5-2016

In Vitro Re Generation and Marker Assisted Evaluation of Genetic Fidelity In Endangered Tree Species Moringa Peregrina (Forsk) Fiori

Salama Mohammed Al Dhaberi

United Arab Emirates University

College of Food and Agriculture

Department of Aridland Agriculture

IN VITRO REGENERATION AND MARKER ASSISSTED EVALUATION OF GENETIC FIEDILITY IN ENDANGERED TREE SPECIES *MORINGA PEREGRINA* (FORSK) *FIORI*

Salama Mohammed Al Dhaheri

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Horticulture

Under the Supervision of Dr. Shyam S. Kurup

May 2016

Declaration of Original Work

I, Salama Mohammed Al Mutawa Al Dhaheri, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*In vitro Regeneration and Marker Assisted Evaluation of Genetic Fidelity in Endangered Tree Species Moringa Peregrina (Forsk) Fiori*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Shyam Kurp in the College of Food and Agriculture at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: Salama Date: 27/6/2016

Advisory Committee

1) Advisor: Dr. Shyam S. Kurup

Title: Associate Professor

Department of Aridland Agriculture

College of Food and Agriculture

2) Co-advisor: Dr. Abdul Jaleel Cheruth

Title: Associate Professor

Department of Aridland Agriculture

College of Food and Agriculture

Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Dr. Shyam S. Kurup
   Title: Associate Professor
   Department of Aridland Agriculture
   College of Food and Agriculture

Signature _____ Date 24/5/2016

2) Member: Dr. Abdul Jaleel Cheruth
   Title: Assistant Professor
   Department of Aridland Agriculture
   College of Food and Agriculture

Signature _____ Date 24/5/2016

3) Member (External Examiner): Dr. Amad Mohammed Jamil Al- Azzawi
   Title: Chairperson of Pharmaceutical Chemistry
   RAK Medical & Health Science University, Ras al Khaimah, UAE

Signature _____ Date 24/5/2016

This Master Thesis is accepted by:

Dean of the College of Food and Agriculture: Professor Afaf Kamal-Eldin

Signature Afaf Kamal-Eldin Date 27/6/2016

Dean of the College of the Graduate Studies: Professor Nagi T. Wakim

Signature Nagi Wakim Date 27/6/2016

Copy 10 of 10

Abstract

Moringa peregrina is a native plant in the United Arab Emirates; it is called "*Shua*". It is an endangered species with important nutritive and medicinal properties. It shows high adaptability to the arid condition with short germination time after irrigation. Unmanaged grazing and slow regeneration rate after browsing leads to the endangerment of the species causing erosion in biodiversity. One way to protect these rare species is by multiplying the planting material through tissue culture system and re-introducing at the habitat. Microproagation is an *in vitro* method used for plant clonal multiplication under controlled condition. This technique allows conserving the genetic characteristics of the plants which are usually affected by environmental restrictions. In the present study different parts of *M. peregrina* explants will be used for standardizing the mass propagation.

Direct organogenesis of nodal explants collected from the in vitro germinated seedlings were inoculated in MS media supplemented with different hormone treatment (TDZ, BA and NAA) with several concentrations. Thidiazuron treatment in the media observed to be better than BA and NAA combinations. It has been observed that media supplemented with TDZ 1 µM induced an average of 2.4 axillary shoots per explant. For callusing the results show that MS media supplemented with 2,4-D 2 µM/l and TDZ 0.5 µM/l found to have the highest callusing percentage and size. The rooting of *in vitro* developed plantlets obtained from nodal ex plants were induced through media supplemented with BA in combination with NAA and IBA. The number of roots was high in BA and NAA medium compare to BA and IBA. The genetic fidelity of the clones produced from

the in vitro protocol was assessed using molecular markers. The similarity index between the progenies and parent was above 94.9%.This shows that *in vitro* developed plantlets are identical with its mother plant. This will facilitate standardizing future conservation programs to limit the erosion of the genetic biodiversity in *Moringa peregrina* and similar species.

Keywords: *In vitro* propagation, *Moringa pergrina*, endangered tree species, ISSR analysis.

Title and Abstract (in Arabic)

الإكثار النسيجي المخبري للنبات المهدد بالإنقراض (مورينغا بيريجرينا) وتقييم المادة الوراثية للنباتات التي تم إنتاجها في المختبر بإستخدام بروتوكول الواسمات الجزيئية

الملخص

Moringa peregrina)) المورينغا بيريجرينا هو من أهم النبات الأصلية المحلية في دولة الإمارات العربية المتحدة؛ يطلق عليه إسم "الشوع". و هو من الأنواع المهددة بالانقراض و هو المعروف بخصائصه الغذائية والطبية الهامة. و للمورينغا بيريجرينا القدرة العالية على التكيف والنمو في الظروف القاحلة حيث ان لها القدرة على الإنبات بعد مدة قصيرة من الري. و يعتبر الرعي الغير منظم ومعدل التجديد البطيئ هو من أهم الاسباب التي تؤدي إلى تعريضها للخطر و تسبب تآكل في التنوع البيولوجي. هناك عدة طرق لحماية هذه الأنواع النادرة من الإنقراض من ضمنها اكثارها من خلال نظام زراعة الأنسجة ثم إعادة زراعتها في بيئتها الطبيعية. (Microproagation) هو طريقة في المختبر تستخدم لأكثار النسيج النباتي تحت ظروف خاضعة للرقابة. هذه التقنية تتيح الحفاظ على الخصائص الجينية للنباتات التي تتأثر عادة بسبب القيود البيئية.

في هذه الدراسة سيتم إستخدام أجزاء مختلفة من الانسجة العقدية للنبات و التي سيتم زراعتها في البيئة الغذائية MS و ستم معالجتها بتركيزات وتركيبات مختلفة لبعض الهرمونات مثل (TDZ, BA, NAA) بغرض اكتشاف البيئة الغذائية الأكثر ملائمة لعملية الأكثار النسيجي المخبري.وقد لوحظ أن البيئة الغذائية المزودة بمادة TDZ و بنسبة 1 ميكرومول نجحت في انتاج براعم إبطيه بمتوسط 2.4 للنسيج الواحد المزروع في البيئة الغذائية .أظهرت النتائج أيضا أن البيئة الغذائية المناسبة لإنتاج (callus) هي (MS) مزودة ب 2 ميكرومول / لتر من هرمون 2,4-D و0.5 ميكرومول / لترمن هرمون TDZو التي أدت الى أعلى نسبة(callusing) و أكبرها حجما. البيئة الغذائية المثالية التي أستخدمت لإنتاج الجذور تحتوي على (BA و NAA وIBA) . وقد لوحظ زيادة عدد الجذور في البيئة التي تحتوى على (BA) و (NAA) مقارنة بتلك التي تحتوي على (BA) و(IBA).

تم تقييم المادة الوراثية للنباتات التي تم إنتاجها في المختبر باستخدام بروتوكول الواسمات الجزيئيةوتبين أن مؤشر التشابه بين السلالات والأم يفوق 94.9٪ مما يظهر تطابق السلالات

التي تم إنتاجها في المختبر مع النباتات الأم . و من الجدير بالذكر أن هذه الدراسة ستساعد في تسهيل و توحيد برامج المحافظة المستقبلية للحد من تآكل التنوع البيولوجي الوراثي في المورينغا بيريجرينا والأنواع المماثلة.

مفاهيم البحث الرئيسية: الأنسجة النباتية، مورينقا بيريجرينا، النباتات المهدده بالإنقراض، بروتوكول الواسمات الجزئية.

Acknowledgements

First of all, I would like to thank Allah, without his grace and care I wouldn't be where I am today. I would like to express my deep appreciation for the leaders of my country, 'the United Arab Emirates', who provided all I needed to complete my studies.

I would like to thank my loving family (my Mom Fatima, my husband Mohammed, my brothers (Hamad, Ali, Sultan and Nasser), my sisters (Aisha, Amna, Maryam, Mai and Fatima) and my kids Ahmed and Maryam for always being there, for encouraging me to successes, and for their love and support.

My thanks go to all my teachers throughout my life who guided me in my classes and in my life. Many thanks to college of food and agriculture, food science department (Dr. Aisha boushlibi, Dr. Oya, Dr. Adam, Dr. Louis, Dr. Hanan, Mr. Jobe, Mr. Abdulgader and all others) and to the college of chemistry specially Dr. Salman Ashraf. I would like to thank my committee for their guidance, support, and assistance throughout my preparation of this thesis. Special thanks to Mr. Fayas our research assistant.

Dedication

I dedicate my thesis to my late father, my wise mom, my lovely husband and kids.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

M. peregrina	*Moringa peregrina*
WHO	World Health Organization
M.hildebrandtii	*Moringa hildebrandtii*
MS medium	Murashige and Skoog medium
Kin	Kinetin
TDZ	Thidiazuron
NAA	1-Napthalene acetic acid
BA	6-Benzyladenine
IBA	Indole- 3-Butyric acid
ISSR	Inter-Simple Sequence Repeat
2,4-D	2,4-Dichlorophenoxyacetic acid
ISSR	Inter simple sequence repeat
PCR	Polymerase chain reaction
ANOVA	Analysis of variance

Chapter 1: Introduction

1.1 Overview

United Arab Emirates is a country that appreciates the flora of life. H.H Sheikh Zayed Al Nahyan, the late President of the UAE, was a renowned environmentalist who spearheaded a number of tree-planting programs in his lifetime and helped transform swaths of barren desert landscape into pockets of lush greenery. There are many valuable native plants in UAE and the *Moringa peregrina* is one of the most important of these native plant species. The use of *Moringa* species has been reported in more than 80 countries all over the world as well as in the ancient Roman, Greek, Egypt, and Indian civilizations and many others for thousands of years. They are recognized in over 200 local languages. In ancient time kings and queens included *Moringa* leaves and fruit in their diet to maintain healthy skin and mental alertness (Daljit et al., 2013)

In some countries of Asia and Africa, 80% of the population is dependent on medicinal plants to maintain their health and cure diseases (WHO, Media center 2008). *Moringa* species are one of the most beneficial trees in the tropics and subtropics of Asia and Africa, with a multiple uses. The Moringacae family has a genus known as *Moringa* which contains 13 species (Steinitz et al. 2009). *Moringa oleifera* and *Moringa peregrina* are the most valuable and widely consumed *Moringa* species (Lalas et al., 2012). Almost all parts of *Moringa* like the flower, roots and fruits are edible, and have long been consumed as food and medicine to treat various diseases such as hysteria, abdominal tumors, paralytic attacks, scurvy, prostate troubles, helminthic bladder, skin infections and sores (Rahman et al., 2009). It has

been reported that, the leaves are highly nutritious and contain more calcium than milk, more vitamin A than carrots, more vitamin C than oranges and more iron and more protein than milk and eggs as well as more potassium than bananas (Sreelatha et al., 2011). Additionally, fruits and seeds are a rich source of protein, vitamins (vitamin A, C and E) and essential elements (Ca, Mg, K and Fe). The seed oil is rich in fatty acids similar to olive oil (except linolieic acid) and was used as acceptable substitute (Lalas and Tsakinis, 2002). In addition, *Moringa* oil also used in salads, machine lubricant in industry, perfume manufacture and hair care products.

Ethnobotanical researches show that *M. peregrina* is used to treat headache, muscle pain back pain, gut pain, burns, and fever (Miller et al., 1989). Other possible uses include treatment of stomach disorders, hypertension, asthma, malaria, and for labor pains (Mekonnen et al, 1999, Elbatran et al., 2005). Moreover, *M. peregrina* has shown to have antihyper-glycemic activity (El-Alfy et al., 2011). Pharmacological studies suggested that *M. peregrina* shows antioxidant, analgesic, anti-inflammatory, anticancer, anti-hyperglycemic, anti-hyperlipidemic, antibacterial and antifungal activities (El‐Batran et al., 2005, Dehshahri et al., 2012, Kohei et al., 2011, El‐Alfy et al., 2011, Rouhi‐Broujeni et al., 2013, Hajar et al., 2014).

