**DOMESTICATION IN MARAMA BEAN (*TYLOSEMA ESCULENTUM*): AGRONOMY, PHENOTYPIC AND MOLECULAR CHARACTERIZATION FOR ITS IMPROVEMENT**

A DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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BY

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## Abstract

Marama bean [*Tylosema esculentum* (Burchell) Schreiber] belongs to the family Fabaceae and is a candidate for domestication in arid zones. It is indigenous to the Kalahari regions of Southern Africa thriving in low nutrient and low moisture soils. Marama bean seeds exhibit high oil (up to 48%) and protein (up to 42%) content comparable to peanut and soybean respectively. The main purpose of this study was to determine the usefulness of previously developed microsatellite markers in distinguishing phenotypically characterized marama plants and plants treated with gamma irradiation for improvement. The chromosome number was determined to lay a foundation for molecular mapping. Further to this, the study sought to establish the effect of improved nutrients, moisture and hormone treatments on vegetative growth of marama bean. Grafting was explored as a propagation method to side step juvenility and molecular identification of potential fungal pathogens of leaf tissue was achieved. SSR primers were screened using DNA isolated from phenotypically characterized individuals representing 13 marama bean ecotypes. Two microsatellite markers MARA 039 and MARA077 were found to be candidates for use in detecting differences in internode length as well as distinguishing seeds treated with gamma radiation from untreated seeds. The chromosome number in *T. esculentum* was found to be n=22 (2n=44) and this will be useful in future mapping efforts. A completely randomized block design was used in assessing the possibilities of enhancing vegetative growth with fertilizer, hormones and water: Lucky plant fertilizer (LS004990-00-00) (1g/L), 100µg/L of hormone Gibberellin ($GA_3$), 200mL water for

the control, 400mL water for the high water treatments were applied. The results obtained were analysed by one way ANOVA and showed no significant difference in internode length ($p=0.362>0.05$), stem length ($p=0.256>0.05$) and number of leaves ($p=0.466>0.05$) suggesting the treatments had no effect on the vegetative growth of *T. esculentum*. Marama bean was observed to be non-responsive to grafting in the trials carried out prompting the exploration of tissue culture methods for future studies. The overall inoculations of PDA and PDB with leaf tissue showing signs of necrosis and DNA isolation together with the internal transcribed spacer (ITS) region amplification of the total 8 single spore cultures plus sequencing followed by a comparison of the DNA sequences with GenBank revealed the presence of a complex with 8 known species: *Penicillium brevicompactum, Epicoccum sorghi, Rhizopus stolonifer, Alternari solani, Fusarium equiseti, Penicillium olsonii, Fusarium chlamydosporum* and *Fusarium incarnatum*. This study has made several contributions to knowledge and current understanding of plant sciences. To our knowledge, this is the first report describing the presence of these fungi on marama bean seedling leaf tissue. This study has made a major contribution to mapping efforts as it identified regions of the genome that can be used in these studies. This study has also confirmed the marama bean chromosome number. Molecular markers linked to internode length or germination rate or any other trait that may be of agronomic importance had not been reported previously. The results from the work on grafting and plant growth regulators will prevent farmers from using high cost agricultural practices in cultivation of marama bean. Based on the findings of this study, the recommended next steps would be to explore Next Generation Sequence data with genome walking to identify genes in marama, particularly genes

controlling flower formation, as well as establishing F1 populations for mapping studies.

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## Abbreviations

| | |
|---|---|
| **AFLP** | Amplified Fragment Length Polymorphism |
| **ANOVA** | Analysis of Variance |
| **BLAST** | Basic Local Alignment Search Tool |
| **DNA** | Deoxyribonucleic acid |
| **γ** | Gamma |
| **GM** | Genetically modified |
| **Gy** | Grays |
| **ITS** | Internal transcribed spacer |
| **kb** | kilo base |
| **LSU** | Large subunit |
| **MAS** | Molecular Marker Assisted Selection |
| **PCR** | Polymerase Chain reaction |
| **PDA** | Potato Dextrose Agar |
| **PDB** | Potato Dextrose Broth |
| **QTL** | Quantitative trait locus |
| **RAPD** | Random Amplified Polymorphic DNA |

| | |
|---|---|
| **rDNA** | ribosomal DNA |
| **SSR** | Simple Sequence Repeat |
| **UV** | Ultra violet |

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Ndatenda

## Dedication

I dedicate this work as a tribute to my late mother, Margaret Gloria Takundwa (nee Mucheri) who left us in 2000, 13 years later your memory and inspiration lives on. I would also like to dedicate this work to my entire family who are my never ending support.

## Declarations

I, Mutsa Takundwa, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institute of higher education.

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……………………… Date…………………..

Mutsa Takundwa

## Publications and Conference Proceedings

***Some of the work presented in this dissertation has been in part or in full been published under the titles*:**

1. **Takundwa M**, Ruzvidzo O, Uzabakiriho JD, Titus PE, Chimwamurombe PM. (2013, November 24-27). **Molecular identification of fungal species associated with leaf lesions of Marama bean seedlings in Namibia.** Paper presented at South African Society of Microbiology (SASM) Conference 2013, Bela-Bela, Limpopo, South Africa**.**

2. **Takundwa M**, Ruzvidzo O, Nambahu F, Kawadza DT, Chatukuta P, Chimwamurombe PM. (2014). **Molecular characterization of gamma irradiated seeds of Kalahari marama bean.** Submitted for publication (*Molecular Biology International*).

3. **Takundwa, M**., *et al*., (2012). **Development and use of microsatellites in Marama bean.** *Crop Science Journal* 20(2) pp. 95-105.

4. **Takundwa, M**., Chimwamurombe, P.M. and Cullis, C.A. (2012). **A chromosome count in Marama bean (*Tylosema esculentum*) by Feulgen staining using garden pea (*Pisum sativum* L.) as a standard.** *Research Journal of Biology* 2(6) pp.177-181.

# CHAPTER 1: INTRODUCTION

## 1.1 General introduction

Marama bean [*Tylosema esculentum* (Burchell) Schreiber], a legume occurring naturally in the arid, dry parts of Southern Africa has high nutrient value in the seeds and tubers, rich in protein, oil and starch. It is a potential crop for arid areas where few conventional crops can survive and there is increasing interest in the cultivation of marama bean (Chimwamurombe, 2008; Nepolo, Takundwa, Chimwamurombe, Cullis & Kunert, 2009). In order to meet future demands for the seed, high yielding cultivars need to be developed and farms established for the cultivation of marama so as to decrease pressure on the natural populations and conserve the existing wild germplasm.

All the crops supplying the worlds needs for food today as domesticates or semi-domesticates were once wild species like marama bean. With breeding and selection the development of cultivars was made possible. The yield and environmental barriers of the natural or wild types of the present day domesticated crops had to be overcome in order for domestication to be successful. These cultivars are now able to grow in wide ranges of environments, overcoming biotic and abiotic stresses while still providing economic yield (Simpson & Ogorzaly, 2001; Thrupp, 2002).

Traditional breeding programs that depend solely on phenotype selection are time consuming (Andjelkovic & Thompson, 2006; Duvick, Smith & Cooper, 2004). At present, more powerful and efficient strategies for producing "ideal" crops are possible with the development of molecular biology (Ribaut, Jiang, Gonzalez-de-Leon, Edmeades & Hoisington, 1977). According to Tuberosa et al., (2002) applying molecular marker techniques on traditional breeding programs can improve the efficiency of breeding crops. Molecular marker assisted selection breeding programs are therefore being established for marama bean.

During the last decades, considerable progress has been made in the development of molecular markers and their use in mapping and/or tagging of genes or quantitative trait loci (QTLs) controlling important agronomic traits in all major crops (Varshney, Mohapata & Sharma, 2004). Molecular markers closely associated with desirable traits are being utilized to increase the efficiency and effectiveness of conventional breeding by indirect selection of the desirable plants in segregating populations (Collard & Mackill, 2008). However, this has not been the case in many other plant species, which are considered to be of "less importance", the so-called orphan crops.

Microsatellite markers had been developed for marama bean and these markers needed to be linked to traits of the bean that are of agronomic interest (Takundwa, Chimwamurombe, Kunert & Cullis, 2010). This study had several aims, namely, first and foremost to determine the link between the microsatellite markers that were

available and traits of agronomic interest. This research aimed to determine which of previously developed Simple Sequence Repeats (SSR's) were potentially informative for genotyping.

Secondly, the study sought to confirm the number and structure of chromosomes in marama bean. The chromosome number was determined in comparison with garden pea, *Pisum sativum*, in order to lay a foundation for QTL identification and molecular mapping efforts in future studies. The standard Schiff's method described later was used to achieve this aim.

In addition, the use of grafting and plant growth promoting factors was investigated as methods to side step juvenility in this legume. Regeneration by grafting was also explored to side step juvenility. These methods of propagation and improvement of the growth environment for marama bean were investigated to answer some basic fundamental questions about the biology of germination and generation of marama bean plants, and determine if the agricultural practices used in other plants to improve growth and yield, could be applied to marama bean.

A reliable method of growing plants under greenhouse conditions was established and potential fungal agents causing leaf necrosis in marama bean were identified. The overall future aim will be to develop high yielding, early germinating and disease resistant cultivars in the near future based on the findings of studies such as

the one carried out here. This would eventually lead to the adoption of marama bean as a viable commercial crop at the end of a domestication process.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Domestication, plant breeding, orphan crops and climate change

For domestication to occur there must be human selection and propagation, both of which can be either intentional or unintentional (Anderson, 2005). Two types of domestication can conceptually be distinguished: landscape domestication and plant (or animal) domestication. Only the latter will be discussed here because plant domestication can now be examined with new genetic techniques. The two kinds of domestication are intimately related because domesticated populations require some kind of landscape management, especially cultivation (Clement, 1999).

Plant domestication can be viewed as a co-evolutionary process by which human selection on the phenotypes of plants of interest results in changes in the descendants that make them more useful to humans and better adapted to human management of the landscape. The degree of change in populations can vary along a continuum from wild (the baseline, with no human-mediated change), through incipiently domesticated, to semi-domesticated, to domesticated. Incipiently domesticated populations have gone through a founder event (defined as human selection of a small sample of the wild population and propagation of descendants from this sample; also called a bottleneck) that reduces its genotypic diversity and its phenotypic diversity varies only somewhat from the ancestral wild population in the traits selected by humans (Clement, 1999; Clement, de Cristo-Anaujo, d'Eeckenbrugge, Pereira & Picanço-Rodrigues, 2010). Founder effects have been

reported in wild populations of marama bean, as low inter population diversity has been observed in studies by several groups using ribosomal DNA (rDNA), SSR, Amplified Fragment Length Polymorphism (AFLP) and Random amplified Polymorphic DNA (RAPD) markers (Nepolo, Chimwamurombe, Cullis, Kandawa-Schulz, 2010; Takundwa et al., 2010; Halloran & Monaghan, 1996; Naomab, 2004 )

Semi-domesticated populations have gone through several founder events sequentially resulting in reduced genotypic diversity, but phenotypic diversity is enhanced by accumulation of diverse alleles for the preferred traits selected by humans. Semi-domesticated populations tend to have more geographic distributions than incipient domesticates, which may permit introgression with other wild, incipient or semi-domesticated populations of the same species; in turn, such introgression may offer additional alleles for selected traits, thus somewhat enhancing genetic diversity. The ample geographic distribution may include areas where wild populations do not exist, which reduces introgression of wild-type alleles and permits more rapid response to human selection (Clement, 1999; Clement et al., 2010).

Domesticated populations have been further selected for adaptation to human-modified landscapes such as gardens and fields losing their original ecological adaptations for survival without humans, especially the original dispersal mechanisms and survival capabilities. The loss of dehiscent capsules is a frequent process in domestication and has been seen between wild and domesticated forms of the common bean *Phaseolus vulgaris* L (Gepts, 1998; Gioia et al., 2012).

Observation is that domestication is a process that occurs at the population level, not the species level, so that it is incorrect to affirm that species X is a domesticate, unless all wild populations have become extinct, which is an uncommon occurrence; it is most generally correct to affirm that species X exhibits domesticated populations (Clement, 1999, Clement et al., 2010). Exceptions to this generalization exist, for example, when the end-result of the domestication process is a new species; a particular case of the latter is interspecific hybridization followed by chromosome doubling, resulting in the formation of allopolyploids (Hancock, 2004).

Plant breeding has its origins in ancient times, starting off simply as humans discriminating among plant types to select and retain plants with the most desirable features. Remarkably, the practice of selection still remains the primary strategy for crop improvement, even though many technologically advanced techniques have been added to the collection of the modern plant breeder. Plant breeding differs from evolution in that the former is planned and purposeful (Acquaah, 2007). Plant breeding is a deliberate effort by humans to nudge nature, with respect to the heredity of plants, to take advantage of new combinations of genes. The changes resulting from these new combinations, since they are in the genes are permanent and heritable.

A recent review of the impacts of climate change on food security in Sub-Saharan Africa concludes that all of the different dimensions of food security, including food availability, access, stability and utilization, will be affected (Schimdhuber & Tubiello, 2007). Swinnen and van Herk (2011) illustrate in their report how

projections indicate that, on current trends, both demand and supply factors are likely to exert pressure on both the level of food prices and their volatility. The demand for animal protein and bio-fuel is believed to be rising, global food stocks are at alarmingly low levels, resources used for food production, particularly water, are stressed, and climate change has caused more uncertainty for food availability (Swinnen & van Herck, 2011). There is an urgent need to increase agricultural production under these pressures by improving current cultivars and looking to alternative plants with potential to be crops to complement those that exists presently. Marama bean has been identified as one of the plants that could be improved and lifted from its "orphan" status to a crop through breeding using Molecular Marker Assisted Selection (MAS) contributing to the need for agricultural solutions to the challenges of malnutrition and climate change.

