media, pH and surface sterilisation of explants on the germination success of *P. angolensis* and *S. cocculoides* were also investigated to identify optimal tissue culture protocol.

The results show that only seed without seed coat and embryos were successful in *P. angolensis*, while embryos (fresh and dry) yielded the best results with *S. cocculoides*. These were extracted from various explants conducted during the pilot experiment (Figure 3.7). The explants seeds without a coat of *P. angolensis* were obtained by removing the outer-coat on the seeds exposing the cotyledons, whereas fresh embryo and dry embryos were extracted from less than a year old and one year or more harvested seed of *S. cocculoides* respectively.

Buds harvested from mature *P. angolensis* had poor shoot and root development during this study. This differs from previous findings which reported that juvenile buds of *P. angolensis* can produce a good shoot and root development (Chisha-Kasumu *et al*., 2006). This difference may be due to harvested time of the buds, as suggested in tree propagation work by (Hartmann *et al.,* 2014). This work indicated that the time of the year a bud is harvested may determine its germinability. Additionally, the shoots of *P. angolensis* were harvested from mature trees in Rundu (Namibia) and transported to Stellenbosch before inoculation, it is likely that the quality and freshness deteriorated, resulting in the poorer shoot and root development. Moreover, due to logistic constraints (power outage, failure of equipment etc.); the buds could not be inoculated in Rundu. However, seed (without seed coat) and embryos of *P. angolensis* showed a significantly higher germination success (80 and 50% respectively; Figure 4.2) compared to nursery methods (6%). Extraction of the embryo from seeds can be challenging and extra

caution should be taken not to damage the growth tip. Careful controlling of pathogens through surface sterilisation can also improve germination success. Although embryos from *P. angolensis* had a lower germination success than seeds without seed coat, they still yielded a significantly higher germination success (68%) compared to nursery methods (6%).

In *Strychnos cocculoides*, two-month-old buds and apical shoots showed germination potential, although highly susceptible to pathogens as material were very sensitive to surface sterilisation (NaClO). Although both fresh and dry embryos explants showed regeneration potential compared to nursery (traditional) methods, dry embryos differed significantly *(P* = 0.0010) from fresh embryos. Again, the fresh materials were exported between Rundu and Stellenbosch, the freshness of samples could have contributed to severe contamination and regeneration potential. There is few or no literature study in *S. cocculoides* tissue culture, however, previous tissue culture studies on similar species (*Strychnos potatorum*) reported a germination success of 100% for dry seeds (Kagithoju *et al*., 2013). Dry embryos are also not sensitive to NaClO surface sterilisation compared to fresh embryos, hence there is a lower level of contamination in dry embryos (Kagithoju *et al*., 2013).

Agar medium were investigated, this is because they influence the germination success of the explant (Hartmann, 2014). Thus, a combination of explant and agar media were investigated to optimise the germination success of each species (Figure 4.6). Two types of agar media were tested: agar with no hormones as control (A) (Appendix A), and agar with added hormones (A+H) (Appendix B). The added hormones were auxin-IBA (0.7g/1000ml) to improve cell elongation (root formation), while cytokinins-kinetin (0.7g/1000ml) was added for cell division (shoot formation). Results indicated that agar medium A had a better shoot and root development than the A+H medium for each species (Figure 4.7). Similar results from a previous study on *P. angolensis* also indicated that shoots on agar media without growth hormones such as auxins have rooted better than those from a media with auxin (Chisha-Kasumu *et al.*, 2006). On growth regulators containing medium (A+H), plantlets stem base, as well as the leaves, becomes brown with a stunted growth. This simply suggests that IBA and Kinetin were not essentially required for rooting and shooting development during *in vitro* tissue culture of *P. angolensis,* or the concentration was too high or low for germination.