Currently the *Moringa* species, including *M. peregrina*, have wide interest because of their important uses in nutrition, medicine and the industrial field. *Moringa* species are in danger of extinction; some have already become extinct in nature eg. *M. hildebrandtii* (Olson et al., 2000). All over the world, *M peregrina* is becoming an endangered plant species due to over-stressing demand, unmanaged grazing and uprooting (for fuel or medicinal uses) and also due to human interventions. The result is reduction in population size thus lead to erosion of genetic diversity. Many current researches are aiming to explore new techniques and

cultivation methods to save *Moringa peregrina* from extinction (Zaghloul et al., 2010).One of the methods to protect endangered plant species is by adopting tissue culture techniques. Plant micro-propagation is a technique used to multiply plants under controlled conditions. This rapid method saves the plant physiological, biological, and genetic material of endangered plant species (Kant et al., 2010). *Moringa peregrina* is a great candidate as a future crop in developing countries because undernourishment and hunger is a growing concern, particularly in arid regions where temperature and water availability are two of the limiting factors of plants (Iyan et al., 2014)

1.2 Moringa peregrina in United Arab Emirates

Moringa peregrina is a native plant in the United Arab Emirates, it is called "Shua". It is scattered in the mountainous areas in the UAE growing on the slopes of the mountains and the edges of the valleys. Locally *Moringa peregrina* has been used as animal feed particularly for camels. The wood from this tree is used as fuel to cook meat because its ambers can tolerate very high temperature. However, it is not recommended to climb *Moringa peregrina* tree because its branches and stems are fragile and susceptible to breakage. Moreover, edible oil is extracted from the seeds. The kernel of *Moringa peregrina* seed is rich in oil, and contains up to 54% (Afsharypuor et al., 2010). Furthermore, the oil of "Shua" traditionally was used to treat skin problems such as scabies, itches and freckles. The leaves have been used to flavor meat to make traditional smoked meat called "tanour". This tradition is still being practiced by the natives of UAE, especially in Eid Al Adha festival. "Shua" tree has always been very important for the Bedouins in the past and still enjoys tremendous popularity among Emiratis, not only for the many beneficial uses of this

tree but because it provides a spiritual connection with both the nation's past and it is natural habitat. Overall, it can be concluded that *Moringa peregrina* is one of the valuable native trees of the U.A.E.

1.3 Moringaceae family

The Moringacae family has a genus known as *Moringa* it contains 13 species of shrubs and trees originated in tropics and subtropics region (Steinitz et al., 2009). *Moringa peregrina* and *Moringa oleifera* are the most famous and utilized *Moringa* species (Lalas et al., 2012).There are various names of *Moringa* for example the horseradish tree in Florida, the drumstick tree in India and the Benz olive tree in Haiti. In the Philippines *Moringa* is called a "mothers best friend" because babies are fed with cooked *Moringa* leaves (Somali et al., 1984).

Table 1: Taxonomic classification of *Moringa peregrina*
(Olson ME, 1999)

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Eudicots
Subclass	Rosids
Order	Brassicales
Family	Moringaceae
Genus	Moringa

The synonyms of *Moringa peregrina* (Forssk.fori) are *hyperanthera peregrina* Forssk., *Moringa aptera* Gaertn.,Fruct., and *Moringa arabica* (Lam.) Pers. (Boulos, L., 1999).

1.4 Distribution

The distribution of *Moringa peregrina* includes semiarid, arid, and hyper arid regions of North East Africa (Somalia, Ethiopia, Eritrea, Djibouti, Sudan, and Egypt) and South Middle East (Saudi Arabia, Yemen, and Oman) (Bolous et al., 1999). *Moringa peregrina* is a native plant in the United Arab Emirates scattered in the mountainous desert growing on the slopes of the mountains and the edges of the valleys. It can be clearly seen around Al Toween road on the way to Dibba.

1.5 Morphology

Moringa peregrina is an extremely fast growing tree with grayish-green bark, 5-15 m in height with a diameter of 20-40cm. It usually has leaves with 20-70cm in length with many leaflets that eventually drop when the main leaf matures (Iyan et al., 2014). The flowers are usually yellowish white to pink colored, 10-15mm long, slightly scented, bisexual and have insect pollination characteristics (Täckholm, 1974; Boulos, 1999; Gomaa and Pico, 2011). The flowering period is from March to April and the fruiting season can last up to 3 months (Hegazy et al., 2008).

The fruit size is 10-25 x 1-1.5 cm and has 5-15 ovoid, trigonous, hard coated seeds. The withering rate of the flowering bud is between 40-50% and fruit set is extremely low ranging between 0.05-0.07 percent. Despite thousands of flower buds

emerging during the flowering period, the number of seeds being produced by *Moringa peregrina* trees is very low thus it has low fecundity (Hegazy et al., 2008).

Moringa peregrina is a drought resistant tree which can grow in soil with deep water table according to Cossalter (1989). *M. peregrina* tree shows xerophytic characteristics which appear when available soil moisture becomes low making the plant drought resistant. Al-Gohary and Hajar (1996) found that Arabian. *M. peregrina* shows modification in its leaves and stem following water deficit treatment. These xerophytic modifications include reduce surface to volume ratio of leaf and stem, leaf rolling, hair covering, and increased frequencies and indexes of stomata. These drought resistant techniques were also observed in newly germinated *M. peregrina* as shown by a study done by Hajar (1997). *Moringa peregrina* tree is highly suitable for cultivation in arid and semi-arid regions where water scarcity is the major problem.

1.6 Nutrient content

The *Moringa* tree is most known for its nutritional values as it consists of vitamin and mineral concentrations. It is commonly known as the miracle tree (Ghodsi et al., 2014). In Middle East *M. peregrina* is used for nutritional and medicinal purposes (Abdullah, 2015). The seeds and leaves of *M. peregrina* are famous for having valuable nutrients for human diet. The seeds in Saudi Arabia contain 49.8% to 57.25% oil (Osman and Abohassan 2012; Tsaknis 1998). Research done by Iyan Robiansyah et al., (2014) shows that linolenic acid (C18:3) is not detected in *Moringa peregrina* oil which makes it more resistant to oxidation compared to olive oil. Linolenic acid is known to turn oil becoming rancid quickly because it is more vulnerable to oxidation (Warner & Mounts 1993).

Moringa peregrina oil has also higher tocopherol concentrations consisting of α- β- and δ- tocopherol which protects the oil during storage and processing (Tsaknis, 1998). A recent study by Lalas et al., (2012) conclude that *M. peregrina* seed oil could resist up to 10.5 hours at 120 °C, while extra virgin olive oil can resist 8.9 hours only. All these characteristics make *M. peregrina* oil ideal for human consumption and similar industrial applications.

Moringa peregrina seeds have lower percentage of carbohydrates and protein than *M. oleifera*. *M. peregrina* seeds contains 18.9% and 23.8% of carbohydrate and protein, respectively (Al Kahtani & Abou-Arab, 1993), while *M. oleifera* have around 21.12% carbohydrate and 33.25% protein (Oliveira et al. 1999). *M. peregrina* seeds have 17 amino acids the most dominant amino acids are Glutame and Arginine.

In Saudi Arabia, the leaves of *M. peregrine* are good source of sulfur-containing amino acid cystine in addition to other amino acids. The iron (Fe) content of *M. peregrina* leaves is much higher compared to common cereals and vegetables. Also it is composed of 23.31%, proteins, 5.81% fat and 6.39% fiber. (Osman & Abohassan, 2012).

1.7 Medicinal properties

Traditionally *M. peregrina* has both medicinal and industrial uses. In Greece, Egypt and Romania for example, seeds oil is used to make perfume while its wood is used in furniture making due to its resistance to termites. In addition, *Moringa* is used as medicine for abdominal pain, headache, and skin protection as well as a

laxative (Steinitz et al., 2009). Recently, it has been shown that *Moringa* seeds contain potential anti-inflammatory and anti-oxidant effects (Koheil et al. 2011).

The roots, leaves, flowers and seeds of *M. peregrina* are used in folk remedies for tumors (Hartwell et al., **1967**). A research done by Lalas et al., 2012 shows that the oil has inhibitory effects on many bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli*. The plant pharmaceutical functions were investigated and proven to have a significant role as an anti-cancer drug for colon and breast cancer cells (El-Alfy et al., 2011).

The seeds also show antimicrobial activity against fungi and bacteria, antitumor, anti-inflammatory, antispasmodic, diuretic and larvicidal activity against the mosquito that transmits dengue and yellow fever (Donli and Dauda, 2003). Seeds of *M. peregrina* have anti-oxidant effects and play role in improving the health and resistance of diseases.

1.8 Uses of Moringa peregrina

1.8.1 Plant growth hormone

Juice extracted from *M. peregrina* leaves can be used as plant growth hormone, increasing yield by 25-30% for nearly any crop. One of the active substances in *M. peregrina* leaves is Zeatin. It is a plant hormone related to the cytokinins (Fuglie, 2001a).

1.8.2 Green manure

Moringa peregrina can be used as a green manure to enrich the agriculture land. First the land is tilled then *Moringa* seed is planted (1-2 cm deep, spacing of 10x10

cm). After 25 days, the seedlings are turned over into the soil at depth of 15 cm deep. Then that the field is prepared for the desired crop (Fuglie, 2001).

1.8.3 Water purifier

M. peregrina seed powder can be used to clean dirty rivers. The powder attaches to the impurities in the water and sinks to the bottom. This treatment also removes 90-99% of bacteria present in water. *M. peregrina* seed powder contains a water-soluble cationic coagulant that has the ability to reduce the turbidity of the water treated. It can be used as a primary coagulant in drinking water clarification and wastewater treatment. (Ghebremichael et al., 2005).

1.8.4 Source of oil

Moringa oil is a concentrated source of food energy and nutrients. It worth to be added to our diet. The seeds of *M. peregrina* were the source of "bean oil" used by the Egyptians during Old and Middle Kingdoms. The refined oil obtained from *Moringa* seeds are used for preparing cosmetics because of its yellow color, sweet taste and its odorless properties (Lucas, 1962). The bright yellow oil with a pleasant taste has been compared in quality with olive oil. The oil contains 60% protein, which means it can be used as a soil fertilizer; further studies are looking at how it could be used as part of animal and poultry feed. (VonMaydeU, 1986; Folkard & Sutherland, 2005)

1.8.5 Food source

The leaves and seeds of *M. peregrina* known to have valuable nutrient content for human diet. Nutritional analysis shows that the leaves and pods of *M. peregrina* contain all essential amino acids as well as disease preventing nutrients (Verma et al.,

1976; Freiberger et al., 1998). It is great source of protein and a very low source of fat thus making it a good food for low fat diet.

1.9 Micropropagation of *Moringa* species

Eufrocinio (2010) showed that a clonal *in vitro* micropropagation method of *Moringa oleifera L.* initiated from nodal sections of young, aseptically-grown seedlings is feasible. Axillary shoot growth was induced by supplementing Murashige and Skoog's (MS) medium with a cytokinin. Of the three cytokinins tested, namely benzylaminopurine (BAP), kinetin (Kin) and thidiazuron (TDZ), BAP at 2.5 μM was found to be optimal in inducing bud break. Optimal rooting of individual shoot culture was obtained with application of naphthaleneacetic acid (NAA) at 0.25 μM).