## 2.2 Marama bean- plant description and potential.

Marama bean belongs to the family Fabaceae, subfamily Caesalpinioiodae and genus Tylosema. There are 5 species described by taxonomists for Tylosema in Africa to date as shown in Figure 1 below.

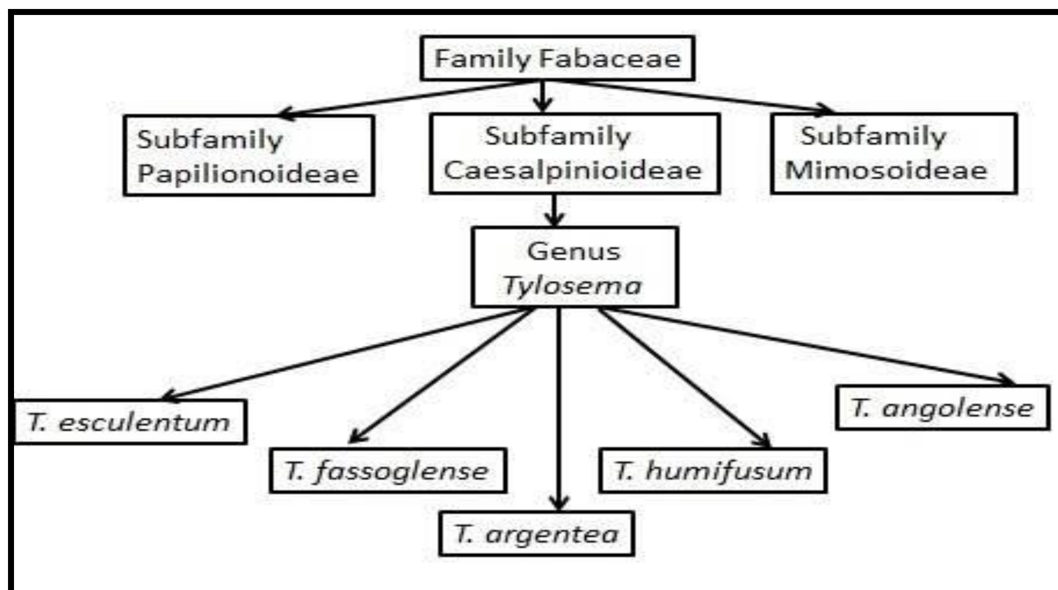


**Figure 1**: Taxonomy of *Tylosema esculentum* (based on Castro, Silveira, Pereira-Coutinho. & Figueiredo, 2005).

*T. esculentum* (marama bean) is a woody prostrate trailing plant and the only species of the genus Tylosema specific to arid regions of Southern Africa. The tuber producing and non-nodulating legume has seeds and tubers edible after roasting and cooking respectively. It is endemic to the Southern parts of Africa where it grows in the grasslands and bushveld (Coetzer & Ross, 1977), with large populations in Botswana (around the central Kgalagadi) and Namibia, smaller populations in the provinces of Limpopo, North-west and Gauteng of South Africa, and Zimbabwe (National Research Council, 2006; Castro et al., 2005). Figure 2 below shows a marama bean plant growing in the wild with a stem length of approximately 10 meters.

**Figure 2**: Marama bean growing in the veld, Omaheke region of Namibia.

The plant thrives in poor quality soil and the harsh climatic conditions of the Kalahari Desert (Hartley, 1997). It is believed that marama beans, and other edible parts of this plant, may have been in our diet as long as humans have been in contact with this plants, and even today it is an important dietary component for people living in the southern parts of Africa which include the Herero, Tswana, Khoi san and other Bantu speaking peoples (National Research Council, 2006).

The primary agronomic potential of marama bean is based upon the high nutritional value of the seeds. The protein content is comparable to that of soybeans while the oil content is twice that of soybeans (Mmonatau, 2005; Jackson et al., 2010). It has a protein content of 29.6 - 41.8% and an oil content of 32.1 - 45.3% (Halloran & Monaghan, 1996). In addition marama bean also contains minerals and vitamins, which include potassium, phosphorus, thiamin, riboflavin and nicotinic acid. A clear, golden yellow oil, which has a nutty odour with an agreeable taste similar to almond

oil, has been extracted from marama bean (Hartley, 1997) and the quality of the oil has been compared to canola oil (Francis & Campbell, 2003). The advantage of plant oils over other sources is that they contain no cholesterol (Bouic, 2003).

There is an increasing interest in the cultivation of marama bean, due to its potential as a cash crop and food source in the face of climate change. The seeds and tubers of this plant are not only used as a food source for both the natives of the areas in which it grows but they also have major health benefits. The marama bean plant has been shown recently to have anti-bacterial and anti-retroviral properties and since Retroviruses (RV's) are a major source of diarrhoea in infants, the plant is traditionally used as a treatment against diarrhoea (Chingwaru et al., 2011). Several products are under development from marama bean including marama flour, marama butter, marama cookies, marama yoghurt, marama milk and snack roasted nuts (van der Maesen, 2006; Holse, Husted, & Hansen, 2010; Jackson et al., 2010 and van Wyk, 2011).

Malnutrition is an increasing global issue. Certain plant foods are deficient in some essential nutrients to the extent that, where these foods constitute the bulk of a staple diet, diseases associated with nutritional deficiency are often common. Cereals tend to be low in lysine and threonine, while legumes tend to be low in cysteine and methionine (both sulphur-containing amino acids). Cereal and grain legume flours can be made in composite to supplement each other and provide nutritious meals (Mmonatau, 2005).

Breeding conventional and future crops is needed to augment the nutritional quality of food crops. The marama bean is widely believed to potentially play a significant role in solving the world's malnutrition problems due to its high content of unsaturated fatty acids, proteins and other nutrients (Chimwamurombe, 2011). Despite the high nutritional value of the marama bean, the only food application currently is roasting the dry beans and consuming it as a snack (Jideani, vanWyk & Cruywagen, 2009).

## 2.3 Phenotype and Molecular Marker Assisted Selection (MAS)

### 2.3.1 Phenotypic characterization

The "phenotype", i.e. the characters made manifest, must be distinguished from the "genotype" or genetic constitution, which alone can transmit changes to the offspring (Klug, Cummings, Spencer & Palladino, 2009). Developmental traits of leaf size, leaf number, stem length, number of flowers, number of pods, number of internodes and internode length were identified as the important phenotypic characters of marama bean for domestication. The work of Gregor Mendel (Mendel, 1866) and the further advances in science that followed his discoveries established that plant characteristics are generally controlled by hereditary factors or genes that consist of DNA (deoxyribonucleic acid, the hereditary material). These genes are expressed in an environment to produce a trait. It follows then that in order to change a trait or its expression, one may change the nature of its genotype, and/or modify the nurture (environment in which it is expressed) (Yu, Arbelbide & Bernado, 2005).

An important factor contributing to overall levels of phenotypic variation is the extent to which individual genotypes affect the phenotype differently depending on the environment (Klug et al., 2009). Many agriculturally important variations such as productivity and quality, tolerance to environmental stresses, and some of the forms of disease resistance are controlled by polygenes and “multifactorial” that greatly depends on genetic × environmental (G×E) interactions (Abdurakhmonov & Abdukarimov, 2008).

Changing the environment essentially entails modifying the growing or production conditions. This may be achieved through an agronomic approach, for example, the application of production inputs (e.g., fertilizers, irrigation). Whereas this approach is effective in enhancing certain traits, the fact remains that once these supplemental environmental factors are removed, the expression of the plant trait reverts to the *status quo*. On the other hand, plant breeders seek to modify plants with respect to the expression of certain attributes by modifying the genotype (in a desired way by targeting specific genes). Such an approach produces an alteration that is permanent (i.e. transferable from one generation to the next) (Acquaah, 2007).

### 2.3.2 Molecular Marker Assisted Selection

The analysis of the genetic bases of morphological variation has become a powerful tool for plant and animal improvement. Recent complete genome sequencing of model species and some orphan legumes, both monocots and dicots will greatly increase the potential of marker approaches in all higher plants, due to homology and

synteny conservation in the plant kingdom (de Vienne, 2003). The most commonly used genetic markers are morphological markers, molecular markers (at DNA level), and biochemical markers (isozymes, proteins). Molecularly, we can classify polymorphism into two types: (1) sequence polymorphism and (2) number of tandemly repeated sequences in the repeated regions.

Some complications are encountered when using visible phenotypes to select superior plants in a breeding program, because although visual inspections of phenotypes by breeders can often be useful in eliminating plainly undesirable phenotypes in variable breeding populations, the genotypes that are ultimately likely to be successful can less often be identified by visual inspection alone (Allard,1999). These challenges may be overcome by using DNA markers that generally co-segregate, that is closely linked, with the gene(s) of interest. The development of DNA (molecular) markers has irreversibly changed the disciplines of plant genetics and plant breeding (Kumar, 2008). Genetic markers, the heritable entities that are associated with economically important traits, can be used by plant breeders as indirect selection tools. By using molecular markers and confirming their association with the trait of interest, breeders can by-pass traditional phenotype-based selection methods, which involve growing plants to maturity and closely observing their physical characteristics, in order to infer the underlying genetic make-up through most of the breeding cycle (Varshney et al., 2004).

While there are several applications of DNA markers in breeding, the most promising for cultivar development is called “Molecular Marker Assisted Selection

(MAS) (Varshney et al., 2004). MAS refers to the use of DNA markers that are tightly-linked to target loci as a substitute for, or as an aid to, phenotypic screening (Collard & Mackill, 2008). According to Varshney, Nayak, May and Johnson (2009a), it is a method that uses molecular markers associated with the traits of interest to select plants at the seedling stage, thus speeding up the process of conventional plant breeding and reducing the cost involved in maintaining fields. By determining the allele of a DNA marker present, plants that possess particular genes or quantitative trait loci (QTLs) may be identified based on their genotype rather than their phenotype (Collard & Mackill, 2008). Allele mining entails identification of allelic variation of relevant traits within genetic resource collections, such as germplasm collections, ecotypes and pathotypes. Alleles are defined as alternative forms of a gene. Different alleles may produce differences in the phenotype of an organism (Klug et al., 2009). Plant breeding is thus becoming more precise and faster through the use of molecular markers. MAS is facilitating the improvement of traits that cannot easily be selected using conventional breeding methods (Varshney et al., 2009a).

The advent of molecular markers has facilitated the detection of genomic regions controlling quantitative characters (Avila et al., 2005). Genetic variation in nature often takes the form of a quantitative phenotypic range, with an approximately normal distribution, compared to qualitative phenotypes, which fall into discrete categories (Paran & Zamir, 2003). A QTL is a genome region that appears to contribute to a quantitative trait, which can be localized by marker linkage mapping as first demonstrated by Paterson et al. (1988). It can be a single gene or it might be a

cluster of several genes linked to one another that affect the trait. Mapping of QTL allows a statistical description of the effects of each genome region on quantitative traits (Vinod, 2009). QTL analysis usually studies the genetic variation in a controlled cross to locate the genes responsible and to explore their effects and interactions (Kearsey, 1998).

Complex traits such as yield can be dissected as Mendelian factors with molecular markers through molecular mapping. The identification of quantitative trait loci is the basis of MAS (Yu et al., 2005). Given the context of current yield trends, predicted population growth and pressure on the environment, traits relating to yield stability and sustainability should be a major focus of plant breeding efforts. These traits include durable disease resistance, abiotic stress tolerance and nutrient and water-use efficiency (Mackill, Nguyen & Zhang, 1999; Slafer, Araus, Royo & Del Moral, 2005). Furthermore, there is a need to develop varieties for cultivation in marginal land areas, especially in developing countries, and give greater emphasis to improving minor or ‘orphan’ crops (Naylor et al., 2004).

As stated earlier, molecular markers rely on a DNA assay, in contrast to morphological markers that are based on visible traits, and biochemical markers based on proteins produced by genes (Ruane & Sonnino, 2006). Microsatellites were the maker of choice since these simple sequence repeat (SSR) markers are widely used in plants because of their abundance, hyper-variability, high information content and suitability for high-throughput analysis (Kumar, 2008). Microsatellites

are derived from short (usually <6 bp) tandemly repeated sequences (Zhao & Kochert, 1993; Cullis, 2004). The most frequent are $(A)_n$, $(TC)_n$, $(TAT)_n$, $(GATA)_n$ and so on with *n* ranging from a few to several units. In higher plants it can be estimated that on average there is one dinucleotide SSR for every 30 to 100kb, with tri- and tetra-nucleotides occurring with similar density (Morgante & Olivieri, 1993).

The Polymerase Chain Reaction (PCR) is useful in revealing SSRs individually, providing co-dominant and highly polymorphic locus-specific markers. The technology requires only small amounts of DNA and the reproducibility of SSRs is such that they can be used in different laboratories to produce consistent data (Rosseto et al., 1999) Even if the SSR itself is not specific to a locus, the adjacent regions in the genome are. A pair of primers adjacent to the region of interest can therefore be used to amplify the SSR (Maguire, Edwards, Saenger & Henry, 2000). Another major advantage of SSRs is that the sequences flanking the repeat regions can be highly conserved. Polymorphisms in the length of the repeat regions are revealed by agarose gel electrophoresis, if the differences in length between alleles are high enough, or acrylamide gels for lower differences in length, also more and more frequently, automatic sequencers are coming into use. Microsatellites are excellent genetic markers with the advantage of routine PCR detection (de Vienne, 2003).

```
1   AGACGCACTCAGCACTCTCCAGCCTCTCACCGATTACAAGCTTTATTGAATGATCAAGTG
           >>>>>>>>>>>>>>>>>>>>

61  GCCACGCCCAATAGAGAGAGCTTCTCTAATGGCTTTTCAGAATACAAGAAAGAAGAAAGA
                                                            ****

121 AGAAGAAGAAGAGAGAACAAGTCCCAAGGAAGAAGTTGGACTTAACAACTCATAACTATG
    ***********

181 CCTAACTCCCTCTTCAACCAACCCCTACTTATATTTACAATAAAGCCTCAACCATCAACT
        <<<<<<<<<<<<<<<<<<<<

241 ACCCTCCTTTGCCTAATTACAATTGA
```

**Figure 3**: Microsatellite $(AAG)_5$ is an example of a SSR that is amplified by primer pair MARA 077 in marama bean.

### 2.3.3 Mutation breeding and detection

Several constraints that limit crop production or quality of grain legumes such as biotic stress in the form of diseases have been addressed by conventional breeding and enhanced management, but there are situations where the existing germplasm lacks the required traits. In Northern India, drastic reductions in areas of chickpea cultivation occurred due to ascochyta blight and pod borer (Pande, Sharma & Rao, 2008; Sarker et al., 2008). The full genetic potential of crops is seldom realized due to the cultivation of susceptible cultivars paired with improper crop management practices employed in coping with biotic or abiotic stress factors. Genetic transformation could help provide solutions to constraints as the aforementioned, thus improving food security in developing countries (Popelka, Terryn & Higgins,

2004). With population growth, the demand for food and feed is growing, while natural resources are becoming more limited. Erratic rain falls, and severe drought conditions as well as excessive floods, often related to climate change contribute to the deterioration of crop production conditions. The actualization of yield potential of crop plants has to be significantly increased through tapping into genetic transformation to combat the worsening food security situation (Reynolds et al., 2009).

