TISSUE CULTURE OF *Moringa oleifera* Lam. AND DETERMINATION OF ANTIFUNGAL ACTIVITY OF ITS CALLUS EXTRACTS

By

OMWOMO KEVIN OCHIENG (Bsc, UON)

I56/81077/2015

Thesis submitted in partial fulfillment for the award of the degree of Master of Science in Genetics at the School of Biological Sciences

UNIVERSITY OF NAIROBI

JUNE 2020

DECLARATION

I make a declaration that this is my original work and that it has not been presented in any university or institution for a degree award.

Omwomo Kevin Ochieng Signature……

I56/81077/2015 Date……...

Supervisors' approval

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Dr. Nelson O. Amugune Signature……..

Senior Lecturer Date…….

School of Biological Sciences, University of Nairobi.

Prof. Sheila A. Okoth Signature…….

Associate Professor Date…….

School of Biological Sciences, University of Nairobi.

ACKNOWLEDGEMENTS

My acknowledgements go to my supervisors, Dr. Nelson Amugune, and Prof. Sheila Okoth for their support through advice, guidance, insights, criticism and encouragement during project conceptualization, implementation, and development of this work. I would also wish to express my gratitude to my classmates for the support during the study and also all the laboratory staff in the school of Biological Sciences especially Mr. Peter Waita for the support both in material form and socially.

DEDICATION

I dedicate this work to my lovely wife Susan and my son Bradley for the support they have given me so far and to the Almighty God for His sufficient grace so far.

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LIST OF ABBREVIATIONS AND ACRONYMS

WHO	World Health Organization
PKM-1	*Moringa oleifera* cultivar 1
PKM-2	*Moringa oleifera* cultivar 2
KEFRI	Kenya Forestry Research Institute
USAID	United States Agency for Intenational Development
KHDP	Kenya Horticultural Development Programme
UV	Ultraviolet
DNA	Deoxy-ribonucleic acid
2,4-D	2, 4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
KIN	Kinetin
IAA	Indole Acetic Acid
NAA	Naphthalene acetic acid
GA_3	Gibberellic acid
MS	Murashige and Skoog
IBA	Indole butyric acid
JIK	commercial bleach
TDZ	Thidiazuron
MTR	Metatopoline Ribonucleotide
PDA	Potato Dextrose Agar

SDA	Sabouraud Dextrose Agar
DCM	Di-chloro methane
KEMRI	Kenya Medical Research Institute
DMSO	Dimethyl sulfoxide
MIC	Minimum Inhibitory Concentration
ANOVA	Analysis of variance

ABSTRACT

Moringa oleifera is both a tropical and sub-tropical tree with medicinal and nutritional properties valuable to humans. However, the tree is under threat due to drought and excessive harvesting of its parts (seeds, bark, leaves and pods) for food and medicine. Therefore there is need to explore alternative propagation methods to cope with increasing demand. *In vitro* propagation is therefore significant for rapid and enhanced production of planting materials free from diseases and for production of secondary compounds. This study aimed at regenerating *Moringa oleifera in vitro* and determining if its callus extracts can inhibit fungal growth. Dehusked and intact seeds were sterilized in 30% commercial bleach (JIK) for 15 minutess followed by 70% ethanol for 2 minutes and rinsed 3 times in sterile distilled water. The seeds were inoculated on Murashige and Skoog, (1962) (MS) media and half MS media and MS supplemented with 0.5, 2.5, 4.5 and 6.5 mg/l Gibberellic acid (GA_3) to germinate. Callus was generated from inter nodal segments, leaf discs and hypocotyls of *in vitro* germinated seedlings on MS supplemented with 0.25, 0.5, 1.0 and 2 mg/l 2,4 di-chloro phenoxyacetic acid (2,4D), naphthalene acetic acid (NAA), thidiazuron (TDZ) or benzyl amino purine (BAP). Callus was subcultured to MS medium supplemented with different concentrations of BAP, NAA, metatopoline ribonucleotide (MTR), TDZ and kinetin (KIN) or their combinations to obtain shoots through somatic embryogenesis. Axillary shoots were induced from nodal explants on MS media supplemented with 0.1, 0.5, 1.0, 2.0 and 3 mg/l BAP, NAA, TDZ, MTR or KIN singly or their combinations. Micro shoots were rooted on MS basal or MS supplemented with 0.01, 0.1, 0.5, 1.0 and 2 mg/l NAA, BAP or MTR. The rooted shoots were then acclimatized and hardened on a mixture of vermiculite and peat moss (1:3) in the glass house. Callus and leaf extracts were tested against *Candida albicans*, *Aspergillus flavus* and *Fusarium semitectum*. The results were then subjected to two way analysis of variance (ANOVA) and means separated by Turkey's HSD test at $p \leq 0.05$. Intact seeds on either MS or half MS medium germinated within 8-14 days. Seeds with seed coats removed started germinating 3 days post inoculation. Over 90% of seeds without seed coats germinated, while less than 25% of GA_3 treated intact seeds germinated in 5 days. Callus induction was optimum at 0.5 mg/l 2,4D, giving friable callus in between 4 to 6 days in hypocotyls, inter-nodal segments and leaf discs. Callus from hypocotyls gave the most weight (12 g) on NAA 1 mg/l after 4 weeks. Callus regeneration on MS basal, or MS supplemented with BAP, KIN, NAA or MTR singly or combined developed only green spots or shoot like structures on the surface and later turned brown and died after 14-28 days. Metatopoline ribonucleotide 1.0 mg/l initiated 9.3 shoots from nodal explants in 3 days which increased to 11.3 shoots when combined with 0.5 mg/l KIN. Best rooting occurred on MS basal with 6 roots on average per explant compared to MS supplemented with NAA, BAP or MTR. On hardening and acclimatization 55% of the total plants survived but after 4 months of growth in the nursery dried out. Extracts from callus and leaves of field grown plants inhibited fungal growth with a Minimum Inhibitory Concentrations of 62.5mg/l on *Fusarium semitectum* and 125mg/l on *Aspergillus flavus* but could not inhibit *Candida albicans* growth. This study has demonstrated the regeneration potential of *Moringa oleifera* and the antifungal activity of the callus extract.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information to the study

Moringa oleifera Lam. originated from sub-Himalayan areas of Northern India, Pakistan and Bangladesh (Shahzad *et al*., 2013). World Health Organization (WHO) recognises *Moringa oleifera* tree as important mostly because it has 18 out of the available 20 amino acids required by the human body and also its seeds contain approximately 30-42 % oil content (Oriabi, 2016). In the developing world, the tree is considered a vegetable, medicine and source of oil (Bennett *et al*., 2003).

Moringa oleifera roots, barks, seeds, leaves and immature pods are considered as having circulatory and cardiac stimulants.They also possess anti-inflammatory, antihypertensive, antioxidant and antimicrobial activities (Kumar *et al*., 2010), root-bark yields alkaloids, moringine and moringinine (Anwar *et al*., 2007). Tender leaves are eaten when cooked or used in salads and soup as they contain vitamins A, B and C and also contain potassium, calcium and sulphur containing amino acids (Foidl *et al*., 2008). The by-products obtained during oil extraction from *Moringa oleifera* seeds contain high protein levels that can neutralize colloids in dirty water as they are negatively charged. Therefore they can be used in water purification, coffee fermentation (Garde *et al.*, 2017).

Moringa oleifera is the mostly propagated of the *Moringaceae* family with its cultural conditions known (Sanchez *et al*., 2006), and the tree is today found in both tropical and sub-tropical regions (Palada, 1996).

Moringa oleifera tree was introduced in Kenya more than 100 years ago, by coolies who constructed the Kenya-Uganda railway, and is common in North Eastern parts of Kenya in areas like Garissa, Wajir and Moyale (Tsaknis *et al*., 1999), but exist in patches and does not regenerate itself, probably because of fungal infections, pests, excessive drought and over harvesting by residents. Commonly the tree is known as moringa, drumstick tree, horseradish tree, ben oil tree (Parotta, 1993).

Due to drought and excessive harvesting, *Moringa oleifera* tree requires conservation from ethno-botanical, pharmacological and dietary perspectives because of its use for medicinal and nutritional purposes (Stephenson and Fahey, 2004). Propagation is usually through seeds or cuttings which mutilates the plant and can lead to its "death" (Nouman *et al*., 2012).

Tissue culture can therefore be exploited for enhanced production of planting materials that are free from diseases and also manipulated environment of *in vitro* cultures may lead to enhanced synthesis of bioactive compounds.

1.2 Statement of the problem

Although *Moringa oleifera* seems to hold a lot in aiding hunger, malnutrition and disease alleviation, the tree is under threat because of changing weather conditions and excessive harvesting of its parts for medicinal and nutritional values (Lewis, 2003; Nouman *et al*., 2012). Mass multiplication of disease free planting materials is a general problem in *Moringa oleifera*

propagation as its seeds are harvested before maturity for medicine and nutrition hence little is available for planting and also not enough cuttings are available for planting as the tree is not commercially cultivated in Kenya. *Moringa oleifera* seeds do not exhibit dormancy but their germination is delayed as if they were dormant may be because of the presence of the seed coat (Albrecht, 1993; Msanga, 1998). Also minimal data on *Moringa* species rate of exploitation in Kenya is available, therefore its natural habitats are disappearing and conservation abilities of this tree in Kenya is poor (Phiri and Mbewe, 2010).

1.3 Justification of the study

Moringa oleifera crude extracts have been shown to have antimicrobial activity (antifungal and antibacterial) leading to over exploitation. There is therefore need to explore alternative propagation methods to cope with increasing demand. *In vitro* propagation is significant for rapid and mass production of clones that can also benefit farmers with disease free planting materials and also help minimize dependence on seeds and vegetative cuttings as only sources of planting materials. In addition to developing an *in vitro* propagation protocol, this study also tested if extracts from leaf callus material can exhibit antifungal activity. Three important and widely occurring fungal species were used as representatives; *Aspergillus flavus* and *Fusarium semitectum* which are plant pathogens of agricultural importance and *Candida albicans* which is a human pathogen. *In vitro* derived materials e.g callus from various *Moringa* explants have been previously shown to allow for the enhanced synthesis of targeted secondary compounds through *in vitro* elicitation (Khaliluev *et al*., 2014; Reshi *et al*., 2013; Sharma and Vashistha, 2015; Wu *et al*., 2013). Therefore callus cultures of *Moringa oleifera* can be employed in suspension cultures for generation of secondary compounds (Ochoa-Villarreal *et al*., 2016, Hellwig *et al*., 2004). *In*

vitro seed germination can also be employed to curb the delay in germination of *Moringa oleifera* seeds.

1.3 Objectives

1.3.1 General objective

To regenerate *Moringa oleifera* through tissue culture and to determine the antifungal activity of its callus extracts

1.3.2 Specific objectives

1. To germinate *Moringa oleifera* seeds in Murashige and Skoog basal media and media with Gibberellic acid

2. To determine generation of callus from leaf discs, inter-nodal segments and hypocotyls of *Moringa oleifera*

3. To obtain *Moringa oleifera* plantlets through somatic embryogenesis and organogenesis

4. To determine the antifungal action of *Moringa oleifera* callus extracts

1.4 Hypotheses

1. *Moringa oleifera* seeds can be germinated on Murashige and Skoog basal media and media with Gibberellic acid.

2. Callus can be generated from leaf discs, inter-nodal segments and hypocotyls of *Moringa oleifera*.

3. *Moringa oleifera* plantlets can be regenerated through somatic embryogenesis and organogenesis

4. *Moringa oleifera* callus extract exhibit antifungal activity.

1.5 Research questions

1. Can *Moringa oleifera* seeds be germinated on Murashige and Skoog basal media and media with Gibberellic acid?

2. Can callus be generated from leaf discs, inter-nodal segments and hypocotyls of *Moringa oleifera*?

3. Can *Moringa oleifera* plantlets be regenerated through somatic embryogenesis and organogenesis?

4. Does *Moringa oleifera* callus extract contain antifungal activity?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin, distribution and habitat of *Moringa oleifera*

Moringa oleifera tree originated from sub-Himalayan parts of Northern India, Pakistan and Bangladesh but today it is also found in both tropical and sub-tropical regions including India, Sudan, Philippines, Latin America, South Africa, tropical Asia and Pacific islands (Palada, 1996). In Kenya the tree was introduced more than 100 years ago, by coolies who constructed the Kenya-Uganda railway, and is common in North Eastern areas of Kenya from Wajir, Garissa and Moyale but has spread and today is found along the lake regions, Central Kenya and parts of the lower Eastern (Tsaknis *et al*., 1999).

The tree has no preference to a specific habitat, but is favored in a hot and a humid region hence is common in several ranges of ecological zones from savannas to the rainforests. It prefers altitudes lower than 600 m, but in protected zones it can tolerate altitudes of up to 2000 m; (Palada, 1996). *Moringa* trees require annual rainfall from 1000 mm to 2000 mm because of their tuberous taproots (Radovich, 2011). The tree tolerates light frost but can be injured at -5°C and below. The *Moringa oleifera* tree survives in most of the soil types but with pH ranging from 4.5-9 and therefore mostly prefers alluvial and sandy soils but vulnerable to water logging therefore requires adequate drainage (Foidl *et al*., 2001; National Research Council, 2006).

2.2 Taxonomy of *Moringa oleifera*

Moringa oleifera is the most cultivated species in the *Moringacea* family but other species in the genera include *Moringa stenopetala, Moringa arborea, Moringa longituba, Moringa drouhardii, Moringa ovalifolia, Moringa borziana, Moringa pygmaea, Moringa ruspoliana, Moringa rivae, Moringa peregrine, Moringa concanensis* and *Moringa hildebrandtii* (Mahmood *et al*., 2010). *Moringa borziana, Moringa arborea, Moringa longituba, Moringa stenoptella* and *Moringa rivae* are indigenous to Kenya.

Most of the cultivars of *Moringa oleifera* are developed in India for the purposes of improving the production of pods, fruits and leaves. The varieties are broadly classified into perennial and annual types. Perennial types like Moolanur and Valayapatti are older but take long before maturity of pods. Annual types include PKM-1 and PKM-2 which have been developed recently through plant breeding research and are seed propagated, rapid in maturation and produce high yields (Agriculture Forum, 2012). Other varieties of *Moringa oleifera* in India include Coimbator-1 and 2, Durga, Chem *Moringa*, and Anupama, but little or no research has been done in other parts of the world (Agriculture Forum, 2012). Other few varieties available from other regions include Chavakacheri and Chemmrungai (Srilanka), Congo-Brazzaville (Democratic Republic of Congo), Malawi (Malawi) and Mbololo which was developed by Kenya Forestry Research Institute (KEFRI) (Nzikou *et al*., 2009; Lalas and Tsaknis, 2002; Tsaknis *et al.*, 1999).

2.3 Cultivation, growth and development of *Moringa oleifera*

Moringa oleifera tree is threatened and can be endangered due to its many uses and weather hanges and therefore there is need for its genetic conservation for biodiversity, ethno-botanical,

dietary and pharmacological perspectives (Stephenson and Fahey, 2004). Propagation is usually through cuttings of 1-2 m long shoots or through seeds (Nouman *et al*., 2012). The tree grows fast and is deciduous and is able to grow upto a height of 12 m with a trunk reaching 45 cm in diameter (Parotta, 1993). It has a whitish to grey bark surrounded with thick cork. The droop is an open crown and branches are fragile and the leaves build up feathery foliage and are tri-pinnate. Flowers are bisexual and with fragrance occurring 4-12 months after planting while fruits are hanging, brown capsules with three sides and measuring 20–45 cm in size and with dark brown, globular seeds 1 cm in diameter. Seeds contain three whitish papery wings and the mode of dispersion is wind and water (Plant Resources of Tropical Africa, 2013).

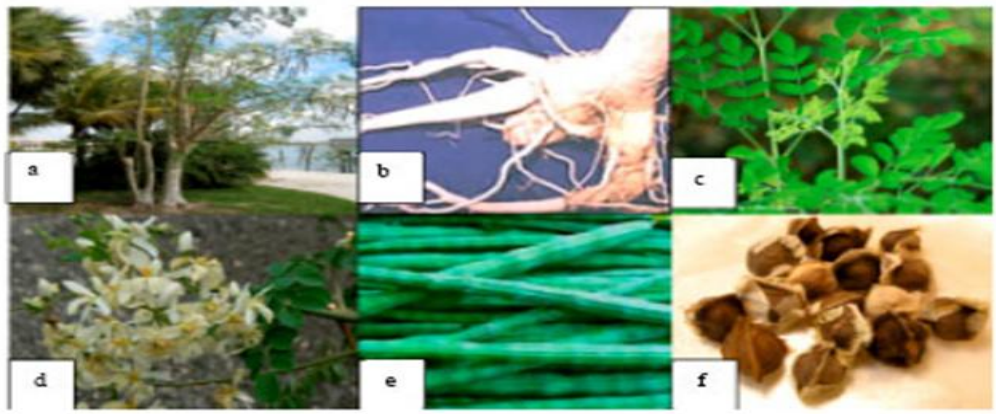

Figure 1: *Moringa oleifera* (a) mature tree (b) roots (c) leaves (d) flowers (e) pods and (f) seeds (Source: Plant Resources of Tropical Africa, 2013)

2.4 Uses of *Moringa oleifera*

Moringa oleifera despite being known by the old world was just recently "rediscovered" as a potential multipurpose tree. It is among the most utilized tropical trees and World Health Organization (WHO) considers it important because it contains 18 out of 20 amino acids

required by the human body and its seeds contain between 30-40% oil content (Foidl *et al*., 2001; Oriabi, 2016).

2.4.1 *Moringa oleifera* as source of food and water purifier

Young leaves and green pods of *Moringa oleifera* can be cooked and eaten, used in making salads and soup due to their high levels of vitamins A, B and C, Iron and proteins. *Moringa oleifera* leaves are also used as cattle feed because of their rich nutritional characteristics and therefore increase milk production (Fuglie, 2000; Fahey, 2005).

The de-hulled seeds of *Moringa oleifera* have approximately 42 % oil content which has lubrication property for machinery, as well as cooking and stabilizing of scents in perfume industry and production of biofuel (Rashid *et al.*, 2008).

By-products obtained during *Moringa oleifera* seed oil extraction contain high levels of proteins which are cationic polyelectrolytes which can be used to neutralize colloids in water. These proteins can therefore work as primary coagulants in beer and juice industries and as organic particles in drinking water purification, or for coffee fermentation (Garde *et al*., 2017). Studies using *Moringa oleifera* seeds as a water purifier have shown both the removal of solid contaminants and also the reduction of harmful bacteria. A study at the University of Newcastle, United Kingdom documented that *Moringa oleifera* seeds also reduce water hardness to between 60-70% in addition to purification but after coagulation and two hours of settling (Sani, 1990).

2.4.2 *Moringa oleifera* as a plant growth enhancer

Moringa oleifera leaves extracted in 80 % ethanol are considered to contain hormones of the

cytokinin type. This extract may be sprayed to plants to enhance their growth. This can also lead to the plants being more resistant to diseases and pests and also become more firm. Plants treated using this growth hormone can also produce larger and more fruits, therefore leading to higher yields (Makkar and Becker, 1996).

2.4.3 *Moringa oleifera* as a medicinal plant

Moringa oleifera tree parts including bark, roots, fruits, seeds, flowers, leaves and immature pods are considered having medicinal activities and are used to treat rheumatism and ascites. They also act as cardiac stimulants, have anti-inflammatory, antitumour, anti-hypertensive, antidiabetic antioxidant and antimicrobial activities (Kumar *et al*., 2010). They therefore treat inflammations, bronchitis, urinary discharges, obstinate asthma, eye conditions, and enlargement of the spleen, head complications, Leucoderma and pain (Nath *et al*., 1992).

2.5 *Moringa oleifera* commercial production

Rajangam *et al*. (2001) describes India as leading in production of *Moringa oleifera* worldwide with 1.1 to 1.3 million tonnes of fruits produced annually. Production of *Moringa oleifera* in Africa is still very low, and the size of land under production and the volume of the trees cannot be quantified. However there is some functioning farming systems in Nigeria, South Africa, Kenya, Ethiopia and other African countries influenced by the growing demand for *Moringa* products including oil and leaf powder by consumers in developed and emerging economies (Saint Sauveur, 2001).

In Kenya, Earth Oil Kenya, an oil processing company situated in Athi River was importing 90% of the *Moringa oleifera* seeds from Uganda and Tanzania by 2003. Today the company is partnering with the local farmers to grow *Moringa oleifera* for seed production. The current production of 50 metric tonnes monthly is only about 30% of requirements. This still leaves a shortfall of 70% (600 tonnes) per year of which is imported from the neighbouring countries therefore there is still potential for farmers to cultivate this tree (http://www.new-ag.info/en/focus/focusItem.php?a=454). The United States Agency for International Development (USAID), is also working with the Kenya Horticultural Development Programme (KHDP) offering extension and market information to *Moringa* farmers in Kenya to promote its cultivation (http://www.newag.info/en/focus/focusItem.php?a=454). In South Africa, *Moringa* was introduced in 2006 to rural areas by the *Moringa* project called Lammangata, and only three provinces are engaged in production and this only occurs in poverty and malnutrition affected areas e.g Kwa Zulu-Natal, Limpopo and Mpumalanga and here *Moringa* is mainly grown by households (van Jaarsveld, 2006).

2.6 Antimicrobial activity of *Moringa oleifera*

In Africa phyto-medicines have been used even long before modern medicine in treatment of various diseases. Herbal medicines are today widely in use worldwide especially in areas with high cost and no access to modern medicine and in recent years plant use to obtain vital compounds in combating microbial infections is prominent because of high cost, reduction in efficacy and increasing microbial resistance to conventional medicine (Ajibade *et al*., 2005). Synthetic fungicides are expensive and also leads to adverse environmental effects, therefore environmentally friendly natural products are being explored to replace these hazardous

chemicals (El–Mohamedy and Aboelfetoh, 2014). Therefore there is need for research to determine the potential activity of secondary compounds in medicinal plants as this may promote the use of natural products (Adde-Mensah, 1992). One such plant, *Moringa oleifera*, has intensively received the attention of researchers worldwide because of its wide pharmacological activities.