Wesam et al, (2012) recorded In vitro germinated seedlings were cultured on Murashige and Skoog (MS) medium supplemented with different levels of either 6-benzyladenine (BA) or kinetin (Kin). The maximum shoot proliferation of 6.5 shoots per explant with 100 % shoot proliferation rate was observed on MS medium supplemented with 1.0 mg/l BA. On the other hand, MS medium supplemented with 1 mg/l indole- 3-butyric acid (IBA) resulted in the maximum number of roots. Micropropagated plants were successfully acclimatized. Genetic stability of micropropagated plants was assessed using Inter-Simple Sequence Repeat (ISSR).

Lalida et al., (2013) found that multiple shoot induction was present in the stem explants of drumstick tree plantlets that were cultured on the MS medium supplemented with BA at the concentration range of 1.0 -4.0 mg/l. For plant regeneration, the shoot explants were cultured on MS agar medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.5 mg/l for callus production.

Friable callus were transferred to MS agar medium supplemented with different concentrations of 1-napthalene acetic acid (NAA) or BA for 4 weeks. Callus grown in MS medium supplemented with BA (0 – 2.0 mg/l) did not produce either shoots or roots, but showed green spots on their surfaces. Callus grown in MS medium containing NAA 0.5 mg/l produced both shoots and roots.

1.10 Conservation status

The conservation status of Arabian *M. peregrina* is unknown due to absence of studies assessing the population size and structure, population trend, occurrence and occupancy areas, and traits associated with the species. In general, the species has also not been assessed for the IUCN Red List. Populations in Egypt, however, are reported to have experienced a rapid decrease mainly due to over-harvesting for fuel and medicinal uses, over-grazing by animal and habitat destruction by unmanaged human activities (Abd El-Wahab et al., 2003). The same population decrease may already have happened to the Arabian populations as human populations is steadily increasing, which in turn may affect the natural ecosystems of the region.

Moringa pregrina is becoming an endangered plant species as a result of unmanaged grazing and slow regeneration rate after browsing (Gomma NH and Pico FX 2011). Many recent studies are aiming to find efficient cultivation methods to save *M. peregrina* from extinction (Zaghloul MS et al., 2010).Due to its valuable nutrient and tolerant to sever drought, the plant could become an important future crop to maintain a sustainable agriculture in arid and semi-arid regions. The loss of plant genetic resources, particularly those of wild species, has led to the development

of '*ex situ*' conservation methods to maintain genetic diversity. One way to protect rare species is by multiplying plants using tissue culture.

In vitro propagation of plants is a technique used for clonal multiplication under controlled conditions. This rapid propagation method saves plant biological, physiological and genetic specifications which are usually affected by environmental restrictions especially for endangered plant species (Kant T et al., 2010). On the other hand, *in vitro* propagation provides new ways to improve plant cultivation conditions and plant genetic characteristics. Changing the growing conditions, such as hormone composition and concentration, light intensity and temperature, may produce more vigorous plants (Kapai V et al., 2010)

Over the last few years much research has been conducted, aiming to overcome the problems involved in *Moringa* tissue culture. In *Moringa olifera* immature seeds were used for *in vitro* propagation using liquid medium (Stephenson and Fahey, 2004). Islam et al., (2005) reported the use of *Moringa olifera* shoot nodes as suitable explant for *in vitro* propagation using medium supplemented with 1.0 mg/l benzylaminopurine for shooting and hormone free MS media for root formation. Wesam et al., (2013) investigated the use of *Moringa peregrina* shoot tips from in vitro germinated seedlings for regeneration using medium supplemented with benzylaminopurine (1.0 mg/l) with a maximum 6.5 shoots per explant. The root formation was achieved by using 1 mg/l indole- 3-butyric acid (IBA).

The present study was conducted to utilize the different explants of *Moringa peregrina* via nodes, shoot tip and shoot cuttings for rapid *in vitro* regeneration and clonal fidelity studies between tissue cultured plants and their parents.

Objectives

1. Standardization of *in vitro* production protocol for *Moringa peregrina.*
2. To establish the clonal fidelity of *in vitro* regenerated progenies of *Moringa peregrina* and the parents.

Hypothesis

Explants of *Moringa peregrina* under *in vitro* conditions will respond to simulated media combinations to produce clonal plantlets, and the genetic uniformity will be maintained in the regenerated plantlets.

Chapter 2: Methods

2.1 Plant material

Healthy mature fruits of *Moringa peregrina* were collected from College of Food and Agriculture farm, UAEU, Al-Foah during the summer of 2015 (Fig.1 and 2). Fruits were cut open, the seeds were separated from pods (Fig.3). Seed coats were removed aseptically and seeds were washed thoroughly in running tap water several times then surface sterilized by immersion in 20% sodium hypochlorite (v/v) and 2 drops of Tween 20 detergent for 10 minutes, followed by rinsing three times in sterile distilled water under aseptic conditions to remove traces of chemicals (Fig.4).

Fig.1: *Moringa peregrina* tree in Al Foah farm

Fig.2: Mature pods of *Moringa peregrina* fruit

Fig.3: Pods and seeds of *Moringa peregrina*

Fig.4: Disinfecting *Moringa peregrina* seeds aseptically

2.2 Media and culture conditions

2.2.1 Seed germination

For *in vitro* seed germination, sterilized seeds of *Moringa peregrine* were inoculated on full strength (FMS) and half strength MS (HMS) medium containing 3% sucrose and 0.8% agar. After adjusting the pH to 5.8, 20 ml of medium was dispensed into each tube. The culture tubes were plugged with non-absorbent cotton wrapped in cheese cloth and autoclaved at 121°C for 15 minutes. The cultures were maintained in the dark at 25 ± 2 °C until germination (Fig.5). The germination percentage was recorded 4 weeks after inoculation. Thirty seeds were used for each treatment and the experiment was repeated three times. The germinated seedlings will be used for extracting explants for culturing.

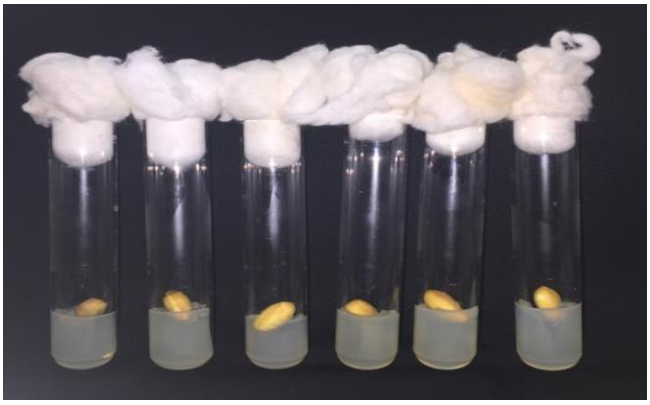

Fig.5: Inoculation of *Moringa peregrina* seeds in FMS and HMS media

2.3 Direct organogenesis

2.3.1 Effect of TDZ on direct shoot induction from nodal explants

Nodal explants were extracted from the *in vitro* germinated seedlings and inoculated in different concentrations of Thidiazuron (TDZ) medium (0.5, 1 and 2

µM/l) and MS hormone free media as control. Cultures were maintained at dark conditions for one month. After germination the cultures were kept in16 hour's photo period and 8 hours dark at 25±2ºC. Sub-culturing was done within 20 days. The germination percentage and the average number of shoots in each treatment were recorded (Eufrocinio, 2010).

2.3.2 Effect of BA and NAA combination on direct shoot induction from nodal explants

Nodal explants, from *in vitro Moringa peregrina* tree plantlet, were cultured on MS agar medium supplemented with BA (0.5, 1, 2) and NAA (0.5, 1,1.5) mg/l combination and MS hormone free media as control. Cultures were maintained at dark for one month. After germination the cultures were maintained in16 hours photo period and 8 hours dark at 25±2ºC. Sub culturing was done within 20 days. The percentages of shoot germination and average number of shoots per explants were recorded.

2.4 Indirect Organogenesis

2.4.1 Shoot cuttings

Shoot parts of *Moringa* seedlings collected from the *in vitro* developed seedlings were chopped into small pieces and were inoculated in different concentration of 2,4 D (2,4 and 8mg/l) and MS hormone free media as control for callus induction. Cultures were maintained at dark at 25±2ºC. The response was recorded after 3 weeks of culture initiation and the sub-culturing was done in 4 weeks. The percentage of response and the size of the callus were recorded.

2.4.2 Shoot tip

Shoot tips collected from the *in vitro* germinated seedlings were chopped and inoculated in different concentration of Thidiazuron (TDZ) (0.5, 1 and 2 µM/l), Benzyl amino purine (BAP) (0.5, 1 and 2mg/l) and Kinetin (Kin) (0.5, 1 and 2mg/l) in combination with 2,4-D (2 mg/l). Hormone free medium served as control. Different combinations of media were prepared and the sterile shoot tip extracted from *in vitro* developed seedlings was inoculated in each media. The cultures were maintained at 25±2ºC under dark for 4 weeks. Sub culturing was done once in four weeks. The percentage of callusing and the size of callus were recorded after 8 weeks of culture initiation (Peddaboina, 2006).

2.5 Regeneration

The regeneration of *in vitro* developed shoots from nodal explants were conducted in medium supplemented with different combination BAP, NAA and IBA. Different concentration of NAA (0.5, 1 and 2 mg/l) and IBA (0.5, 1 and 2mg/l) in combination with BAP (1mg/l) were prepared and the cultures were maintained in 16 hour light period and 8 hour dark at 25±2º. The shoot length, number of roots was recorded after 5 weeks of culture initiation. The regeneration of callus obtained from shoot tip explants were conducted in media supplemented with different combinations of BA (0.5, 1 and 2mg/l), NAA (0.5, 1 and 2mg/l). The cultures were maintained in 16 hour light period and 8 hour dark at 25±2º.

2.6 Acclimatization

Five week old rooted in *vitro* plants collected from the culture were thoroughly washed with sterile water for 10 min to remove the agar. The plants were

transferred in 10 cm pots filled with sterile potting soil and peat moss (1:1). Irrigation was done with sterile distilled water for 2-3 weeks under the culture room conditions (25±2º C).

2.7 Genetic fidelity study

2.7.1 DNA isolation

For clonal fidelity studies four progenies were selected from the *in vitro* developed plants. The DNA was extracted from the four progenies and mother plant using DNA isolation kit (Promega). Immature leafs were collected and the extract was prepared in motor and pestle using liquid nitrogen. The isolated DNA quantity was measured in Nanodrop and was checked in Agarose gel electrophoresis using 1% agarose gel (Fig.7).

2.7.2 ISSR Analysis

Genetic uniformity of the *in vitro* propagated plants was tested using ISSR markers (Bhatia et al. 2011). Ten ISSR primers were used for the study. The PCR amplification was performed using master mix kit (Qiagen). PCR amplifications were carried out in a total volume of 20 µl containing 10 µl master mix, 2 µl primer (0.2µM), 2 µl of 25 ng template DNA and 6 µl of DNase free water. PCR amplification was performed in a Master cycler (Nexus gradient-Eppendorf) with the following cycling profile. An initial denaturation at 94 °C for 2 min, followed by 35 cycles of 40 s at 94 °C,45 s at 40 °C and 1:45 s at 72 °C with a final extension for 10 min at 72 °C (Fig.6).

The amplified products were checked using agarose gel electrophoresis on 1.5 % agarose gels in 1X TBE buffer and ethidium bromide was added for staining the DNA. The electrophoresis was done at 100 V for 3 h and was visualized under UV light using a gel documentation system. PCR reactions were repeated twice to check the reproducibility of the banding patterns. 1 kb DNA ladder was used as the molecular standard in order to confirm the appropriate ISSR markers. The ISSR bands were scored in excel sheet and were used to construct phylogenetic tree using NtSys software.