Based on the findings for *S. cocculoides,* agar media A performed better than A+H. This findings were different from the findings of a previous study on a related species *Strychnos*

potatorum (Kagithoju *et al.*, 2013). During the study on *S. potatorum*, multiple shoot formation were obtained with two different cytokinins: 1.0 to 2.0mg/L 6-Benzylamino purine (BAP); 3.0mg/L Kinetin(Kn); on Murashige Skoog's medium (Kagithoju *et al.*, 2013). It is possible that the differences in germination success between these similar species may be due to differences in concentrations of the growth hormones as well as the environment of the experiments. Additionally, poor germination in agar medium A+H may also be caused by erroneous hormones concentrations. Research indicated that, although there are different forms of hormones, IBA and Kinetin is likely to promote germination/regeneration of woody species (Chisha-Kasumu *et al*., 2006; Moon *et al*, 2010; Lobine *et al.*, 2015). The study used a semisolid and see-through agar because it is best to use when collecting data on root development and for imaging, in contrast to i.e. agar with activated charcoal additions (Hartmann *et al*., 2014). The application of growth hormones can be complex and costly, especially at local community level (George and Manuel, 2013).

Other aspects that were considered during the project where the pH (5.5 and 5.8) of the two agar media (A and A+H) used during the tissue culture experiments. During the pH experiment, different explants (10 from each species) were inoculated onto agar media adjusted at two different pH levels (5.8 and 5.5). This was to study the effect on germination success of *P. angolensis* and *S. cocculoides*. The results showed that there was a highly significant difference ($P = 0.001$, $R^2 = 0.31$) in the average germination between the two explants (seeds and embryo) inoculated in pH 5.5 and pH 5.8 of *P. angolensis* (Figure 4.8). This show high average germination in seeds (38%) and embryo (28%) explants of pH 5.8 than that of pH 5.5 seeds (22%) and embryo (10%) in *P. angolensis*. These findings were for both agar media (A and A+H). The study could not establish a concrete reason why a difference in germination success between the two-pH levels existed. However, woody species can germinate at a pH range of 5.0 to 7.0 (FAO, 2017; Cornell University, 2017). There was no significant difference in germination between the fresh embryo and dry embryo inoculated in agar at pH 5.5 and pH 5.8 for *S. cocculoides* (Figure 4.8). As limited published literature is available in the tissue culture pH requirements for the two-studied species, future studies need to explore the effect of different pH levels on germination potential further.

Lastly, on the aspects evaluated, the level of contamination in the Petri dishes were investigated and rated during this study. Different sterilisation media, such as distilled water, 70% ethanol and NaClO were used to control pathogens (Dunaeva and Osledkin, 2015). The results show

that sterilisation with NAClO supplement with dishwashing detergent resulted in lower contamination compared to 70% ethanol or distilled water for both species. These findings similar to the finding of previous studies, which indicate that dipping seeds onto a 2.5% NaClO for 3 minutes lessen the level of contamination compared to 70% ethanol which resulted in explants decay in Mellisa officinalis (Kiani *et al.,* 2017). Furthermore, surface sterilisation using only distilled water seemed too weak to control pathogens. Studies argued to make surface sterilisation in the *in vitro* less harmful and accessible (Soghra Younesikelaki *et al.,* 2016). A basic protocol was henceforth, developed to control pathogens on *P. angolensis* and *S. cocculoides* seeds and buds (Appendix C). Contaminations during the study was mainly caused by an unhygienic environment during the procedures and had a negative impact on the success if not well controlled. The study therefore, aimed at eliminating all possible pathogens so that healthy plantlets are produced.

Furthermore, the present work was aimed at developing a robust tissue culture method that can be utilised and implemented successfully even at the local level. This was achieved by applying minimum equipment and materials as possible, without compromising results, adopted from a previous study (George and Manuel, 2013). The earlier findings indicated, despite the numerous plant benefits obtained from the technique, there are constraints that affect the successfulness of tissue culture (Ogero *et al.,* 2012). Numerous documentation have indicated that the costs of laboratories, equipment's and skilled personnel are one of the major constraints to tissue culture implementation. These have impediments for the adoption of the technique in some countries like India and Sub-Saharan African (George and Manuel, 2013). As such, the techniques are considered as one of a capital-intensive industry, because the unit cost per product can be unaffordable. Given this, George and Manuel (2013) argue that it is necessary to review strategies of low-cost tissue culture techniques and to adopt them so that we decrease the cost per plant unit. Low-cost plant technology means adoption of the affordable practices techniques without compromising the yield and quality of the products (Ogero *et al.,* 2012).