Increasing crop yields to ensure food security is a huge and very important challenge world-wide (Azam-Ali, 1996). Plant breeding requires genetic variation of useful traits for crop improvement but the desired variation is frequently lacking in available germplasm (Ruane & Sonnino, 2006). Mutagenic agents, such as radiation and certain chemicals, can be used to induce mutations and generate genetic variations from which desired mutants may be selected (Datta, Datta & Chatterjee, 2009). Mutation induction has become a proven way of creating variation within crop varieties and mutagenesis is indeed an important tool in crop improvement and is free of the regulatory restrictions commonly imposed on genetically modified (GM) organisms (Seetohul, Puchooa & Ranghoo-Sanmukhiya, 2009). This forward genetic approach enables the identification of improved or novel phenotypes that can be exploited in conventional breeding programmes (Parry et al., 2009).

Exploiting natural or induced genetic diversity is a proven strategy in the improvement of all major food crops, and the use of mutagenesis to create novel variation is particularly valuable in those crops with restricted genetic variability

(Seetohul et al., 2009). Although high intra-population diversity has been shown in marama bean, its natural populations are unfortunately still under immense pressure from both grazing and the human exploitation of its seed (Naomab, 2004; Chimwamurombe, 2010). Thus under this very delicate circumstance, it is therefore also important to generate strategies for conserving and further developing the remaining wild germplasm of this very important and highly promising leguminous resource.

Whilst mutations occasionally occur spontaneously in nature, the frequency of such mutations is usually too low to be relied on and even more so for the accelerated plant development and breeding systems. However, advantage can be taken of mutations induced by physical and chemical mutagens applicable to all plant and animal species (van Harten, 2007). Mutations may be gross, resulting in large-scale deletions of DNA, or only involve point mutations. Mutation can be induced by irradiation with non-ionizing (e.g. UV) or ionizing radiation (e.g. X and gamma rays, alpha and beta rays, fast and slow neutrons). These physical mutagens often result in the larger scale deletion of DNA and changes in chromosome structure (Girija & Dhanavel, 2009). By contrast, chemical mutagens most often only affect single nucleotide pairs (Parry et al., 2009). For plants, some of the most widely and commonly used mutagens include ethyl methane sulphonate (EMS), methyl methane sulphonate (MMS), hydrogen fluoride (HF), sodium azide, N-methyl-N-nitrosourea (MNU), and hydroxylamine (Netto et al., 2011). The degree of mutation is dependent on the type of tissue and the level of mutagen exposure. Mutations at single nucleotide pairs are generally of the most interest to breeders because large-scale

changes to chromosomal structures usually have severely negative results (Parry et al., 2009). Critically, mutations in important traits or genes involved in nutritional quality, resource use efficiency, architecture or phenology can be readily exploited by plant breeders without the legislative restrictions, licensing costs, and societal opposition applied to GM approaches. This is despite the fact that transcriptomic analyses have since shown that large-scale plant mutagenesis may induce greater changes in gene expressional patterns than transgene insertion (Batista, Saibo, Lourenco & Oliveira, 2008).

A molecular exploration on the potential use of gamma radiation in the induction of useful and most preferable germline mutational changes of marama bean, for the possible adoption and its subsequent future participation in mutational and marker-assisted breeding programmes, was explored with the use of existing SSR markers for detecting these mutations.

## 2.4 Karyotyping

The Fabaceae are the third largest family of flowering plants and they contribute substantially to the sustenance of humans. The Fabaceae include major legumes and oilseeds such as soybean (*Glycine max*, $2n=4x=40$), peanut (*Arachis hypogaea*, $2n=4x=40$), mung bean (*Vigna radiata*, $2n=2x=22$), chickpea (*Cicer arietinum*, $2n=2x=16$), and lentil (*Lens culinaris*, $2n=2x=14$), as well as vegetable crops such as common bean (*Phaseolus vulgaris*, $2n=2x=22$) and pea (*Pisum sativum*, $2n=2x=14$) and forages such as alfalfa (*Medicago sativa*, $2n=4x=32$) (Paterson et al., 2000).

Detailed genetic maps have been assembled in at least eight genera of the Fabaceae (Arachis, Glycine, Lens, Medicago, Phaseolus, Pisum, Vicia, and Vigna) (Paterson et al., 2000; Varshney et al., 2009b). Grain legumes are important in human nutrition in several parts of the world and they contribute substantially to the total protein intake, mainly in vegetarian diets (Doyle, Chappill, Bailey & Kajita, 2000).

Every species of eukaryote has a characteristic number of chromosomes in each cell nucleus. Each chromosome is one very long, linear DNA molecule containing thousands of genes. The secret of differences between individual organisms lies in their genetic material, DNA, packaged into chromosomes in the nuclear material of cells (King & Stansfield, 1990). A genetic marker can be defined in one of the following ways: (a) a chromosomal landmark or allele that allows for the tracing of a specific region of DNA, (b) a specific piece of DNA with a known position on the genome, or (c) a gene whose phenotypic expression is usually easily discerned, used to identify an individual or a cell that carries it, or as a probe to mark a nucleus, chromosome or locus. Genetic markers may not have a biological function, and they are inherited from one generation to next (King & Stansfield, 1990; Premkrishnan & Arunachalam, 2012).

The term chromosome comes from the Greek words for colour (*chroma*) and body (*soma*). Scientists gave this name to chromosomes because they are cell structures, or bodies that are strongly stained by colourful dyes used in research. The structure of chromosomes keeps DNA tightly coiled around histone proteins, since, without this packaging, DNA molecules would be too long to fit inside cells. Since interphase

chromosomes are difficult to study, most research describing their structure has been done on mitotic cells. The arrangement of chromosomes in a metaphase plate is then assumed to reflect the interphase ordering. To analyse the relative positions of chromosomes, squash or air dried preparations have been commonly used to determine chromosome localization (Oud et al., 1989). In previous studies, chromosome counts made from the root tips of marama bean seedlings ranged from 42 to 50 showing a relatively high number of chromosomes, which is consistent with chromosomes count of 52 from its closely related species, *Tylosema fassoglense* (Goldblatt & Davidse, 1977; Monaghan, 1995). The present study sought to identify the chromosome number in *Tylosema esculentum* as part of an effort to initiate a molecular map for the marama bean. In this thesis, the effective use of Feulgen staining to identify chromosomes in *Tylosema esculentum* will be described.

## 2.5 Plant growth environment, growth promoting factors and regeneration

### 2.5.1 Growth environment and growth factors

*T. esculentum* grows in sandy soils, which typically have limited water-holding capacity, and is frequently exposed to very high light intensity, extreme temperature and prolonged drought (Mitchell, Keys, Madjwick, Parry & Lawlor, 2005). Soils, which support growth of marama bean, are generally low in organic matter and nutrients, including nitrogen. Marama bean roots do not nodulate and it does not obtain its nitrogen from symbiotic fixation with soil *Rhizobia*, nor from a source different from that utilized by associated plant species in the same site. It probably scavenges nitrogen efficiently from low concentrations in the soil and rapidly builds

reserves in the large tubers to serve as a buffer from formation of protein-rich organs (Dakora, Lawlor & Sibiuga, 1999).

It grows at altitudes of between 1000 and 1500 m with 300 to 700 mm rainfall, and at a minimum temperature above 15ºC and a maximum of approximately 33ºC (Müseler & Schönfeldt, 2006). Marama bean is dormant in winter and re-grows from the tuber in spring. The plant grows in well drained, fine, generally calcareous sands but also in regions of harder calcareous conglomerates, at pH 6-8 with very little organic matter, nitrate or phosphate (Lawlor, 2004).

Although tropical grain legumes generally have the ability to grow in a wide range of environmental conditions, water stress is considered as a major environmental constraint limiting their vegetative and reproductive growth and yield (Acosta-Gallegos & Adams, 1991; Ramirez-Vallejo & Kelly, 1998). Travlos and Karamanos (2008) found that water shortage significantly reduced leaf and stem growth as well as dry matter production of marama in comparison with well-watered controls and shifted dry matter allocation from shoots to roots. Furthermore, Mitchell et al. (2005) found that marama plants grown in the greenhouse exhibited early stomatal closure with minor leaf water potential fluctuations under drought.

The manipulation of growth and increasing productivity and quality of plants is the basis for most plant related research. As a result many compounds are used to accelerate flowering and fruiting in young plants. The most widely available plant growth regulator is $GA_3$ or gibberellic acid, which induces stem and internode

elongation, seed germination, enzyme production during germination and fruit setting and growth (Davies, 1995). Marama bean seeds grown under greenhouse conditions in this study were tested for response to high water treatment, fertiliser and $GA_3$ to determine if any of these treatments would enhance vegetative growth and accelerate the time to flowering.

### 2.5.2 Regeneration

Travlos, Economou & Karamano (2007) found that marama seeds have no physiological dormancy and that speed and percentage of *Tylosema esculentum* seed germination was greatly increased by mechanical scarification of the seeds with sand paper. Their results showed that the highly positive responses of marama seeds to the mechanical treatment clearly indicate that there is moderate coat imposed dormancy in this species. Immersion in water for 20 hours and in concentrated sulphuric acid for 20 minutes were also some of the most effective treatments. Germination rate was determined in three settings: pre-treatment by soaking in water, scarification of seeds with sand paper before planting in soil and inducing germination in an incubator before transferring to soil.

With the objective of improvement of plants, humans have devised various methods of propagating plants by vegetative reproduction in addition to regeneration from seeds. A twig or bud from one plant can be grafted onto a plant of a closely related species or a different variety of the same species in a modification of vegetative reproduction from cuttings known as grafting. Grafting is a technique that joins

material from 2 compatible plants, which will grow together as one making it possible to combine the best qualities of different species or varieties into a single plant (Reece et al., 2011).

The part that will grow to become the roots of the grafted plant is called the “root stock”. The part that will grow to become the stem and branches is called the “scion”. The seedling providing the root stock should be well developed and not soft even though a graft is usually done when the plant is young. The scion is the part where the leaves fell off and where the fruits will grow again. The properties of the fruits of the grafted plant should be the same as the fruits from the plant from where the scion originates. This is why it is important to cut the scion from the plant with the type of fruit or properties that you want to have. Grafting can be very useful because it can help you to grow a plant with qualities that you want (Hartmann et al., 2011). Grafted plants can also bear fruit much quicker than other young plants (NFRC, 2011).

Gulati, Schryer and McHughen (2001) reported that cultivated lentil micrografts established 4 weeks earlier than rooted cuttings. For faba bean several scion x rootstock combinations showed scion-rootstock interaction affected percent survival and flowering time of grafted scions. Significant changes in days to flowering for grafted scions in comparison with selfgrafts occurred for some scion-rootstock combinations. For example, scion FB 20-5 flowered significantly earlier on rootstocks FB 25-26 ZT and FB 18-20 compared with self-grafts. Scions of cultivar Gloria also flowered significantly earlier when grafted to FB 18-20 rootstocks

(Gurusamy, Warkentin & Vandenburg, 2012). The possibility of using grafting to side step juvenility thought to be 18 months in marama bean in trial plots and greenhouse experiments was explored.

## 2.6 Potential pathogens of marama bean and their molecular identification

Marama bean has been demonstrated to have vast agronomic potential and surveys of plant diseases caused by microbes such as fungi become very crucial in understanding the biology of this plant and potential threats to domestication efforts. Fungi grow in nearly every ecological niche commonly as free living saprophytes, however, a few are on occasion found as accidental, often opportunistic, pathogens (Madigan & Martinko, 2006). The absence of photosynthetic pigments in fungi leads them to have a saprophytic or a parasitic existence among other organisms. Harmful effects of saprophytic fungi on human economy are often seen when food, timber and textiles are rotted. As parasites, fungi cause diseases in plants and animals (Webster & Weber, 2007). Great plant losses caused by natural factors can be suffered in natural ecosystems but by bringing crops together into fields, agriculture creates the ideal conditions necessary for the spread and perpetuation of plant diseases. Furthermore, the more selective farming is and the closer crops come to being true monocultures, the greater is the extent of agricultural losses due to any single agent like a specific plant disease (Moore, Robson & Trinci, 2011).

Microbial diseases of plants of which 70-80% are fungal, cause 16% of annual crop losses worldwide (Oerke, 2006). In 2011, one in every eight crop plants on average,

failed to yield because of fungal diseases (Moore et al., 2011). Many species of the genus *Cercospora* (Ascomycota) cause plant diseases, mostly of the leaf spot variety. Leaf spots are rounded blemishes occurring on the leaves of infected plants; a typical spot has a defined edge with a darker boarder central zone varying from yellow to brown (Khan et al., 2008). Leaf tissue in wild populations of marama bean has been observed to display leaf spots.