Fig.6: PCR amplification using a Master cycler (Nexus gradient-Eppendorf)

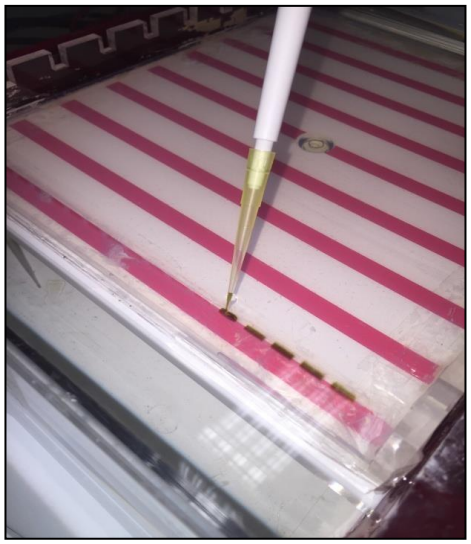

Fig.7: Agarose gel electrophoresis

2.8 Statistical Analysis

The experiment was repeated twice and three replications were made for each treatment. Data were analyzed by analysis of variance (ANOVA) followed by Tukey’ t-test for mean comparison.

Chapter 3: Results

3.1 Seed germination

Seed germination was conducted *in vitro* to extract explant from the germinating seeds in sterile condition. The seed germination studies were conducted with Murashige & Skoog (MS) under different strength showed significant variation in germination rate. After 4 weeks the percentage of seeds germinated on HMS medium was 70%, while the percentage of seeds germinated on FMS was 60% (Fig.8). Half strength MS medium proved to be more suitable than full strength MS medium for the germination of *Moringa peregrina* seeds.

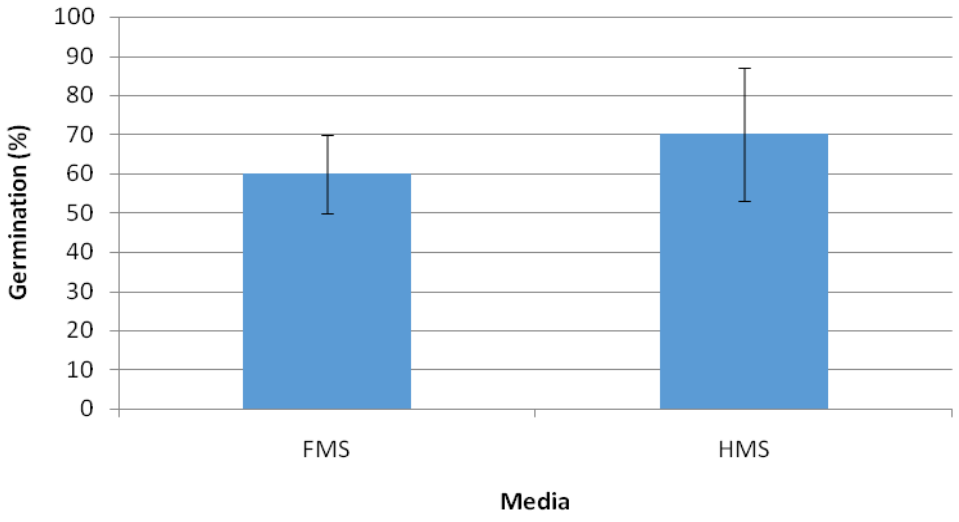

Fig.8: Germination percentage of *Moringa peregrina* seeds inoculated in full strength MS medium (FMS) and half strength MS medium (HMS)

3.2 Direct organogenesis

3.2.1 Effect of TDZ on direct shoot induction from nodal explants

The effect of Thidiazuron (TDZ) on nodal explants of *Moringa peregrina* germinated seedlings was conducted using different concentrations of TDZ (0.5, 1 & 2 µM/l) in MS media. The germination of nodal explants was observed within two weeks of culture initiation. The germination percentage and number of shoots were recorded 4 weeks after inoculation (Fig.10). The percentage of nodal germination

was compared with the media combination of different concentrations of TDZ. (Table 2).The germination was found to be high in TDZ 0.5 µM/l (90.4%) compared to other concentrations. Both TDZ 1.0 µM/l and TDZ 2.0 µM/l showed 81% of germination. The germination of nodes was the lowest in hormone free medium.

The shoot formation was observed within second subculture. The number of axillary shoots per explant induced by TDZ at various concentrations is shown in Fig.9. The highest number of axillary shoots per explant induced by TDZ was at 1 µM/L which induced an average of 2.4 axillary shoots per explants. In TDZ 0.5 (1.714±0.64) and TDZ 2 µM/l (1.85±0.96) media combinations, there was no significant change in the shoot number. The hormone free medium showed significantly low shooting compared to TDZ supplemented media**.**

Media(µM/l)	Germination (%)	number of shoots (mean)
TDZ 0.5	90.4	1.714 ± 0.64^{bc}
TDZ 1	81	2.47 ± 1.03^{a}
TDZ 2	81	1.85 ± 0.96^{b}
MS	66.6	0.809 ± 0.26^{c}

Table 2: Germination percentage and number of shoots per explants influenced by media supplemented with TDZ treatments

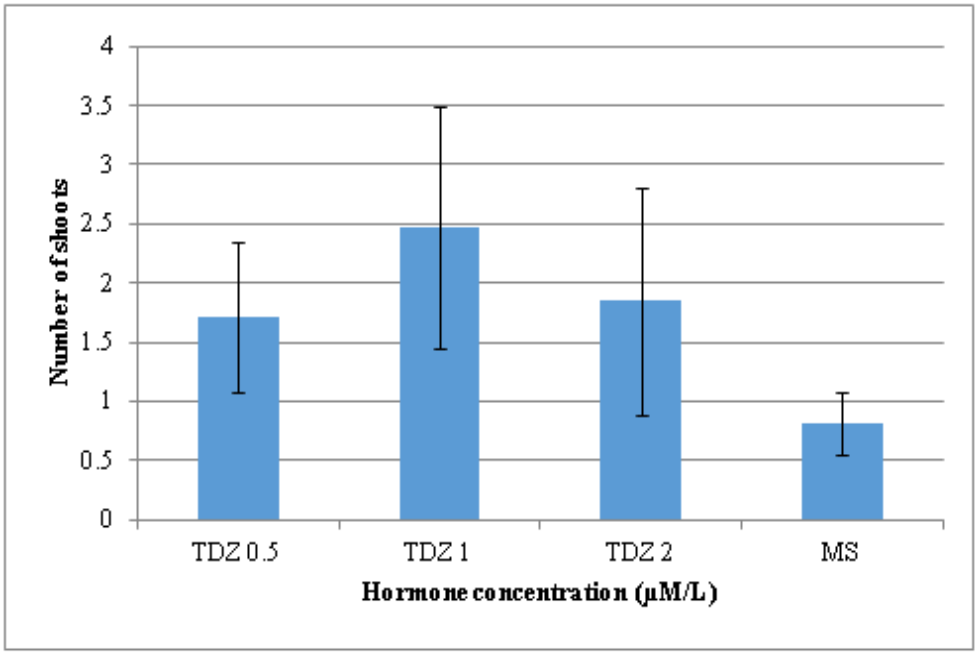

Fig.9: Germination and shooting of *Moringa peregrina* nodal explant on media containing different concentration of TDZ

3.2.2 Effect of BA and NAA combination on direct shoot induction from nodal explants

It has been observed that BA and NAA combination produced less germination percentage of *Moringa peregrina* nodal explants ranging from 13 to 58% (Table 3). The highest germination percentage of shoot was in MS media without growth regulator (as a control) inducing 100% germination followed by MS media supplemented with BA 0.5 and NAA 1.5 mg/l and MS media supplemented with BA 1 and NAA 1 mg/l inducing 58% germination with number of shoots per explants 0.41 and 0.58 respectively.

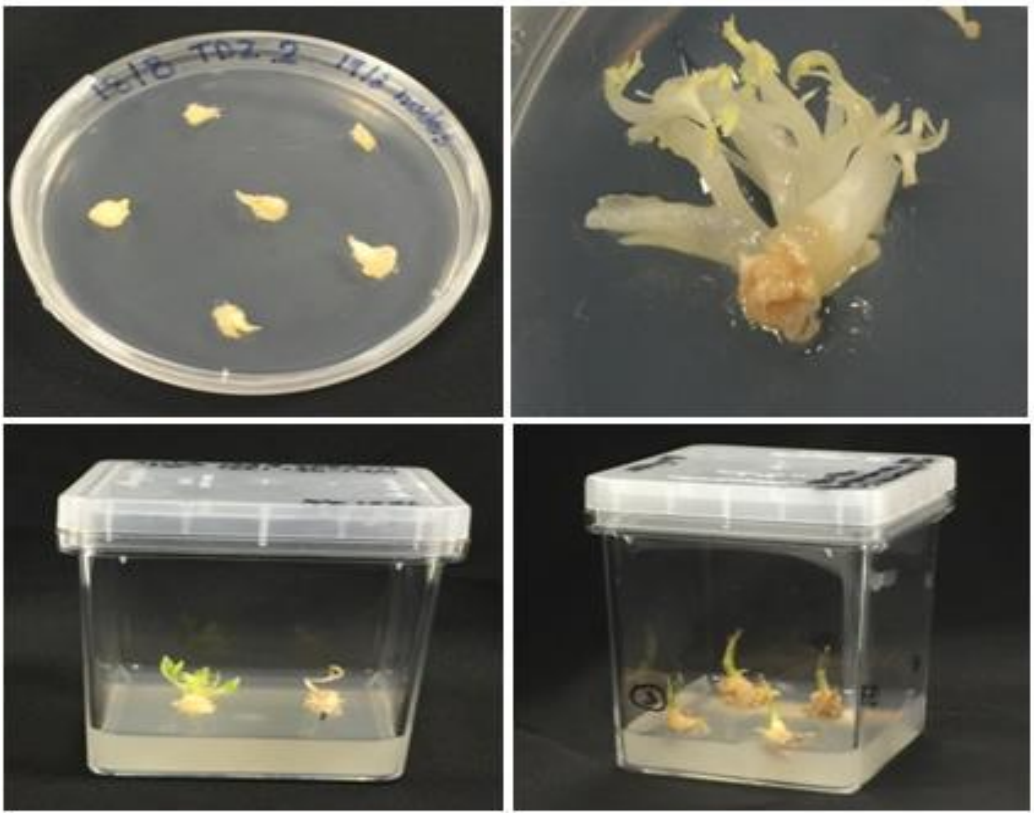

Fig.10: Number of shoots per ex-plant influenced by TDZ concentrations in media

Media (mg)	Germination (%)	No. of shoots
BA 0.5+ NAA 0.5	13	0.16±0.13cd
BA 0.5+ NAA 1	41	0.41±0.13bc
BA 0.5+ NAA 1.5	58	0.416±0.12bc
BA 1+ NAA 0.5	50	0.5±0.22bc
BA 1+ NAA 1	58	0.58±0.26b
BA 1+ NAA 1.5	16	0.16±0.26cd
BA 2+ NAA 0.5	16.6	0.16±0.13cd
BA 2+ NAA 1	16.6	0.16±0.13cd
BA 2+ NAA 1.5	0	0d
MS	100	1.08±0.13a

Table 3: Regeneration percentage and number of shoots per explant influenced by media supplemented with TDZ treatments