Moringa oleifera is known to contain several phytochemicals including tannins, anthocyanins, flavonoids, vitamins, alkaloids, carotenoids, polyphenols, phenolic acids, glucosinolates and saponins. But pharmaceutical benefits of its different pharmacology and toxicity profiles are not yet fully exhausted (Bose, 2007). Methanol, water, hexane and ethanol *Moringa oleifera* leaf extracts have demonstrated the ability to contain *in vitro* antimicrobial activities against several bacteria and fungi including *E.coli, Pseudomonas, Aspergillus, Salmonella, Streptobacillus, Penicillium notatum, Streptococcus, Candida albicans and Staphylococcus* (Singh *et al.*, 2006).

2.6.1 Human and animal diseases

Fungi and bacteria are among the microbes of veterinary and human importance known to cause diseases. *Bacillus cereus* causes food-borne infections (Granum and Lund, 1997) while *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* cause illnesses including upper respiratory infections and mastitis (Masika and Afolayan, 2002). *Aspergillus niger* causes lung infections while *Aspergillus flavus* is associated with lung aspergillosis, corneal, otomycotic and nasoorbital infections in humans and livestock. *Fusarium semitectum* causes locally invasive, superficial or disseminated infections in humans and also allergic diseases in immuno-compromised people (Nucci and Anaissie, 2002; Wickern, 1993) and mycotoxicosis due to

ingestion of toxin contaminated food by humans and animals (Nelson *et al*., 1994). *Candida albicans* is an opportunistic fungus growing both as yeast and filamentous. The fungi is the common cause of oral and genital infections in immuno-compromised humans and its biofilms are commonly found forming on medical devices surfaces leading to hospital acquired infections (Ryan and Ray, 2004).

Bennett *et al*. (2003) reported that an active compound 4α L-rhamnosyloxy-benzyl isothiocyanate was isolated from seeds of *Moringa oleifera* and has been identified as being an antimicrobial agent. Galeotti *et al.* (2008) demonstrated growth inhibition of *Candida albicans* using *Moringa oleifera* hexane seed extract and corroborated that this might be because of the high flavonoid content in the extract. Ncube *et al.* (2008) used methanol to extract saponins from *Moringa oleifera* and reported they contained antimicrobial activity.

2.6.2 Plant pathogens

Aspergillus flavus also causes problems in plants, stored grains, fabrics and other materials. It produces aflatoxin enough to cause aflatoxicosis (Samson *et al*., 2001; Klich, 2007). The fungi belongs to phylum *Ascomycota*, class *Eurotiomycetes*, order *Eurotiales* and when cultured gives greenish colour in cultures with a distinct conidiophore and a long stalk which supports an inflated vesicle.

Fusarium a plant pathogenic fungus causes infections in both plants and humans. It's in the family Nectriaceae of the order Hypocreales. *Fusarium semitectum* is known to cause root rots, wilts, cankers and blights in plants. Its colonies are floccose with dense aerial mycelium that canturn to brown. It has septate hyphae which is hyaline and with simple or branched conidiophores, and with sparse or absent micro-conidia (Sutton *et al*., 1998).

Fungicidal ability of *Moringa oleifera* extracts have been tested on a number of fungi such as *Fusarium, Rhizoctonia* and *Pythium* spp (Moyo *et al*., 2012). *Moringa oleifera* methanol extracts (75 % v/v) significantly inhibits mycelial growth of *Fusarium solani* (Dwivedi and Enespa, 2012). The tree is rich in rare combinations of quercetin, zeatin, kaempferol and b-sitsterol, which contains antifungal activities (Anjorin *et al*., 2010).

Aqueous extracts of seeds of *Moringa oleifera* have been tested as bio fungicides (Adandonon *et al*., 2006). Antifungal activity of *Moringa oleifera* against *Aspergillus niger* and *Mucar mucedo* was more strong than on *Rhizoctonia solani and Aspergillus tamari* (Jamil *et al*., 2007). Boyd and Beveridge (1981) and Huang *et al*. (2000) also concluded that antifungal activity of crude extracts of *Moringa oleifera* plant parts could be because of the presence of lipophilic compounds which binds onto the cytoplasmic membrane, affecting growth of filamentous fungi by membrane permeability.

Moringa oleifera Callus extract on the other side has never been explored in such assays to determine their potential in harboring bioactive compounds important in eliminating pathogenic microbes, and they may also contain active compounds like those from native plants, hence the need for the study. Callus extracts has been employed in some other areas, Oriabi (2016) reported that ethanolic callus extract significantly reduced level of blood glucose of the treated albino mice. Lalida *et al*. (2013) reported that *Moringa oleifera* callus cultures had higher peroxidase activity than that obtained from native plants. Djande *et al*. (2019) demonstrated

differential influence of kinetin and 2,4-D on callus cultures of *Moringa oleifera* for secondary metabolites production.

2.7 *Moringa oleifera* genetics and breeding

Moringa oleifera tree is a true diploid that possess a 2n = 28 chromosome number, but its genetic diversity pattern important for successful breeding and improvement is not yet well established (Mgendi *et al*., 2010). Developing DNA markers of medicinally important plants can help generate molecular data base important in utilizing the information systematically (Khanuja *et al*., 2000). These markers are capable of detecting polymorphisms which are beneficial in improving the efficiency and precision in conventional breeding (Sarikamis, 2010). Seven *Moringa oleifera* populations in Kenya showed high levels of differentiation under amplified fragment length polymorphism (AFLP) (Muluvi *et al*., 1999). In Eastern parts of the world where *Moringa oleifera* most likely originated, wild species have large diversity (Raja *et al*., 2013), which is useful in breeding programs. In other regions especially Africa, where *Moringa oleifera* is not native, diversity is minimal among the cultivars. There are different breeding aims for different *Moringa oleifera* farmers as per the different uses of the tree and these results in the selection criteria in various regions. In India, *Moringa oleifera* is cultivated as an annual tree for large production of pods and dwarf or semi-dwarf varieties are preferred. Genetic erosion is much smaller with perennial cultivation (Raja *et al*., 2013). In Pakistan, *Moringa oleifera* breeders were selecting on varieties based on the nutritional composition of their leaves in different locations (Iqbal and Bhanger, 2006).

2.8 Tissue culture of *Moringa* spp

Tissue culture of plants generally is the aseptic culture of tissues or cells or organs in known chemical and physical conditions (*in vitro*) that depends on the cells self-regeneration ability. It is helpful in micro-propagation, genetic conservation, understanding of genes and conservation which helps in improvement of plants through transgenic technology (Jaskani *et al*., 2008). *Moringa* is unexplored group of plants with nutritional and pharmaceutical potential and can be conserved and improved through tissue culture (Sanchez *et al*., 2006). Only *Moringa oleifera* cultural practices and requirements have been widely developed within the genera (Sanchez *et al*., 2006). Islam *et al*. (2005) obtained regeneration response in nodal explants of *Moringa oleifera* using 1.0 and 1.5 mg/l BAP and also found that hypocotyls were highly responsive to callusing. Hussain *et al*. (2010) reported that callus induction in *Moringa oleifera* explants increased with increased 2,4-D concentration. Low BAP and Kinetin concentrations (below 0.5 mg/l) produced more shoots in *Moringa oleifera* (Riyathong, 2010; Abdellatef and Khalafallah, 2010). Khalafallah *et al*. (2011) also demonstrated that low 2,4-D concentration (below 0.5 mg/l) was efficient in inducing callus from *Moringa oleifera* leaves. Nieves and Aspuria (2011) initiated callus from cotyledons of *Moringa oleifera* in 14 -28 days on 1, 2.5 and 5 mg/l 2,4-D. Shank *et al.* (2013) obtained optimal callusing from *Moringa oleifera* explants on MS with 0.5 mg/l 2,4D. Lalida *et al*. (2013) also obtained 100% callus induction from *Moringa oleifera* explants on 0.5 mg/l 2,4-D and reported that this was the optimal concentration.

Shahzad *et al*. (2014) obtained callus from *Moringa oleifera* explants in 2-4 weeks in all the concentrations (0.1- 2.0 mg/l) of IAA and NAA used. They also found that shoot regeneration from *Moringa oleifera* explants is highly depended on type and level of growth regulators.

Umbreen *et al*. (2014) obtained good callusing in cotyledons of *Moringa oleifera* on 2,4-D in 6 days and reported that hypocotyls responded well to callusing in 7 days but leaf explants gave callus in 10 days. Fatima and Perveen (2016) reported that leaf explants of *Moringa oleifera* formed callus at the edge of the explant (part of the injury) that is directly in contact with the medium. Zulaliya (2017) reported fewer shoots per explants on 2.0 mg/l BAP than on 0.25 mg/l BAP.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental design

Tissue culture experiments were carried out in the tissue culture laboratory at the School of Biological Sciences, University of Nairobi. Antifungal assays were carried out in the mycology laboratory, Centre for Respiratory Diseases Research, Kenya Medical Research Institute (KEMRI) Nairobi. All the antifungal experiments were replicated three times while in tissue culture, experiments were replicated six times with five explants in each replicate. The inoculated jars and plates were randomly arranged in the culture shelves and incubator respectively. All the experiments were carried out inside the safety hood to maintain sterile conditions.

3.2 Culture media preparation

Murashige and Skoog (1962) (MS) media consisting of macronutrients, micronutrients, vitamins, inositol, sucrose and agar was used for tissue culture (Appendix 1). In fungal study, Potato Dextrose Agar (PDA) consisting of potato starch, dextrose and agar (Appendix 2) and Sabouraud Dextrose Agar (SDA) consisting of mycological peptone, dextrose and agar (Appendix 3) were used. One litre of Murashige and Skoog basal media was constituted in a beaker each time (hormones were added according to the experiment), 30 ml of the constituted media was poured into each jar, autoclaved at 121°C, 15 psi for 15 minutes and kept in refrigerator at 4 °C ready for use. One litre each of PDA (39 g/l) and SDA (65 g/l) was prepared in media bottles and autoclaved at 121°C, 15 psi for 15 minutes. Sterilized media was poured into Petri dishes (10 ml) inside the safety hood, left to cool, covered and kept in a refrigerator at 4 °C ready for use.

3.3 Explant preparation and sterilization

Certified seeds of *Moringa oleifera* were obtained from seed centre, Kenya Forestry Research Institute (KEFRI) Muguga. The seeds were brought to the laboratory and stored at room temp until when needed. Seeds were used either in intact form (with seed coat) or with seed coat removed. Seed coats were removed by use of a scapel blade. The procedure involved cutting off one edge followed by splitting of the husk open followed by surface sterilization. Intact seeds were surface sterilised when whole. Surface sterilization of the seeds was evaluated using different concentrations of commercial bleach (JIK). In one experiment, 10% (v/v) commercial bleach was used for 10, 15 or 20 minutes followed by ethanol 70% (v/v) for two minutes. The same was repeated using 20% and 30% (v/v) commercial bleach for 10, 15 and 20 minutes followed by ethanol 70% (v/v) for two minutes respectively inside safety hood, and rinsed three times with sterile distilled water after each wash with sterilant.

3.4 Culture conditions

During seed germination, callus initiation and regeneration, jars inoculated with explants were covered with cling film and kept in a growth room at 26°±2 in light at 3000 lux provided by fluorescent tubes or in darkness for 4-6 weeks. For antifungal assays, plates were covered with parafilm and incubated at 30 °C for 48 hours.

3.5 *In vitro* seed germination

Intact seeds and seeds with seed coats removed were separately inoculated into jars with MS basal and MS half strength media. Each treatment consisted of 6 repliacates each with 5 seeds. To evaluate the effect of giberrellic acid on germination, only intact seeds were used.

Murashige and Skoog (1962) with Gibberellic acid (0.5, 2.5, 4.5 and 6.5 mg/l) was tested. The inoculated jars were labelled, sealed and darkness was achieved by wrapping the jars with aluminum foil. Inoculated jars were kept at 26 ± 2°C in a growth room for 4-6 weeks. Number of days to initiate germination and germination percentage was recorded. Also germination of seeds without seed coats and intact seeds were compared.

3.6 Generation of callus from hypocotyls, inter-nodal segments and leaf discs

One centimeter long of hypocotyls from a 5 day old germinating seed, one centimeter long inter-nodal segments and one centimeter square leaf sections excised from 4-6 weeks old plants of *Moringa oleifera* grown *in vitro* were used to generate callus. Explants were inoculated into jars with MS medium supplemented with (0.25, 0.5, 1.0 and 2.0 mg/l) of Naphthalene Acetic Acid (NAA), 2,4 di-chloro phenoxyacetic acid (2,4-D), Thidiazuron (TDZ) or Benzyl Amino Purine (BAP). Explants were also inoculated on MS basal medium (control). Each jar was inoculated with 6 explants and 5 replicates were used each. The inoculated jars were labeled, sealed and darkness was achieved by wrapping the jars with aluminum foil. Inoculated jars were kept at 26 ± 2°C in a growth room for 4-6 weeks. The number of days taken to generate callus, callus weight and effect of TDZ combined with 2,4D or NAA or BAP on weight of callus from hypocotyls were recorded. Sub-culturing of leaf calli onto fresh medium with same composition was done after every 4 weeks for calli proliferation and calli were maintained for 8 weeks for antifungal assay.

3.7 Obtaining *Moringa oleifera* plantlets from callus on different media

Approximately 1gm of 4-6 weeks old callus from hypocotyls, inter-nodal segments and leaf discs were each transferred to jars with MS basal medium and MS media supplemented with (0.5, 1.0,

2.0 and 3.0 mg/l) Kinetin (KIN), Benzyl Amino Purine (BAP), Metatopoline ribonucleotide (MTR), Naphthalene Acetic Acid (NAA) or their combinations to obtain plantlets. The jars were then wrapped in aluminum foil to provide dark conditions and then incubated at 26 ±2 °C in a growth room for 4-6 weeks. Number of shoots obtained from each treatment and days taken to obtain shoots were recorded.

The experiment was also done using MS with the following hormone combinations; 0.5 mg/l BAP plus 0.5 mg/l KIN; 1 mg/l BAP plus 1 mg/l KIN; 2 mg/l BAP plus 2 mg/l KIN and 3 mg/l BAP plus 3 mg/l KIN. In another experiment, NAA and BAP were combined as follows; 0.1 mg/l NAA plus 0.5 mg/l BAP; 0.5 mg/l NAA plus 1 mg/l BAP and 0.5 mg/l NAA plus 3 mg/l BAP. The effect of NAA combined with KIN at; 0.5 mg/l NAA plus 1 mg/l KIN, 0.5 mg/l NAA plus 2 mg/l KIN and 0.5 mg/l NAA plus 3 mg/l KIN was also tested. Also MTR combined with BAP or NAA or KIN was tested; 0.5 mg/l BAP plus 0.5 mg/l MTR, 0.5 mg/l BAP plus 1 mg/l MTR; 0.25 mg/l NAA plus 1 mg/l MTR, 1 mg/l MTR plus 0.5 mg/l NAA and 0.5 mg/l KIN plus 1 mg/l MTR, and 1 mg/l KIN plus 1 mg/l MTR). The inoculated jars were labeled, sealed and darkness was achieved by wrapping the jars with aluminum foil. Inoculated jars were kept at 26 ± 2°C in a growth room for 4-6 weeks. Each treatment comprised of 6 replicates each with 5 explants.

3.8 Obtaining *Moringa oleifera* plantlets from nodal explants on different media

Nodal segments of *Moringa oleifera* measuring 2 cm long were cut from 4-6 weeks old *in vitro* germinated seedlings and inoculated in jars with MS media supplemented with 0.1, 0.5, 1.0, 2.0 and 3 mg/l of BAP, NAA, TDZ, MTR or KIN. The segments were also inoculated into jars with MS basal medium (control). Also a combination of MTR with BAP, MTR with NAA, MTR with

TDZ and MTR with KIN at different concentrations (0.01 and 0.01, 0.5 and 0.1, 1.0 and 0.5, 2.0 and 1.0, 3.0 and 2.0 mg/l) was tested for shoot induction.

The inoculated jars were labeled, sealed with cling film and covered with aluminum foil to provide for dark conditions and then incubated in a growth room at 26 ±2 °C for 4-6 weeks for shoots to emerge. Each treatment comprised of six replicates each with 5 explants. Days to initiate shooting and number of shoots induced per explant were recorded.

3.9 Rooting of plantlets obtained from nodal explants

The 4 weeks old plantlets from nodal explants were excised and inoculated onto jars with MS media supplemented with 0.01, 0.1, 0.5, 1.0 and 2.0 mg/l of NAA, BAP or MTR for rooting. The plantlets were also inoculated onto jars with MS basal medium (control). Inoculated jars were incubated at 26 ±2 °C for 3-4 weeks and number of roots obtained recorded. Each treatment comprised of six replicates each with 5 explants.

3.10 Hardening and acclimatization of the rooted plantlets

The rooted planlets were transferred to jars with sterile vermiculite and left in the growth room for 2 weeks. They were later transferred to a mixture of sterile vermiculite and peat moss (1:3) in plastic pots respectively, covered with polythene and left under dim light in a growth room for 2 weeks. The pots were held on a tray where water was supplied from the holes in the bottom of the plastic containers. After 2 weeks the polythene was punctured to allow gradual exposure to the external environment. After the third week the polythene was withdrawn and the plants left in the growth room for another week and later put in the green house for acclimatization and later transferred to the soil.

3.11 Antifungal assay of the callus and leaf extracts

3.11.1 Preparation of the callus and leaf extracts

Eight weeks old calli generated from leaf discs were collected, dried at room temperature and ground with mortar and pestle to form powder. Callus powder (150g) was soaked in a mixture of methanol: Di-chloro methane (DCM) in the ratio 1:1 for 2-3 days and filtered with Whatman paper No (1). The compound was evaporated using Rota-vap to remove the solvent and concentrate the compound.

Leaves from *Moringa oleifera* trees growing in the fields were randomly collected, air dried at room temperature and ground on mortar and pestle to form powder. The leaf powder was also extracted in a mixture of methanol: Di-chloro methane (DCM) in the ratio 1:1 for 2-3 days and filtered with Whatman paper No (1). The compound was evaporated using Rota-vap for removing the solvent and compound obtained was concentrated. The residue was dried inside an oven at 45°C to prevent contamination and for complete evaporation. The extract was kept in sterile screw capped universal bottles and refrigerated ready for use.

3.11.2 Fungal isolation

Three fungi *Candida albicans*, *Aspergillus flavus* and *Fusarium semitectum* were used to test for the antifungal action of *Moringa oleifera* callus and leaf extracts. These are human and plant pathogens that cause damage and lose in animals and plants. The isolates were pure cultures obtained from the Mycology laboratory, Centre for Respiratory Diseases Research, Kenya Medical Research Institute (KEMRI).

They were maintained on Potato dextrose agar (PDA) at 4°C. The fungi were cultured on Petri plates with previously prepared PDA and plates kept at 30°C to reactivate the isolates. After the

reactivation, the inocula was made by scooping the conidia and/or hyphae from the plates using a sterile wire loop and then transferred to 1ml of sterile distilled water inside a universal bottle. The fungi was standardized to 0.5 Mc Farland standards by gradually adding normal saline to 1ml of the subculture and compared to theMc Farland standards.

3.11.3 Growth inhibition test of callus and leaf extracts

The fungal growth inhibition activity of the methanol: Di-chloro methane (1:1) leaf and callus extracts was performed using agar well diffusion technique. On already cooled SDA media on Petri dishes, the inoculum was spread using a sterile glass bent rod inside the safety hood. Four equidistant wells were made in the agar plates with sterile cork borers, two for the test extracts, one for 1% (DMSO) and one for 250mg/ml fluconazole (positive control).

One gram of the extracts were each dissolved in 1ml of 1% dimethyl sulfoxide (DMSO) in separate tubes, then serially diluted to obtain final concentrations of (80%, 60%, 40% and 20%). To the wells for the test extracts, 100 µL of the methanol: Di-chloro methane extract of the *Moringa oleifera* callus and leaf extract was introduced. Plates were kept for 48 hours at 30 ℃. Clear zones of inhibition were measured in millimeters to show relative susceptibility of the microorganisms to the extract.

The extract with an inhibition zone of less than 8 mm was described as inactive, that with 9-15 mm was described as being active while that with more than 16 mm was described as being very active. The fungi were categorized as Susceptible (S), Intermediate (M) and Resistant (R) based on the zones of inhibitions recorded. This was repeated three times and the mean of the triplicates results taken.

3.11.4 The Minimum Inhibitory Concentration test

One gram of callus and leaf extracts were dissolved in 1ml of 1% dimethyl sulfoxide (DMSO) in separate tubes, then serially diluted to obtain final concentrations of (80%, 60%, 40% and 20%). A mixture of 100 µl 1% DMSO, 100 µl of the extract and 10 µl of the inoculum in the dilution factor of 1:2 were spread onto the previously prepared SDA media using a sterile glass bent rod inside the safety hood and incubated for 72 hours at 30 ℃. The MIC of the extracts (mg/l) was determined by counting the colony forming units and recording plates with minimum concentrations of the extracts and recorded the lowest colony forming units.

CHAPTER FOUR

4.0 RESULTS

4.1 *In vitro Moringa oleifera* seed germination on Murashige and Skoog media

From preliminary results on sterilization, commercial bleach 10% and 20% (v/v) for 10 and 20 minutes followed by 70% ethanol for 2 minutes had contaminations in seeds with seed coats, but seeds without seed coats were sterile and germinated. Therefore complete sterilization of seeds was achieved in 30% (v/v) commercial bleach (JIK) for 15 minutes followed by ethanol 70% for 2 minutes and seeds inoculated on MS basal and MS half strength media.