A disease in plants results only if the following three factors occur simultaneously: a susceptible host, a disease-causing organism (the pathogen) and a favourable environment for the disease; otherwise if one or more of these factors is not present, then the disease does not occur. The symptoms observed in a diseased plant depend on the effect of the pathogen on the physiological and biochemical processes of the plant. Photosynthesis is an essential function of plants and any pathogen that interferes or tampers with it will cause a disease that may appear as chlorosis and/or necrosis of the leaves and stems (Moore et al., 2011).

DNA sequencing of the internal transcribed spacer (ITS) and the large subunit (LSU) regions of rRNA, followed by comparative sequence analysis, has been the standard for molecular identification of most fungi, particularly of culturable fungi. This strategy is fast and accurate, but is dependent on sequence quality in existing reference databases (Tsui et al., 2011). In this study and during a greenhouse experiment at the University of Namibia (later referred to as Group 2), some signs of necrosis were observed on leaf tissue of marama seedlings and the leaves were immediately removed from the plants in order to investigate and determine the exact

causative agent(s) of such a leaf spotting and necrosis effect. Herein, we outline and present the findings of that study.

## 2.7 Statement of the problem

The main problem identified when this study was initiated was a gap in knowledge of the usefulness of SSR markers for genotyping and a lack of QTL identification as a foundation for mapping the marama bean genome. The chromosome number and form needed to be verified, as the literature did not provide conclusive data for the karyotype of marama bean. Further to this, the factors affecting the reproductive maturity and regeneration of marama bean were not well known. The time it takes to reach reproductive maturity, thought to be about 18 months was considered to be long for legumes by many farmers and researchers. Any early maturing, higher yielding genotypes needed to be identified. The effect of growth promoting factors needed to be investigated for their effects on the growth of marama bean as well as possible methods that could help side step juvenility. The potential pathogens to marama were not known and this was deemed crucial to cropping efforts. Without this knowledge, domestication of marama bean cannot be achieved with precision and in a shorter time than traditional breeding methods would require.

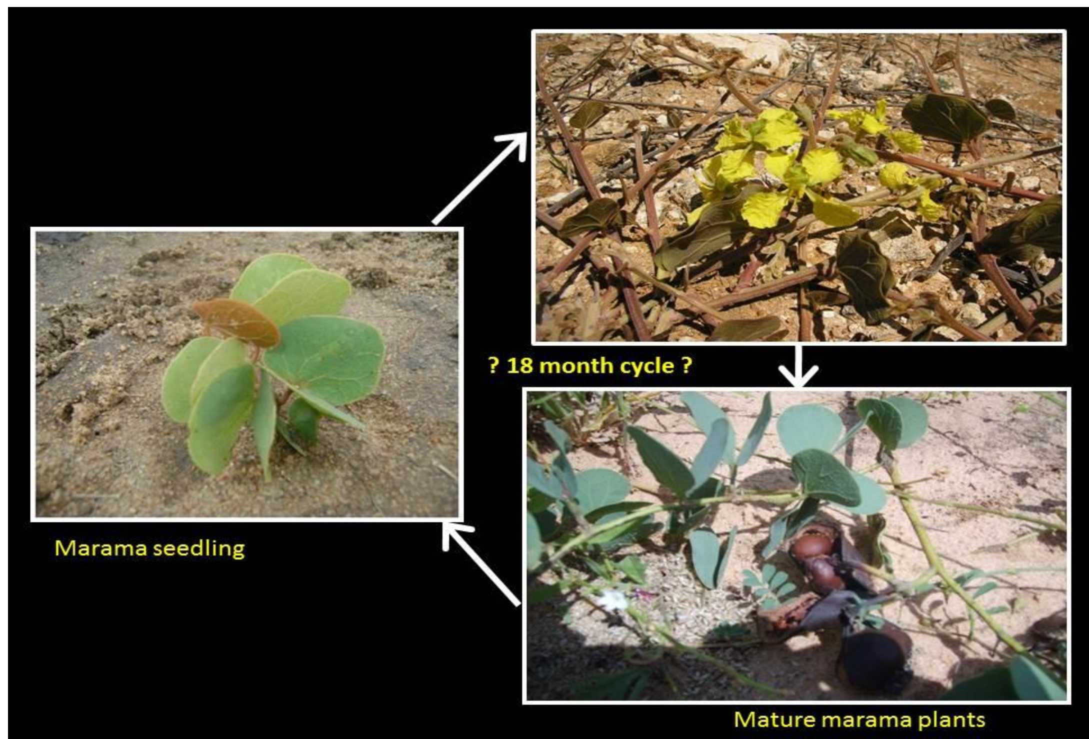


**Figure 4**: Marama bean life cycle thought to be about 18 months.

### 2.8 Research questions and objectives of the study

The research questions guiding this study were:

1. Are any of the previously developed polymorphic SSRs linked to phenotypic traits in marama bean?
2. What is the number and structure of chromosomes in *Tylosema esculentum*?
3. Is grafting a possible method to side step juvenility?
4. What is a reliable and robust method for growing plants in a glasshouse.
5. What is the effect of nutrient (Nitrogen, Phosphorus and Potassium), moisture and hormone ($GA_3$) treatments on vegetative growth in marama bean?

6. What is the causative agent(s) of leaf spotting and necrosis effects observed on leaf tissue of marama seedlings and leaves grown in the greenhouse?

The objectives thereof were:

1. To determine genetic diversity present in characterized marama plants using SSRs linking molecular markers of marama bean to phenotypic traits.
2. To determine the number and structure of chromosomes in *Tylosema esculentum*.
3. To explore the possibility of grafting to side step juvenility.
4. To establish a reliable and robust method for growing plants in a glasshouse.
5. To determine the effect of nutrient (Nitrogen, Phosphorus and Potassium), moisture and hormone ($GA_3$) treatments on vegetative growth to shorten juvenility.
6. To determine the exact causative agent(s) of leaf spotting and necrosis effects observed on leaf tissue of marama seedlings and leaves grown in the greenhouse.

## 2.9 Significance of the study

The study explored the potential of modern DNA based technologies as an aid in marama breeding programs. Prior to this study, microsatellites for marama bean had been developed. However, there was no link between traits of interest and molecular markers that could reveal variations in the traits. This study was able to characterize the different genotypes of marama bean based on phenotypic traits at the vegetative stage of the plant or seedling stage which is the stage at which breeders would typically wish to screen plants for further work in order to determine if they carry the desired traits they seek. Furthermore, the trait of internode length was linked to a microsatellite locus and a second locus was linked to early germination through experiments with irradiated seeds. The characterization of the chromosomes in marama achieved through this study will provide useful information for mapping efforts as Next Generation Sequence data is becoming available. A robust method for the germination of marama bean under greenhouse setting was established and it was also determined if grafting and growth-promoting factors would be useful. This study was able to determine that grafting may not be possible in marama bean due to an inbuilt mechanism which preferentially causes resprouting from the tuber rather than having the graft grow when the scion was added to a root stock. The effect of nutrient or hormone treatments had not been investigated in marama bean before this study and the information gathered here will be useful in future breeding and domestication efforts.

# CHAPTER 3: METHODOLOGY

## 3.1 SSR screening for Molecular Marker Assisted Selection

### *3.1.1 Germplasm collections, phenotypic characterization and SSR screening*

Marama bean seeds from eight different localities were collected and raised in the greenhouse at the Department of Biological Sciences at the University of Namibia in Windhoek or at the National Forestry Research Centre (NFRC) in Okahandja. These plants will be referred to as Group 1, Group 2, Group 4A as well as Group 4B and the origin of seed in each case will be specified. Group 3 were field plants in trial cultivation plots in Omaheke region while Group 5 were leaves and seeds collected in wild populations. The seedlings generated from seeds in Group 1, 2 and 4A were soaked to induce germination before planting while Group 4B seeds were sown directly into the soil without pre-treatment.

**Table 1:** Geographical positions of the sites of *Tylosema esculentum* from which seeds used in Group 1 – 4B were collected.

| Location | GPS coordinates | Code | Group |
|---|---|---|---|
| Aminius | S23 38.00<br>E019 22.00 | A | Group 1, Group 2 |
| Uitschot | S23 43.000<br>E19 10.600 | U/UIT | Group1, Group 2, Group 4B |
| Tsumkwe | S19 21.000<br>E20 16.000 | T | Group 1 |
| Okomombonde | S20 57.000<br>E018 55.000 | OK/OKO | Group1, Group 2, Group 4A, Group 4B |
| Omipanda | S21 19.355<br>E020 04.553 | OMP | Group 3 |
| Otjovanatje | S21 19.080<br>E020 07.321 | OTJ | Group 3 |
| Osire | S21 02.031<br>E017 21.244 | OSI | Group 4A |
| Ghanzi | S21 34.002<br>E021 46.9998 | B | Group 1 |

The following map (Figure 5 below) shows where these localities are found in Namibia and Botswana.

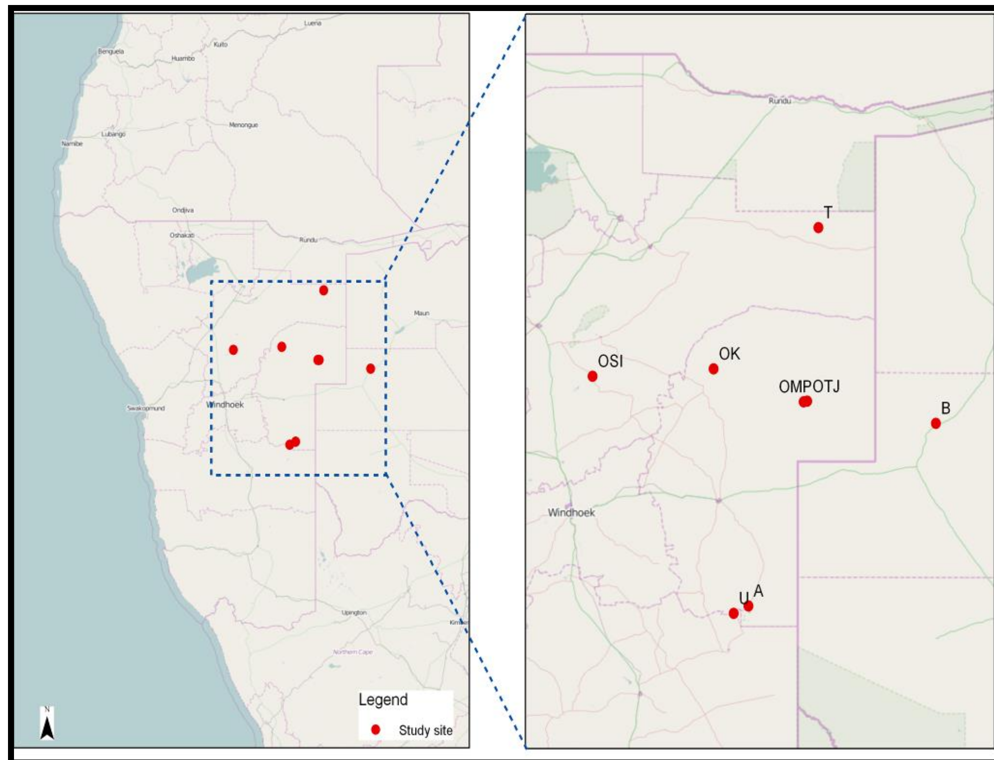


**Figure 5:** Map showing the origin of seeds in the collection used in Group 1-4B.

<u>Group 1</u>

Seeds from Aminius (**A**), Tsumkwe (**T**), Uitschot (**U**) and Okomombonde (**OK**) were used in this first experiment (Group 1). A total of 240 seeds with 60 each from the 4 subpopulations were planted on the 28$^{th}$ November 2010. The seeds were soaked in jars of water overnight to induce germination and then planted in seedling trays with potting soil.

**Figure 6:** Marama seed soaking before planting

The seeds were watered every 2nd day and temperatures recorded daily over a 7 week period (28th November 2010-16th January 2011) and monitored for emergence. The trays were moved to the Molecular biology laboratory at the University of Namibia after 3 weeks when it was noted that the mid-day temperatures there were rather high (ranging 45°C-61°C). Seedlings emerged from 31st December, 2010 in the laboratory, where temperatures ranged 24°C-28°C. The plants showed signs of phototropism as well as shown in figure 7 below. Phenotypic characters were recorded for stem length (primary vine length), leaf number, internode number and internode length on the 15th of February, 2011 at 12 week seedling stage and the characters were analysed in SPSS 16.0 to determine variation. Leaves were harvested when the plants were 14 weeks old for DNA extraction and molecular analysis to determine if some of the microsatellite markers were useful for genotyping.

**Figure 7**: Marama bean seedlings from Group 1 growing in the laboratory.

The plants were sown in trays to determine how long it generally takes for marama bean to germinate. In this group, days to seedling emergence ranged from 33 days to 62 days in the 79 out of 240 plants that germinated (33%). Days to emergence, stem length, number of leaves, number of internodes and length of the first internode were monitored from week 8 to week 12 for each of the plants in this group. Seeds from the four locations A, T, U and OK germinated at 35%, 3%, 58% and 35% respectively. The germination data from this experiment led to the exclusion of seeds from Tsumkwe in the second experiment where 150 seeds were planted from Aminius, Uitschott and Okomombonde (Group 2) in 18cm diameter pots. The following phenotypic characters were followed for each of 48 plants that had enough leaf material for DNA extraction: stem length, number of leaves on the plant, number of internodes, length of internode. The data were analysed for phenotypic variation with SPSS 16.O and correlation coefficients were assessed among traits using Microsoft Office Excel 2010.

Group 2

Group 2 plants were sown on 26th January 2011. Seeds from three locations: Aminius (AMI), Uitschot (UI) and Okomombonde (OKO) were collected from the seed collection kept at the University of Namibia. Tsumkwe (T) seeds were not included in this group as the germination rates from Group 1 were too low to be useful. Fifty seeds with size variation from each of the 3 locations were picked at random. Seeds were then placed in petri dishes, 1 seed in each petri dish and the petri dish was flooded with water for 24 hours before sowing the seeds to induced germination.