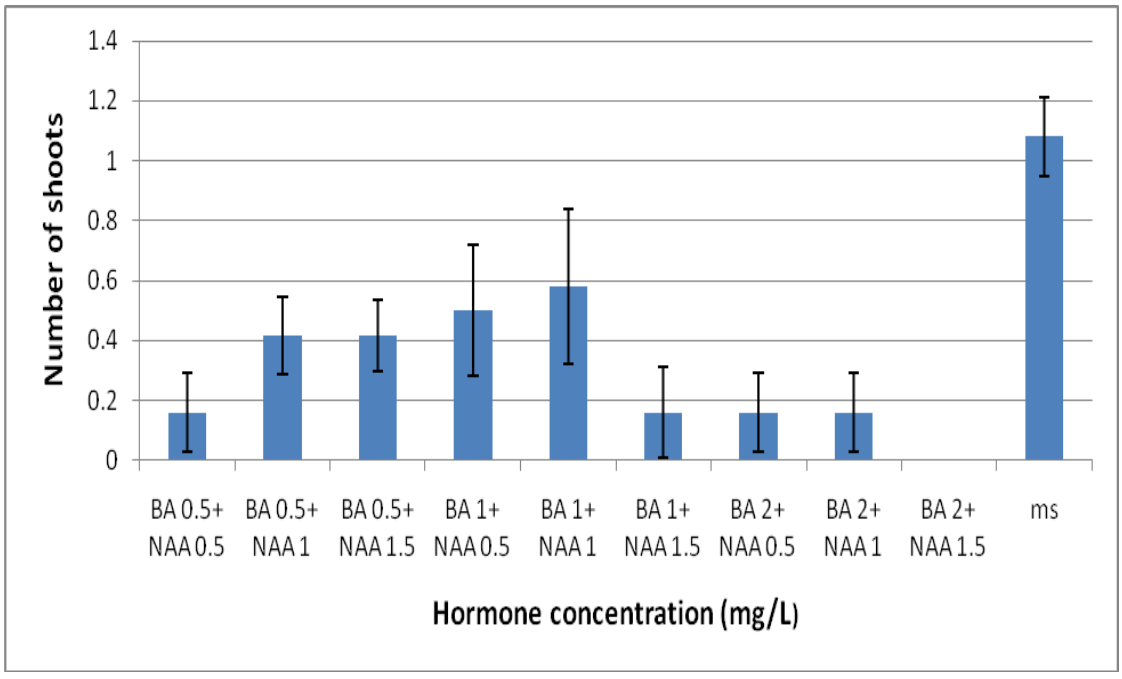

Fig.11: Effect of BA and NAA on number of shoots per explant

3.3 Indirect Organogenesis

3.3.1 Callus induction from shoot cuttings using 2, 4-D

Moringa peregrina callus were induced from shoot explants cultured on the MS agar medium supplemented with 2, 4 and 8 mg/l of 2, 4 D and MS hormone free media as control. The results showed that the MS medium containing 2 mg/l of 2,4-D produced the highest percentage of callusing (100%) with the highest average size of callus per explants 1.8 cm (Table 4), followed by those treated with 4 mg/l of 2,4-D (91.6% of callusing and 1.4 cm callus size per explants). It has been observed that the callus formation of MS media with 8 mg/l of 2, 4-D recorded 8.3% lower than the MS media with 4 mg/l of 2,4-D. Hormone free media induced 50% callus with the lowest average size of callus 0.38 cm per explants (Fig.12) . The size of callus was affected by different concentrations of 2, 4-D supplemented media (Fig13).

Media	Callusing (%)	Size of callus (Cm)
2,4-D 2	100	1.8^{a}
2,4-D 4	91.6	1.408^{b}
2,4-D 8	83.3	1.108^{c}
MS	50	0.38^{d}

Table 4: Callusing percentage and size of callus (cm) as influenced by media containing different 2,4-D concentrations (mg/l)

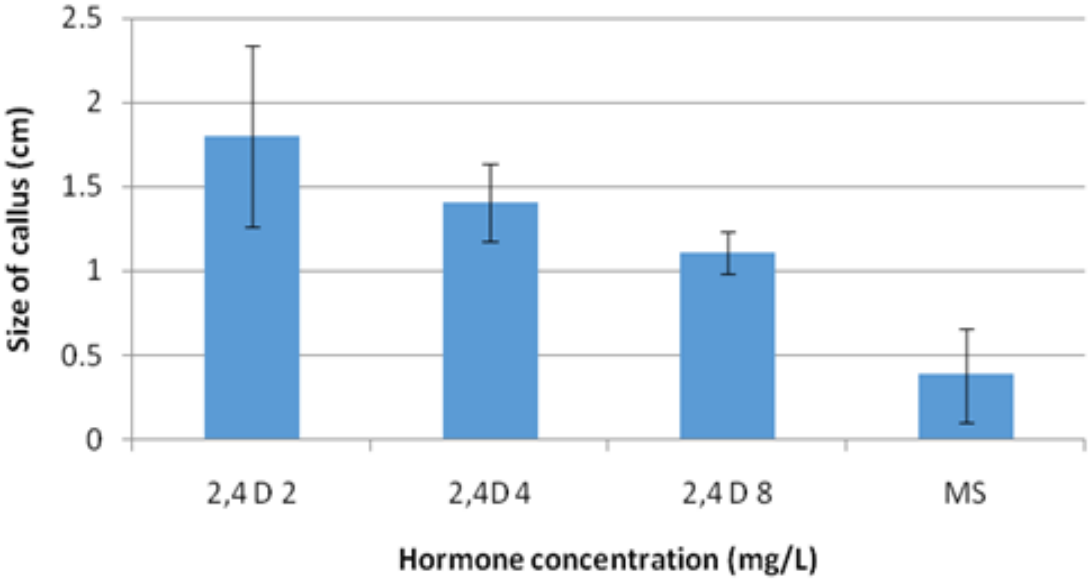

Fig.12: Size of callus (cm) affected by 2, 4-D hormone concentrations in media (mg/l)

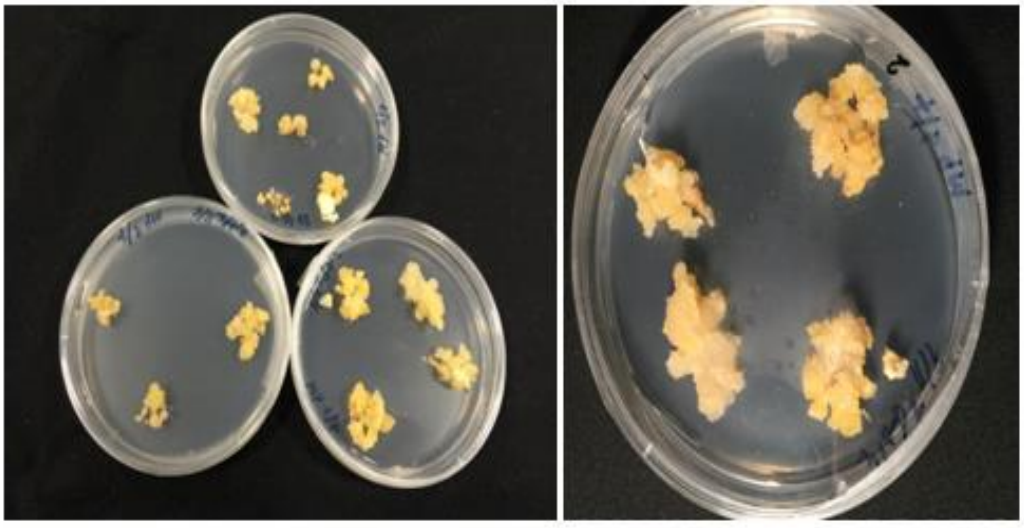

Fig.13: Callus induced by different concentrations of 2,4-D from *Moringa peregrina* shoot cuttings

3.3.2 Callus induction from shoot tip with the combination of 2,4-D,TDZ, BA and Kinetin

2, 4- D in combination with TDZ, BA and Kinetin were used to induce the callus induction from the juvenile shoot tips of germinated seedlings. The callusing percentage was found to be high in all media combinations except in hormone free media. The callusing size was significantly high in medium supplemented with 2, 4-D 2 mg/l and TDZ 0.5 µm/L combination (2.966±0.492) compared to other treatments (Fig.14). The combination of 2, 4-D 2mg/l and TDZ 2 µm/L (2.76±0.186) was on par with the above combination and significantly superior to hormone free media (0.086±0.072) (Table 5). Among the other combinations 2,4- D with BA was found to be better for callusing compared to 2,4-D with Kinetin combination.

Media	Callusing (%)	Size of callus (cm)
2,4-D 2+ TDZ 0.5	100	2.966±0.492[a]
2,4-D 2+ TDZ 1	100	1.7±0.09d[e]
2,4-D 2+ TDZ 2	100	2.76±0.186[ab]
2,4-D 2+ BA 0.5	93.3	2.26±.86b[c]
2,4-D 2+ BA 1	100	1.93±0.40[cd]
2,4-D 2+ BA 2	100	1.86±0.27[cd]
2,4-D 2+ KIN 0.5	100	1.23±0.136[ef]
2,4-D 2+ KIN 1	86.6	0.92±0.123[f]
2,4-D 2+ KIN 2	60	0.39±0.045[g]
Hormone free	13.3	0.086±0.072[h]

Table 5: Callusing percentage and size of callus (cm) as influenced by of 2, 4-D in combination with TDZ, BA and Kin in media

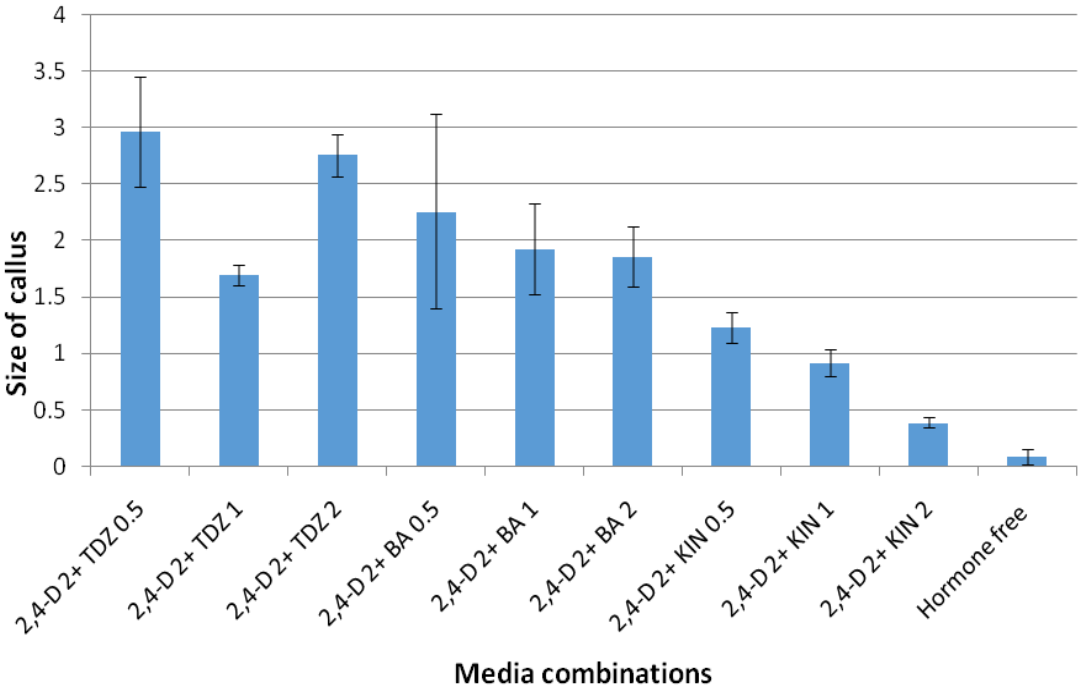

Fig.14: Effect of 2, 4-D in combination with TDZ, BA and Kin on callus size (cm)

3.4 Regeneration

3.4.1 Effect BA and NAA combination on regeneration of callus cultures

The callus cultures obtained from the shoot tip and shoot cutting explants were subcultured in different combinations of BA and NAA media. The result showed that the callus regeneration was found to be less in all media combinations. The callus nature was friable in some media and compact in some. Some of the callus in the BA 2mg/l and NAA 1mg/l showed greenish structure with leaf primodia. The greenish nature was observed within 3 weeks of culturing in light conditions (Fig.15). Further experiments are in progress using different media combinations to achieve more promising result from the callus.