Germination of intact seeds on MS basal medium from day 8, 9, 10, 11, 12, 13 and 14 had the following means; 5.00±0.42, 5.10±0.43, 7.40±0.51, 8.08±0.50, 10.20±0.61, 13.10±0.68 and 16.30±0.74 respectively. Germination was not observed on day 1, 2, 3, 4, 5, 6 and 7 (Table 1). Germination of seeds without seed coats on the other hand recorded the following means; 8.20±0.51, 17.20±0.61, 20.3±0.64, 25.1±0.69, 26.1±0.68, 26.1±0.68, 27.0±0.69, 29.4±0.70, 29.2±0.70, 29.3±0.70, 29.2±0.70 and 29.1±0.70 from day 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 respectively on MS basal medium. There was no germination observed on day 1 and 2 (Table 1).

On half MS media, the following results were obtained; intact seeds had the following means of germination; 5.07±0.40, 5.00±0.40, 7.10±0.43, 8.10±0.48, 10.20±0.52, 13.08±0.55 and 16.20±0.57 from day 8, 9, 10, 11, 12, 13 and 14 respectively. From day 1, 2, 3, 4, 5, 6 and 7 no germination was observed. On the other hand seeds with their seed coats removed recorded the following means of germination; 6.67±0.60, 17.2±0.73, 20.0±0.74, 25.0±0.76, 26.1±0.76, 26.1±0.76, 27.09±0.76, 29.00±0.78, 29.07±0.78, 29.10±0.78, 29.00±0.78 and 29.10±0.78 from

day 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 respectively on MS half strength medium. There was no germination observed on day 1 and 2 (Table 1).

Table 1: Number of *Moringa oleifera* intact seeds and seeds without seed coats germinated on MS and half MS media

Number of seeds germinated

Days	MS media		Half MS media	
	With seed coat	Without seed coat	With seed coat	Without seed coat
1	0	0	0	0
2	0	0	0	0
3	0	8.20±0.51i	0	6.67±0.60k
4	0	17.20±0.61e	0	17.2±0.73e
5	0	20.3±0.64d	0	20.0±0.74d
6	0	25.1±0.69c	0	25.0±0.76c
7	0	26.1±0.68bc	0	26.1±0.76bc
8	5.00±0.42k	26.1±0.68bc	5.07±0.40l	26.1±0.76bc
9	5.10±0.43k	27.0±0.69b	5.00±0.40l	27.09±0.76b
10	7.40±0.51j	29.4±0.70a	7.10±0.43j	29.00±0.78a
11	8.08±0.50i	29.2±0.70a	8.10±0.48i	29.07±0.78a
12	10.20±0.61h	29.3±0.70a	10.20±0.52h	29.10±0.78a
13	13.10±0.68g	29.2±0.70a	13.08±0.55g	29.00±0.78a
14	16.30±0.74f	29.1±0.70a	16.20±0.57f	29.10±0.78a
	$p<0.05$	Df 2,13	$p<0.05$	Df 2,13

Germination referred to here is marked by the presence of radicle and plumule. A significant difference ($p\leq0.05$) existed in seed germination between intact seeds and those without seed coats. While there existed no difference in the seeds germinated between MS and half MS media. *Moringa oleifera* intact seeds germinated on both MS basal and half strength MS media after 8 days of inoculation showed only radicle, while seeds with their seed coats removed showed radicle transforming into a root and plumule into a shoot (Fig. 2).

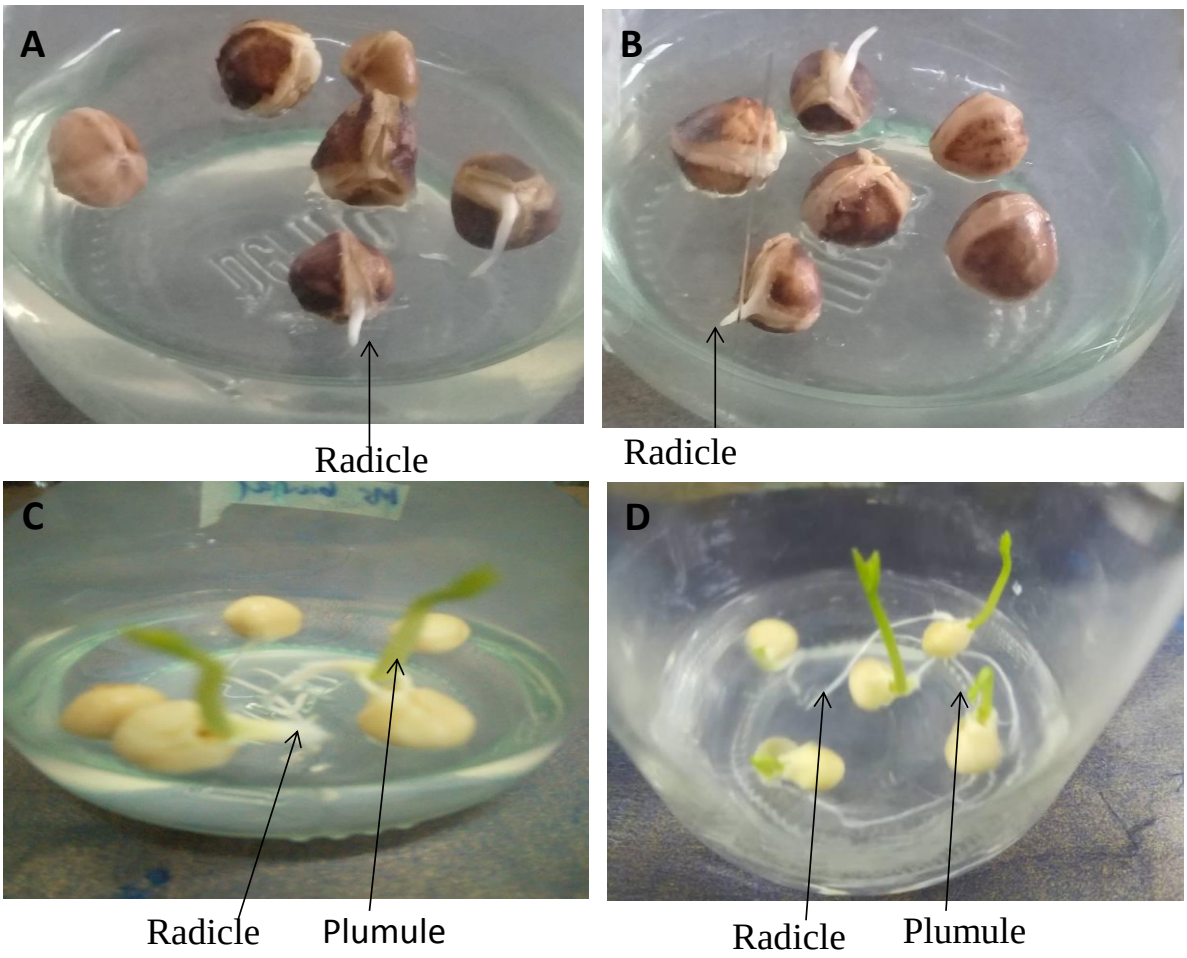

Figure 2: Germination of Moringa oleifera seeds with and without seed coats after 8 days of inoculation on basal MS and half MS media; A and B intact seeds on basal MS and half MS media respectively, C and D seeds without seed coats on basal MS and half MS media respectively.

4.1.1 Effects of Gibberellic acid on germination of *Moringa oleifera* seeds

Germination of *Moringa oleifera* seeds without seed coats on MS medium with 0.5 mg/l Gibberellic acid **(**GA_3) resulted in 100% germination with 30.00±0.00 seeds germinated (Table 2). Medium with 2.5 mg/l GA_3 had 27.33±0.11 seeds without seed coats germinated representing 90% of the total seeds inoculated, while in 4.5 mg/l and 6.5 mg/l 29.33±0.11 and 28.67±0.11 seeds germinated respectively after 5 days of inoculation representing 96.7% of the total seeds inoculated. Basal MS medium (control) had 28.33±0.11 seeds without seed coats germinated (Table 2).

Intact seeds on the other hand had few seeds germinated 5 days of inoculation with 0.5 mg/l GA_3 having 3.33±0.00 seeds germinated representing 13.6% of total seeds inoculated. While MS medium with 2.5 and 4.5 mg/l GA_3 had 4.67±0.11 and 5.67±0.22 intact seeds germinated with 17.0% and 23.3% of total seeds inoculated represented respectively. On MS medium with 6.5 mg/l GA_3, 7.67±0.11 intact seeds germinated representing 24.6% of inoculated seeds. Basal MS medium had 15.00±0.33 seeds germinated after 5 days of inoculation (Table 2) showing only radicle (Fig.3).

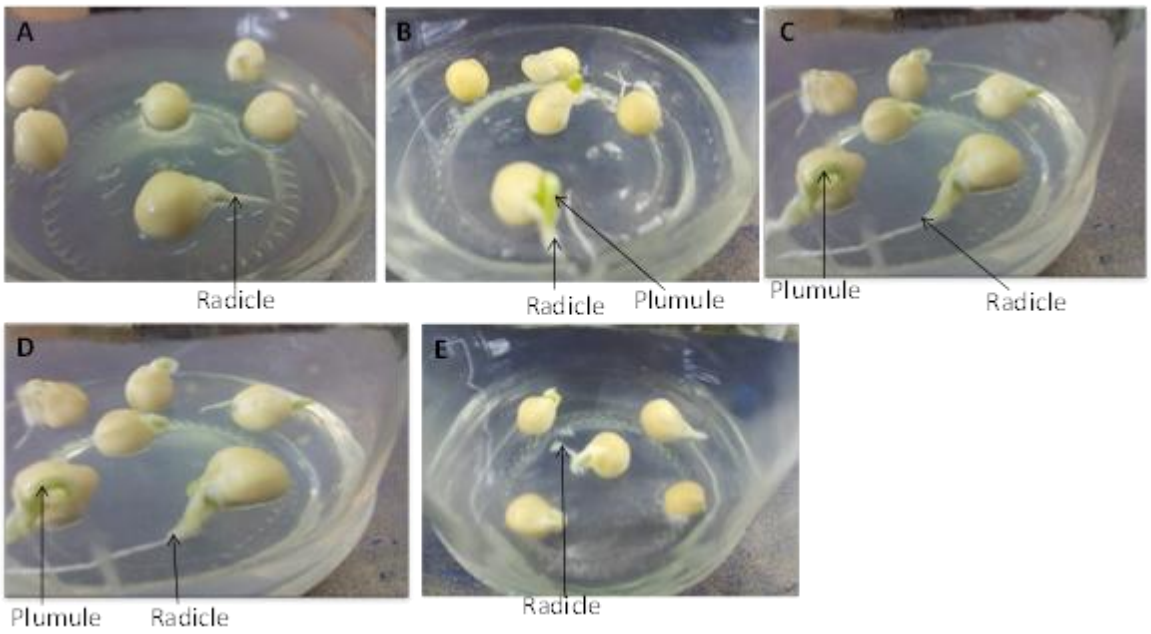

Figure 3: Germination of *Moringa oleifera* seeds without seed coats after 5 days of inoculation on MS media with Gibberellic acid (A) 0.5 mg/l, (B) 2.5 mg/l, (C) 4.5 mg/l, (D) 6.5 mg/l and (E) MS basal

In overall a significant difference ($p \leq 0.001$) was observed in seed germination in intact seeds and seeds without seed coats.

Table 2: Effects of Gibberellic acid on germination of *Moringa oleifera* seeds 5 days after inoculation

GA_3 Con	Seeds with no seed coats	Seeds with seed coats	F	Df	P
0.5mg/l	30.00±0.00e	3.33±0.00a	6400	1,4	<0.001
2.5mg/l	27.33±0.11e	4.67±0.11ab	2312	1,4	<0.001
4.5mg/l	29.33±0.11e	5.67±0.22ab	1008	1,4	<0.001
6.5mg/l	28.67±0.11e	7.67±0.11c	1984	1,4	<0.001
MS basal	28.33±0.11e	15.00±0.33cd	160	1,4	<0.001

Moringa oleifera seeds without seed coats after 5 days of germination on basal MS and MS with 0.5 mg/l of Gibberellic acid showed only radicle, while on MS medium with 2.5, 4.5 and 6.5 mg/l showed both radicle and plumule (Fig.4).

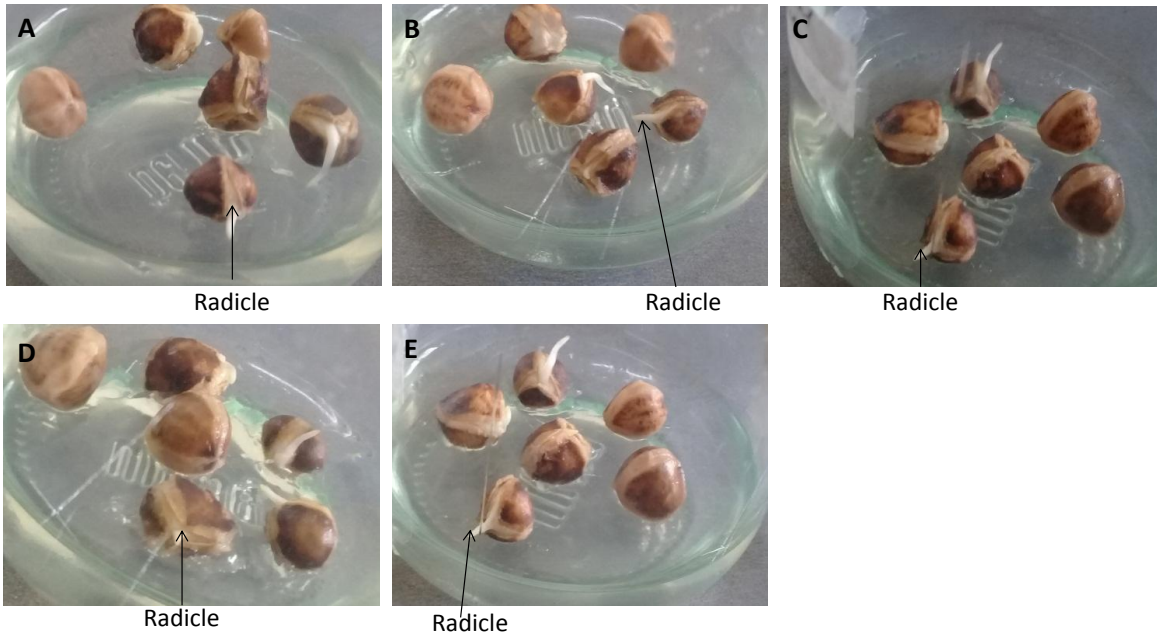

Figure 4: Germination of *Moringa oleifera* intact seeds after 5 days of inoculation on MS media with Gibberellic acid (A) 0.5 mg/l, (B) 2.5 mg/l, (C) 4.5 mg/l, (D) 6.5 mg/l and on (E) MS basal

4.2 Generation of callus from hypocotyls, inter-nodal segments and leaf discs of *Moringa oleifera*

4.2.1 Generation of callus from hypocotyls

Callusing on MS with 0.25 mg/l NAA started in 4.3±0.27 days and on 0.5 mg/l NAA it started in 4.0±0.27 days, while 1 mg/l and 2.0 mg/l NAA initiated callusing in 2.7±0.3 and 4.7±0.27 days respectively. Callus initiation on 0.25 mg/l 2,4D started in 5.0±0.3 days and on 0.5 mg/l 2,4D it gave callus in 4.0±0.3 days, while 1.0 and 2.0 mg/l 2,4D had callus in 4.7±0.3and 5.7±0.3 days respectively. With 0.25 mg/l TDZ callusing was initiated in 5.7±0.27 days while 0.5 mg/l TDZ gave callus in 5.3±0.3 days, 1.0 and 2.0 mg/l TDZ gave callus in 4.7±0.27 and 6.3±0.3 respectively. When BAP was used, 0.25 and 0.5 mg/l BAP initiated callusing in 5.3±0.3 and 4.7±0.27 days respectively, while 1.0 and 2.0 mg/l gave callus in 4.0±0.27 and 5.7±0.3 days respectively. MS basal medium (control) also initiated callus formation but in an average time of 7.8 days (Fig.5).

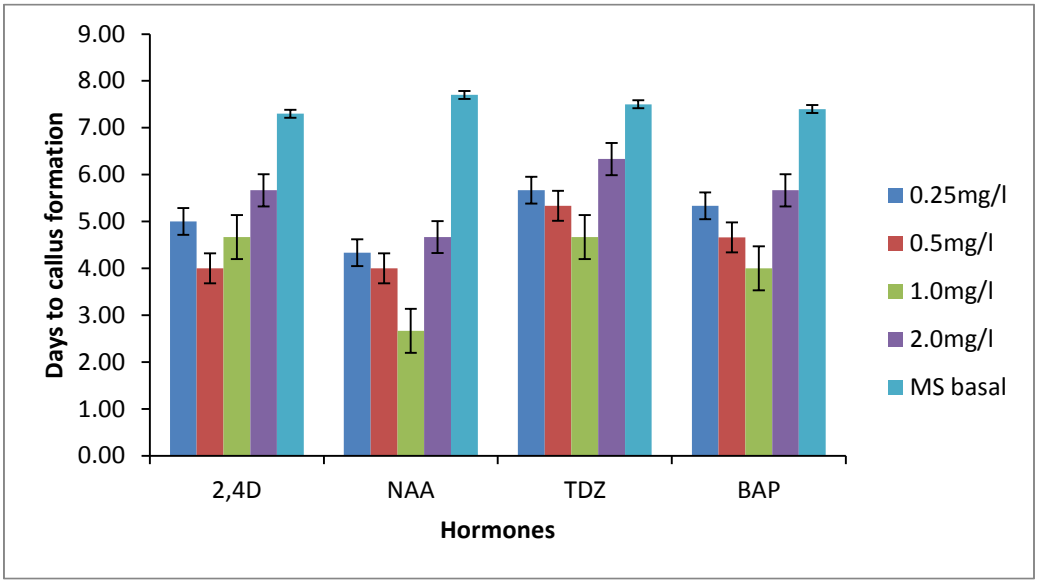

Figure 5: Effects of different hormones on number of days to callus generation in hypocotyls of *Moringa oleifera*

Medium with 2,4D and NAA only callus was produced but the callus was whitish while callus from NAA was in addition watery and sticky. Basal MS and MS with BAP developed shoots

from nodes together with callus while TDZ on the other hand also developed whitish and sticky callus (Fig.6).

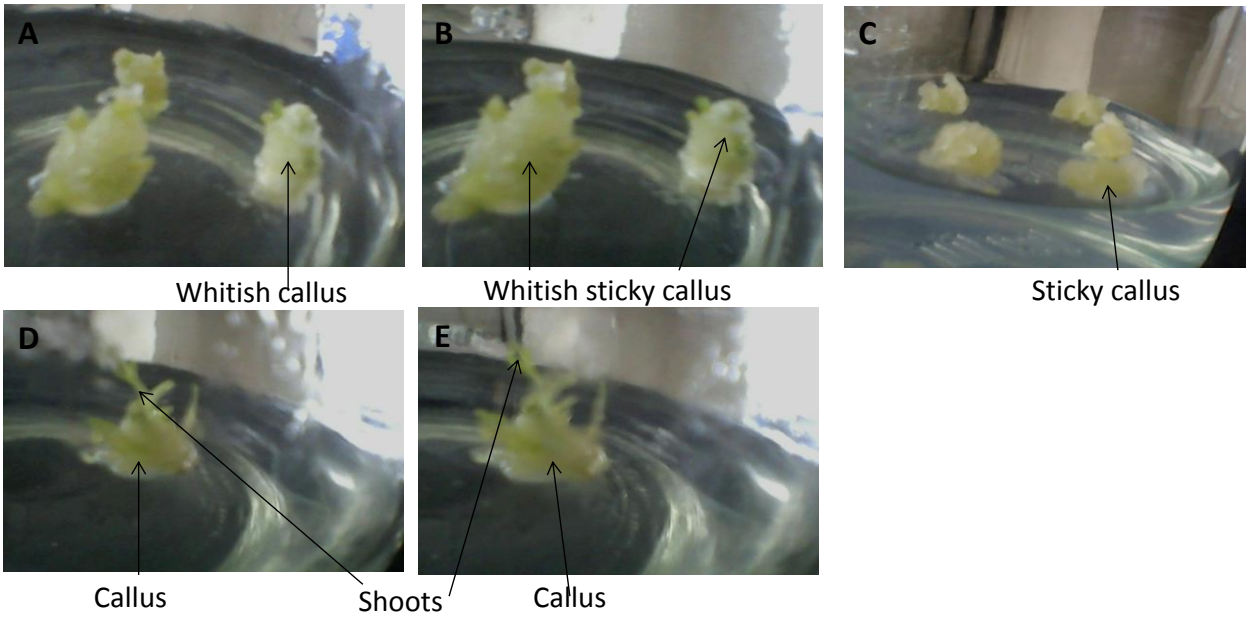

Figure 6: Generation of callus from hypocotyls of *Moringa oleifera* 14 days after inoculation on MS media with (A) 0.5 mg/l 2,4D, (B) 1 mg/l NAA, (C) 1 mg/l TDZ, (D) 1 mg/l BAP and (E) MS basal

4.2.2 Generation of callus from inter-nodal segments

Callusing on MS media with 0.25 g mg/l NAA occurred in 5.0±0.3 days, while on 0.5 mg/l NAA callusing started in 4.3±0.27 days. On media with 1.0 and 2.0 mg/l NAA callusing was initiated in 3.7±0.27 and 5.3±0.3 days respectively. In MS supplemented with 0.25 mg/l 2,4D, callus formation started in 5.3±0.3 days while 0.5 mg/l 2,4D gave callus in 4.7±0.27 days. On 1.0 and 2.0 mg/l 2,4D callus started forming in 5.0±0.3 and 5.7±0.3 days respectively. With 0.25 mg/l TDZ callusing was initiated in 6.3±0.3 days while 0.5 mg/l initiated callusing in 6.0±0.3 days. On 1.0 and 2.0 mg/l TDZ callusing was initiated in 5.3±0.27 and 5.7±0.27 days respectively. When BAP was used, 0.25 and 0.5 mg/l BAP initiated callusing in 6.0±0.3 and 5.7±0.3 days

respectively, while 1.0 and 2.0 mg/l gave callus in 4.7±0.27 and 5.0±0.3 days respectively. MS basal (control) initiated callusing in mean number of 7.5 days (Fig.7).