**Figure 8**: Marama plants (Group 2) pre-planting treatment by soaking in water for 24 hours in petri dishes.

Daily temperatures were recorded and dates when each seedling emerged were noted. Daily temperatures ranged from 24°C-34°C. Out of the 150 seeds sown 67

germinated including 9 plants that germinated and wilted off (47% germination). Seeds from the three locations AMI, UI and OKO germinated at 34%, 44% and 56% respectively. Treatment of the seeds with fertilizer, hormone and normal and high water regimes was carried out with 32 of the healthy plants that had developed a shoot by 24$^{th}$ May 2011 as disease symptoms appeared visible in some of the plants (see *section 3.6*). Monitoring of all plants was stopped on the 8$^{th}$ of June as some had died off at this stage. These plants could only be used for phenotypic diversity assessment and leaf material could not be used for DNA extractions or to determine the effect of the treatments.

**Figure 9**: Marama bean plants (Group 2) at 13 weeks old.

The tubers of plants from Group 2 that were suspected to be root bound or infected by fungi in 2011 were planted in a garden in July 2011 and re-sprouted in the rainy season in January 2012, with healthy stems and leaves. Tuber regeneration was

established, however, these plants did not flower until October 2013. The plants are being observed if any pod set will take place.

Group 3

Group 3 phenotypic data was from a field trip that took place between December 16th, 2011 and January 4th, 2012 to make crosses to generate an F1 population from phenotyped plants at Omipanda and also collecting phenotypic data of plants at Omipanda (OMP) and Otjovanatje (OTJ). At the 2 cultivation plots in Omaheke, 30 plants at Omipanda and 14 at Otjovanatje were characterized for stem length, internode length, number of leaves, number of internode, stem colour, number of inflorescences and number of pods as these were the characteristics measurable. Taking these measurements took up to 45 minutes per plant as the number of leaves for example ranged from 17 to as many as 3113. Stem length, leaf number, internode number, internode length, number of pods and number of flowers were analysed for variation with SPSS 16.O and correlation coefficients were evaluated among traits using Microsoft Office Excel 2010.

**Figure 10**: Marama bean plant in cultivation plot at Otjovanatje, Omaheke.

Group 4A

Seeds from Okomombonde (OKO) and Osire (OSI) were germinated in petri dishes. The seedlings had germination induced by growing on filter paper and water in a petri dish between 9th December, 2011and 15th December, 2011 in an incubator set at 30°C. The seeds were transferred to soil in trays on the 17th December, 2011. The seedlings were watered every second day and were transferred to black bags and grown at the Okahandja nursery. A total of 120 seeds, 60 each from the 2 ecotypes were germinated to obtain a total of at least 50 seedlings. As the seedlings emerged in the petri dishes they were transferred to seeding trays with potting soil. After 8 weeks the seedlings that were established in the trays were transplanted into black bags and kept at the nursery with the Department of Forestry at Okahandja, Figure 11

below. At 15 weeks the transplanted seedlings were measured for basic phenotypic characters: widest leaf width, leaf number, stem length, internode length and internode number. The phenotypic traits were analysed for variation with SPSS 16.0 and simple correlation coefficients were assessed among traits using Microsoft Office Excel 2010.

**Figure 11**: Transplanting marama bean plants from seedling trays to black bags.

Group 4B

A separate group of seedlings was raised straight from seed into soil in black bags. Seeds from Okomombonde (OKO)-30 and Uitschot (UIT)-10 were sown on 7$^{th}$ February, 2012 at the Okahandja nursery. The plants were 8 weeks old when phenotypic characters were measured. Stem length, internode length, internode number, number of leaves and the width of the widest leaf were the characteristics measured. The phenotypic traits were analysed for variation with SPSS 16.O and

simple correlation coefficients were assessed among traits using Microsoft Office Excel 2010.

<u>Group 5</u>

The screening of SSRs was expanded to a larger data set using natural wild populations. Leaf material and seed embryonic tissue was collected from 521 individuals from 13 ecotypes: Eiseb (EI), Omitara (OMT), Okamatapati (OKM), Okaramapunga (OKR), Okondjatu (ONT), Harnas (HNS), Otjovanatje (OTJ), Okatumba (OKT), Steinhaussen (STN), Buitepos (BTP), Otjiwarongo (OTW), Otjozombanui (OZM), Ehungiro (EHU). The location of the 13 areas where seeds and leaves were collected in Namibia is shown in Figure 12 below.

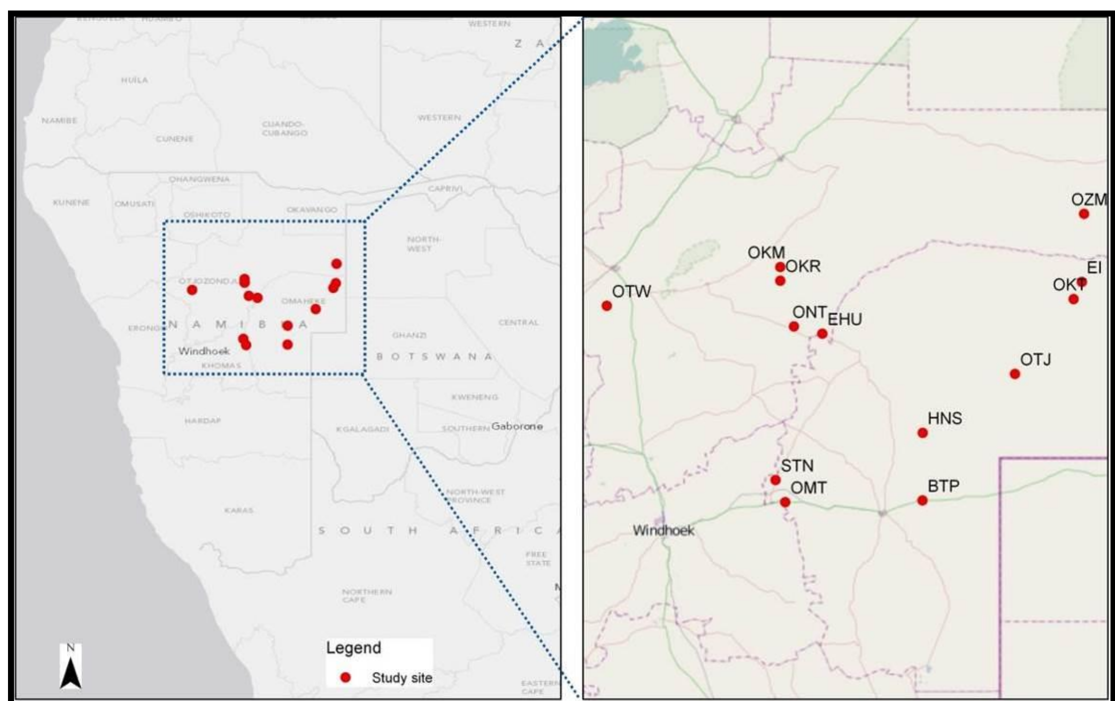


**Figure 12:** Geographic location of the 13 ecotypes in which leaf and seed material were collected in February 2013.

Nuclear SSRs were amplified with DNA extracted as described in the Qiagen DNA Plant Mini Kit according to the manufactures instructions. The extracted DNA was then resolved onto a 1% agarose gel stained with 1µg/mL ethidium bromide in Tris-borate-EDTA (TBE) buffer and viewed under UV illumination to check the quality and concentration of the extracted product for subsequent PCR application. SSR amplification was performed as described by Takundwa et al., 2010 for Group 1 and Group 5 leaf tissue. PCR amplifications were performed in 25 µl reaction volumes using 2X PCR master mix from Fermentas. Each PCR reaction contained 1 µl template genomic DNA, 1 µl of 10µM SSR forward primer, 1 µl of 10µM SSR reverse primer, 12.5 µl of the 2X PCR master mix and 9.5 µl nuclease free water. The PCR reaction profile used involved an initial denaturation step of 95 $^{o}$C for 4 minutes, followed by 35 cycles of denaturation at 95 $^{o}$C for 30 sec, an annealing at between 55 $^{o}$C and 65 $^{o}$C (primer sequence dependent) for 60 sec and an extension at 72 $^{o}$C for 2 minutes, a final extension at 72 $^{o}$C for 5 minutes and then held at 4 $^{o}$C. Agarose gel (2.5%) visualization of PCR products was then used to determine if a primer pair was polymorphic or monomorphic based on its separation of amplification products and banding patterns generated on the agarose gels in the different DNA templates. A total of 80 microsatellite primers were screened and described as polymorphic or monomorphic with regards to Group 1 traits. The SSR markers: MARA 001, MARA 020, MARA 037, MARA 039, MARA 065, MARA 068, MARA 072, MARA 074, and MARA 077 were used to screen further different genotypes in Group 5 plants for the trait of internode length based on the data from the Group 1 screens to see if any of the SSR's were linked to the trait. Allele scoring of the gels was performed in Excel with 1 (presence) of an allele and 0 (absence) of

an allele. The sample data was transferred into Primer 5. Dendograms were plotted from Bray-Curtis Similarity matrices using a group averages in Primer 5. The proportion of different classes for each character was calculated. The Shannon-Weaver diversity index H', as described by Hutcheson (1970) was used to determine phenotypic diversity in the group 1 collection for internode length, internode number, leaf number and stem length. For days to seedling emergence and leaf width the diversity index was obtained from group 2 plants and for reproductive characters of pod number and flower number group 3 was used. The index is defined as:

$$H' = -^{n}\Sigma\ p_i * \ln p_i$$

Where $n$ is the number of phenotypic classes for a character and $p_i$ is the proportion of the total number of entries in the $i^{th}$ class. H' was estimated for each character.

*3.1.2 SSR screening for mutation detection*

*(Manuscript submitted to Molecular Biology International)*

*3.1.2.1 Seed material and germination regimes*

Seeds for this work were collected from a wild population (OMP) of Namibian marama bean plant and the irradiation process was then kindly done for us by the International Atomic Energy Agency (IAEA) Laboratories in Vienna (Austria). Experimental groups for this study were three: 50 seeds irradiated with 50 grays gamma rays (50 Gy), 50 seeds irradiated with 100 grays gamma rays (100 Gy), and 50 non-irradiated seeds (controls). Irradiation levels beyond 100 Gy were found to stop seeds germinating. For each experimental group, five batches of seeds in

collections of 10 seeds per batch were surface-sterilized by transferring the 10 seeds into a sterile 50 mL Falcon tube followed by their flood-soaking with 10 mL 70% ethanol for 30 seconds. The seeds were then vortexed for 90 seconds and allowed to stand for another one and half minute. Ethanol was discarded and the seeds repeatedly washed (5 times) with sterile distilled water. The seeds were then submerged into 10 mL of sterilization buffer (50% bleach and 0.1% Triton X-100) and immediately vortexed for 3-5 minutes. The sterilization buffer was removed and seeds then thoroughly washed (5 times) with sterile distilled water. About 10 mL of 0.1% Type 'M' agar were then added to the washed seeds and to almost fill to the brim of the Falcon tube, and ensuring that all the seeds were fully submerged. The tube was then incubated for 3 days at 4°C and in order to stratify the seeds for rapid and improved uniform germination rates. After stratification, the seeds were transferred to vials lined with sterile moist filter papers and then incubated (as is shown in Figure 13 below) for 14 days (the natural germination period of marama bean seeds) in a Plant Growth Chamber (Labex-Labcon GC-300, Maraisburg, RSA) set at a constant temperature of 23°C, under an 18-hour light and 6-hour dark cycle. The time of emergence as well as the percentage germination of each of the three experimental groups were then evaluated and determined by respectively monitoring the exact time at which each of the various embryonic axes was produced as well as noting the exact number of embryonic axes that had been successfully produced by each group.

**Figure 13**: Germination of the marama seeds. The surface-sterilized seeds were germinated in transparent plastic vials lined with sterile moist filter papers under growth chamber conditions.

*3.1.2.2 Extraction of total genomic DNA and its purification for Polymerase Chain Reaction*

The DNA material that was used for the molecular screening of any possible gamma ray-induced genetic mutations in the treated seeds was extracted from their emerging embryonic axes (Figure 14 below). Each embryonic axis coming out of a germinating seed was physically dissected with a sterile scalpel blade to expose its active mitotic tissue, and from which the genomic DNA was then extracted. This tissue (embryonic axis mitotic tissue), which includes the initial shoot and initial root of a new plant, has a very high DNA content to fresh weight ratio and thus the

overall yield of DNA from this tissue is usually very high. In our case, the targeted total genomic DNA was extracted using a Zymo Research Plant/Seed DNA Mini-prep kit and in accordance with the manufacturer's instructions (Zymo Research Corporation, California, USA). The extracted DNA was then quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Maryland, USA), and also further resolved onto a 0.8% agarose gel stained with 1 µg/mL ethidium bromide in Tris-borate-EDTA (TBE) buffer and viewed under a UV illumination system (UV Transilluminator 2000, Bio-Rad Laboratories, Munich, Germany), before being used for the subsequent PCR amplifications.

**Figure 14**: Extraction of total genomic DNA from active mitotic region of embryonic axes. An embryonic axis emerging from a germinating seed shown here.