Overall the results show that the nursery experiment could only produce *S. cocculoides* and *P. angolensis* seedlings after 21 to 30 days. Therefore, the tissue culture protocol is a promising technique for high and fast multiplication and conservation of *P. angolensis* and *S. cocculoides* trees. These results are similar to those of Chisha-Kasumu *et al.* (2006) that show that the tissue culture techniques can provide better germination success for *P. angolensis*. Although tissue culture techniques are not easily implemented, it is one of the most important

technologies for the production of high quality and fast growing plants under controlled conditions (Abdullahi, 2013; Bhojwani and Razdan 1989). *In vitro* tissue culture is also complimentary to *ex situ* methods such as seed banking because both methods are important at conserving indigenous species (Sarasan, 2010). The current study was precisely aimed at producing a baseline protocol of tissue culture in *P. angolensis* and *S. cocculoides*. Other important aspects such as hardening off, also known as acclimatization, were not investigated. A follow-up study is therefore recommended.

By contrast, in case of the statistical findings, the coefficient of determination (R^2) of tissue culture and nursery methods was slightly low ($R^2 = 0.59$). This is possibly an indication of not considering other factors or aspects which may have an equal impact on tissue culture success, resulted from a weakness in experimental set-up. Low coefficient of determination was also obtained in various results, implying that the model used could only explain some of the variation of the response data around its mean. For instance, in figure 4.3, the regression model for the effect of explant type selection on germination only accounted for 28.0% of the variance ($p=0.12$, $R^2 = 0.28$).

The current study outcomes may possibly contribute to the replanting of the two tree species and may prevent further depletion of these species from the wild. Also, the tissue culture protocols developed can now be optimised and applied to enhance germination of this important tree species in the field.

Chapter 6:
Conclusions and Recommendation

1 Conclusions

This study compared nursery and *in vitro* propagation methods of *Pterocarpus angolensis* (kiaat) and *Strychnos cocculoides* (monkey orange). *Pterocarpus angolensis* and *Strychnos cocculoides* are two important indigenous tree species of the Namibian woodland currently on the verge of extinction due to high exploitation. *Pterocarpus angolensis* provides quality wood materials utilised in and outside of Namibia. *Strychnos cocculoides* provides wood and fruit products consumed and sold in and outside of Namibia. The protection of these species through plant regeneration is very important.

Propagation of any indigenous tree species is important to increase population size and to support the local livelihoods. Nursery germination of *P. angolensis* and *S. cocculoides* have been carried out in Namibia, but yielded limited success to date. Poor results are likely to be attributed to the poor seedling establishment and lack of propagation knowledge, for example, the application of seed pre-treatments before sowing. Therefore, tissue culture was investigated as an alternative propagation method for *P. angolensis* and *S. cocculoides* and compared to traditional nursery methods. Tissue culture is an *in vitro* vegetative technique known to produce and multiply hardly propagated plants. The technique is vastly reported to have good potential for producing plants of superior quality. However, tissue culture in *P. angolensis* and *S. cocculoides* in Namibia has not been investigated and its potential is yet to be exploited.

To promote regeneration and germination potential in *P. angolensis* and *S. cocculoides* tissue culture, a baseline protocol for both species was developed. The following factors were investigated: agar medium (with and without hormones), pH of agar medium (5.5 and 5.8), explants (i.e. axially buds, seeds, and embryo) and surface sterilisation (distilled water, ethanol and NaOCl).