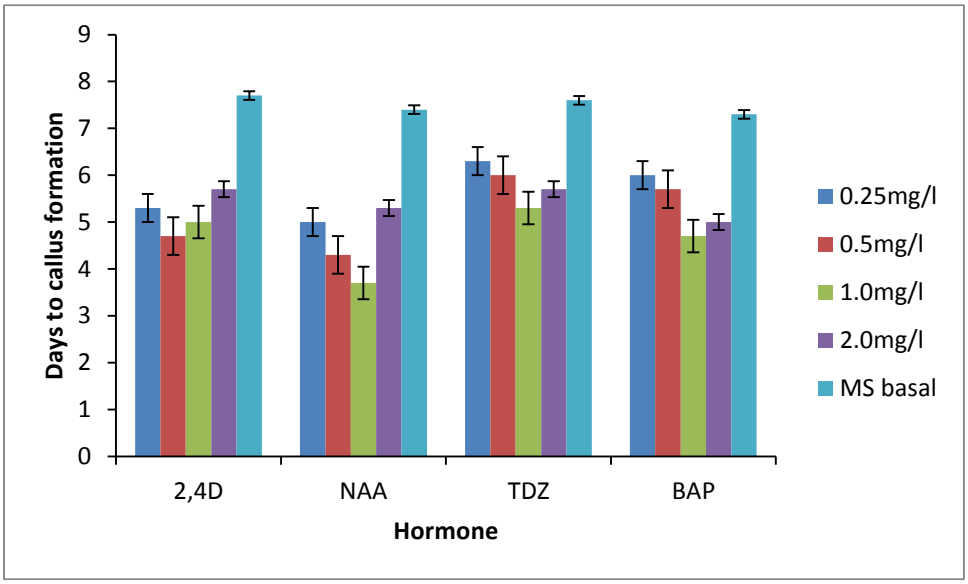

Figure 7: Effects of different hormones on number of days to callus generation in inter-nodal segments of *Moringa oleifera*

Callus from media with 2,4D and NAA started developing from the cut edges of the explant and callus formed was whitish and friable, TDZ also developed whitish and friable while in MS basal callus developed from both the cut end and the surface of the inter-nodal segments (Fig.8).

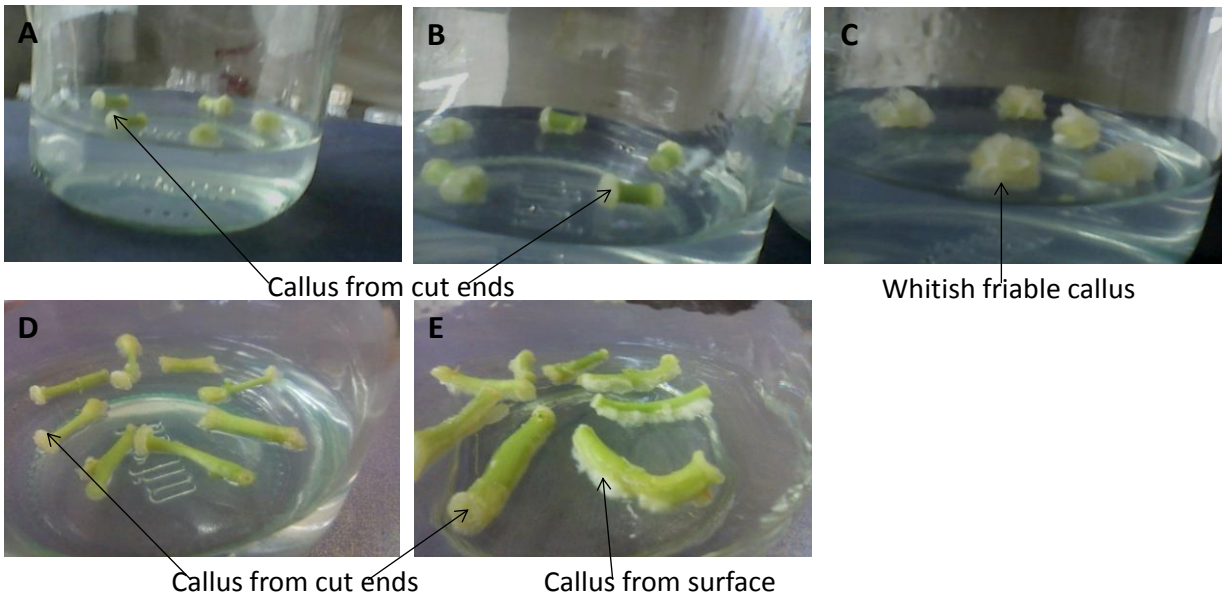

Figure 8: Generation of callus from inter-nodal segments of *Moringa oleifera* 7 days after inoculation on (A) 0.5 mg/l 2,4D, (B) 1 mg/l NAA, (C) 1 mg/l TDZ, (D) 1 mg/l BAP and (E) MS basal

4.2.3 Generation of callus from leaf discs

Callus formation from leaf discs of *Moringa oleifera* cultured on MS with 0.25 mg/l NAA took 5.7±0.27 days, while 0.5 mg/l NAA initiated callusing within 5.3±0.27 days. For those on 1.0 and 2.0 mg/l NAA, callusing occurred in 4.3±0.47 and 6.0±0.3 days respectively. When 2,4D 0.25 mg/l was used it gave callus in 6.7±0.3 days, 0.5 mg/l 2,4D formed callus in 6.0±0.3 days while 1.0 and 2.0 mg/l of 2,4D initiated callus in 6.3±0.3 and 7.3±0.3 days respectively. With TDZ 0.25 mg/l callusing was initiated in 7.3±0.3 days while 0.5, 1.0 and 2.0 mg/l TDZ initiated callus formation in a mean of 6.3±0.3, 6.0±0.3 and 6.7±0.3 days respectively. When BAP was used, 0.25 and 0.5 mg/l BAP initiated callusing in 6.3±0.3 and 5.7±0.3 days respectively, while 1.0 and 2.0 mg/l gave callus in 5.0±0.27 and 5.3±0.3 days respectively. MS basal (control) also initiated callus formation with an average mean of 7.33 days (Fig.9). Over 90% of the explants cultured responded by giving callus both in hypocotyls, inter-nodal segments and leaf discs.

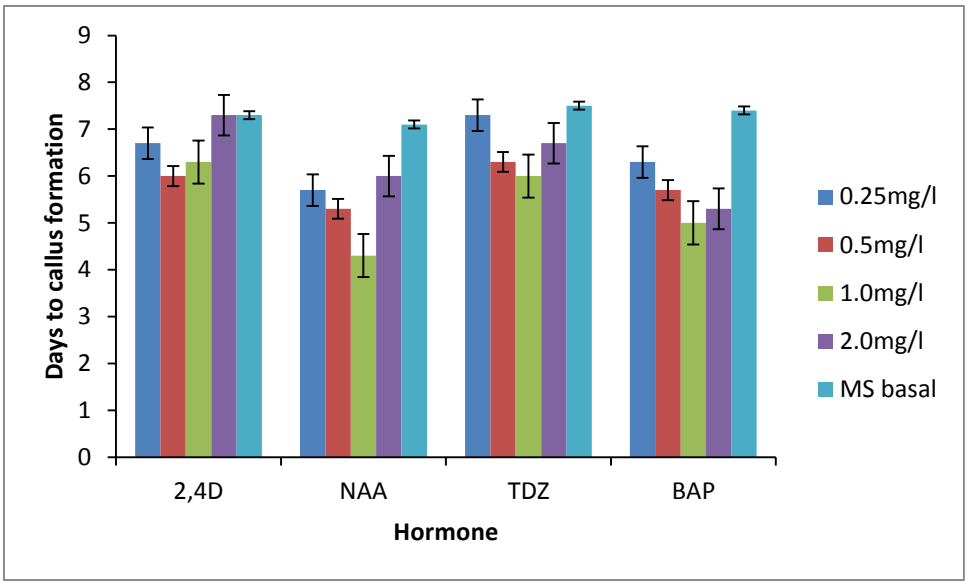

Figure 9: Effects of different hormones on number of days to callus generation in leaf discs of *Moringa oleifera*

Callus formation in 2,4D and NAA started from the leaf petiole and the leaf explants turned yellowish but the callus produced in 2,4D was whitish and friable. MS basal produced whitish friable callus from the leaf discs of *Moringa oleifera* (Fig.10).

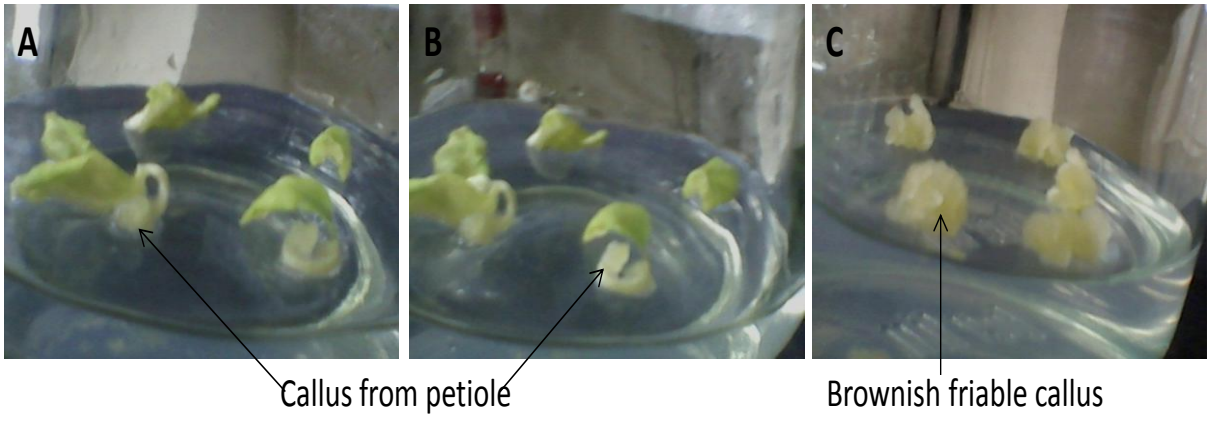

Figure 10: Generation of callus from leaf discs of *Moringa oleifera* 14 days after inoculation on MS media with (A) 0.5 mg/l 2,4D, (B) 1 mg/l NAA and (C) MS basal

4.3 Effects of different hormones on fresh weight and growth of callus from hypocotyls, inter-nodal segments and leaf discs of *Moringa oleifera*

Preliminary results had all hormones used in the study produce callus including MS basal but the callus weight varied with the hormone concentrations and the type of the explants used (Table 3). In MS with 0.25, 0.5, 1.0 and 2.0 mg/l TDZ, callus weights of 3.41±0.03, 4.16±0.12, 7.85±0.13, 4.43±0.32 g were recorded respectively in hypocotyls. In MS with 0.25, 0.5, 1.0 and 2.0 mg/l 2,4D callus weights of 4.78±0.18, 9.78±0.26, 7.68±0.23 and 3.66±0.05 g were recorded respectively in hypocotyls.

In MS with 0.25, 0.5, 1.0 and 2.0 mg/l BAP mean callus weights recorded were 3.82±0.04, 4.45±0.06, 6.82±0.02 and 5.46±0.04 g respectively in hypocotyls. On MS with 0.25, 0.5, 1.0 and 2.0 mg/l NAA callus mean weight of 8.07±0.22, 11.0±0.21, 12.2±0.09 and 7.90±0.34 g were recorded respectively in hypocotyls. MS basal had callus with mean weights of 2.26±0.09, 2.81±0.10, 2.30±0.09 and 2.29±0.10 g when used as control for 0.25, 0.5, 1.0 and 2.0 mg/l in hypocotyls.

Table 3: Weight (g) of 4 weeks old callus from hypocotyls of *Moringa oleifera* in different hormones

Conc.	Hormone					F	Df	p
	2,4D	BAP	Ms basal	NAA	TDZ			
0.25mg/l	4.78±0.18c	3.82±0.04b	2.26±0.09a	8.07±0.22d	3.41±0.03b	241.3	4,10	<0.001
0.5mg/l	9.78±0.26c	4.45±0.06b	2.81±0.10a	11.0±0.21d	4.16±0.12b	470.3	4,10	<0.001
1.0mg/l	7.68±0.23c	6.82±0.02b	2.30±0.09a	12.2±0.09d	7.85±0.13c	678.8	4,10	<0.001
2.0mg/l	3.66±0.05b	5.46±0.04c	2.29±0.10a	7.90±0.34d	4.43±0.32b	91.78	4,10	<0.001

Callus obtained from hypocotyls and inter-nodal segments in MS basal and MS with TDZ were friable and in addition developed embryonic structures on the surface. Callus from 2,4D and NAA were also friable but callus from NAA in addition was profuse. Callus from hypocotyls and inter-nodal segments cultured on media with BAP was compact (Fig. 11).

In inter-nodal segments, MS with TDZ 0.25, 0.5, 1.0 and 2.0 mg/l callus had weights of 4.46±0.01, 5.38±0.08, 7.99±0.14 and 4.99±0.18 respectively. With MS supplemented with 2,4D 0.25, 0.5, 1.0 and 2.0 mg/l, the callus weights recorded were 5.49±0.07, 9.97±0.20, 8.05±0.08 and 4.76±0.07 g respecyively in inter-nodal segments. When MS medium was supplemented with BAP 0.25, 0.5, 1.0 and 2.0 mg/l, the weights recorded were 4.62±0.07, 4.53±0.06, 6.88±0.02 and 5.75±0.02 g respectively in inter-nodal segments (Table 4). With MS supplemented with NAA 0.25, 0.5, 1.0 and 2.0 mg/l, callus weights of 8.80±0.01, 11.47±0.25, 12.80±0.04 and 8.64±0.03 g were recorded respectively in inter-nodal segments. MS basal had callus with mean weight of 3.70±0.08, 3.85±0.05, 3.37±0.03 and 3.34±0.03 g when used as control for 0.25, 0.5, 1.0 and 2.0 mg/l in inter-nodal segments (Table 4).

Table 4: Weight (g) of 4 weeks old callus from inter-nodal segments of *Moringa oleifera* in different hormones

Conc	Hormone							
	2,4D	BAP	Ms basal	NAA	TDZ	F	Df	p
0.25mg/l	5.49±0.07c	4.62±0.07b	3.70±0.08a	8.80±0.01d	4.46±0.01b	1153	4,10	<0.001
0.5mg/l	9.97±0.20c	4.53±0.06a	3.85±0.05a	11.47±0.25d	5.38±0.08b	528.9	4,10	<0.001
1.0mg/l	8.05±0.08c	6.88±0.02b	3.37±0.03a	12.80±0.04d	7.99±0.14c	1987	4,10	<0.001
2.0mg/l	4.76±0.07b	5.75±0.02c	3.34±0.03d	8.64±0.03a	4.99±0.18b	504.8	4,10	<0.001

In leaf discs, MS with TDZ 0.25, 0.5, 1.0 and 2.0 mg/l had callus with weights of 3.23±0.01, 4.01±0.01, 5.71±0.04 and 3.33±0.02 respectively. With MS supplemented with 2,4D 0.25, 0.5, 1.0 and 2.0 mg/l, the callus weights recorded were 3.48±0.01, 6.15±0.08, 5.95±0.06 and 3.26±0.02 g respecyively in leaf discs. When MS was supplemented with BAP 0.25, 0.5, 1.0 and 2.0 mg/l, the weights recorded were 3.12±0.01, 3.70±0.01, 4.87±0.01 and 4.83±0.05 g respectively in leaf discs (Table 5).

With MS supplemented with NAA 0.25, 0.5, 1.0 and 2.0 mg/l, callus weights of 4.74±0.02, 6.87±0.02, 7.63±0.03 and 4.84±0.07 g were recorded respectively in leaf discs. MS basal medium on the other hand had callus with mean weight of 2.23±0.02, 2.88±0.04, 2.37±0.02 and 2.50±0.04 g when used as control for 0.25, 0.5, 1.0 and 2.0 mg/l in leaf discs (Table 5).

Table 5 Weight (g) of 4 weeks old callus from leaf discs of *Moringa oleifera* in different hormones

	Hormone							
Conc	2,4D	BAP	MS basal	NAA	TDZ	F	Df	p
0.25mg/l	3.48±0.01c	3.12±0.01b	2.23±0.02a	4.74±0.02d	3.23±0.01b	387.4	4,10	<0.001
0.5mg/l	6.15±0.08c	3.70±0.01b	2.88±0.04a	6.87±0.02d	4.01±0.01b	264.6	4,10	<0.001
1.0mg/l	5.95±0.06c	4.87±0.01b	2.37±0.02a	7.63±0.03d	5.71±0.04c	236.6	4,10	<0.001
2.0mg/l	3.26±0.02b	4.83±0.05c	2.50±0.04a	4.84±0.07c	3.33±0.02b	64.28	4,10	<0.001

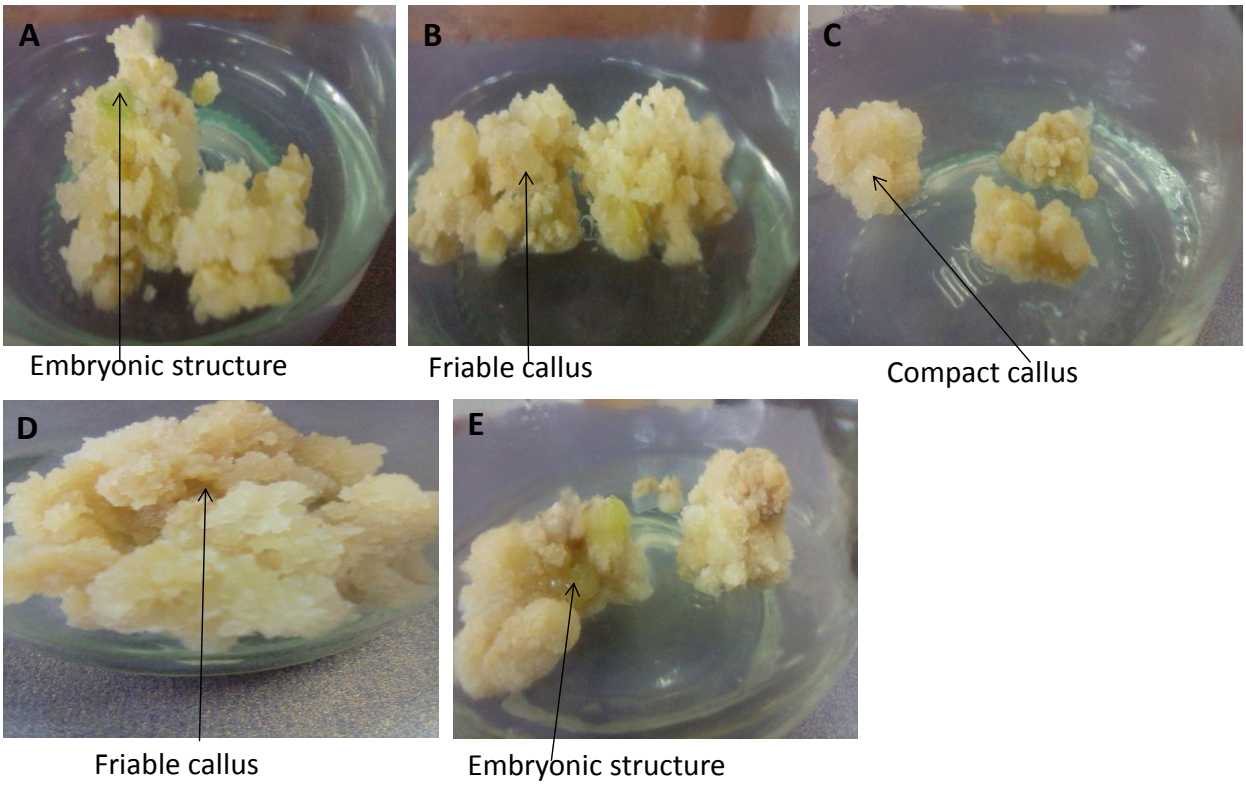

Figure 11: Four weeks old callus from hypocotyls and inter-nodal segments of *Moringa oleifera* on MS media with (A) TDZ, (B) 2,4D, (C) BAP, (D) NAA and (E) MS basal

Callus obtained from leaf discs was little in amount compared to those obtained from hypocotyls and inter-nodal segments. Callus from leaf discs in MS basal and MS supplemented with TDZ was friable. On medium with 2,4D and NAA, leaf callus was friable but in addition callus in NAA developed roots after 14 days of initiation With BAP and MS basal there were some green structures on the callus surface (Fig. 12).