*3.1.2.3 Polymerase Chain Reaction*

Polymerase chain reaction (PCR) amplifications were performed using a Thermo Scientific DreamTaq Green PCR Master mix (2X) (Thermo Scientific, Maryland,

USA) in 25 µL reaction volumes using the following reconstitutions: 12.5 µL 2X DreamTaq Green PCR Master mix, 1.0 µL of 10µM forward primer (MARA039; L-TCATTAAAGGGCTCCATTGC), 1.0 µL of 10µM reverse primer (MARA039; R-ATGCCCAAAATCACCAACAT), 8.5 µL sterile water and 2.0 µL DNA template (10ng/µL). The used primer set (MARA039) specifically targeted and amplified the AGA repeat and microsatellite region of the extracted seed embryonic axis DNA. The PCR conditions on the thermocycler (C1000 Touch™, Bio-Rad Laboratories, Munich, Germany) were as follows: 1 cycle of Pre-denaturation: 95°C for 4 minutes; 35 cycles of Denaturation: 95°C for 30 seconds, Annealing: 55°C for 1 minute, and Extension: 72°C for 2 minutes; 1 cycle of Final Extension: 72° for 5 minutes and a sample holding period at 4°C. The amplified PCR products were viewed on a 2% agarose gel in TBE buffer stained with 1 µg/mL ethidium bromide under UV illumination to check their sizes and qualities. The yielded amplicons were then cleaned up and concentrated for subsequent sequencing using a Zymo Research, DNA Clean & Concentrator-5 kit (Zymo Research Corporation, California, USA). Sequencing was then carried out at Inqaba Biotechnology in Pretoria, South Africa.

*3.1.2.4 Sequence analysis*

The nucleotide sequences obtained by Inqaba Biotechnology (Pretoria, South Africa) for the primer set (MARA039) were manually edited by eye from their established chromatograms in the Chromas Light and BioEdit program. In order to obtain the regions of overlaps, the forward and reverse sequences of the amplicons were aligned using complements of their sequences. All overlapping sequences were then trimmed

and the resulting consensus sequences created in the BioEdit Sequence Alignment Editor for each of the obtained samples. The consensus sequences were then used to create BLAST searches of the NCBI database using CLUSTAL X to align with organisms in the database and seeking the most likely gene identity of the obtained amplicons.

*3.1.2.5 Rates of emergence and percentage germinations*

For all the three experimental groups (controls, 50 Gy, and 100 Gy), the exact time of embryonic axis emergence per seed as well as the actual number of germinated seeds per group were physically monitored and manually recorded over the 14-day experimental period. For each group, the exact time of emergence was noted and then regarded as the rate of emergence while the total number of germinated seeds per group was expressed as the percentage germination.

## 3.2 Karyotyping

*(Published: Takundwa et al., (2012). A chromosome count in Marama bean (Tylosema esculentum) by Feulgen staining using garden pea (Pisum sativum L.) as a standard. Research Journal of Biology 2(6) pp.177-181)*

Germination of the seeds was performed in Petri dishes, on filter paper moistened with distilled water, at 22 ± 2°C. Root tips from *Tylosema esculentum* and *Pisum sativum* L were obtained from germinating seedlings. To determine the number of chromosomes, actively growing root tips of 5 - 10 mm were excised from

germinating seeds. The root tips were given a cold shock in ice cold water for 24 hours at 0°C or pre-treated with 0.2% and 0.05% colcemid. The root tips were fixed in ethanol: acetic acid (3:1). The fixed tips were rehydrated by successive incubations in 70%, 50%, 30% ethanol and finally in water, with a water rinse between each step. The rehydrated root tips were hydrolysed with 1N HCl for 10minutes at 60°C, followed by staining with Schiff's reagent for 2hours at 25°C. The root tips were be squashed on a microscope slide and counterstained with 2% acetic orcein. The slides were examined using 100X oil immersion objective giving a total magnification of 1000X. Pea root tips were used to compare with results for marama bean. Well-scattered metaphase plates were obtained. Chromosome numbers per cell were recorded and photographs were captured with an Olympus BX60 photomicroscope with an Olympus DP72 digital camera mounted on it. The software used for photographing and image analysis was cellSens Entry version 1.5 software.

### 3.3 Regeneration- grafting

Stems for scions for grafting were collected at Omitara on the 28$^{th}$ of November 2012. The stems were newly resprouted with young buds and having no flowers. A scion is the end of a branch where leaves fell off and fruits will grow again. The scion stems were wrapped in wet newspaper to keep them moist overnight and kept at 4°C. All leaves were removed from scion and stock the following morning. The scion only had buds remaining. A sterile Swiss knife was used to prepare the root stock and the scion for grafting. The stock plants were from plants germinated at Omipanda in December 2011 (Group 4A) and the scions were the stems collected at

Omitara. All the leaves and branches that had started to grow on the root stock were removed and a sharp, straight cut was made at a 45° angle on the root stock ensuring at least 15cm were kept under the cut. A cut with exactly the same angle was made on the scion to ensure the 2 pieces would fit together perfectly. The root stock and scion were put together and grafting tape washed in water with bleach to sterilise was used to tie the pieces together and covered with tree wound sealing gel. The grafted plants were watered immediately and every second day thereafter. 5 grafts were made and monitored over a 3 week period. The plants were kept in the shaded greenhouse (Figure 15 below).

**Figure 15:** Preparation of root stock and scion for grafting.

### 3.4 Germination and transplantation of marama bean under greenhouse setting

Group 1, Group 2, Group 4A and Group 4B plants were all generated from seed under greenhouse settings as described in section 3.1. Germination rates and practices used in these plants were used to establish best practice for generating marama bean from seeds in a greenhouse setting. Seeds were tested for regeneration

with a pre-treatment of soaking in water and without pre-treatment to break dormancy.

### 3.5. Investigating growth promoting factors

Marama bean was thought to experience prolonged time to first flower due to water stress and nutrient deficiencies in its natural environment. Potting experiments were set up to assess if plants would respond positively to increased water and nutrient access as well as hormone treatment. Seeds from Omipanda were placed in Petri dishes with water to induce germination on the 15$^{th}$ of December 2012. A total of 100 seeds were prepared in this way as 40 were needed to sow in the bags of soil that had been prepared. On the 21$^{st}$ of December, 40 of the 100 seeds had germinated and these were sown in the bags of sand (Figure 16 below). On the 10$^{th}$ of January 2013, 22 out of the 40 seedlings had emerged from the soil and these were used for the 4 treatments. The experiment had 5 plants per treatment in a complete block design.

**Figure 16**: Germination and growth of marama plants for treatment with water, fertilizer and hormone.

The following treatments were applied: 200ml of 1g/L Lucky plant fertilizer (LS004990-00-00) or 100µg/L of hormone Gibberellin $GA_3$ or water for the control. The high water treatment received 400ml of water. The treatment was applied once a week and started on the 19th of February, 2013 for 4 weeks till the 16th of March, 2013 (Figure 17 below). Three traits were followed: number of leaves, stem length and internode length. The baseline data for each plant was collected before treatment and the change in growth rate after application of the treatments was used to determine their effect. Phenotypic traits were analysed for variation with SPSS 16.O and simple correlation coefficients were assessed among traits using Microsoft Office Excel 2010.

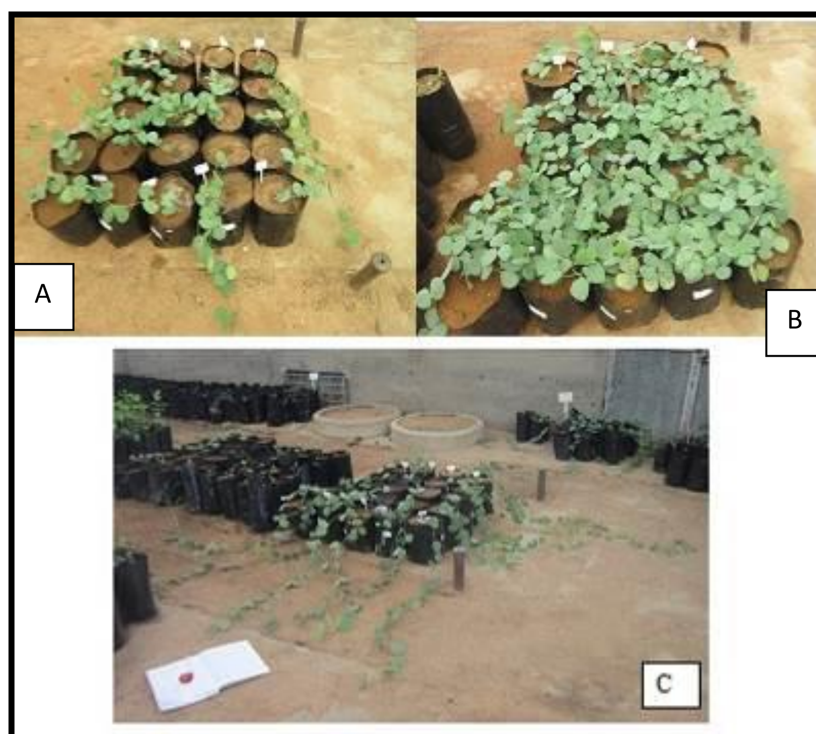


**Figure 17**: The seedlings are shown in insert A before application of treatments. After 7 weeks the seedlings are shown in insert B and measurements of changes in the 3 traits followed were measured as shown by insert C.

## 3.6. Investigating the causal agent(s) of leaf spotting and necrosis

### *3.6.1 Plant growth and development*

A group of marama plants indigenous to Namibia were grown and developed at the University of Namibia under glass house conditions as described earlier for Group 2 plants. Seeds were then sown in sterilized potting soil once they had germinated. Daily temperatures were taken and as well as noting the dates at which each seedlings was emerging. Out of the 150 seeds sown 67 germinated (46.7%) while 83 failed to germinate (53.3%). Monitoring and treatment of the seedlings with fertilizer, hormones and normal and high water regimes was routinely carried out up until disease symptoms were detected on seedling leaf tissues on the 24$^{th}$ of May, 2011. The monitoring of all plants was eventually terminated on the 8$^{th}$ of June, 2011 as most of the plants had died off at this stage.

### *3.6.2 Fungal isolations*

Leaf tissues from 30 infected seedlings (2-3 leaves per plant) were randomly collected and washed individually in running tap water before being moved to a laminar flow hood where disc sections measuring approximately 2 mm x 2 mm were then cut with a sterile scalpel blade. The disc sections were surface-sterilized by dipping into 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and rinsing in sterile distilled water followed by drying on a sterile filter paper. Concurrently, potato dextrose agar (PDA) was prepared by resuspending 39 g of PDA powder into 1 L of distilled water. The mixture was brought to boil in a conical flask over a Bunsen burner and until the powder had completely been resuspended. The media

was then sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the media was allowed to cool for 5 minutes before being poured into sterile plastic petri dishes and allowing for a further cooling to solidify the agar. The cut leaf sections were then individually placed into the PDA containing petri dishes as is shown in Figure 18 below.

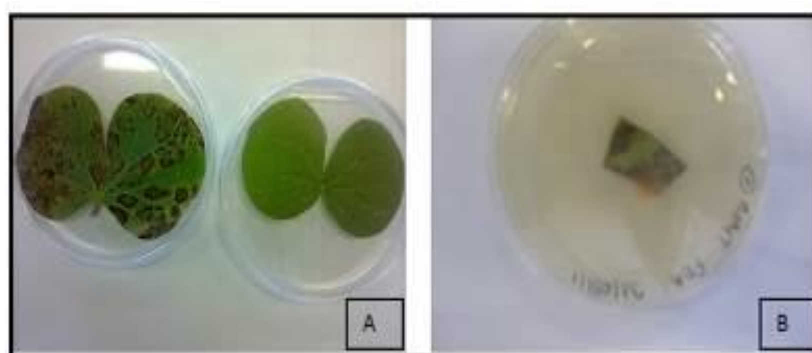


**Figure 18:** Seedling leaves used for the study of possible fungal co-infection in marama bean. (A) Petri dishes containing infected (left) and non-infected (right) marama seedling leaves. (B) A PDA petri dish inoculated with a piece of surface sterilised infected marama leaf disc.

The inoculated plates were subsequently incubated at room temperature (25°C) under white fluorescent light for 5 days. Isolation of pure cultures was then achieved by sub-culturing from hyphal edges and repeatedly inoculating onto fresh PDA media at 25°C up until the desired pure cultures were obtained. Control leaves (showing no symptoms) were also similarly treated but no fungal cultures were observed to grow on the inoculated media. From each of the isolated pure cultures, some single spore cultures were prepared by aseptically scraping the mycelium edges with a sterile

needle under a microscope and then dissolving the scrapped edges into individual drops of sterile distilled water on clean microscope slides. The slides were left to air-dry and then aseptically viewed under a microscope at 100X objective. Magnification was then lowered to 60X in order to isolate single spores from each of the scraped and prepared mycelium edges with a sterile needle. The isolated spores were then aseptically placed onto sterile PDA media to grow as single spore cultures at 25°C. From each of the prepared single spore cultures, some 4 mm X 4 mm segments of the morphologically distinct mycelia were aseptically cut and sub-cultured into sterile conical flasks containing potato dextrose broth (PDB) at 25°C for 14 days or up until their mycelia had reached the stationary phase. The resulting mycelia were then harvested and filtered under gravity through Whatman paper in Buchner funnels. The filtered mycelia were then collected in Whatman paper and air-dried for two days in a HEPA filtration cabinet. The collected mycelia were once more dried at 60°C in an oven for 24 hours and subsequently ground into a fine powder with a pestle and mortar for further downstream applications.

*3.6.3 DNA isolation*

Total genomic DNA was extracted from each of the prepared fungal mycelial powder using the Zymo Research, ZR Fungal DNA Mini prep kit and according to the manufactures instructions. The extracted DNA was then resolved on a 1% agarose gel stained with 1µg/mL ethidium bromide in Tris-borate-EDTA (TBE) buffer and viewed under UV illumination to check the quality and concentration of the extracted product for subsequent PCR application.