Results indicated that agar medium without hormones (A) promoted a higher germination success in both *P. angolensis* and *S. cocculoides* than agar medium with added hormones

(A+H). The added hormones were auxin-IBA (0.7g/1000ml) and cytokinin-kinetin (0.7g/1000ml). Five different types of explants were investigated, namely buds, dry and fresh seeds (with and without seed coat), apical shoot and embryo. The results show that the embryo and seeds without seed coat produced a higher germination such, compared to buds and apical shoots. To control contamination in buds and apical shoots was also difficult which may contribute to bud's explants poor success. However, contamination was easier controlled in the seeds and embryo explants as material were not so sensitive to the chemical solutions such as 70% ethanol and NaOCl. As such, improving the surface sterilisation of buds and apical shoots by other alternatives need to be investigated further. Additionally, there was no evidence of statistically significant effects in the germination success between a pH of 5.5 and 5.8 in *S. cocculoides* as opposed to *P. angolensis* which showed significant differences.

Overall, the study concludes that tissue culture can successfully promote and shorten the germination rate of *P. angolensis* and *S. cocculoides* compared to traditional nursery procedures. This is because the tissue culture germination appeared to out-perform nursery germination methods. Therefore, tissue culture procedures offers i*n vitro* propagation protocols of economically important tree species in Namibia, and therefore this method has potential to contribute to modern sustainable forestry in Namibia. The protocol would facilitate the tree improvement programme using transformation technology and can be related to any other indigenous species around the world. Henceforth, the study served as a baseline information toward indigenous tree propagation using tissue culture techniques.

2. Recommendations

Based on results from this study, the following recommendations are made for future projects:

- There is a need to undertake different projects in and outside Namibia to determine whether tissue culture can be used on a commercial scale to conserve indigenous species. There is therefore a great need for further information on the development of protocols of tissue culture for indigenous tree species.
- This study was unable to encompass the entire tissue culture aspects, hence other tissue culture factors that could be investigated are temperature, light intensity, different hormone balances and concentrations, as well as photoperiod.
- Although Agar medium was employed and investigated, in-depth study on specific nutrients contents required by the species still need to be quantified and outlined.
- Other investigations should attempt to explore other different explants other than those that were investigated by this study (e.g. root tips and leaves blade).
- Continued studies should investigate the survival of plantlets at hardening off stage and/or survival of tissue culture plants in the nursery as this study only concentrated on the establishment of the plantlets.
- The current study has tested and developed a protocol of the aspects that promote germination in tissue culture of *P. angolensis* and *S. cocculoides.* A follow up study to observe interactions and relationships between all these variables is recommended.
- Other studies can also conduct other similar analysis on the development of protocols for propagation, rooting and shooting for other species.
- Continued research can also look at cost-effective of conducting tissue culture and how to fully implement low-cost tissue culture without compromising the success.

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Appendices

Appendix A:

1. Phytosanitary Certificate from Republic of Namibian

REPUBLIC OF NAMIBIA

GOVERNMENT REPUBLIC OF NAMIBIA
PHYTOSANITARY CERTIFICATE

ORIGINAL

Serial Number

Certificate Number 14472

To: The Plant Protection Organisation of	Place of Issue
SOUTH AFRICA	Windhoek, NAMIBIA

DESCRIPTION OF CONSIGNMENT

1. Name and Address of Exporter	2. Declared name and address of consignee
Lisias Tjaveondja P. O. Box 396 Okahandja, Namibia	HLENI HEITA TN Stellenbosch Republic of South Africa
3. Number and description of packages Seeds 1Kg	4. Distiguishing marks
5. Place of origin Namibia	5. Declared means of conveyance Road

7. Declared point of entry: Ariamsvlei/Noordoewer

8. Name of produce and quantity declared

Name of produce	Botanical name	Quantity
Seeds	*Strychos Cocculoides*	0.5Kg
Seeds	*Pterocarpus Angolensis*	0.5 Kg