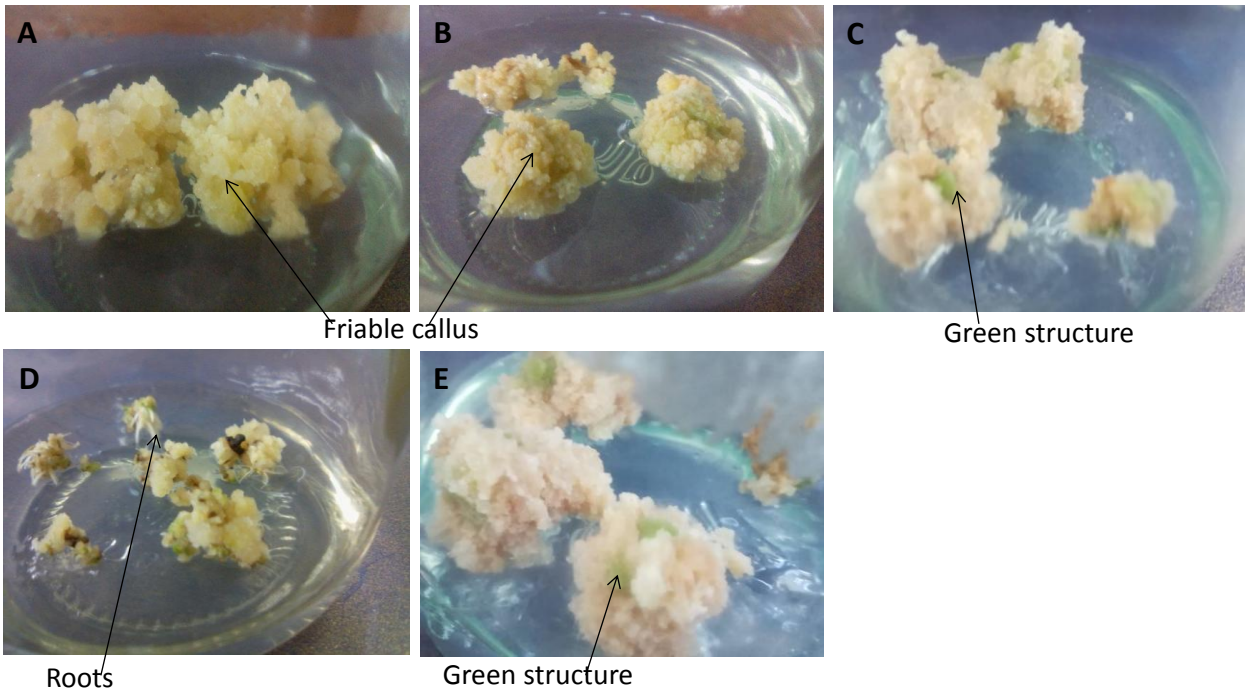

Figure 12: Four weeks old callus from leaf discs of *Moringa oleifera* on MS media with (A) TDZ, (B) 2,4D, (C) BAP, (D) NAA and (E) MS basal

4.3.1 Effects Thidiazuron on the weight and growth of callus from hypocotyls of *Moringa oleifera*

From the preliminary results callus weight increased significantly after 4 weeks of inoculation when 2,4D, NAA and BAP were combined with TDZ compared to when the hormones were used singly. When 0.25 mg/l 2,4D plus TDZ 0.25 mg/l was used callus weight increased from 4.78±0.18 g to 6.58±0.11 g. The callus weight increased to 12.66±0.05 g from 9.78±0.26 g when 2,4D 0.5 mg/l plus 0.5 mg/l TDZ was used. At 1.0 mg/l 2,4D plus TDZ 1.0 mg/l the callus mean weight increased to 15.41±0.10 g from 7.68±0.23 g, while a concentration of 2.0 mg/l 2,4D plus 2.0 mg/l TDZ recorded a mean weight of 4.98±0.05 g from 3.66±0.05 g (Table 6).

With NAA 0.25 mg/l plus 0.25 mg/l TDZ the callus mean weight increased to 10.75±0.08 g from 8.07±0.22 g, while at 0.5 mg/l NAA plus TDZ 0.5 mg/l an increase to 12.33±0.07 g from

11.0±0.21 g was recorded. When NAA 1.0 mg/l plus 1.0 mg/l TDZ and 2.0 mg/l NAA plus TDZ 2.0 mg/l was used, an increased mean callus weight of 20.83±0.07 g from 12.2±0.09 g and 8.68±0.10 g from 7.9±0.34 g was recorded respectively (Table 6).

With BAP 0.25 mg/l plus 0.25 mg/l TDZ an increase in mean callus weight to 5.64±0.04 g from 3.82±0.04 g was obtained, while 0.5mg/l BAP plus TDZ 0.5 mg/l gave an increased mean weight of 6.47±0.03 g from 4.45±0.06 g. With 1.0 mg/l BAP plus 1.0 mg/l TDZ and BAP 2.0 mg/l plus 2.0 mg/l TDZ mean callus weights of 8.36±0.04 g from 6.82±0.02 g and 8.36±0.06 g from 5.46±0.04 g respectively was observed (Table 6).

Table 6: Fresh weight of callus from hypocotyls of *Moringa oleifera* after 4 weeks in 2,4D, NAA and BAP combined with TDZ

Conc	Hormone				F	Df	P
	2,4D+TDZ	BAP+TDZ	Msbasal	NAA+TDZ			
0.25+0.25	6.58±0.11c	5.64±0.04b	3.33±0.03a	10.75±0.08d	6.56	3,8	<0.001
0.5+0.5	12.66±0.05c	6.47±0.03b	3.48±0.08a	12.33±0.07c	1221	3,8	<0.05
1.0+1.0	15.41±0.10c	8.36±0.04b	3.31±0.03a	20.83±0.07d	7214	3,8	<0.05
2.0+2.0	4.98±0.05b	8.36±0.06c	3.27±0.03a	8.68±0.10d	739.8	3,8	<0.05

4.4 Regeneration of plantlets of *Moringa oleifera*

4.4.1 Effects of different hormones on regeneration of plantlets of *Moringa oleifera* through somatic emryogenesis

Callus from hypocotyls, inter-nodal segments and leaf discs in MS medium with 2,4D showed normal growth and proliferation on fresh hormone free MS medium 2 weeks after sub-culturing.

As the callus continued growing, green structures were observed on the surface a week later but the callus later turned brown and dried out (Fig. 13)

Callus obtained from hypocotyls, inter-nodal segments and leaf discs in TDZ and NAA separately also resumed growth on subculture to basal MS without differentiation, but leaf callus from NAA in addition developed roots two weeks after subculture (Fig. 13)

Callus from hypocotyls and inter-nodal segments in BAP on the other hand was compact and therefore was not explored further, while leaf callus from BAP was friable but did not regenerate on MS basal. The embryonic like structures observed on callus from basal MS and TDZ turned brown two weeks after sub culture to MS basal medium (Fig 13).

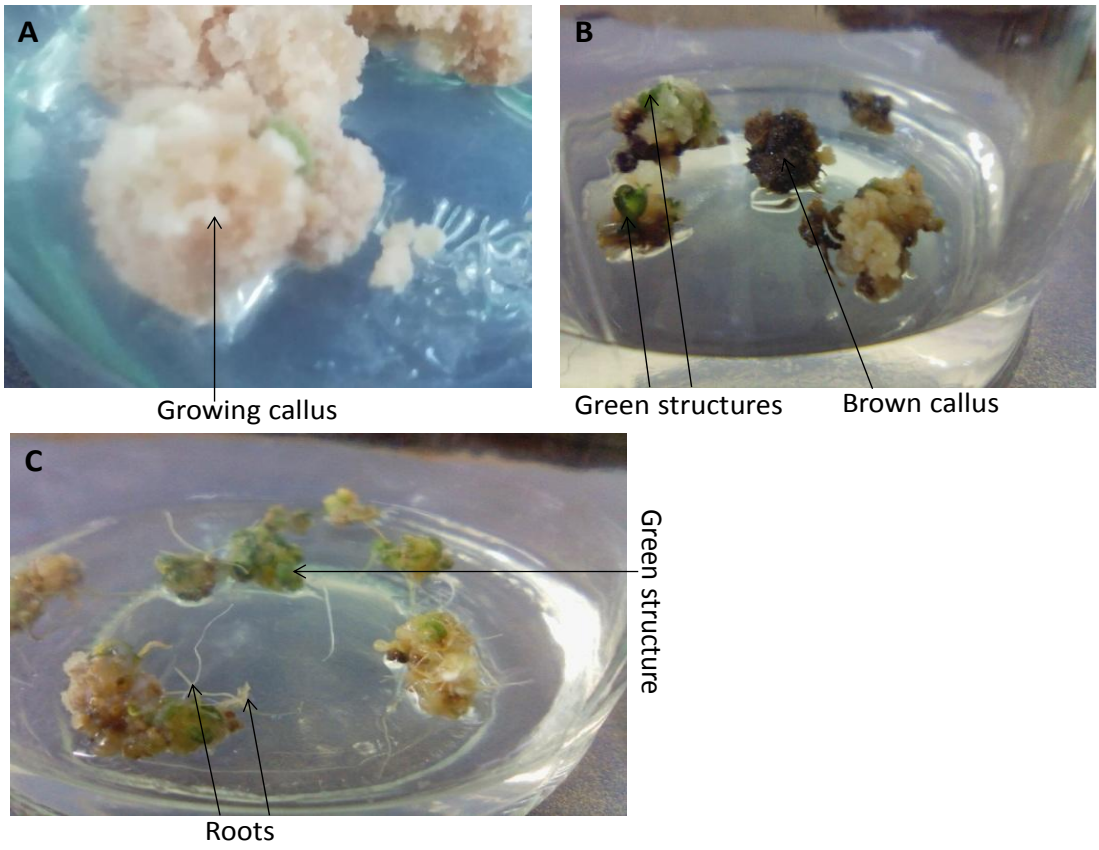

Figure 13: Four weeks old callus of *Moringa oleifera* from (A) inter-nodal segments (B) hypocotyls and (C) leaf discs regenerating on MS basal medium

Following lack of regeneration on hormone free media, attempts were made to improve callus regeneration on MS with BAP and KIN. After three weeks of incubation callus from hypocotyls, inter-nodal segments and leaf discs generated from 2,4D, TDZ and NAA developed green structures on the surface in 2 mg/l BAP, later shoot like structures started forming but a week later turned brown and dried (Fig.14). Those cultured on 2 mg/l KIN formed few green structures and the callus later turned brown and dried. On 0.5, 1.0 and 3.0 mg/l, BAP and KIN callus only resumed growth four weeks after sub-culturing and were not explored further. When 2 mg/l BAP and KIN was combined shoot like structures developed on the surface increased but later turned brown 4-5 weeks after inoculation (Fig.14). In other combinations of BAP and KIN at 0.5 mg/l, 1 mg/l and 3 mg/l callus resumed growth without differentiation.

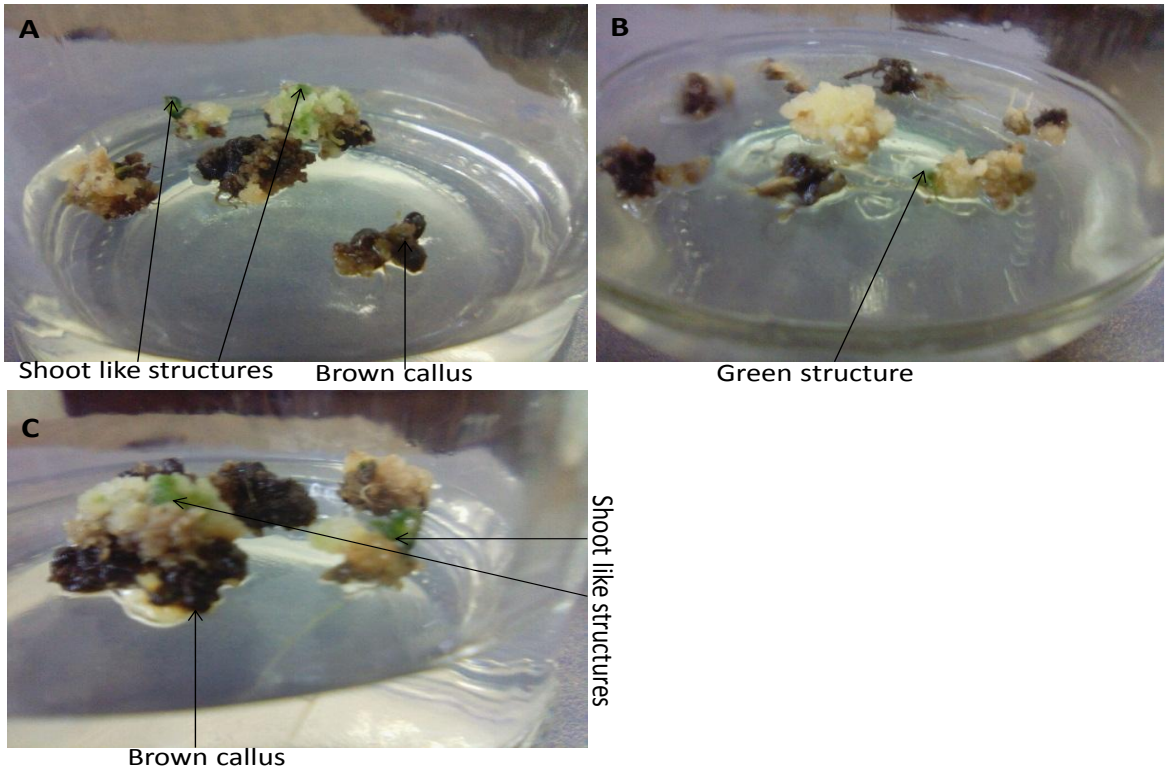

Figure 14: Four weeks old callus of *Moringa oleifera* regenerating on MS media with (A) 2 mg/l BAP, (B) 2 mg/l KIN and (C) 2mg/l BAP plus 2 mg/l KIN

When MS with NAA 0.5 mg/l plus 2 mg/l BAP was used, callus from hypocotyls, inter-nodal segments and leaf discs obtained from media with 2,4D, TDZ and NAA started to develop shoot like structures after 3 weeks but later roots developed, turned brown and died. The other combinations of NAA and BAP used (0.1 mg/l NAA plus 0.5 mg/l BAP, 0.5 mg/l NAA plus 1 mg/l BAP and NAA 0.5 mg/l plus 3 mg/l BAP) gave similar results. The same result was also observed when KIN 2 mg/l plus NAA 0.5 mg/l, KIN 1 mg/l plus 0.5 mg/l NAA and KIN 3 mg/l plus 0.5 mg/l NAA) was used (Fig.15).

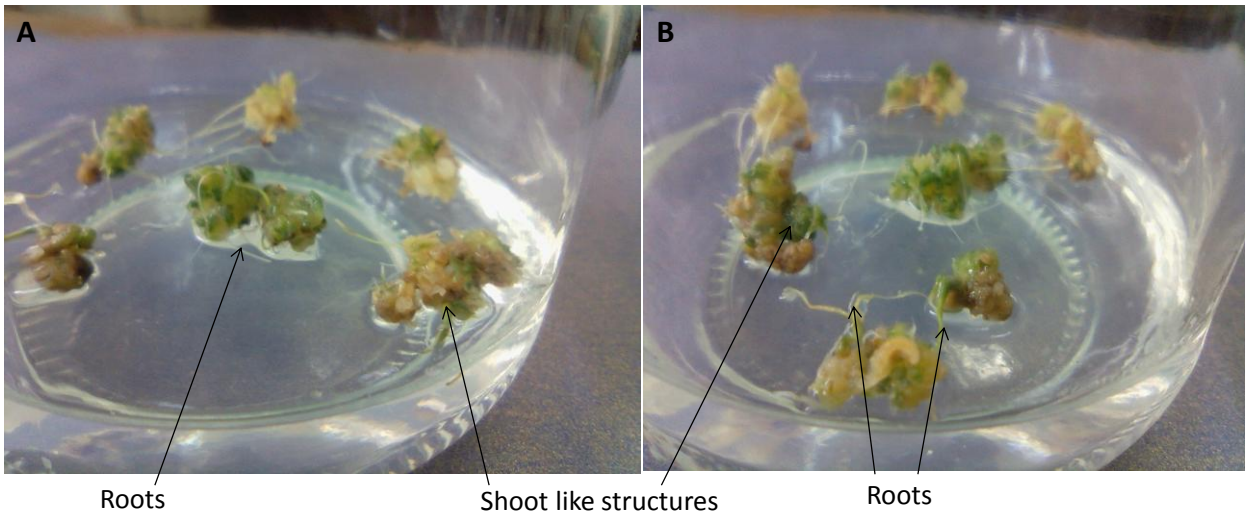

Figure 15: Four weeks old *Moringa oleifera* callus regenerating on MS media with (A) NAA 0.5 mg/l and BAP 2 mg/l (B) NAA 0.5 mg/l and KIN 2 mg/l

Callus regeneration on MS with TDZ 0.5, 1.0 and 2 mg/l, led to increased growth in callus without any sign of differentiation and therefore was not explored further.

Callus on sub cultured to MS media with 0.25 mg/l Metatopoline ribonucleotide (MTR) callus resumed growth without any sign of differentiation. At 0.5 mg/l and 1 mg/l MTR several green structures developed on callus surface two weeks post inoculation, and after the third week shoot like structures started developing but after the fourth week the developed shoots turned brown

and dried (Fig.16). When 2.0 mg/l MTR was tested, callus resumed growth without regeneration and it was not further explored. MS with 0.5 mg/l of BAP plus 0.5 mg/l MTR, 0.5 mg/l of BAP plus 1.0 mg/l MTR developed green structures on the callus surface which developed to shoot like structures after 3 weeks only on the latter. When MTR 1 mg/l plus 0.5 mg/l NAA was used callus developed green structures on the surface but later roots formed and regeneration could not continue any further (Fig. 16). All other combinations used (0.5 mg/l KIN plus 1.0 mg/l MTR, 0.25 mg/l NAA plus 1.0 mg/l MTR and 1.0 mg/l KIN plus 1.0 mg/l MTR) showed no sign of regeneration.

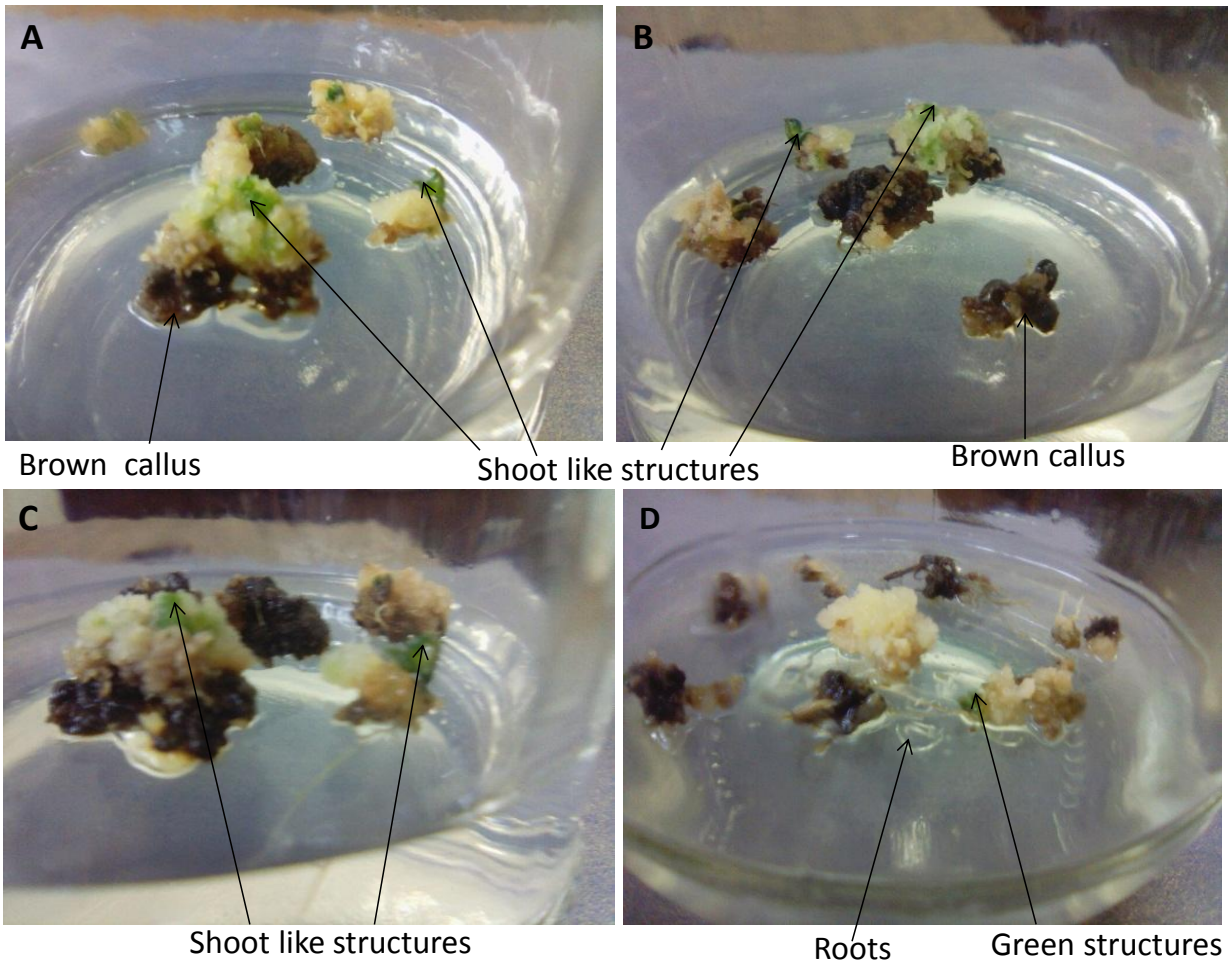

Figure 16: Four weeks old *Moringa oleifera* callus regenerating on MS media with (A) MTR 0.5 mg/l (B) MTR 1.0 mg/l (C) 0.5 mg/l BAP plus 1.0 mg/l MTR and (D) MTR 1 mg/l plus NAA 0.5 mg/l

Callus from media with 2,4D and TDZ, BAP and TDZ and NAA and TDZ when sub cultured resumed growth and did not show any sign of differentiation.

4.4.2 Effects of different hormones on the number of days to obtain shoots from nodal segments of *Moringa oleifera*

Axillary shoot induction from nodal segments of *Moringa oleifera* on 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l BAP happened in 9.7, 8.7, 8.3, 6.3 and 8.3 days respectively (Fig.17). On MS with 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l NAA, shoots were induced in 7.7, 7.3, 7.0, 5.3 and 6.7 days respectively. When 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l TDZ was tested, shoot induction was initiated in 10.7, 7.3, 9.3, 8.7 and 8.7 days respectively (Fig.17).