*3.6.4 Polymerase Chain Reaction*

Polymerase chain reaction (PCR) amplifications were performed using a Thermo Scientific DreamTaq Green PCR Master mix (2X) in 25 µL reaction volumes using the following volumes: 12.5 µL 2X DreamTaq Green PCR Master mix, 1.0 µL of 10µM forward primer (ITS 1 or 4), 1.0 µL of 10µM reverse primer (ITS 4 or 5), 8.5 µL sterile water and 2.0 µL DNA template. The ITS regions of the obtained fungal isolates were then amplified using the following primer sets: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′- GGAAGTAAAAGTCGTAACAAGG-3′). The PCR profile on the thermo cycler was: Pre-denaturation (1 cycle): 95°C for 4 minutes; 35 cycles of Denaturation: 95°C for 30 seconds, Annealing: 55°C for 1 minute, and Extension: 72°C for 2 minutes; a final Extension (1 cycle): 72° for 5 minutes and samples were then held at 4°C. The PCR products were viewed on a 2% agarose gel in TBE buffer stained with 1µg/mL ethidium bromide under UV illumination to check the sizes of the amplified products. The yielded amplicons were then cleaned up and concentrated for subsequent sequencing using a Zymo Research, DNA Clean & Concentrator-5. Sequencing was then carried out at Inqaba Biotechnology in Pretoria, South Africa.

*3.6.6 Sequence analysis*

The nucleotide sequences obtained by Inqaba Biotechnology were manually edited by eye from their determined chromatograms. In order to obtain the regions of overlaps, the forward and reverse sequences were aligned using the complements of their sequences. All overlapping sequences were then trimmed and the resulting

consensus sequences created in the Bio Edit Sequence Alignment Editor for each of the obtained fungal isolate. The consensus sequences were then used to create BLAST searches at the NCBI database using CLUSTAL X to align with organisms in the database and seeking the most likely species identities of the obtained fungal isolates.

# CHAPTER 4: RESULTS

## 4.1 SSR screening for Molecular Marker Assisted Selection

### *4.1.1 Germplasm collections, phenotypic characterization and SSR screening*

Group 1

*Internode length*

Phenotypic characterisation for internode length was possible for 45 out of the 50 samples and the internode length was found to be not normally distributed in this instance by the Shapiro-Wilk test ($p=0.003<0.05$). The internode length ranged from 5mm-69mm. Short internodes, s≤ 20mm, medium internodes, 21mm≥m≤40mm, large internodes, l≥41mm were defined as described. The mean for short internodes was s=13.60±1.62, medium internodes m=27.71±1.08, and long internodes, l=54.14±4.19. There was a significant difference between short, medium and long internodes as shown in Figure 19 below.

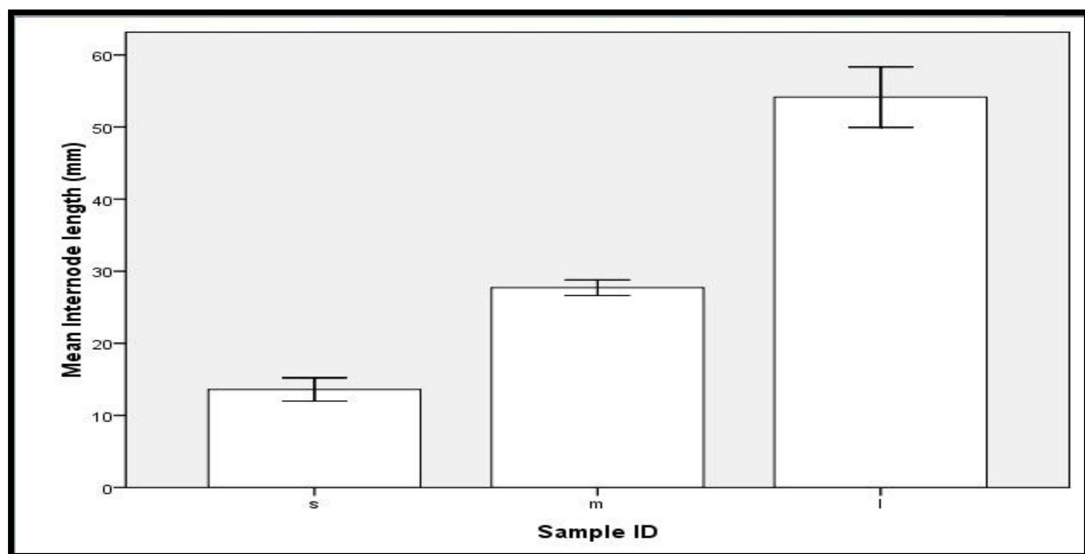


**Fig 19**: Group 1 differences between short (s), medium (m) and long (l) internodes found to have a significant difference by the Kruskal-Wallis test ($p= 0.000<0.05$).

*Internode number*

The number of internodes were not normally distributed by the Shapiro-Wilk test ($p=0.004<0.05$). The internode number ranged from 2-12 in these young vegetative plants. Low and high internode number were specified as, low internode number, $l\leq5$ while high internode number, $h>5$. The mean for low internode number was $l=3.75\pm0.750$ and high internode number $h=8.44\pm0.259$ (Figure 20). There was a significant difference between low and high internode number in this instance according to the Mann-Whitney test results ($p=0.001<0.05$).

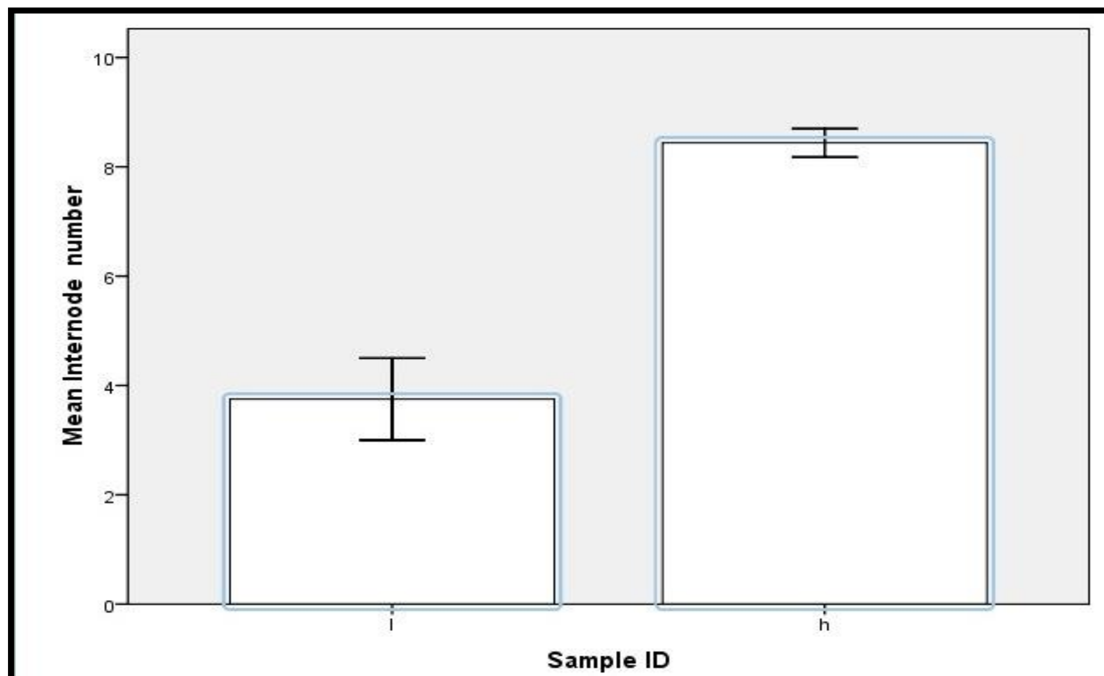


**Fig 20**: Group 1 differences between low (l) and high (h) internode number found to have a significant difference by Mann-Whitney ($p= 0.001<0.05$).

*Leaf number*

The number of leaves were normally distributed by the Shapiro-Wilk test ($p=0.584>0.05$). The leaf number ranged from 1-10 in the young seedlings. Low and

high leaf number were specified as, low leaf number, l≤5 while high leaf number, h>5. The mean for low leaf number was l=3.79±0.366 and high leaf number h=8.12±0.307. There was a significant difference between low and high leaf number according to an independent samples T-test (p=0.000<0.05) as depicted in Figure 21 below.

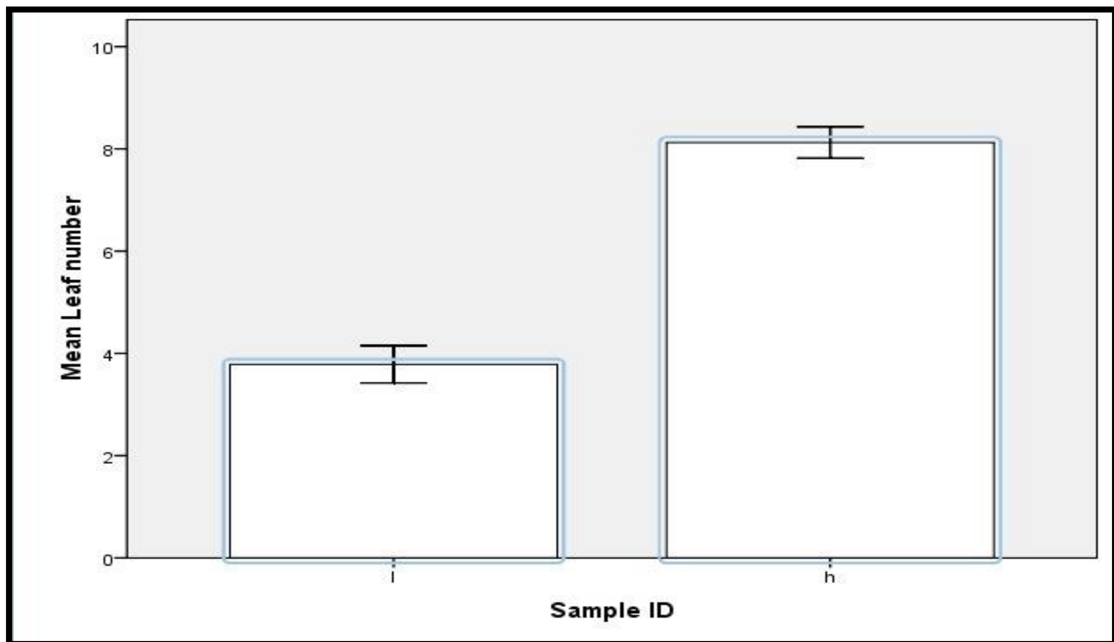


**Fig 21**: Group 1 differences between low (l) and high (h) leaf number found to have no significant difference by an independent samples T-test (p= 0.000<0.05).

*<u>Stem length</u>*

The length of stems in this group were normally distributed by the Shapiro-Wilk test (p=0.275>0.05). The stem length ranged from 23mm-606mm in the young seedlings. Short and long stem length were specified as, short stem length, s≤200mm while long stem length, l>200mm. The mean for short stem length was s=105.33±27.881 and mean long stem length was l=401.32±17.729 (Figure 22). There was a

significant difference between short and long stem length according to an independent samples T-test (p=0.000<0.05).

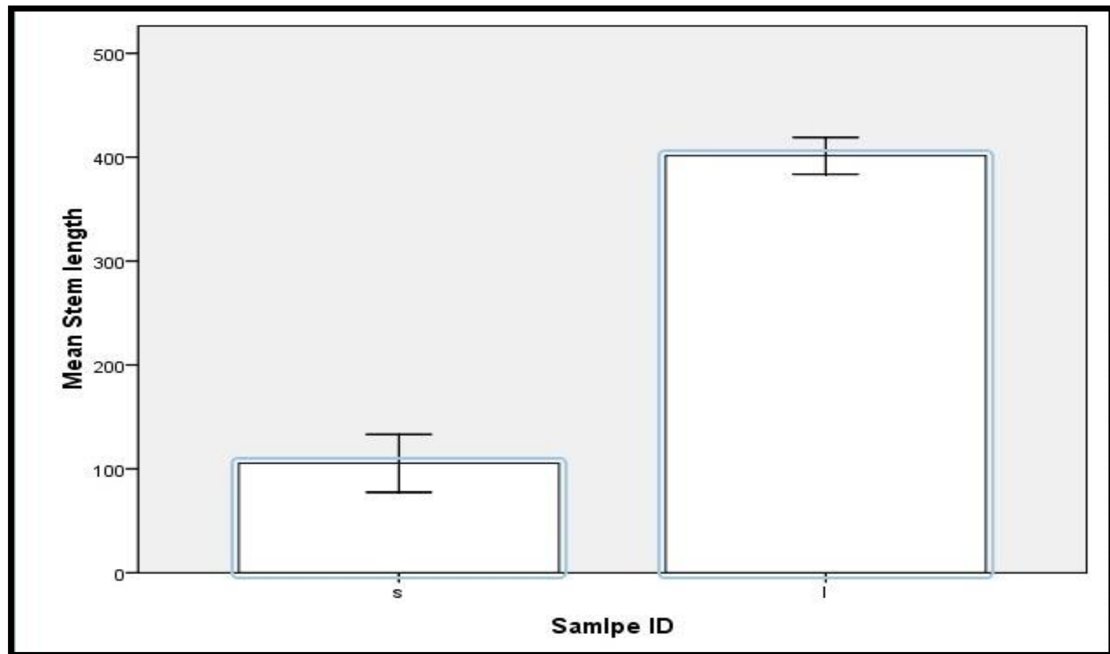


**Fig 22**: Group 1 differences between short(s) and long (l) stem length found to have significant difference by an independent samples T-test (p= 0.000<0.05).

*Correlations*

Correlation coefficient measures the linear relationship between two quantitative variables and these were determined for the traits measured in Group 1 plants as shown in the following series of figures, Figure 23 to Figure 28 that follow. There was a strong positive correlation between stem length and leaf number; stem length and internode number; internode number and leaf number. A weak positive correlation was found between stem length and internode length; leaf number and internode length. A negative correlation was found between internode number and internode length.