3.4.2 Effect of BA, IBA and NAA combination on root formation of regenerated plantlets from nodal explants

The regenerated plantlets from nodal explants were cultured on MS agar medium supplemented with combination hormone concentrations of BA, IBA and NAA for 5 weeks. It was found that all media (except hormone free) induced root formation with different percentages (Fig.16). The MS medium containing 1 mg/l of BA and 2 mg/l of NAA produced the highest percentage of rooting per explant, followed by those treated 1 mg/l of BA and 2 mg/l of IBA (Fig. 16). The lowest rooting percentage of regenerated plantlets on MS-hormone combination medium was 1 mg/l of BA with 1 mg/l of IBA. There was increase by 16.7% of roots per shoot in 1 mg/l of BA media supplemented with 2 mg/l of NAA compared to 1 mg/l of BA media containing 1 mg/l of NAA (Table 6). There was no significant difference in number of roots per shoot between 1 mg/l of BA with 1 mg/l of NAA (0.766±0.051) and media 1 mg/l of BA with 2 mg/l of IBA media (0.93±0.273). Hormone-free media showed the lowest rooting percentage per shoot (17.2%). Media containing 1 mg/l of BA and 2 mg/l of NAA was the highest significant in number of roots per shoot compared with other media combination with hormones (Fig.17)

Media	Rooting (%)	Number of roots
BA1+IBA1	26.6	0.4±0.18c
BA1+IBA2	40	0.93±0.273ab
BA1+NAA1	33.3	0.766±0.051ab
BA1+NAA2	50	1.23±.051a
Hormone free	17.2	0.266±0.103d

Table 6: Rooting percentage and number of roots of regenerated plantlets from nodal explants influenced by BA, IBA and NAA combinations

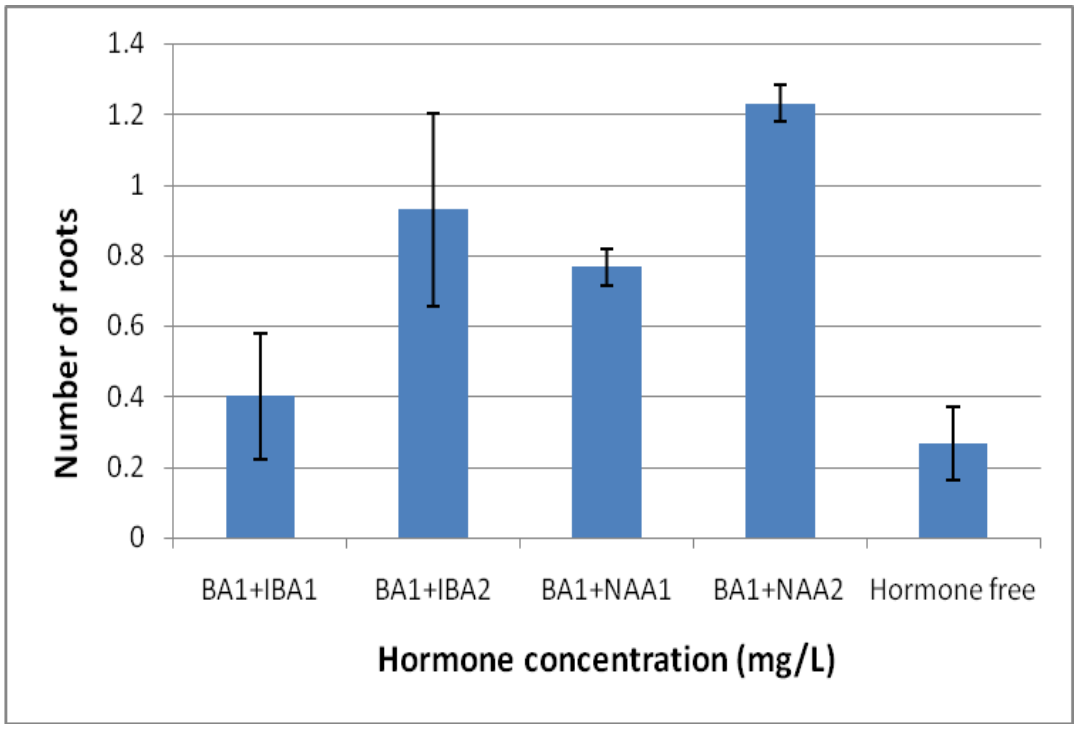

Fig.15: Effect of BA, IBA and NAA combinations on number of roots per explant

Fig.16: Regeneration of *Moringa peregrina* callus culture

Fig.17: Root formation of *Moringa peregrina* regenerated plantlets from nodal explants

3.5 Acclimatization

The *in vitro* developed platlets with shoot and roots were selected for hardening and acclimatization. The complete plantlets were removed from culture media and washed with steril water. The washed plantlets were transferred to sterilized potting mixture which contains soil and peat moss 1:1 in 10 cm diameter (Fig. 18).The plantlets were grown in at 25±2º C in sterile for few weeks. The hardened plants were then transferred to the green house conditions.

Fig.18: *In vitro* developed plantlets of *Moringa peregrina* in pots

3.6 Clonal fidelity study

The ISSR profiles of the amplification products of the ten ISSR primers are shown in Figure (Fig.19). 10 ISSR primers were used to produce a total number of 284 amplified DNA products were generated across all the four progenies and the mother plant selected. The genetic similarity coefficients based on the bands generated from ISSR primers were calculated using NtSys software (Fig 20). The similarity index value illustrated that the highest similarity was 100% which recorded between progeny -1 (P1), progeny -2 (P2) and progeny- 3 (P3). The lowest similarity was 94.91 between Progeny -4 and all the other progenies. Similarity coefficient matrices were used to generate a dendrogram of all the four progenies with its mother plant based on UPGMA analysis (Fig.21). The dendrogram resulted in three clusters in which p1, p2 and P3 grouped in one cluster which showed a 98.24 similarity with parent. Among the progenies only P4 was separated in cluster which

showed 96.6 similarity with parent and 94.91 with all the other progenies. These results showed that all the progenies obtained from tissue culture were found to be similar to the mother plant.

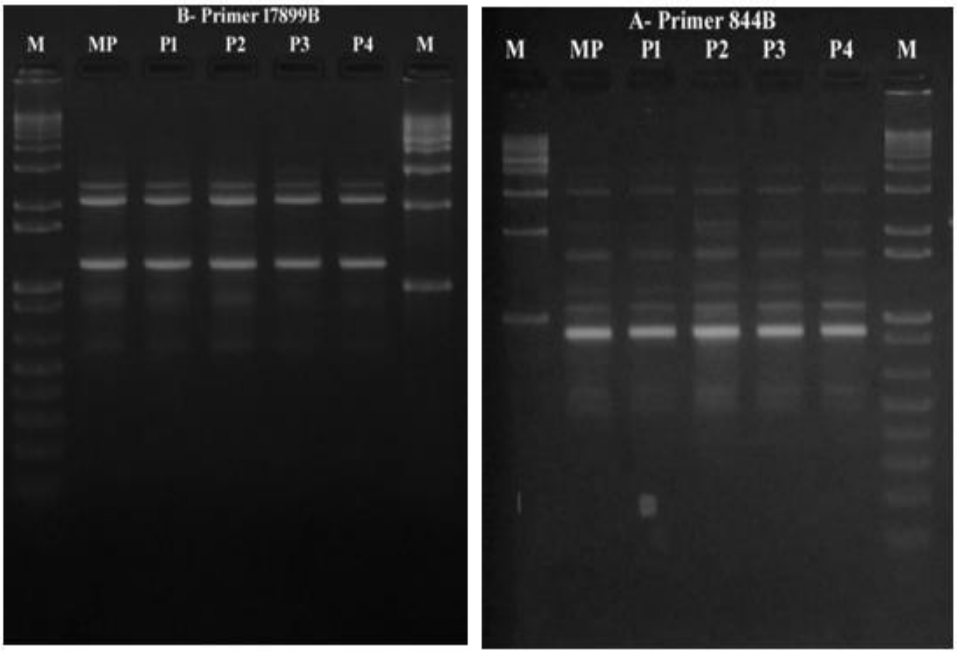

Fig.19: DNA fingerprinting patterns generated by ISSR fingerprints of two selected primers (844B & 17899B) of *in vitro* micropropagated *Moringa peregrina*

M- Marker, MP- mother plant, P1- progeny 1, P2- progeny 2, P3- progeny 3, P4- Progeny 4

	p	P1	P2	P3	P4
p	1.0000000				
p1	0.9824561	1.0000000			
p2	0.9824561	1.0000000	1.0000000		
p3	0.9824561	1.0000000	1.0000000	1.0000000	
p4	0.9661017	0.9491525	0.9491525	0.9491525	1.0000000

Fig.20: Similarity coefficient of all the four progenies with its parent plant based on ISSR analysis

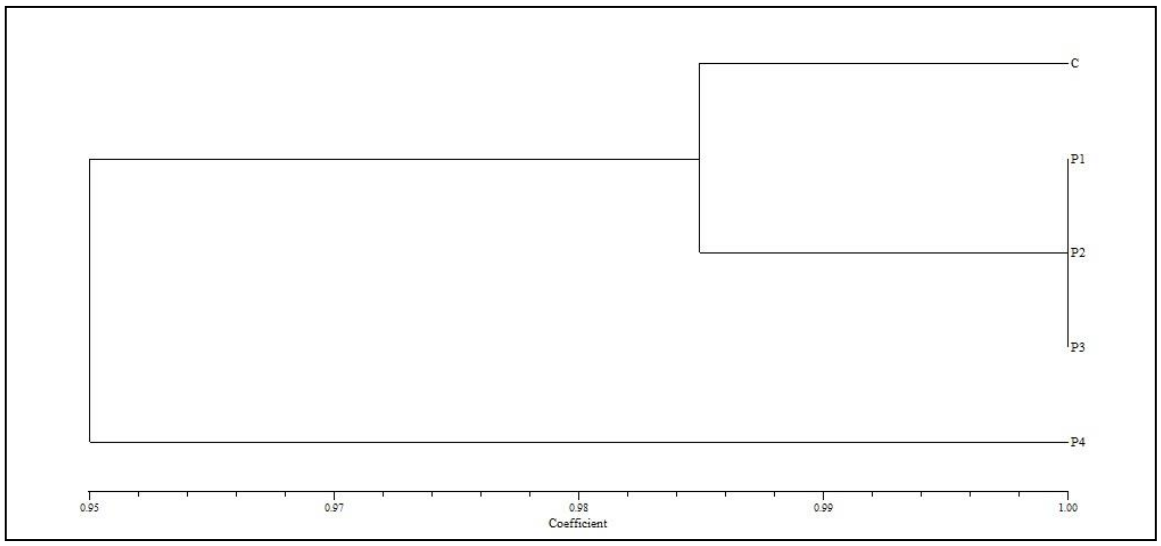

Fig.21: UPGMA dendrogram indicating the genetic relationships among the progenies and the mother plant based on ISSR markers

Chapter 4: Discussion

4.1 Seed germination

It is well know that *in vitro* culture medium has great influence on responses of plant tissues as it is responsible to provide necessary stimulus for triggering processes of differentiation and it provides nutrients for formation of new cells and tissues. Composition of culture medium has great influence on performance of in vitro cultures and directly affects complexity of its preparation (Schneider et al., 2014).