This is to certify that the plants, plant products or other regulated articles described herein have been inspected and /or tested accordingly to appropriate official procedures and are considered to be free from the quarantine pests specified by the importing contracting party and to conform with the current phytosanitary requirements of the importing contracting party, including those for regulated non-quarantine pests

DISINFESTATION AND / OR DISINFECTION TREATMENT

9. Date	10. Treatment
11. Chemical *(Active ingredient)*	12. Duration and Temperature
13. Concentration	14. Additional Information

15. Additional declaration: For research purpose only

Stamp of Organisation

16. Date of issue: 22/06/2016

17. Signature of Authorized Officer

18. Name of Authorized Officer: E.TJIHURO

No financial liability with respect to this Certificate shall be attached to the Government Republic of Namibia or to any of its officers or representatives.

2. Phytosanitary Certificate from Republic of South Africa

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Plant Health

Permit No. **P0072724**

PERMIT FOR THE IMPORTATION OF CONTROLLED GOODS

In terms of the provisions of section 3(1) of the Agricultural Pests Act, 1983 (Act 36 of 1983) and subject to the conditions stated here under, authorisation is hereby granted to-

MS HANNEL HAM **Tel No: 021 808 3301**

STELLEBOSCH UNIVERSITY
PRIVATE BAG X 1
MATIELAND
7602

to import into the Republic the following controlled goods **SEED FOR RESEARCH/ PLANTING PURPOSES**

PTEROCARPUS SPP **750** **GRAM (S)**

Name and address of foreign supplier **NAMIBIA**

Conditions **1. AS ATTACHED**

Port of Entry : **VIOOLSDRIFT**
NAKOP
CAPE TOWN INTERNATIONAL AIRPORT

Import authorized from **2015/08/26** TO **2016/08/26**

IMPORTANT : This permit does not exempt the holder from the provisions of any other Act, ordinance or agreement

DEPARTMENT: AGRICULTURE FORESTRY AND FISHERIES
DIRECTORATE PLANT HEALTH
2015 -08- 27
DIRECTORATE PLANT HEALTH
P.O. BOX 40024, ARCADIA, 0007

Date

Executive Officer

Reference Number **3/15/272**

INQUIRIES : TEL.: (012)319 6102 (Solomon Matsa) **FAX: (012)319 6370**

3. Phytosanitary Certificate from Republic of South Africa

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Plant Health

Permit No. **P0069913**

PERMIT FOR THE IMPORTATION OF CONTROLLED GOODS

In terms of the provisions of section 3(1) of the Agricultural Pests Act, 1983 (Act 36 of 1983) and subject to the conditions stated here under, authorisation is hereby granted to-

MS HANNEL HAM **Tel No: 021 808 3301**

STELLEBOSCH UNIVERSITY
PRIVATE BAG X 1
MATIELAND
7602

to import into the Republic the following controlled goods **SAMPLES FOR ANALYSIS**

STRYCHNOS SPP **10** **KG/CONSIGNMENT**

Name and address of foreign supplier **NAMIBIA**

Conditions **1. AS ATTACHED**

Port of Entry : **VIOOLSDRIFT**
NAKOP
CAPE TOWN INTERNATIONAL AIRPORT

Import authorized from **2015/03/10** TO **2016/03/10**

IMPORTANT : This permit does not exempt the holder from the provisions of any other Act, ordinance or agr

DEPARTMENT OF AGRICULTURE
FORESTRY AND FISHERIES
DIRECTORATE PLANT HEALTH
2015 -03- 11
DIRECTORATE PLANT HEALTH
P.O. BOX 40024, ARCADIA, 0007

.....
Date

.....
Executive Officer

Reference Number **9/14/309**

INQUIRIES : TEL.: (012)319 6102 (Solomon Matsa) **FAX: (012)319 6370**

Appendix B

How to make agar medium A

Materials needed

- Distilled water (1000 ml)
- Beaker /glass bottle- blue cap bottle 1000ml
- Autoclave machine or pressure cooker for smaller quantities
- Scale
- pH meter
- Calibrator
- Weighing plate
- Agar powder
- Buffers (NaOH and HCl) 0.1M