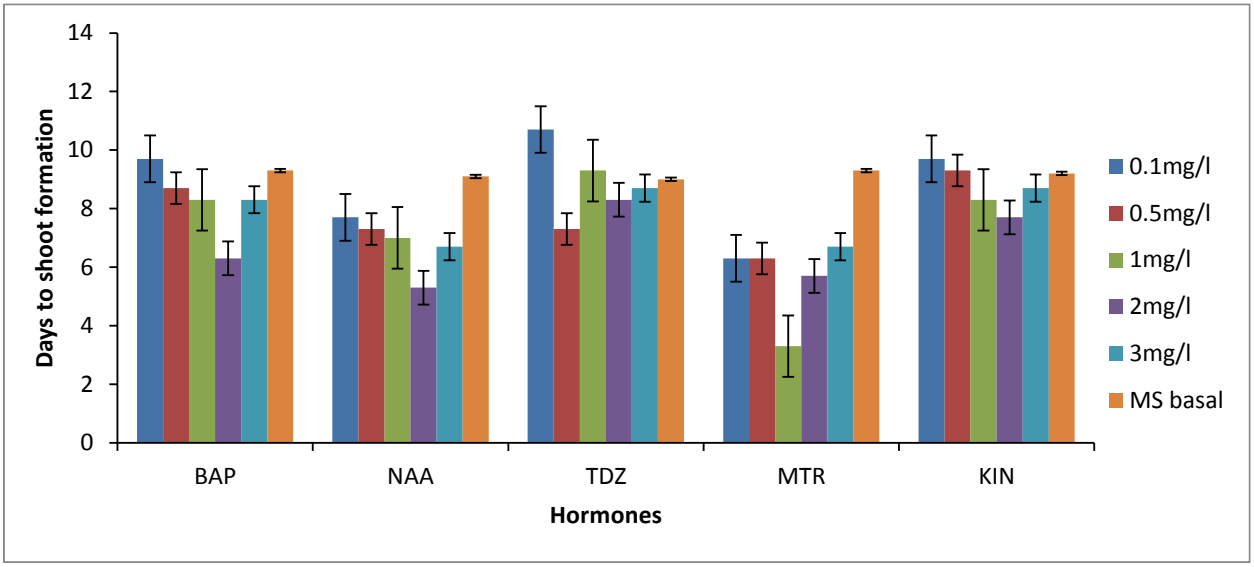

Figure 17: Days taken to obtain shoots from nodal segments of *Moringa oleifera* in different hormones

The cytokinin MTR had shoots initiated in less days than the other hormones used. At 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l MTR, shoots were initiated in 6.3, 6.3, 3.3, 5.7 and 6.7 days respectively (Fig.17). Kinetin 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l initiated shooting in 9.7, 9.3, 8.3, 7.7 and 8.7

days respectively. MS basal medium (control) also initiated nodal shooting but in 9.4 days which was longer time compared to MS media supplemented with hormones (Fig.17).

4.4.3 Effects of different hormones on the number of shoots obtained from nodal segments of *Moringa oleifera*

There existed a significant difference ($p \leq 0.05$) in shoots induced from nodal segments of *Moringa oleifera* among the different hormones tested. On MS with 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l BAP, 2.3, 3.3, 3.7, 4.7 and 2.7 shoots were induced respectively (Fig 18). On MS media with 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l NAA, 3.0, 4.3, 3.0, 3.7 and 3.0 shoots were recorded respectively. On MS with 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l TDZ, 2.3, 3.0, 3.0, 3.3 and 3.3 shoots were induced respectively (Fig.18).

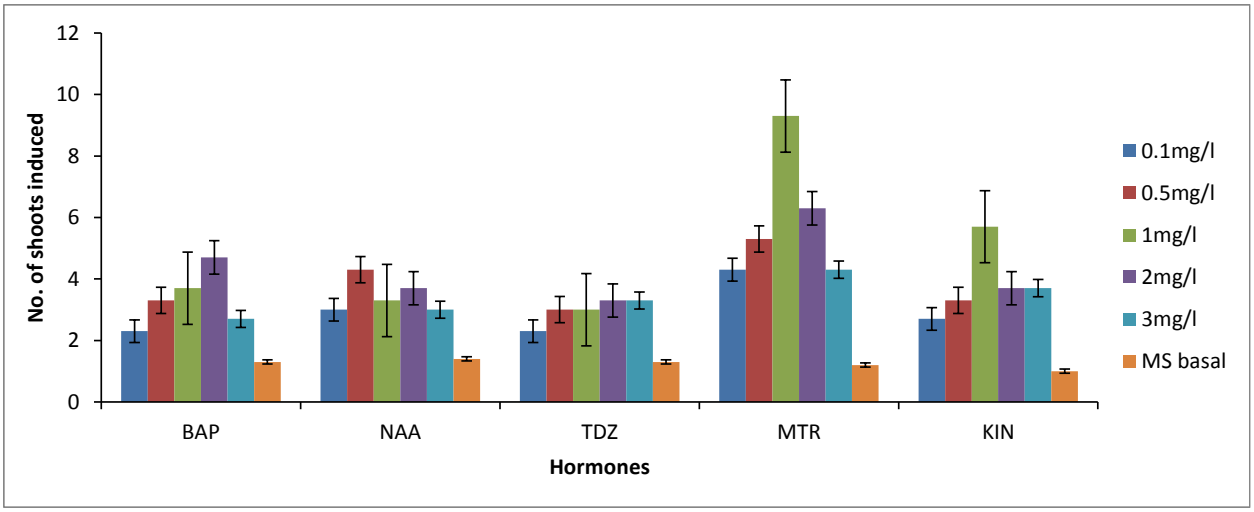

Figure 18: Shoots obtained from nodal explants of *Moringa oleifera* in different hormones

On MS with 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l MTR, 4.3, 5.3, 9.3, 6.3 and 4.3 shoots were induced respectively (Fig. 18). On MS with 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l Kinetin, 2.7, 3.3, 5.7, 3.7 and 3.7 shoots were recorded respectively. Basal MS medium (control) on the other hand also induced shooting with an overall mean of 1.53 shoots (Fig.18).

On MS media wih BAP, each explant produced 4 shoots on average. On NAA each explant gave 3 shoots on average, while TDZ on the other hand produced stunted shoots with each explant producing 2 shoots on average (Fig.19). On average more shoots were regenerated with MS supplemented with MTR compared to the other hormones used with each explant producing an average of 7 shoots per explant. On KIN, few and stunted shoots were produced with each explant giving an average of 2 shoots while on MS basal medium (control) each explant gave only a single shoot. Callus developed on the base of the explant before the shoot were induced (Fig.20).

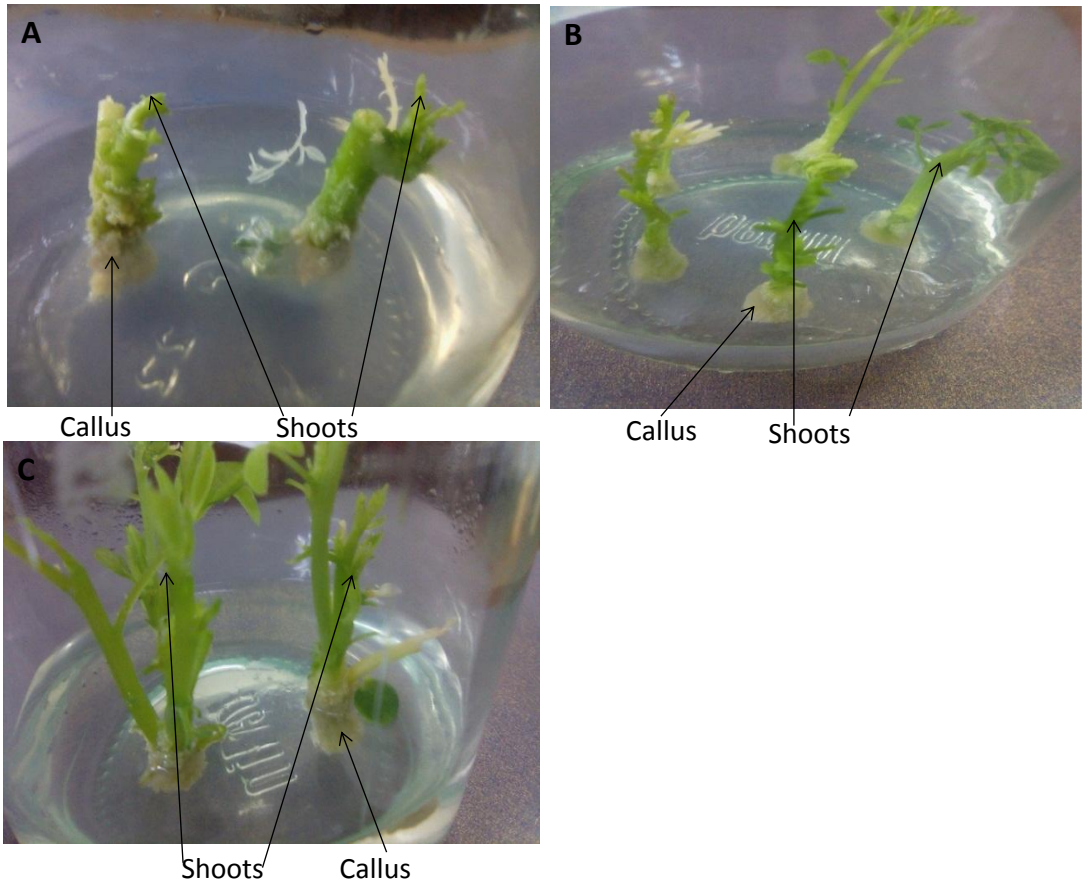

Figure 19: Two weeks old axillary shoots of *Moringa oleifera* on MS media with (A) 2 mg/l TDZ, (B) 0.5 mg/l NAA and (C) 2 mg/l BAP

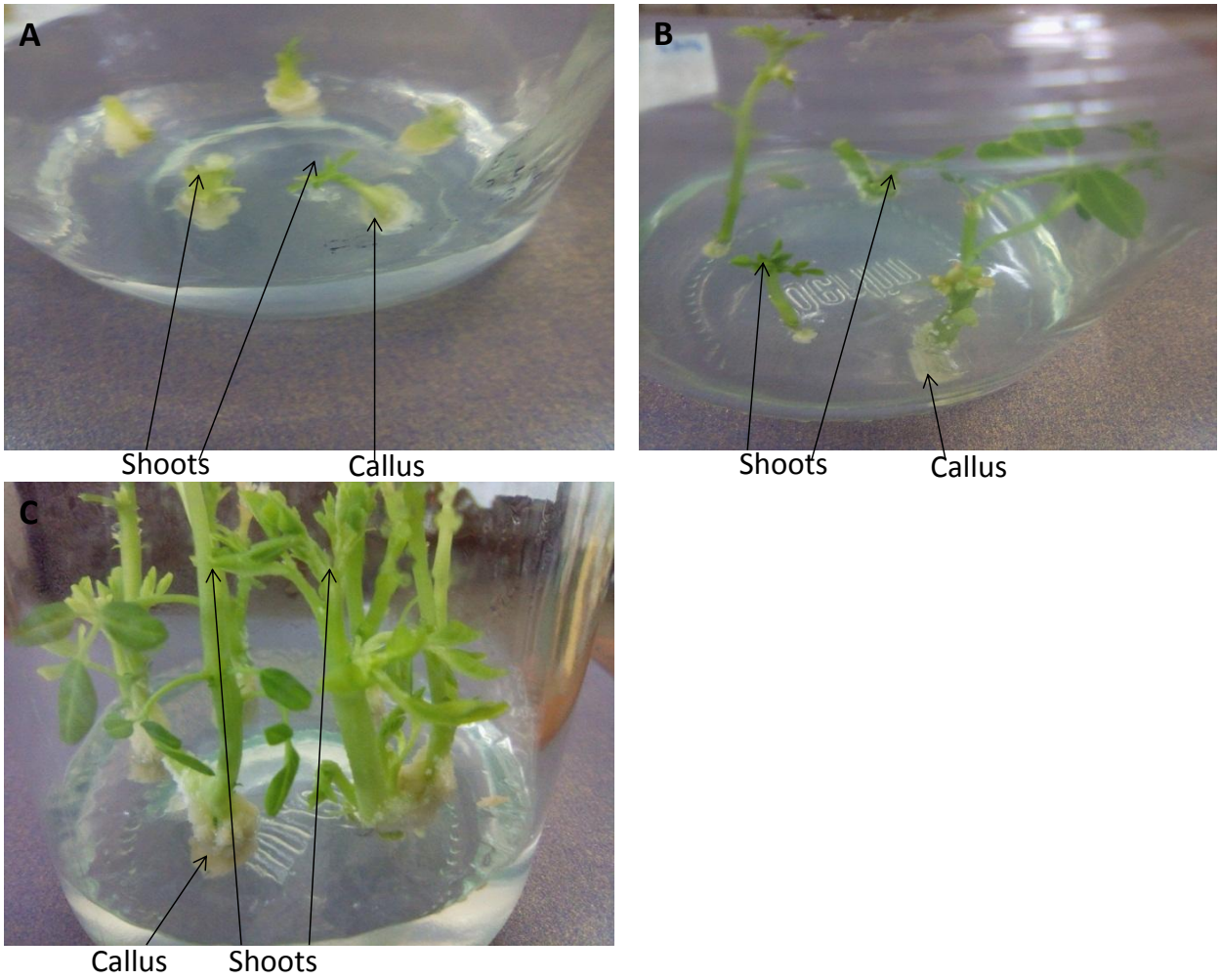

Figure 20: Two weeks old axillary shoots of *Moringa oleifera* on MS media with (A) 1 mg/l KIN, (B) MS basal and (C) 1 mg/l MTR

On MS with MTR combined with other hormones, the number of shoots induced increased significantly ($p \leq 0.05$), with 0.1 mg/l of MTR plus 0.01 mg/l of BAP, 0.5 mg/l of MTR plus 0.1 mg/l of BAP and 1 mg/l of MTR plus 0.5 mg/l of BAP recording 4.3, 5.0 and 6.3 shoots respectively. On medium with 2 mg/l MTR plus 1 mg/l BAP and 3 mg/l MTR plus 2 mg/ l BAP, 8.0 and 5.7 shoots were induced respectively (Fig.21). When 0.1 mg/l MTR plus NAA 0.01 mg/l, 0.5 mg/l MTR plus NAA 0.1 mg/l, 1 mg/l MTR plus NAA 0.5 mg/l, 2 mg/l MTR plus NAA 1 mg/l and 3 mg/l MTR plus NAA 2 mg/l was used, 3.7, 5.7, 7.3, 6.7 and 4.7 shoots were obtained

respectively. MTR With 0.1 mg/l plus 0.01 mg/l TDZ, 0.5 mg/l MTR plus 0.1 mg/l TDZ, 1 mg/l MTR plus 0.5 mg/l TDZ, 2 mg/l MTR plus 1 mg/l TDZ and 3 mg/l MTR plus 2 mg/l TDZ, 3.7, 4.3, 5.0, 5.7 and 4.3 shoots were obtained respectively (Fig.21). When 0.1 mg/l MTR plus 0.01 mg/l KIN, 0.5 mg/l MTR plus 0.1 mg/l KIN, 1 mg/l MTR plus 0.5 mg/l KIN, 2 mg/l MTR plus 1 mg/l KIN and 3 mg/l MTR plus 2 mg/l KIN was tested, 6.0, 6.7, 11.3, 9.7 and 8.3 shoots were induced respectively (Fig.21).

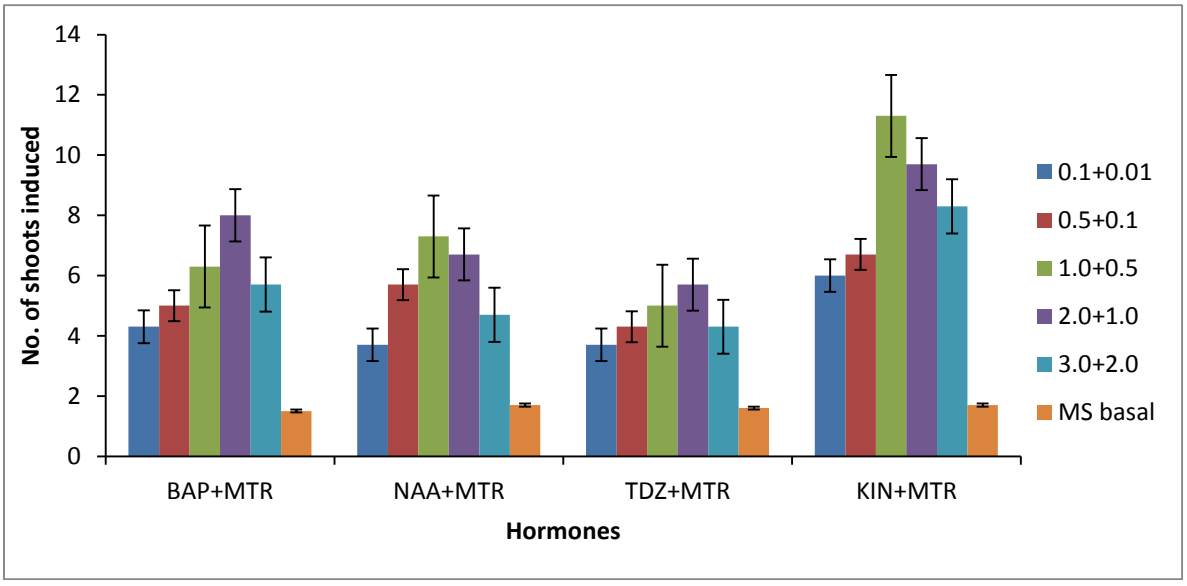

Figure 21: Shoots from nodal explants of *Moringa oleifera* on medium with MTR combined with BAP, NAA, TDZ and KIN

4.5 Effects of Naphthalene acetic acid, Benzyl amino purine and Metatopoline ribonucleotide on rooting of axillary shoots of *Moringa oleifera*

Rooting was tested on MS with NAA, BAP and MTR and roots induced were recorded after four weeks. At 0.01, 0.1, 0.5, 1.0 and NAA 2.0 mg/l, 4.7±0.27, 5.3±0.27, 6±0.47, 6±0 and 4.3±0.27 roots were induced respectively (Table 7). All the explants inoculated on NAA gave roots. With 0.01 and 0.1 mg/l BAP less than one root was observed while BAP 0.5, 1.0 and 2.0 mg/l

produced an average of 1±0, 1.3±0.17 and 1.3±0.17 roots respectively and only 20% of the cultured shoots on BAP gave roots. No roots were induced with MTR. Basal MS medium (control) obtained an overall mean of 5.87 roots and 70% of the cultured explants gave the roots (Table 7), (Fig. 22).

Table 7: Number of roots obtained from axillary shoots of *Moringa oleifera* in different hormones.

	Hormone			
Conc. (mg/l)	NAA	BAP	MTR	MS basal
0.01	4.7±0.27bc	0d	0d	5.3±0.30abc
0.1	5.3±0.30abc	0.7±0.07d	0d	5.7±0.33abc
0.5	6±0.47ab	1±0d	0d	6.7±0.5a
1	6±0ab	1.3±0.17d	0.7±0.07d	6.7±0.5a
2	4.3±0.25c	1.3±0.17d	0d	6ab

F 3.05 Df 4, 3 $p<0.05$

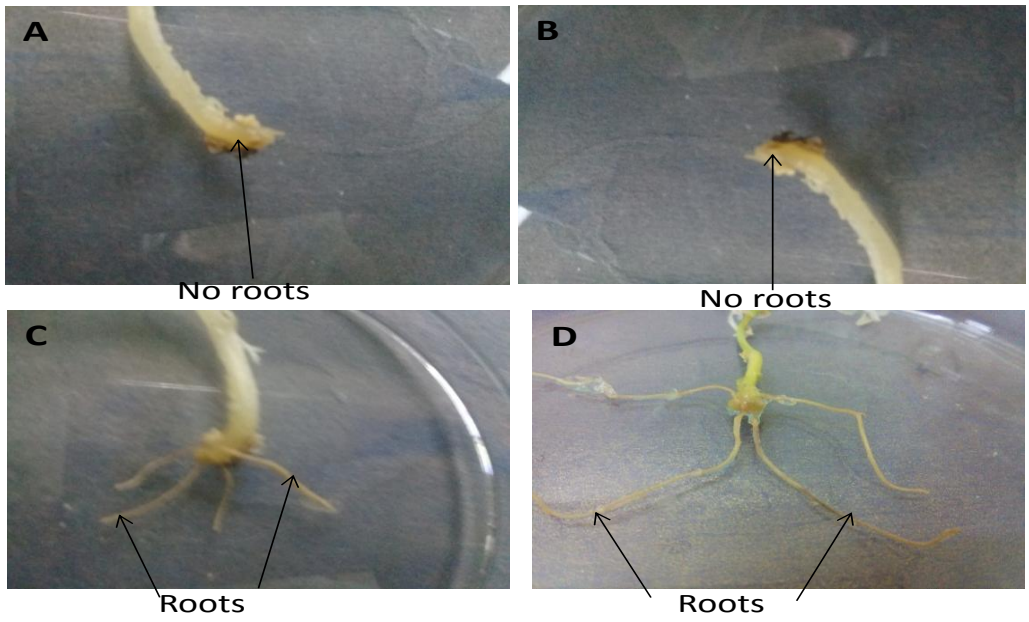

Figure 22: Four weeks old roots from nodal shoots of *Moringa oleifera* on MS media with (A) BAP 0.5 mg/l, (B) MTR 0.5 mg/l, (C) NAA 0.5 mg/l and (D) MS basal

4.6 Hardening and acclimatization of *Moringa oleifera* plantlets from nodal explants

After rooting the planlets were transferred to jars with sterile vermiculite for two weeks (Fig. 23). In the jars 90% of the plantlets survived, and within the first one week after transfer to a mixture of sterile vermiculite and peat moss (1:3), the plantlets remained healthy, but in two weeks 20% of the remaining plantlets suffered from damping off which manifested as falling and sudden death of plantlets. Out of the remaining plantlets (70%), 5% showed evidence of wilting and necrosis one week after partial exposure; even though there were no obvious symptoms of infection macroscopically they turned yellow and died within the second week of exposure. When the remaining plants (65%) were later fully exposed by completely removing the polythene cover after the third week, the survival rate was sharply reduced within one week and 55% of the plantlets survived. These were later transferred to the soil and taken to the glass house (Fig.23), and they remained healthy and continued to grow though with low vigour and these plantlets were phenotypically normal.