In the present study, high germination rate was observed when the seeds of *Moringa peregrina* were inoculated in half-strength Murashige and Skoog medium. This suggested that the organic and inorganic components present in HMS medium are sufficient for the production of plantlets of *M. peregrina* .Similar research reported by Wesam Al Kateeb et al., (2012) on the effect of MS media strength on germination rate of *Moringa peregrina* showed that full strength MS media was better than half strength MS media. These variable responses could be due to different factors including genetic differences, difference in ex-plant, source and the type or age of ex-plants used to establish the culture.

4.2 Direct organogenesis

4.2.1 Effect of TDZ on direct shoot induction from nodal explants

Thidizuron (TDZ) is among the most active cytokinin-like substances for woody plant tissue culture. It facilitates efficient micropropagation of many woody species. Low concentrations (<1 µM) can induce greater axillary proliferation than many other cytokinins (Huetteman 1993). It has been found that, the germination

percentage of nodes was the highest in MS media supplemented with TDZ 0.5 µm/L (90.4%). Hormone free MS media had the lowest percentage of germination and shoot number per explant suggesting that hormones supported higher number of shoots per explant. Direct organogenesis from nodal explants was the highest in medium supplemented with 1µM of TDZ which induced an average of 2.4 axillary shoots per explants followed by medium supplemented with 2 µM of TDZ which induced an average of 1.85 axillary shoots per explants. In Pinus brutia, the direct organogenesis was obtained high in Murashige and Skoog (MS) medium containing 4.5 µM thidiazuron (Jericó et al 2012). Varutharaju et al (2014) reported a maximum number of shoot organogenesis (23.6 ± 0.16) in Aerva lanata on medium containing 1.0 mg L−1 TDZ.

4.2.2 Effect of BA and NAA combination on direct shoot induction from nodal explants

BA is a synthetic cytokinin that enhances plant growth and development responses, and NAA is a plant hormone which is used for the vegetative propagation of plants. The results show that, BA and NAA combination did not show any enhancement in shoot induction of plantlets when added to the media. MS media supplemented with BA and NAA combination showed less germination percentage of Moringa peregrina nodal ex-plant ranging from 13 to 58%. Hormone free MS media produce 100% germination with an average of 1.08 axillary shoots per-explant. Thus, MS media with BA and NAA combination was less effective than TDZ supplemented media.

4.3 Indirect Organogenesis

4.3.1 Effect of 2,4-D on callus induction from shoot cuttings

2,4-Dichlorophenoxyacetic acid (2,4-D) has been used in plant research laboratories as a supplement in plant cell culture media such as MS medium (Murashige and Skoog,1962). 2,4-D is employed in most *in vitro* culture systems. It is classified as an auxin plant hormone derivative that has been found to elicit rapid cell proliferation and callus formation (Zheng and Konzak,1999).

Shoot cuttings were inoculated in MS media containing the synthetic auxin (2,4-D) which will induce the callus formation. The results suggested that MS medium supplemented with 2 mg/l of 2, 4-D was the most effective medium with 100% callus induction from shoot cuttings. Similar research done by Lalida P. et al.(2013) on *Moringa oleifera* to study the effect of MS media supplemented with different concentration of 2,4-D on callus formation found that 0.5 mg/l of 2,4-D was the best medium for callus induction of *M. oleifera* with 100% of callus induction. A research on the effects of 2, 4-D on callus induction from leaf explants of Cornukaempferia larsenii P. Saensouk showed that, the highest number of callus formation, percentage of callus formation and average weight of callus were obtained from young leaves cultured on the medium supplemented with 0.5 mg/l 2,4-D in the light condition (Saensouk et al.,2007). Suitable concentration of 2, 4-D for callus induction varied due to the plant species and the nature of the tissue of the explants.

4.3.2 Effect of 2,4-D in combination with TDZ, BA and Kin on callus induction from shoot tip

Callus induction from shoot tip was also tested using MS media supplemented with 2, 4-D in combination with TDZ, BA and Kin. Kinetin and 6-Benzyladenine (BA) are type of cytokinin, a class of plant hormones that promotes cell division. Thidiazuron has been used successfully *in vitro* to induce adventitious shoot formation and to promote axillary shoot proliferation. Thidiazuron is especially effective with recalcitrant woody species. Shoot numbers produced on medium containing thidiazuron are equivalent to or greater than numbers initiated on medium with purine-type cytokinins (Lu, Chin-Yi,1993)

The callusing was significantly high in 2, 4-D 2mg/l and TDZ 0.5 μM/L combinations compared to other treatments. A research done by Shen et al., 2008, indicate that, the combination of 5 μM TDZ and 1 μ 2,4-D resulted in the greatest callus formation frequency among the four cultivars of Dieffenbachia tested. Data obtained from our research showed that callusing percentage and size were higher when MS media was supplemented with 2, 4-D in combination with TDZ compared with MS media containing 2, 4-D and BA And Kin combinations, suggesting callus induction of *M.peregrina* shoot tip can be enhanced if MS media was supplemented with 2 μM of 2,4-D and 0.5 μM of TDZ. Tang and Newton (2005), suggest the use of TDZ as the cytokinin to stimulate adventitious shoot formation if callus desired to subsequently be used for regeneration.

4.4 Regeneration

4.4.1 Effect BA and NAA combination on regeneration of callus cultures

The regeneration of callus into complete plantlet is one of the major steps in indirect organogensis. The use of BA and NAA media under light conditions resulted changes in the callus colour to green. The formation of shoot primordia was observed in some of the cultures treated with BA2mg/l and NAA 1mg/l. The formation of complete shoots from the callus was less in medium supplemented with BA and NAA. In *Punica granatum*, the highest number of shoots regenerated from calli was observed in media containing 5 mg L^{-1} BA with 0.1 mg L -1 NAA (Alireza Bonyanpour and Morteza Khosh-Khui 2013). Jyothi Abraham and Dennis Thomas (2015) reported that in *Elephantopus scaber* the highest shoot formation from callus was recorded on MS medium supplemented with 6.0 μM N6-benzylaminopurine (BA) and 1.5 μM α naphthalene acetic acid (NAA). These reports suggest that the concentration of NAA should be reduced further in combination with BA for high regeneration potential.

4.4.2 Effect of BA, IBA and NAA combination on root formation of regenerated plantlets from nodal explants

For the regenerated plantlet, it has been found that media containing 1 mg/l of BA and 2 mg/l of NAA was highly significant for root formation per shoot compared with other media hormone combination. The combination of BA with NAA was found to be better than BA with IBA for root formation. A research done by Sultana et al., 2015 suggested that MS media supplemented with 2.0 mg/l NAA and 7.5 mg/l BAP was found to be the best for rapid root formation of *hibiscus cannabinus*. Considering the findings of present study, MS media supplemented with 2mg/l of

NAA and 1 mg/l of BAP resulted in enhanced root formation for the regenerated plantlets of *Moringa peregrina*.

4.5 Clonal fidelity study

ISSR analysis is one the molecular techniques used to assess the genetic similarity between the progenies and its parent. One of the main problems in tissue culture is the loss of genetic identity between the *in vitro* developed clones therefore, it is necessary to evaluate uniformity of the clones to conserve the genetic material. In the present research the clonal fidelity study was done using 10 ISSR primers. Among the four progenies tested three had 98 percent similarity between them and the mother plant. ISSR proved to be more effective and reproducible for detecting genetic uniformity (Martins, 2004). In previous report on *Moringa peregrina* by Wesam et al., (2012), used 10 ISSR primers to assess the similarity between 12 progenies and the mother plant. These results showed that regenerated plants are similar and no genetic variation was detected after *in vitro* culture. In *Tylophora indica*, the banding pattern of each primer was uniform and comparable to mother plant and showed about 93% homology using ISSR analysis to confirm the genetic stability of *in vitro* raised plants (Sharma et al., 2014).

Molecular marker analysis of micropropagated plant been successfully applied in cauliflower (Leroy et al., 2000), almond (Martins et al., 2004), banana (Lakshman et al., 2007; Rout et al., 2009), *Cymbopogon martinii* var motia (Bhattacharya et al., 2010), gerbera (Bhatia et al., 2009) and *Swertia chirayita* (Joshi and Dhawan 2007).

We were able to optimize *in vitro* direct organogenesis through nodal explants for *Moringa peregrina* and observed high similarity percentage between the progenies and the mother plant. This rapid propagation method allow efficient germplasm conservation irrespective of environmental restrictions especially for endangered plant species.

Chapter 5: Conclusion

Moringa peregrina is a native plant in the United Arab Emirates, It is becoming an endangered plant species all over the world (Gomma and Pico, 2011). Many recent researchers are aiming to find effective cultivation methods to protect *M. peregrina* from extinction. In the present study we were able to standardize *in vitro* production protocol through direct organogenesis, and to confirm the clonal fidelity of *in vitro* regenerated progenies of *M. peregrina* and the parents. The seed germination experiment showed that half strength MS basal media found to be better than full strength MS media with 70% germination rate. The explants like shoot tips, shoot cuttings and stem nodes were collected from the *in vitro* developed seedlings for further experiments.. For direct organogenesis of nodal explants, it has been found that MS media supplemented with TDZ was better than MS media with BA and NAAcombinations. The number of shoots was the highest (2.47 per explant) in media containing TDZ 1 µM/l.

In the callus induction experiment media supplemented with 2, 4-D alone under lowest concentration , the callus percentage and size were found to be highest. Also 2,4-D, TDZ, BA and Kinetin combination was tested for callus induction. The results showed that, 2,4-D in combination with TDZ was found to be better for callusing compared to 2,4-D, BA and 2,4-D, Kinetin combinations.

The callus regeneration experiment was conducted with media supplemented with BA and NAA combination. The callus structures turned to green in colour with shoot primordia initiated, but with less shoot formation. The root formation

experiments were conducted in medium supplemented with BA in combination with NAA and IBA. The rooting percentage and the number of roots were found to be high in media supplemented with BA 1mg/l and NAA 2mg/l. Compared to IBA, NAA in combination with BA was better for rooting of *Moringa pergrina* . Both callus induction and plant regeneration from explants require the presence of appropriate combination and concentration of plant growth regulator in the culture media.

The *in vitro* developed plantlets were transferred to pot and acclimatized to survive in ambient conditions.. The assessment of the genetic similarity of the progenies was important in order to conserve the germplasm. The clonal fidelity study using ten ISSR primers revealed that all the four progenies were found to be similar with its parents with a minimum similarity coefficient of 94.9. This suggests that all the progenies are true to type with its parents.

Bibliography

Abdullah, A. (2015).Genetic diversity of *Moringa peregrina* species in Saudi Arabia with ITS sequences. Saudi Journal of Biological Sciences, 22, 186-190.

Afsharypuor, S., Asghari, G., Mohagheghzadeh, A., & Dehshahri, S. (2010). Volatile constituents of the seed kernel and leaf of *Moringa peregrina* (Forssk.) Fiori, Agricolt. Cultivated in Chabahar (Iran). Iranian Journal of Pharmaceutical Sciences, 6(2), 141-144.

Al-Gohary, I.H. & Hajar, A.S. (1996). On the Ecology of *Moringa peregrina* (Forssk.) Fiori Anatomical Responses to Varying Soil Moisture Contents, Journal of Kingdom Abdulaziz University (JKAU), 8: 5-17 pp.

Al-Kahtani, H. A., & Abou-Arab, A. A. (1993). Comparison of physical, chemical, and functional properties of *Moringa peregrina* (Al-Yassar or Al-Ban) and soybean proteins. Cereal chemistry (USA).

Arora, D. S., Onsare, J. G., & Kaur, H. (2013). Bioprospecting of Moringa (*Moringaceae*): microbiological perspective. Journal of Pharmacognosy and Phytochemistry, 1(6).

Bonyanpour, A., & Khosh-Khui, M. (2013). In vitro plant regeneration in *Punica granatum* L. cv Nana from leaf explants. International Journal of Agriculture, Environment & Biotechnology, 6(1).