Procedures

1. Clean the scale, making sure there are no substances on it; weigh an empty plate and tare or zero the scale with the plate
2. Measure 7g of agar powder and put it in a 1000ml blue cap beaker. Or 3.5 in 500ml
3. Add 1000 ml of distilled water
4. Blend/Stir it well with the help of a boiling stone
5. Start calibrating the solution to the right pH e.g. at 5.5 and 5.8 pH
6. Use NaOH (BLUE) 0.1 to increase the pH and HCl (RED) 0.1 to reduce the PH
7. Autoclave the solution at 120˚C for 60 minutes (20 minutes at 40 ˚C and 40 minutes at 60 ˚C).
8. Pour into Petri dishes and let it settle in a laminar flow unit.

Appendix C

1 Agar medium A (Sigma-Aldrich 1996)

					TYPICAL TRACE ELEMENT*				
PRODUCT	NAME DESCRIPTION	GELLING TEMP	~ pH at 1.5%	ASH CONTENT	Ca	Mg	K	P	Na
A1296	Agar Prepared from A7002 **Plant cell culture tested** **Microbiologically tested** **Cell Culture tested** A purified agar for which most research needs. Use at 6-12 g/L.	32-35°C	7.0-7.5	2-5%	0.30	0.10	0.01	0.01	0.50 (%)
A4550	Agar, Type A Prepared from A6549 **Plant cell culture tested** General purpose, good bacteriological grade agar. Use at 6-12 g/L.	26-28°C	7.2-7.7	5-6%	0.01	0.01	0.10	0.17	1.80 (%)
A4675	Agar, Type E Prepared form A6674 **Plant cell culture tested** General purpose agar. Use at 5-10 g/L.	26-28°C	7.5-8.0	3-4%	0.02	0.02	0.07	0.13	1.20 (%)
A4800	Agar, Type M **Plant cell culture tested** General purpose agar. Use at 5-11 g/L.	34-36°C	7.0-7.5	3-6%	0.09	0.14	0.07	0.01	1.40 (%)
A9799	Agar, High gel strength Prepared from A6924 **Plant cell culture tested** Use when firmer gel is required. Use at 4-8 g/L.	34-37°C	6.5-7.0	3-4%	0.03	0.00	0.07	0.09	0.72 (%)
A7921	Agar, Purified Prepared from A7049 **Plant cell culture tested** High purity agar for research and protoplast culture. Use at 6-12 g/L.	30-35°C	6.5-7.0	2.0%	0.02	0.01	0.01	0.01	0.35 (%)
A8678	Agar, Washed **Plant cell culture tested**	25-27°C	7.0-7.5	2.2%	0.15	0.08	N/A	N/A	0.38 (%)

* Done by ICP (Inductively coupled plasma)