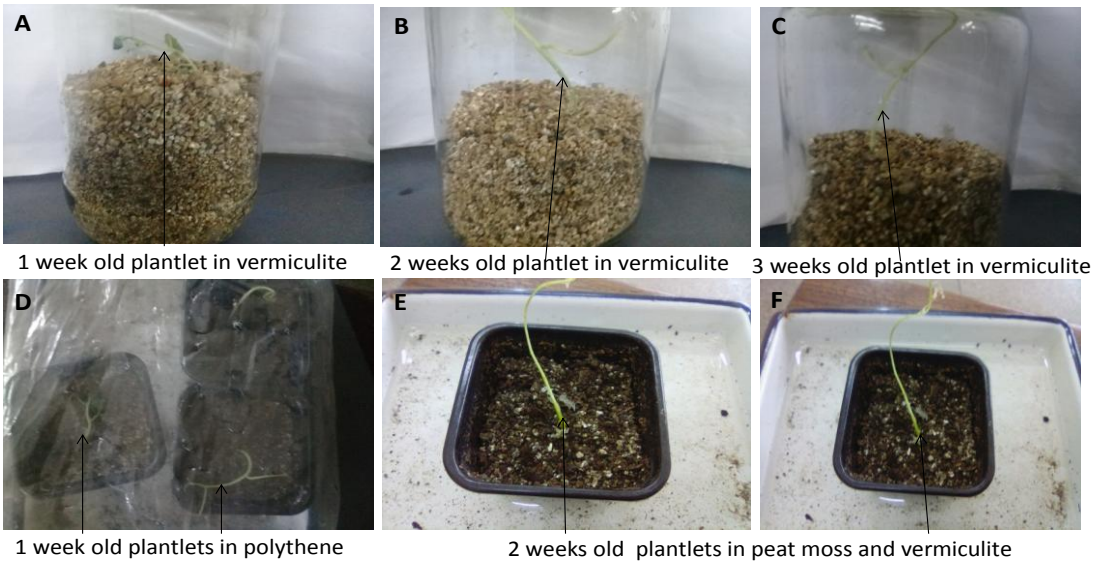

Figure 23: *Moringa oleifera* plantlets (A) 1 week in vermiculite, (B) 2 weeks in vermiculite, (C) 3 weeks in vermiculite, (D) covered in polythene, (E and F) 2 weeks in peat moss and vermiculite for acclimatization and hardening

4.7 Antifungal assays of the callus and leaf extracts of *Moringa oleifera* on *Fusarium semitectum, Aspergilus flavus and Candida albicans*

Methanol: Di-chloromethane (1:1) leaf extracts from field grown *Moringa oleifera* plants and of callus were assayed for their inhibitory activity against *Fusarium semitectum, Aspergillus flavus and Candinda albicans.* All concentrations of callus extract tested were active against *F. semitectum* except at a concentration of 20% as depicted in the inhibition zones. Callus extract at a concentration of 20% had inhibition zone of 8.7±0.11 mm and this was considered inactive. At concentrations of 40%, 60%, 80% and 100% of callus extract the inhibition zones of 9.7±0.11, 10.6±0.11, 12.7±0.22 and 14.3±0.33 mm were recorded respectively against inhibition zone of 17.4±0.52 from 250 mg/ml fluconazole (positive control) (Table 8). Callus extract at concentrations of 40%, 60%, 80% and 100% were considered active because they recorded inhibition zones above 8 mm.

On *A. flavus,* all concentrations of callus extract were considered active except concentrations of 20% and 40% which recorded inhibition zones of 8 mm and below. At 20% and 40% the inhibition zones of 5.0±0.06 and 5.7±0.07 mm were recorded respectively. While at concentrations of 60%, 80% and 100% inhibition zones of 9.0±0.07, 10.7±0.11 and 11.7±0.116 mm were recorded respectively (Table 8). *Fusarium semitectum* was found to be susceptible to the callus extract, *Aspergilus flavus* was found to be intermediate while *Candida albicans* was resistant to the callus extract.

Table 8: Inhibition zones (mm) of *Moringa oleifera* callus extract against *Fusarium semitectum, Aspergillus flavus* and *Candida albicans.*

	Inhibition zones (mm)		
Conc. (%)	F. semitectum	A. flavus	C. albicans
20%	8.7±0.11cd	5.0±0.06def	0
40%	9.7±0.11bcd	5.7±0.07cde	0
60%	10.6±0.11abcd	9.0±0.07cd	0
80%	12.7±0.22abc	10.7±0.11bc	0
100%	14.3±0.33ab	11.7±0.11ab	0
+ve control	17.4±0.52a	17.0±0.52a	16.1±0.33ab
Fungi Category	Susceptible	Intermediate	Resistant
Df 5,24	F 0.77	p=0.05	

Leaf extract against *F. semitectum* had all concentrations considered inactive except at 80% and 100% which recorded inhibition zones above 8 mm. At 20%, 40% and 60% of the extract the inhibition zones were 4.0±0.25, 5.0±0.27 and 5.5±0.27 mm respectively. At 80% and 100% leaf extracts had mean zone of inhibitions of 9.7±0.3 and 10.3±0.30 mm respectively (Table 9). Leaf extract on *A. flavus* also was considered inactive except at 100%; at 20% and 40% inhibition zones were 3.7±0.27 and 4.8±0.47 mm respectively. At 60%, 80% and 100% inhibition zones recorded were 5.0±0.54, 5.4±0.54 and 9.2±0.60 respectively (Table 9). *Fusarium semitectum* and *Aspergilus flavus* were considered intermediate to leaf extract while *Candida albicans* on the other hand was resistant to leaf extract.

Table 9: Inhibition zones (mm) of *Moringa oleifera* leaf extract against *Fusarium semitectum, Aspergillus flavus* and *Candida albicans.*

Leaf extract			
	Inhibition zones (mm)		
Conc. (%)	F. semitectum	A. flavus	C. albicans
20%	4.0±0.25f	3.7±0.27fg	0
40%	5.0±0.27de	4.8±0.47e	0
60%	5.5±0.27d	5.0±0.54de	0
80%	9.7±0.30bc	5.4±0.54d	0
100%	10.3±0.30b	9.2±0.60bc	0
+ve control	17.5±0.64a	17.2±0.68a	16.3±0.58ab
Fungi Category	Intermediate	Intermediate	Resistant
Df 5,24	F 1.25	p<0.05	

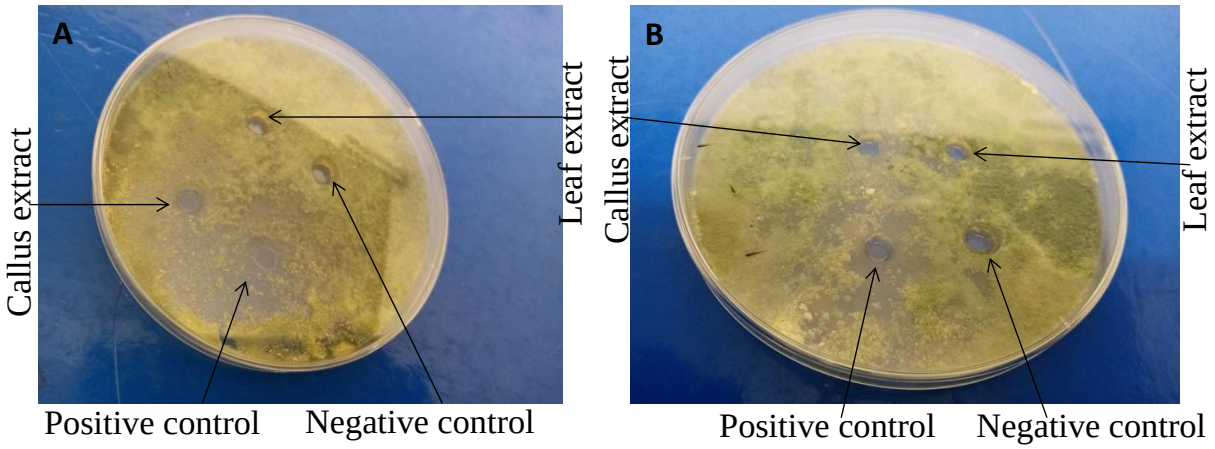

Figure 24: Inhibition zones of *Moringa oleifera* leaf and callus extracts on *Aspergilus flavus*

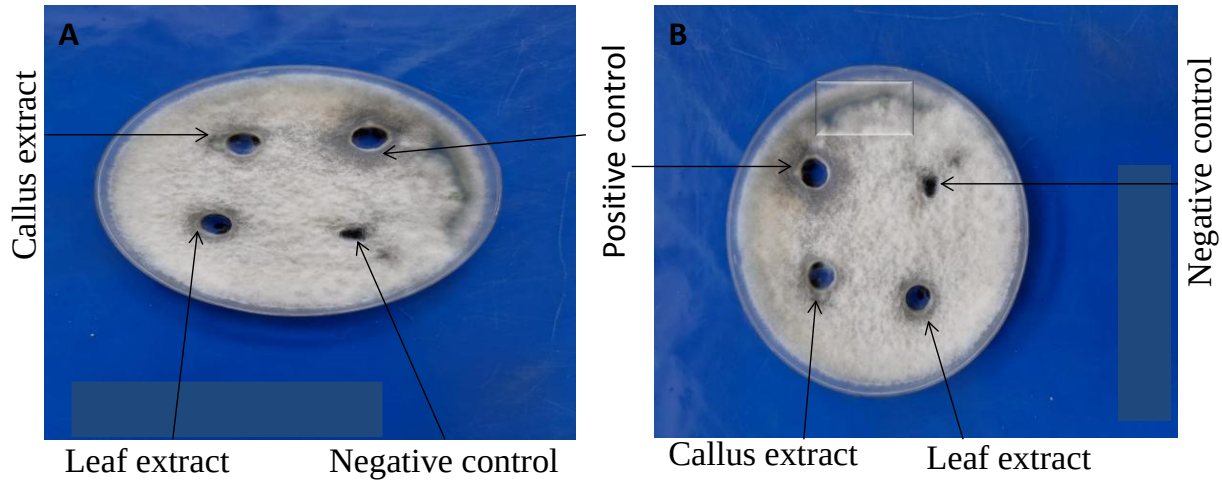

Figure 25: Inhibition zones of *Moringa oleifera* leaf and callus extracts on *Fusarium semitectum*

4.7.1 Minimum Inhibitory Concentration of *Moringa oleifera* callus and leaf extracts on *Fusarium semitectum, Aspergillus flavus* and *Candida albicans*

Minimum Inhibitory Concentration (MIC) was considered to be the least concentration of leaf or callus extract that inhibited growth of the fungi. This was determined by recording the least concentration of the extracts that had the least colony forming units of *F. semitectum* and *A. flavus.* At 10^{-3} dilution, leaf extract recorded 26 x 10^{-3}, 28 x 10^{-3} and 12 x 10^{-3} colony forming units at concentrations of 20%, 40% and 60% respectively against *F. semitectum*. At 80% and 100%, no *F. semitectum* growth was observed (Table 10). Also leaf extract recorded 40 x 10^{-3}, 22 x 10^{-3}, 14 x 10^{-3} and 10 x 10^{-3}colony forming units at concentrations of 20%, 40%, 60% and 80% respectively against *A. flavus* while at 100% no colony forming unit of *A. flavus* was reported (Table 10). Therefore the MIC of the leaf extract against *F. semitectum* and *A. flavus* was reported at 80% and 100% respectively.

Table 10: Colony forming units of *Fusarium semitectum and Aspergillus flavus* on leaf extract of *Moringa oleifera*

	Fungi	
Extract concentration	*F.semitectum*	*A. flavus*
20%	26x10^3	40x10^3
40%	28x10^3	22x10^3
60%	12x10^3	14x10^3
80%	0	10x10^3
100%	0	0
Negative control	>40x10^3	>40x10^3
Positive control	0	0

At 10^{-3} dilution, callus extract recorded 24 x 10^{-3} and 22 x 10^{-3}colony forming units at concentrations of 20% and 40% respectively against *F. semitectum*, while at 60%, 80% and 100% concentrations, no colony forming units were observed (Table 11). Against *A. flavus*, callus extract recorded 32 x 10^{-3}, 16 x 10^{-3} and 10 x 10^{-3}colony forming units at concentrations of 20%, 40% and 60% respectively, while no colony forming units were observed at 80% and 100% respectively (Table 11). Therefore the MIC of the callus extract on *F. semtectum* was 60% and on *A. flavus* was 80%.

Table 11: Colony forming units of *Fusarium semitectum and Aspergillus flavus* on callus extracts of *Moringa oleifera*

Extract concentration	Fungi	
	F.semitectum	*A. flavus*
20%	24x10^3	32x10^3
40%	22x10^3	16x10^3
60%	0	10x10^3
80%	0	0
100%	0	0
Negative control	>40x10^3	>40x10^3
Positive control	0	0

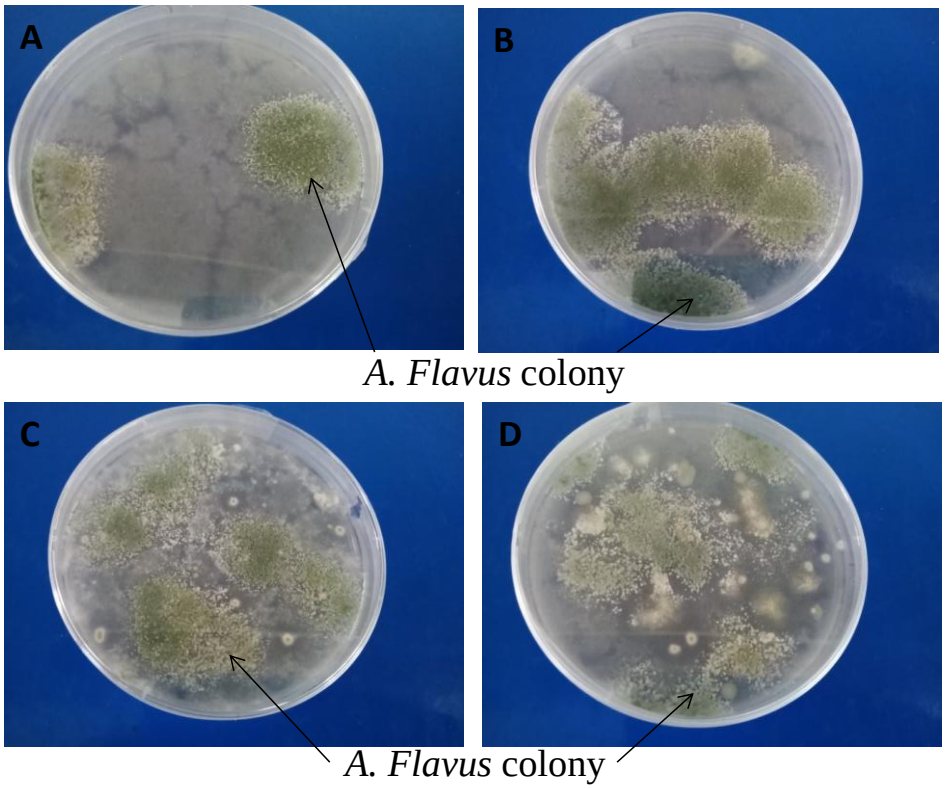

Figure 26: *Aspergilus flavus* colonies on (A) 60% (B) 40% of callus extract, (C) 80% (D) 40% of leaf extract of *Moringa oleifera* after 72 hours

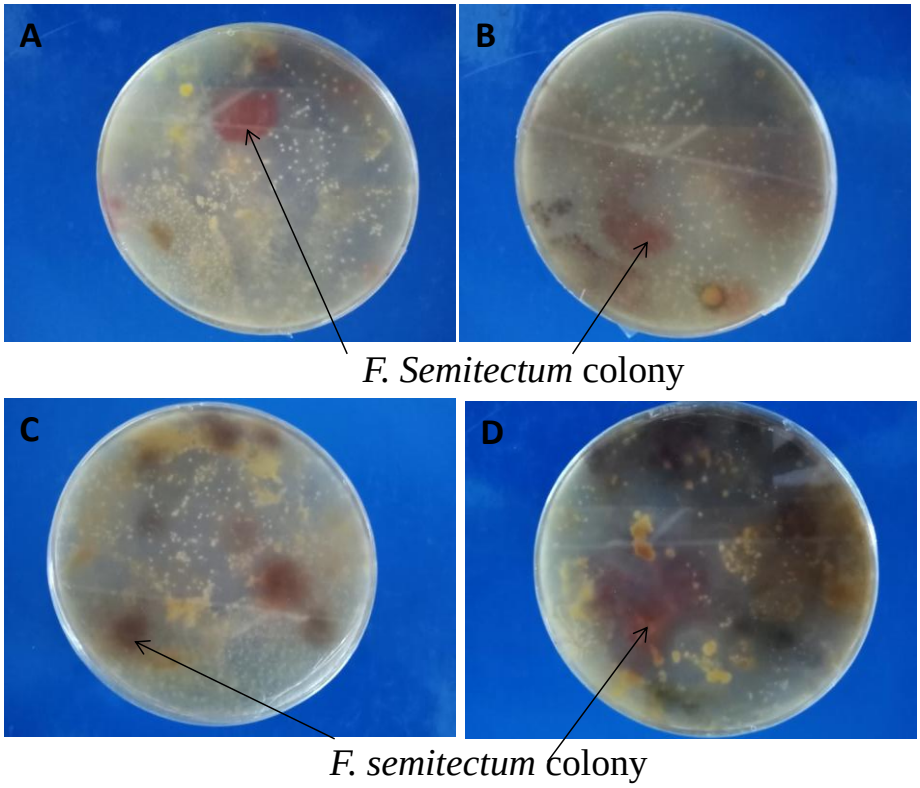

Figure 27: *Fusarium semitectum* colonies on (A) 40% (B) 20% of callus extract, (C) 40% (D) 20% of leaf extract of *Moringa oleifera* after 72 hours

CHAPTER FIVE

5.0 DISCUSSION

5.1 Explant sterilization and *in vitro* seed germination

The best sterilization of *Moringa oleifera* seeds for *in vitro* germination was achieved using 30% commercial bleach (JIK) for 15 minutes followed by 70% ethanol for 2 minutes. This result is in agreement with Allan, (1991) who explained this could be because the sterilants eliminated all the microorganisms that could grow under *in vitro* conditions. Also they guaranteed the viability of the explants and their regeneration capacity, which could be affected by sterilants concentration and their application period. The results also concur with those of Ndakidemi *et al*. (2013) who achieved best sterilization with sodium hypochlorite 3.85% and ethanol 70%, but differed with Förster *et al*. (2013), who could not establish *in vitro* sterile cultures of *Moringa oleifera* seeds with any concentration of sodium hypochlorite. Almost all explants of *Moringa oleifera* exposed to commercial bleach at 10%- 15% were contaminated, this agrees to the findings of this study, while those exposed to concentrations of 35%- 40% lost their viability (Colgecen *et al.*, 2009). Sodium hypochlorite at high concentrations (above 4.5%) proved toxic and resulted in 100% necrosis and death of *Moringa oleifera* explants (Moutia and Doukin, 1999).

Germination of *Moringa oleifera* intact seeds on both MS and half MS media started on day eight post inoculation with 16.7% of seeds germinated while in seeds with their seed coats removed germination started on day three with 27.3% of seeds in MS and 22.3% of seeds in half MS media germinated. The delay in the germination of intact seeds could be because of the effect of seed coat. Many seeds of the tropical trees have no dormancy, but some species (i.e

Moringa oleifera Lam.) have delayed germination as if they were dormant (Albrecht, 1993; Msanga, 1998). By day 10 of culture more than 95% of the seeds without seed coats had germinated, this is an improvement over Avila *et al*. (2017) who achieved *in vitro* germination percentage of 84% of *Moringa oleifera* seeds without seed coats in 10 days on MS medium. In this study germination on half MS and MS media had no significant difference ($p \leq 0.05$). However this disagrees with a similar research done by Wesam *et al*. (2013) who reported that MS basal media was better on germination of *Moringa peregrina* seeds *in vitro* than MS half strength media.

Moringa oleifera seeds without seed coats germinated faster on MS with 0.5 mg/l Gibberellic acid (GA_3) with 100% germination in 5 days, while GA_3 2.5 mg/l reported 90% seeds germinating after 5 days of inoculation. Germination of seeds with seed coats had a lower germination with MS medium containing 6.5 mg/l GA_3 achieving best germination of 24.6% in all the concentrations used (0.5, 2.5, 4.5 and 6.5 mg/l). Giberellic acid concentration did not affect germination of both intact seeds and seeds without seed coats ($p \leq 0.05$). This differs with Sunday *et al*. (2015) who depicted that germination of GA_3 treated seeds was proportional to the concentration of GA_3 dissolved. This could be because GA_3 penetrated the seed coat, activating hydrolytic enzymes thus breaking down stored food resources into metabolically useful chemicals, allowing the cell of the embryo to divide and grow (Raven *et al*., 2005). By day nine, germination was complete in all the treatments and the shoot elongation continued as normal inside the jars without any interference.

5.2 Callus formation and growth

When used, NAA initiated callus formation in the least time of 2- 6 days in hypocotyls, inter-nodal segments and leaf discs, but the callus was sticky, frothy and compact. This is an improvement to Umbreen *et al*. (2014) who achieved callus initiation using NAA in 17 days in all the explants of *Moringa oleifera* tested. Also according to Shahzad *et al*. (2014), callus formation from leaves of *Moringa oleifera* took more than two weeks in NAA 0.5- 2.0 mg/l.

With 2,4D it took 4 days in hypocotyls to form callus, while in inter-nodal segments and leaf discs it took 4.7 and 6 days respectively to form callus. Callusing was best in hypocotyls at 0.5 mg/l 2,4D among the concentrations of 2,4D used (0.25, 0.5, 1.0 and 2.0 mg/l) and among the explants used (hypocotyls, inter-nodal segments and leaf discs). This agrees with the results of Abdellatef and Khalafalla (2010) who reported ability of 2,4-D in callus induction on hypocotyls of *Moringa oleifera,* and documented 2,4-D 0.1-0.5 mg/l to be better for inducing callus (Khalafallah *et al*., 2011).