Boulos, L. (1999). 'Flora of Egypt.' Vol. I (Azollaceae – Oxalidaceae). Al hadara Publishing, Cairo, Egypt, 419 pp. By John. Grasas y Aceites. Vol. 49. (2),170-176

Cossalter, C. (1989). Drought Resistant Trees and Shrubs for Dry Planting in Bahrain, in FAO (1998). Forest Genetic Resources Information No. 17. Food and Agriculture Organization of the United Nations, Via delle Terme di Caracalla, 00100 Rome, Italy.

Dehshahri, S., Wink, M., Afsharypuor, S., Asghari, G., & Mohagheghzadeh, A. (2012). Antioxidant activity of methanolic leaf extract of *Moringa peregrina* (Forssk.) Fiori. Research in pharmaceutical sciences, 7(2), 111-118.

Donli, P. O., & Dauda, H. (2003). Evaluation of aqueous Moringa seed extract as a seed treatment biofungicide for groundnuts. Pest management science, 59(9), 1060-1062.

El-Alfy, T. S., Ezzat, S. M., Hegazy, A. K., Amer, A. M., & Kamel, G. M. (2011). Isolation of biologically active constituents from *Moringa peregrina* (Forssk.) Fiori.(family: Moringaceae) growing in Egypt. Pharmacognosy magazine, 7(26), 109.

Elbatran, S. A., Abdel-Salam, O. M., Abdelshfeek, K. A., Nazif, N. M., Ismail, S. I., & Hammouda, F. M. (2005). Phytochemical and pharmacological investigations on *Moringa peregrina* (Forssk) Fiori. Natural Product Sciences.

Elbatran, S.A., Abdel-Salam, O.M., Abdelshfeek, K.A., Nazif, N.M., Ismail, S.I. and Hamoouda, F.M. (2005). Phytochemical and pharmacological investigations on *Moringa peregrina* (Forssk) Fiori. Nat. Prod. Sci. 11(4), 199-206.

Ghodsi, R., Sadeghi, H. M., Asghari, G., & Torabi, S. (2014). Identification and cloning of putative water clarification genes of *Moringa peregrina* (Forssk.) Fiori in E. coli Xl 1 blue cells. Advanced biomedical research, 3(1), 57.

Gomaa N.H. and Pico X.F. (2011). Seed germination, seedling traits, and seed bank of the tree *Moringa peregrina* (Moringaceae) in a hyper-arid environment. Am. J. Bot. 98, 1024–1030.

Hajar, A. S., & Gumgumjee, N. M. (2014). Antimicrobial activities and evaluation of genetic effects of *Moringa peregrina* (forsk) fiori using molecular techniques. International Journal of Plant, Animal and Environmental Sciences, 4(1), 65-72.

Hajar, A.S. (1997). On the Ecology of *Moringa peregrina* (forssk.) Fiori (2) Germination and Growth responses to water deficit stress. Alexandria Bull. Fac. Sci. Alex. Univ. 37(1), 41-48.

Hartwell, J.L. (1967). Plants used against cancer: a survey. Lloydia's Journal, 32(1):78-107 pp.

Hegazy AK, Hammouda O, Lovett-Doust J, Gomaa NH (2008). Population dynamics of *Moringa peregrina* along altitudinal gradient in the northwestern sector of the Red Sea. J. Arid Environ. 72: 1537- 1551.

http://www.who.int/mediacentre/factsheets/fs134/en/.

Huetteman, C. A., & Preece, J. E. (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant cell, tissue and organ culture, 33(2), 105-119.

Iyan Robiansyah, Hajar, A. S., Al-kordy, M. A., & Ramadan, A. (2014). Current status of economically important plant *Moringa peregrina* (Forrsk.) Fiori in Saudi Arabia: a review. International Journal of Theoretical and Applied Sciences, 6(1), 79.

Jericó, B. B., Lourdes, I. A., Lázaro, S. V., José, C. M., & Nancy, S. B. (2012). *In vitro* regeneration of *Pinus brutia* Ten. var. eldarica (Medw.) through organogenesis. African Journal of Biotechnology, 11(93), 15982.

John Tsaknis.(1998). Characterisation of Moringa peregrina Arabia seed oil

Kant, T., Tomar, U. K., Prajapati, S., & Parmar, A. K. (2010). *In vitro* propagation as a viable conservation strategy for *Commiphora wightii*, an endangered medicinally important desert tree, India. Conservation evidence, 7, 94-99.

Koheil, M. A., Hussein, M. A., Othman, S. M., & El-Haddad, A. (2011). Anti-inflammatory and antioxidant activities of *Moringa peregrina* seeds. Free Radicals and Antioxidants, 1(2), 49-61.

Koheil, M. A., Hussein, M. A., Othman, S. M., & El-Haddad, A. (2011). Anti-inflammatory and antioxidant activities of *Moringa peregrina* seeds. Free Radicals and Antioxidants, 1(2), 49-61.

Lalas S, Gortzi O, Athanasiadis V, Tsaknis J, Chinou I (2012) Determination of antimicrobial activity and resistance to oxidation of *Moringa peregrina* seed oil. Molecules 17:2330–2334

Lalas S, Gortzi O, Athanasiadis V, Tsaknis J, Chinou I (2012) Determination of antimicrobial activity and resistance to oxidation of *Moringa peregrina* seed oil. Molecules 17:2330–2334

Lalas, S., & Tsaknis, J. (2002). Extraction and identification of natural antioxidant from the seeds of the *Moringa oleifera* tree variety of Malawi. Journal of the American Oil Chemists' Society, 79(7), 677-683.

Lu, Chin-Yi (1993). The use of thidiazuron in tissue culture. *In Vitro* Cellular & Developmental Biology-Plant, 29(2), 92-96.

Martins M, Sarmento D, and Oliveira MM (2004) Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. Plant Cell Rep 23: 492–496

Mekonnen, Y., Yardley, V., Rock, P., & Croft, S. (1999). *In vitro* antitrypanosomal activity of Moringa stenopetala leaves and roots. Phytotherapy Research, 13(6), 538-539.

Miller, A. G., & Morris, M. (1988). Plants of Dhofar: the southern region of Oman, traditional, economic and medicinal uses. Oman: Office of the Adviser for Conservation of the Environment, Diwan of Royal Court Sultanate of Oman xxvii, 361p.-col. illus.. ISBN, 715708082.

Murashige, T. and F. Skoog (1962)A revised medium for rapid growth and bioassays with tobaccotissue cultures. Physiol. Plantarum. 15:473-97.

Oliveira, J.T.A., Silveira, S.B., Vasconcelos, I.M., Cavada, B.S. and Moreira, R.A. (1999). Compositional and nutritional attributes of seeds from the multiple

purpose tree *Moringa oleifera Lamarck*. Journal of the Science of Food and Agriculture, 79(6), 815–820. Osman, H.E. (2010).

Olson ME (1999). The home page of the plant family Moringaceae, Available at:

Olson, M. E., and S. G. Razafimandimbison. (2000). *Moringa hildebrandtii:* A tree extinct in the wild but preserved by indigenous horticultural practices in Madagascar. Adansonia sér. 3 22(2) 217-221.

Osman, H. E., & Abohassan, A. A. (2012). Morphological and analytical characterization of *Moringa peregrina* populations in western Saudi Arabia. Int J Theor Appl Sci, 4, 174-84.

Peddaboina, V., Thamidala, C., & Karampuri, S. (2006). In vitro shoot multiplication and plant regeneration in four Capsicum species using thidiazuron. Scientia horticulturae, 107(2), 117-122.

Rahman, I. M., Barua, S., Begum, Z. N., Rahman, A.M. and Hasegawa, H. (2009). Physicochemical properties of *Moringa oleifera Lam.* Seed oil of the indigenous cultivar. Journal Food Lipids, 16: 540–553.

Rouhi-Broujeni, H., Heidarian, E., Darvishzadeh-Boroojeni, P., Rafieian-Kopaei, M., & Gharipour, M. (2013). Lipid lowering activity of moringa pergerina seeds in rat: A comparison between the extract and atorvastatin. Res J Biol Sci, 8(5), 150-4.

Saensouk, P., Theerakulpisut, P., Kijwijan, B., & Bunnag, S. (2007). Effects of 2, 4-D on Callus Induction from Leaf Explants of *Cornukaempferia larsenii P.* Gardens' Bulletin, 59, 183-188.

Schneider, L., & Zaffari, D. S. G. R (2014). Seed Germination of *Cattleyaintermedia* and *Cattleyawarneri* in Alternative culture Media.(23).851-970

Sharma, M. M., Verma, R. N., Singh, A., & Batra, A. (2014). Assessment of clonal fidelity of Tylophora indica (Burm. f.) Merrill "in vitro" plantlets by ISSR molecular markers. SpringerPlus, 3(1), 400.

Shen, X., Kane, M. E., & Chen, J. (2008). Effects of genotype, explant source, and plant growth regulators on indirect shoot organogenesis in *Dieffenbachia* cultivars. *In Vitro* Cellular & Developmental Biology-Plant, 44(4), 282-288.

Somali, M.A., Bajneid, M.A., Fhainian, S.S. (1984): Chemical composition and characteristic of Moriga peregrina seeds and seed oil. – Journal of American chemistry Society 16: 85.

Sreelatha, S., Jeyachitra, A., & Padma, P. R. (2011). Antiproliferation and induction of apoptosis by Moringa oleifera leaf extract on human cancer cells. Food and Chemical Toxicology, 49(6), 1270-1275.

Steinitz B, Tabib Y, Gaba V, Gefen T, Vaknin Y (2009) Vegetative micro-cloning to sustain biodiversity of threatened Moringa species. *In vitro* Cell Dev Biol Plant 45:65–71

Steinitz B, Tabib Y, Gaba V, Gefen T, Vaknin Y (2009) Vegetative micro-cloning to sustain biodiversity of threatened Moringa species. *In vitro* Cell Dev Biol Plant 45:65–71

Steinitz B, Tabib Y, Gaba V, Gefen T, Vaknin Y (2009) Vegetative micro-cloning to sustain biodiversity of threatened Moringa species. In vitro Cell Dev Biol Plant 45:65–71

Sultana, R., Quader, A. K. M. L., Haque, M., Mazumder, S., & Paul, S. K. (2015). Combined effect of hormonal concentrations (naa and bap) on root formation kenaf (*hibiscus cannabinus l.*).

Sybean proteins. – Cereal Chem. 70: 619-626.

Täckholm, V. (1974). Student's Flora of Egypt, Cairo University, Cairo, 1180 pp.

Tang, W., & Newton, R. J. (2005). Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in Virginia pine. Plant Growth Regulation, 46(1), 31-43.

Varutharaju, K., Soundar Raju, C., Thilip, C., Aslam, A., & Shajahan, A. (2014). High Efficiency Direct Shoot Organogenesis from Leaf Segments of Aerva lanata (L.) Juss. Ex Schult by Using Thidiazuron. The Scientific World Journal, 2014.

Warner, K. and Mounts, T.L. (1993). Frying stability of soybean and canola oils with modified fatty acid compositions. Journal of the American Oil Chemists' Society, 70(10), 983-988.

World Health Organization, Media Center 2008.Traditional medicine. Fact sheet N°134.

www.mobot.org/gradstudents/oslon/Mo ringahome.html.

Zaghloul, M. S., Abd El-Wahab, R. H., & Moustafa, A. A. (2010). Ecological assessment and phenotypic and fitness variation of Sinai's remnant populations of *Moringa peregrina.* Applied Ecology and Environmental Research, 8(4), 351-366.

Zheng, M. Y., & Konzak, C. F. (1999). Effect of 2, 4-dichlorophenoxyacetic acid on callus induction and plant regeneration in anther culture of wheat (Triticum aestivum L.). Plant Cell Reports, 19(1), 69-73.