2. The growth regulators used:

2.1 Auxins, Indole-3-butyric acid (IBA)

Product Name	Product No.	Molar Equivalence		Solution Preparation					
		Mol. Wt.	µM for 1mg/L	Solvent	Diluent	Powder Storage	Liquid Storage	Steriliz-ation*	Working Conc. (mg/L)
p-Chlorophenoxyacetic acid (4-CPA)	C0413	186.6	5.36	EtOH	—	RT	2-8°C	CA	0.1-10.0
2,4-Dichlorophenoxyacetic acid	D7299	221	4.53	—	—	RT	2-8°C	CA	0.01-6.0
2,4-Dichlorophenoxyacetic acid Sodium salt	D6679	243	4.12	Water	—	RT	2-8°C	CA	0.01-6.0
Indole-3-acetic acid Free acid (IAA)	I2886	175.2	5.71	EtOH/1N NaOH	Water	-0°C	-0°C	CA/F	0.01-3.0
Indole-3-acetic acid Sodium salt	I5148	197.2	5.07	Water	Water	2-8°C	-0°C	CA/F	0.01-3.0
Indole-3-acetic acid methyl ester	I9770	189.2	5.29	—	—	2-8°C	2-8°C	—	—
Indole-3-acetyl-L-aspartic acid	I9387	290.3	3.45	0.5N NaOH	Water	-0°C	-0°C	F	0.01-5.0
Indole-3-butyric acid (IBA)	I5386	203.2	4.90	EtOH/1N NaOH	Water	2-8°C	-0°C	CA/F	0.1-10.0
Indole-3-butyric acid Potassium salt (K-IBA)	I7512	241.3	4.14	Water	—	2-8°C	-0°C	CA/F	0.1-10.0
alpha-Naphthaleneacetic acid Free acid (NAA)	N0640	186.2	5.37	1N NaOH	Water	RT	2-8°C	CA	0.1-10.0
beta-Naphthoxyacetic acid Free acid (NOA)	N3019	202.2	4.95	1N NaOH	Water	RT	2-8°C	CA	0.1-10.0
Phenylacetic acid (PAA)	P6061	136.2	7.34	EtOH	—	RT	2-8°C	CA/F	0.1-50.0
Picloram	P5575	241.5	4.14	DMSO	—	RT	2-8°C	CA	0.01-10.0
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	T5785	255.5	3.91	EtOH	—	RT	2-8°C	CA	0.01-5.0
2,3,5-Triiodobenzoic acid Free acid (TIBA)	T5910	499.8	2.00	1N NaOH	Water	-0°C	-0°C	F	0.05-5.0

***CA** = coautoclavable with other media components. **F** = filter sterlize. **CA/F** = coautoclavable with other media components, however, some loss of activity may occur. This can be compensated for by increasing component concentration. Component may be filter sterilized.

2.2 Cytokinins – Kinetin

Product Name	Product No.	Molar Equivalence		Solution Preparation					
		Mol. Wt.	µM for 1mg/L	Solvent	Diluent	Powder Storage	Liquid Storage	Steriliz-ation*	Working Conc. (mg/L)
Adenine Free base	A5665	135.1	7.40	1.0 HCl	Water	RT	2-8°C	CA	50-250
Adenine hemisulfate Hemisulfate salt	A2545	184.2	5.43	Water	—	RT	2-8°C	CA	50-250
6-Benzylaminopurine (BA)	B3408	225.3	4.44	1N NaOH	Water	RT	2-8°C	CA/F	0.1-5.0
6-Benzylaminopurine Hydrochloride	B5920	261.7	3.82	Water	—	RT	2-8°C	CA/F	0.1-5.0
6-Benzylaminopurine (BA)	B3274	225.3	4.44	1N NaOH	Water	RT	2-8°C	CA/F	0.1-5.0
N-Benzyl-9-(2-tetrahydropyranyl)adenine (BPA)	B2275	309.4	3.23	EtOH	—	-0°C	-0°C	CA/F	0.1-5.0
N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU)	C2791	247.7	4.04	DMSO	—	2-8°C	2-8°C	F	0.001-1.0
6-(gamma,gamma-Dimethylallylamino)purine (2iP)	D7674	203.2	4.92	1N NaOH	Water	-0°C	-0°C	CA/F	1.0-30.0
6-(gamma,gamma-Dimethylallylamino)purine (2iP)	D5912	203.2	4.92	1N NaOH	Water	-0°C	-0°C	CA/F	1.0-30.0
1,3-Diphenylurea (DPU)	D7535	212.3	4.71	DMSO	—	RT	2-8°C	F	0.1-1.0
Kinetin	K0753	215.2	4.65	1N NaOH	Water	-0°C	-0°C	CA/F	0.1-5.0
Kinetin	K3378	215.2	4.65	1N NaOH	Water	-0°C	-0°C	CA/F	0.1-5.0
Kinetin	K3253	215.2	4.65	1N NaOH	Water	-0°C	-0°C	CA/F	0.1-5.0
Kinetin Hydrochloride	K1885	251.7	3.97	Water	—	-0°C	-0°C	CA/F	0.1-5.0
1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea	P6186	220.2	4.54	DMSO	—	RT	2-8°C	CA/F	0.001-0.05
trans-Zeatin Free base	Z0876	219.2	4.56	1N NaOH	Water	-0°C	-0°C	CA/F	0.01-5.0
Zeatin	Z0164	219.2	4.56	1N NaOH	Water	-0°C	-0°C	CA/F	0.01-5.0
trans-Zeatin Hydrochloride	Z2753	255.7	3.91	Water	—	-0°C	-0°C	CA/F	0.01-5.0
trans-Zeatin riboside	Z3541	351.4	2.85	1N NaOH	Water	-0°C	-0°C	F	0.01-5.0