Callusing was initiated in 14 to 28 days in cotyledon explants of *Moringa oleifera* using 2,4-D 1.0, 2.5 and 5.0 mg/l (Nieves and Aspuria, 2011). Results in this study agree with Shank *et al.,* (2013) and Lalida *et al*., (2013) who found MS medium with 2,4D 0.5 mg/l to induce callus most effectively in *Moringa Oleifera* giving 100% callusing. But it differs from Umbreen *et al*. (2014) who observed good callus initiation with 0.5 mg/l 2,4D in 7 days in hypocotyls and 10 days in leaf discs of *Moringa oleifera*. Shahzad *et al*. (2014) initiated callus in hypocotyls within five days in 2,4-D 2.0 mg/l, this agrees with the findings of this study that reported callus in hypocotyls was initiated in 5.7 days in 2.0 mg/l 2,4D. Shahzad *et al*., (2014) initiated callus in cotyledonary explants of *Moringa oleifera* within four days using 2,4 D 2.0 mg/l.

Callus induction on MS supplemented with BAP happened in 4-6 days in hypocotyls, inter-nodal segments and leaf discs with BAP 1.0 mg/l being the optimum and giving callus in 4.0 days in hypocotyls, 4.7 days in inter-nodal segments and 5.0 days in leaf discs. These differ with Saini *et al*. (2012) who did not produce any callus from *Moringa oleifera* shoots on MS with BAP 1.0 mg/l.

With TDZ it took 4-7 days to initiate callusing in hypocotyls, inter-nodal segments and leaf discs of *Moringa oleifera*. This differs with Shahzad *et al*. (2014) who induced callus from leaves and hypocotyls of *Moringa oleifera* on MS with TDZ at (0.5- 2.0 mg/l) in an average of nine days. Shahzad *et al.* (2014) initiated callus on MS with TDZ at 2.0 mg/l in 4 days but from epicotyls of *Moringa oleifera*.

Callus induction from inter-nodal segments and leaf discs was observed on the cut edges, this was in agreement with the work of Fatima and Perveen, (2016) who reported that growth of callus of *Moringa oleifera* leaf explants formed at the edge of the explant (part of the injury) which touches the medium. This may be because cells from cut surface are directly in contact with nutrient medium leading to better absorption. The emergence of callus on the injured part may be due to the stimulation of cells in the explants to cover the wound (Robbiani *et al*., 2010). Auxins induced callus faster than the cytokinins, this could be due to their ability to stimulate the growth of callus, division and elongation of cells.

Among the auxins used NAA gave callus with higher weight after four weeks of inoculation with NAA 1.0 mg/l recording optimum callus weights, but the callus developed roots. Riyathong *et al*., (2010) also found root production in callus on MS with NAA 1 mg/l while NAA 0.5 mg/l produced both roots and shoots. With 2,4D optimum callus weight was obtained at 0.5 mg/l with

9.78±0.26 g in hypocotyls, 9.97±0.20 g in inter-nodal segments and 6.15±0.08 in leaf discs. This differs with reports by Oriabi, (2016) who found fresh callus weight in *Moringa oleifera* to increase with increased 2,4-D concentration with 0.0, 1.0 and 1.5mg/l recording 0 g, 0.22 g and 0.34 g, while 2.0 and 2.5 mg/l recording 0.38 g and 0.50 g on average of respectively.

Highest callus weight on MS with BAP was recorded at 1.0 mg/l BAP with weights of 6.82±0.02 g in hypocotyls, 6.88±0.02 g in inter-nodal segments and 4.87±0.01 g in leaf discs recorded but the callus was compact and sticky. This differs with Saini *et al*. (2012) who recorded 100% callus formation in medium supplemented with BAP (0.5, 2.0 and 3.0 mg/l), and obtained friable callus, followed by those treated with BAP 4.0 mg/l recording 80%. Oriabi, (2016) obtained highest callus fresh weight of 0.37 g on MS with BAP 0.5 mg/l. He obtained increased callus weight of 0.37 g and 0.33 g with BAP 0.5 and 1.0 mg/l respectively, but got 0.17 and 0.29 g at 0.0 and 1.5 mg/l BAP respectively in shoot explants. The result of this study is an improvement to the previous studies as it reported increased weight of callus.

When 2,4D, NAA or BAP was combined with TDZ at equal concentration of 1.0 mg/l callus weight increased greatly. This is in line with the results of Nieves and Aspuria, (2011) who recorded increase in callus weight in TDZ combined with 2,4-D. They also found callus to be nodular and loose, which differs with this work that reported callus from 2,4D and TDZ and NAA and TDZ to be friable while that of BAP and TDZ to be compact or sticky. Al Dhaberi and Salama, (2016) also found out that callusing was increasingly high on 2, 4-D 2 mg/l in combination with 0.5 μM/l TDZ compared to other treatments (2, 4-D and BA and Kin combinations) in *Moringa peregrina* shoot tips.

The highest callus weight in this study was 15.4 g obtained from hypocotyls on 2,4D 1.0 mg/l combined with TDZ 1.0 mg/l. Preece *et al*. (1991) and Huetteman and Preece (1993) also reported that amino-purines, when combined with TDZ results in profuse callus being formed. Therefore TDZ incorporated with other growth regulators increases callusing in *Moringa oleifera* which leads to an increase in callus weight. Callus weight varied with the hormone concentration and the type of the explant used, with hypocotyls giving callus with the most weight and in less time compared with inter-nodal segments and leaf discs. This is in agreement with Islam *et al*. 2005 who also found out that hypocotyls responded well to callusing and in short period of time in *Moringa oleifera*.

Murashige and Skoog (1962) basal medium also gave callus in hypocotyls, inter-nodal segments and leaf discs of *Moringa oleifera* after 4 weeks. This could be because the plant contains some endogenous growth regulators (i.e. hormones of the cytokinin type) as also observed by Makkar and Becker (1996). The result also agrees with that of Djande *et al*. (2019) who also obtained callus growth on hormone-free medium, and the callus retained ability to grow in several sub-cultures. But this differs with Lalida *et al*. (2013) who found no callus forming from shoot explants of *Moringa oleifera* inoculated on hormone free MS for four weeks. Riyathong *et al*. (2010) also reported 0% callus formation with MS medium without hormone in shoot explants of *Moringa oleifera*.

5.3 Plant regeneration from callus

Efforts to regenerate shoots from callus were not successful in all the growth regulators tested; BAP, KIN, NAA, TDZ and MTR both singly and when combined. In regeneration experiments on MS basal medium, callus resumed normal growth and later developed green spots on the

surface but turned brown and died. This agrees with report of Riyathong *et al*. (2009) that showed callus did not result in the regeneration of shoots when sub-cultured to hormone free MS medium. The green spots observed on the callus surface in MS basal medium could be because *Moringa oleifera* contains endogenous hormones of the cytokinin type which can induce shooting from callus cultures (Makkar and Becker, 1996).

With BAP 2 mg/l the green spots that also developed on callus surface formed shoot like structures that later turned brown and dried. This agrees with Riyathong *et al*. (2010) who also found neither shoots nor roots from callus on MS with BA 0 – 2.0 mg/l, but observed green spots on the surface. When BAP 2.0 mg/l was combined with 2 mg/l KIN, shoot like structures increased but later turned brown and dried.

Callus regenerated on NAA 0.5 mg/l and on media with 0.5 mg/l NAA combined with 2 mg/l BAP, only protrusions and roots developed which later turned brown and dried. This is in agreement with Riyathong *et al*. (2009), who stated that callus grown on MS with NAA did not result in regeneration of shoots. When Riyathong *et al*. (2010) improved on their work; they found out that callus on MS with NAA 0.5 mg/l gave roots and shoots. Nieves and Aspuria, (2011) also reported that when the green compact callus with protrusions were incubated on medium with NAA 0.5, 1.0, 2.5 mg/l or NAA 0.5, 1.0, 2.5 mg/l combined with BA 1.0, 2.5, 5.0 mg/l the protrusions did not grow further after 2 weeks of incubation.

Callus regenerated on medium with 0.5, 1.0 and 2.0 mg/l TDZ, resumed growth without any sign of differentiation. Huetteman, (1998) explained that this could be because when TDZ is incorporated with other hormones it results in excess callusing and shoot ceases to grow. On 0.5 and 1 mg/l MTR, shoot like structures developed which after the fourth week dried and died. The

same was recorded on 0.5 mg/l BAP combined with 1.0 mg/l MTR. On media with 1 mg/l MTR and 0.5 mg/l NAA, green spots appeared on the callus surface but later roots developed. This work could not regenerate *Moringa oleifera* through somatic embryogenesis. The low response of *Moringa oleifera* callus to shoot induction could be because cells of *Moringa oleifera* callus retain the traces of hormones used in callusing when subcultured to shoot induction media and this could inhibit the germination of somatic embryos and shoot formation (Leljak -Levanić *et al*., (2016).

5.4 Obtaining shoots from nodal segments

Shoot induction from nodal segments of *Moringa oleifera* on Benzylaminopurine, Thidiazuron, Metatopolin ribonucleotide, Kinetin and Naphthalene acetic acid was significant ($p \leq 0.05$) among the hormones used. Medium with 1.0 mg/l MTR gave shoots in the least number of days (3.3) followed by 2.0 mg/l NAA with 5.3 days and 2 mg/l BAP with 6.3 days. This is the first work to report on the ability of MTR to induce shoots from nodal segments of *Moringa oleifera*. When 0.5 mg/l TDZ was used, shoot initiation occurred in 7.3 days. In comparison Al Dhaberi and Salama, (2016) reported 0.5, 1.0 and 2 mg/l TDZ germinated nodal explants within two weeks of culture initiation in *Moringa peregrina*. Kinetin 2.0 mg/l initiated shooting from nodal segments in 7.7 days. In this study, basal MS medium (control) also initiated nodal shooting in 9.4 days. Shoots induced from nodal explants of *Moringa oleifera* depended on type and level of hormones used which varied in efficiency and potential.

The most shoots induced in BAP occurred at 2.0 mg/l, with 4.7 shoots which was the highest among the BAP concentrations used (0.1- 3.0 mg/l). This differs from Islam *et al*. (2005); Stephenson and Fahey (2004) who reported 1.0-1.5 mg/l BAP as the best for inducing shoots

from nodes of *Moringa oleifera.* But the results concur with Riyathong *et al*., (2010) and Saini *et al.,* (2012) results that 2.0 mg/l BAP was best for shoot induction with 10.8 shoots per nodal explant of *Moringa oleifera*. Findings of Zulaliya (2017) differ with the findings of this study as she reported slightly lower shoots (1.23) per explant of *Moringa oleifera* on MS with 2 mg/l BAP. The difference could be attributed to differences in *Moringa oleifera* genotypes.

Medium with 0.5 mg/l NAA recorded 4.3 shoots which was the highest among the concentrations of NAA used (0.1- 3.0 mg/l. Thidiazuron 2.0 mg/l and 3.0 mg/l gave 3.3 shoots each which were the highest among the concentrations of TDZ used (0.1- 3.0 mg/l). Al Dhaberi and Salama (2016) in their study reported that direct organogenesis from nodal explants of *Moringa peregrina* was highest on MS with 1 µM of TDZ giving 2.4 axillary shoots in each explant followed by 2 µM of TDZ with 1.85 shoots per explant.

Metatopoline ribonucleotide 1.0 mg/l recorded the highest shoots of 9.3 amongst all the hormones tested. However no data exist on the use of MTR in inducing axillary shoots from *Moringa oleifera* explants. In this study, 1.0 mg/l Kinetin recorded 5.7 shoots, highest shoot numbers in all the KIN concentrations used (0.1- 3.0 mg/l). Riyathong *et al.* (2010) and Abdellatef and Khalafallah, (2010) also produced shoots on KIN 0.5- 1.0 mg/l. Alkhateeb, (2013) on his work obtained 2.6 shoots per explant of *Moringa peregrina* on MS with 1.0 mg/l KIN. The least shoots induced in this study on MS with plant hormones were 2.3 in 3.0 mg/l BAP. This is a slight improvement from Shahzad *et al*. (2014) who reported 1.61 shoots in nodal explant of *Moringa oleifera* on MS medium with Kinetin and BAP singly. The variables may be due to differences in the plant source and the genetic variability of the plant. Murashige and Skoog (1962) basal media also induced shoots with an average of 1.53 shoots per nodal explant of *Moringa oleifera*. This work managed to obtain shoots from nodal segments of *Moringa*

oleifera with hormone free medium (MS basal). This could be because *Moringa oleifera* explants acquire the ability to grow without the presence of externally added hormones because the plant is known to contain hormones of the cytokine type (Makkar and Becker, 1996).

When MTR was combined with other growth regulators shoots increased significantly ($p \leq 0.05$). On MS with 1.0 mg/l KIN plus 0.5 mg/l MTR, 11.3 shoots were induced, the highest among all the hormone treatments used in this study. On 3.0 mg/l TDZ plus 2.0 mg/l MTR, 3.7 shoots were recorded the least among the hormone combinations used in this study.

Stephenson and Fahey, (2004) produced 4.7 shoots on 1 mg/l BA on combination with 1 mg/l GA_3 from seeds of *Moringa oleifera*. Al Dhaberi and Salama, (2016) found out that when BA was combined with NAA the number of shoots did not increase when compared to the two hormones used singly. Fatima *et al*. (2016) reported 19.66 shoots on 0.1 mg/l BAP combined with NAA 0.5 mg/l as the highest from nodal explants of *Moringa concanens*. This study however induced the highest of 11.3 shoots when 1.0 mg/l KIN plus 0.5 mg/l MTR was used, however these were different *Moringa* species.

In all the hormones used both singly and in combination, callus formed at the base of the nodal explants before shooting including those inoculated on MS basal medium. Shahzad *et al*. (2014) and Islam *et al*. (2005) also observed callus development at the base of the cultured nodal explants of *Moringa oleifera* and stated it was mostly prominent on increased hormone concentrations.

The study therefore concluded that cytokinins were good in inducing and proliferation of shoots from nodal explants of *Moringa oleifera.* This could be due to their ability of enhancing shoot initiation, promoting axillary branching or axillary bud proliferation and results from other

studies have also revealed that cytokinins were a key factor for multiple shoot proliferation in *Moringa oleifera* (Riyathong *et al*., 2010). All plant hormones used in the study (BAP, NAA, TDZ, KIN and MTR) showed shoot proliferation, however shoot numbers from nodal explants of *Moringa oleifera* depended mostly on hormone type and concentration.

5.5 Rooting

Best rooting in this study occurred on MS basal medium giving a mean of 5.9 roots per explant. This agrees with Shahzad *et al*. (2014) and Islam *et al*. (2005) who produced more roots on both MS full and half strength media than on media with root inducing hormones in *Moringa oleifera.* They also concluded that rooting was efficient on MS basal medium. In this study, media with NAA ranging from 0.01-2.0 mg/l initiated rooting giving 4-6 roots, but roots were very short and abnormally thick. This differs from the work of Marfori, (2010) who obtained optimal rooting of shoot cultures of *Moringa oleifera* on 0.05 mg/l NAA and explained that this difference could be due to genetic differences, different explant source, hormone type and concentration and/or explant age. Stephenson and Fahey (2004) also reported good root formation on MS with 0.5 mg/l NAA. But Saini *et al*. (2012) achieved best rooting on 0.5 mg/l IAA combined with 1.0 mg/l IBA in *Moringa oleifera*. Benzyl amino purine and metatopoline ribonucleotide recorded between 0-1 roots and this was considered insignificant.

5.6 Acclimatization

On acclimatization and hardening of the rooted shoots, 55% remained healthy and continued to grow though with low vigour but they were phenotypically normal. These results obtained are slightly lower compared to those of Marfori, (2010) and Saini *et al*. (2012) who obtained 80% of plants surviving. But Marfori, 2010 explained that this happens only when the rooted shoots

remained covered with clear polythene and maintained under a shade for 15 days in a greenhouse before exposing them to conducive conditions. Fungal contamination was the main drawback in acclimatization of the plantlets with over 30% of the plantlets dying of either damping off or drooping. The physical conditions including humidity and temperature could also have contributed to the low number of the plantlets successfully acclimatized.

5.7 Antifungal activity

Leaf and callus Methanol: Di-chloromethane (1:1) extracts were found to have activity against *Fusarium semitectum* and *Aspergillus flavus* but the activity differed with different concentrations. All concentrations of callus extract tested were active against *F. semitectum* except at 20% which recorded an inhibition zone of 8.7±0.11 mm and was considered inactive. Callus extract at concentrations of 40%, 60%, 80% and 100% were considered active because they recorded inhibition zones above 8 mm. On *A. flavus*, all concentrations of callus extract were considered active except at concentrations of 20% and 40% which recorded inhibition zones of 8 mm and below. While at concentrations of 60%, 80% and 100% were considered active because they recorded inhibition zones above 8 mm. When leaf extract was tested against *F. semitectum*, it was considered active at concentrations of 80% and 100% recording inhibition zones above 8 mm. At 80% and 100% leaf extracts recorded mean zones of inhibition of 9.7±0.3 and 10.3±0.30 mm respectively. Leaf extract tested on *A. flavus* also was considered active at 100% which recorded mean inhibition zone of 9.2±0.60 mm.

These results agree with those of Oluduro and Aderiye, (2009) who also reported the antifungal properties of *Moringa oleifera* leaf against *Aspergillus flavus, Trichophyton mentagrophyte*, *Pullarium sp.*and *Penicillium sp.* Arowora and Adetunji, (2014) also inhibited growth of *A.niger*

with *Moringa olifera* leaf extracts. Aisha *et al*. (2016) reported inhibition activity of *Moringa oleifera* leaves extracted on methanol but on *Aspergillus niger*. *Candida albicans* on the other hand was resistant to both the callus and leaf extracts. This disagrees with Bauer *et al.* (1966) who reported inhibition activity of *Moringa oleifera* leaves extracted on methanol against *Candida albicans*. Aisha *et al*. (2016) also reported activity of ethanol and methanol extracts of dried leaves of *Moringa oleifera* against *Candida albicans*.

Callus extract had activity against *F. semitectum and A. flavus* in least concentrations of 60% and 80% respectively. The leaf extract from field grown plants showed a less antifungal activity against *F. semitectum* and *A. flavus* compared to callus extract recording activity at 80% and 100% respectively. This finding suggests that *in vitro* products (callus) are still able to synthesize secondary metabolites just like field grown plants as changes may take place in metabolic activities of cells under culture leading to enhanced yield and activity. This can therefore help save on over harvesting of the trees by use of callus as the source material Oriabi (2016).

Callus extract was found to have a Minimum Inhibitory Concentration (MIC) against *Fusarium semitectum* at a concentration of 60% and an MIC against *Aspegillus flavus* at a concentration of 80%. Leaf extract on the other hand had MIC against *Fusarium semitectum* at a concentration of 80% and MIC against *Aspegillus flavus* at a concentration of 100%.

This study confirms that extracts from callus and leaves of field grown plants have antifungal activity. The results of this work could therefore be the basis for future work on the investigations on the activity of callus extracts on inhibition of fungal growth. More work is therefore expected to identify and characterize the secondary compounds present in callus extract

and their activity as the only available report on the use of callus extract of *Moringa oleifera* is on anti-diabetic (Oriabi, 2016) and peroxidase activity Lalida *et al*. (2013).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. Seeds of *Moringa oleifera* were able to germinate *in vitro*, but intact seeds took longer to germinate and recorded lower germination percentage than seeds with their coats removed. Giberellic acid (GA_3) did not have effect on germination of Moringa oleifera seeds.

2. All the explants used (hypocotyls, inter-nodal segments and leaf discs) gave callus with all the hormones tested, but the rate and amount of callus generation depended on the hormone and the concentration. Basal medium (MS) also generated callus in all the explants used.

3. *Moringa oleifera* shoots could not be generated through somatic embryogenesis, but through organogenesis, nodal segments developed shoots and this depended on the hormone types and concentration. The ability of MTR in regeneration of *Moringa oleifera in vitro* through organogenesis was observed as it developed more shoots compared to other hormones tested.

4. Callus extract was able to inhibit growth of *Fusarium semitectum* and *Aspergillus flavus* but was inactive on *Candida albicans*.

6.2 Recommendations

Germination of Moringa oleifera seeds *in vitro* should be carried out with the seed coats removed. There is need for more study on what is reducing the potential of regeneration of *Moringa oleifera* through somatic embryogenesis, exploring physical factors, hormone habituation by callus and plant dependent factors. The ability of MTR in regeneration of *Moringa oleifera in vitro* through organogenesis was observed as it developed more shoots

compared to other hormones tested. However there is need for further work to explore more on MTR, its ability to regenerate callus and also in micro-propagation. Also there is no data on the use of MTR combined with BAP, NAA, TDZ or KIN and combination of two or more hormones to induce shoots in *Moringa oleifera* explants hence the need for more work.Callus extract were discovered to be active against *Fusarium semitectum* and *Aspergilus flavus*. This should also be further explored to understand the phytochemistry of the compounds available in the extract.

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APPENDIX 1

Murashige and Skoog Medium (1962) composition	
Nutrients Concentrations (mg/l)	
Macronutrients	
Ammonium Nitrate	1650
Potassium Nitrate	1900
Calcium Chloride, Anhydrous	332.2
Magnesium Sulphate	180.7
Potassium Phosphate Monobasic	170
Micronutrients	
Potassium Iodide	0.83
Boric Acid	6.2
Manganese Sulphate· H_2O	16.9
Zinc Sulphate· $7H_2O$	8.6
Cupric Sulphate· $5H_2O$	0.025
Cobalt Chloride· $6H_2O$	0.025
Ferrous Sulphate· $7H_2O$	27.8
Na_2-EDTA	37.26
Vitamins	
Nicotinic Acid	0.5
Pyridoxine, HCl	0.5
Thiamine	0.1
Other components	
Myo-Inositol	100
Glycine	2
Sucrose	3000
Agar	8000

APPENDIX 2

Potato Dextrose Agar composition	
IngredientsConcentration (Gms/litre)	
Potato starch	4
Dextrose	20
Agar	15

APPENDIX 3

Sabouraud Dextrose Agar composition	
IngredientsConcentration (Gms/litre)	
Mycological peptone	10
Dextrose	40
Agar	15