*__CA__ = coautoclavable with other media components. __F__ = filter sterlize. __CA/F__ = coautoclavable with other media components, however, some loss of activity may occur. This can be compensated for by increasing component concentration. Component may be filter sterilized.

Appendix D

1. Surface sterilisation of buds

Materials used

- 1-3 cm Buds
- Liquid detergent (sunlight) or Tween
- Sodium hypochlorite (NaCIO)
- Ethanol 70%
- Bunsen burner (flame)
- Laminar flow unit
- Rotary shaker
- Agar medium

1. In both trials, plant materials (including shoots and new suckers) were collected and cut into pieces of length of approximately 1-3 cm. The explant piece included one internode with an enclosed developing bud in the axil of a leaf (**Figure 2.3).** The leaf was carefully removed from the internode, leaving the developing buds. Caution must be taken not to damage the buds.
2. In Trial 1, the internode was washed with autoclaved water and rinsed in a running distilled water at least 5 times. They were then air-dried in a laminar flow prior to inoculation in the agar medium. In Trial 2, explants were dipped in alcohol for 2 second and subjected to a flame for another 1 second before inoculated in the agar. While in trial 3, explants were dipped in a diluted (0.75 ml/l) sodium hypochlorite (NaCIO) containing two drops of detergent (sunlight). They were then incubated for 3 to 5 min on a rotary shaker while observing the colour change on the stock that may imply decay. Hand shaking the solution can also apply as an alternative the rotary shaker.
3. After chemical treatment in Trial 3, all explants were rinsed three times with sterile water for 2 min and air-dried in a laminar flow unit.
4. The buds were then recut at the base before inoculated in the petri dish filled with agar medium
5. Explants from the three trials were then incubated at 24-27°C, with a 12/12 day/night photoperiod, light intensity ~60 µmol m-2 s-1 arranged in a completely randomised design.

Growth parameters including shoot length and numbers and microbial contamination was recorded every three days for up to 10 weeks. Contaminated Petri dishes were then discarded.

2. Surface sterilisation of seed and embryos

Materials used

- Seeds
- Sunlight detergent
- NaClO
- Laminar flow unit
- Rotary shaker
- Agar medium

1. The seeds were soaked overnight (24 hrs) in warm water, depending on the sensitivity of the seeds coat, *S. cocculoides* can go up to 48 hours. This was done to soften the seed for easy embryo and seed coats removals.
2. In trial 1, seeds were subjected to a five times wash with sterile water. In trial 3, the seeds were immersed in a diluted sodium hydrochloride 0.75 ml/l sterile water with two drops of a sunlight detergent. The seeds were then shaken using the rotary shaker to make sure the solution have reached the surface area of the seeds for 3 minutes in *P. angolensis* and 5 minutes in *S. cocculoides* held on a rotary shaker.
3. Trial 3 seeds, were then removed and rinsed at least three times for 2 min and air-dried for another 2 minutes in the laminar flow unit.
4. The embryos from *P. angolensis* and *S. cocculoides* were carefully extracted from the seeds using sterile tweezers before inoculated on the agar. Separate seeds of *P. angolensis* had seed coats removed and (seed without coat) inoculated.

NB: Make sure your hands, the laminar flow surface and every other equipment you are using are clean and well sterilized.