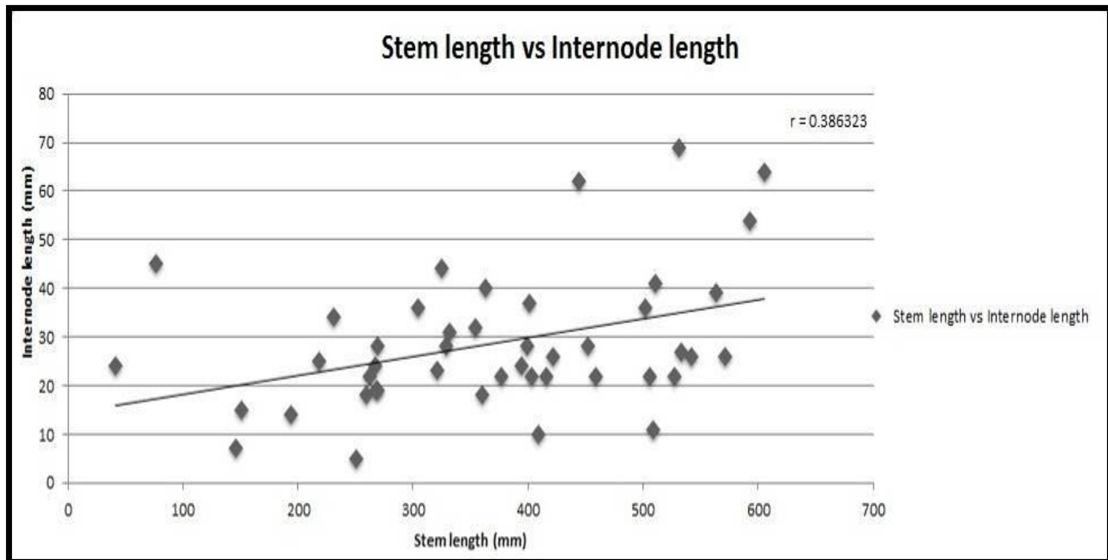


**Fig 23**: Group 1 correlation between stem length and leaf number (r=0.386).

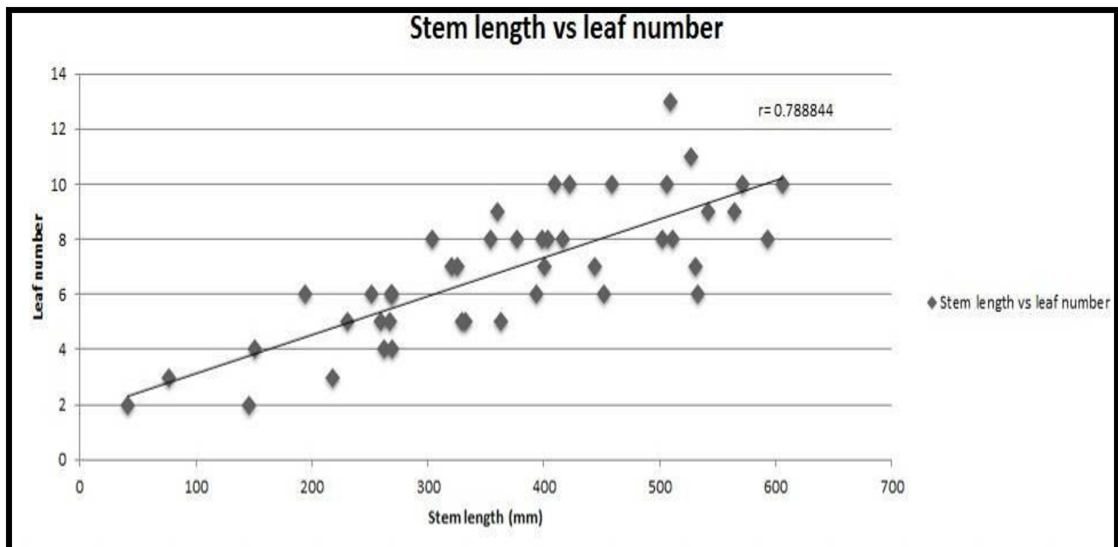


**Fig 24**: Group 1 correlation between stem length and leaf number (r=0.789).

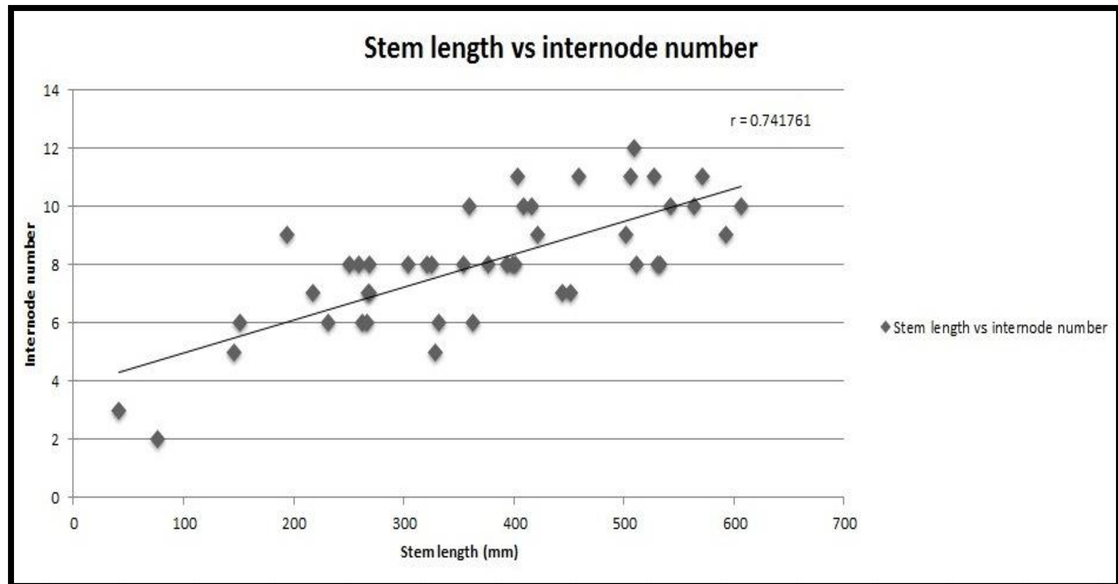


**Fig 25**: Group 1 correlation between stem length and internode number (r=0.742).

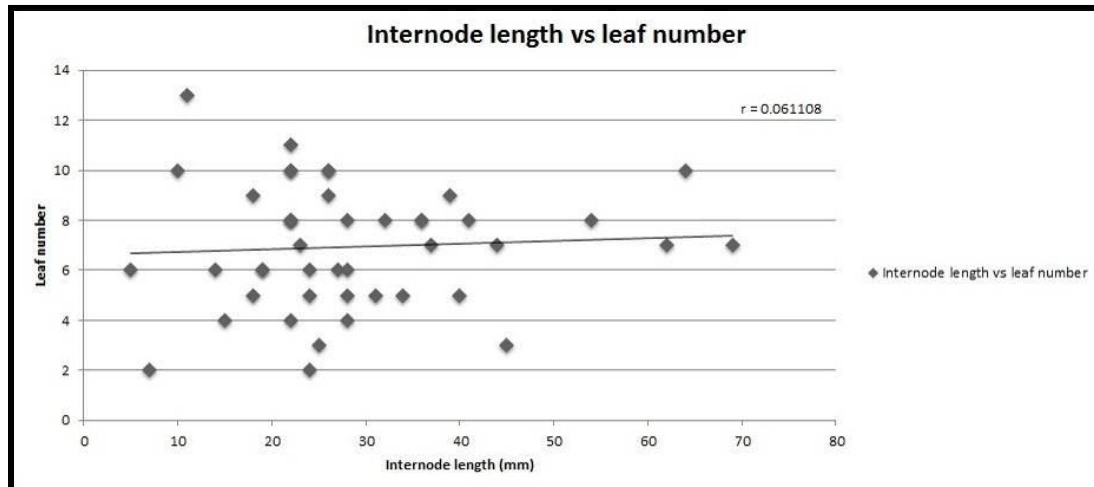


**Fig 26**: Group 1 correlation between internode length and leaf number (r=0.0611).

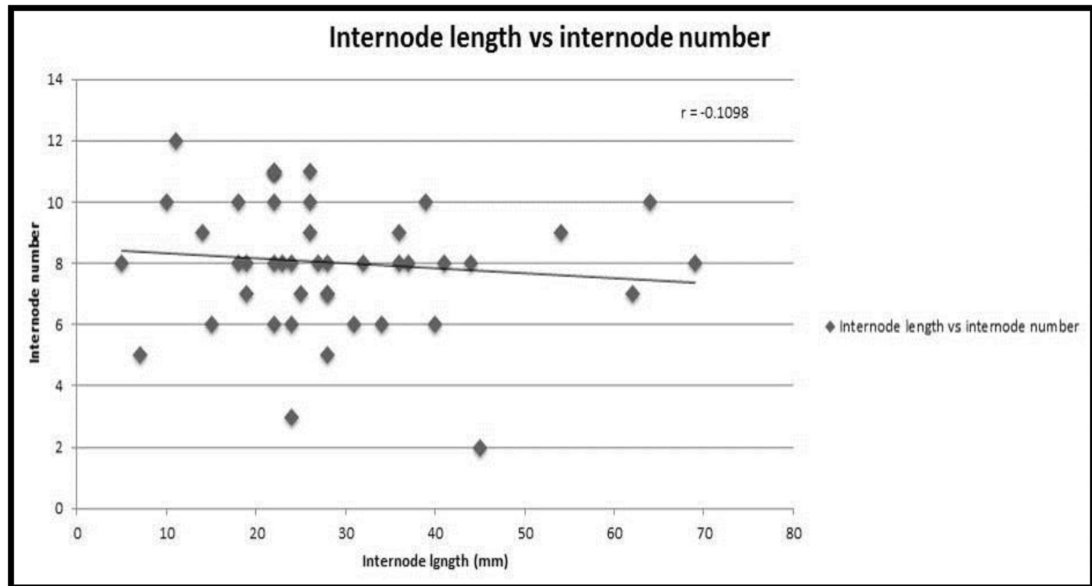


**Fig 27**: Group 1 correlation between internode length and internode number (r= -0.110).

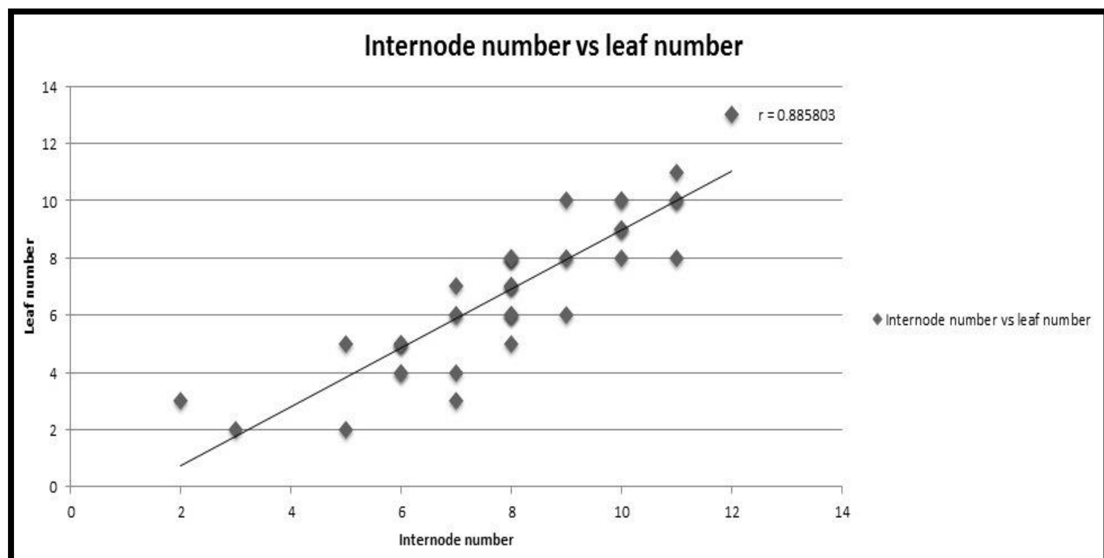


**Fig 28**: Group 1 correlation between internode number and leaf number (r=0.886).

Table 2 below provides a summary of phenotype data from Group 1 to Group 5 demonstrating that the phenotypic characters of marama bean displayed variation.

**Table 2:** Summary of phenotypic variation in Group 1 - 5.

| **Phenotypic trait** | **Group 1 N=45 Vegetative** | **Group 2 N=55 Vegetative** | **Group 3 N=44 Reproductive** | **Group 4A N=30 Vegetative** | **Group 4B N=31 Vegetative** | **Group 5 N=114 Reproductive** |
|---|---|---|---|---|---|---|
| **Internode length(mm)**<br>S (short)<br>M (medium)<br>L (long) | 5mm-69mm<br>13.6±1.62[a]<br>27.7±1.08[b]<br>54.1±4.19[c] | 1mm-134mm<br>14.4±1.53[a]<br>29.6±1.18[b]<br>56.3±6.49[c] | 23mm-77mm<br>-<br>33.2±0.884[b]<br>54.6±3.02[c] | 25mm-115mm<br>-<br>30.8±1.82[b]<br>73.6±3.85[c] | 3mm-90mm<br>-<br>-<br>64.0±2.59[c] | 5mm-69mm<br>18.1±0.35[a]<br>29.1±0.58[b]<br>46.2±0.86[c] |
| **Internode number**<br>L (low)<br>H (high) | 2-12<br>3.75±0.750[a]<br>8.44±0.259[b] | 3-11<br>4.17±0.167[a]<br>7.78±0.194[b] | 25-7568<br>199±27.6[a]<br>2244±475 [b] | 5-16<br>8.29±0.381[a]<br>12.9±0.548 [b] | 5-19<br>-<br>8.00±0.477 [b] | - |
| **Leaf number**<br>L (low)<br>H (high) | 2-13<br>3.79±0.366[a]<br>8.12±0.307[b] | 1-9<br>4.11±0.232[a]<br>7.19±0.200[b] | 17-3957<br>155±21.8[a]<br>1690±284[b] | 2-23<br>3.57±0.369[a]<br>9.70±0.818[b] | 2-11<br>4.00±0.707[a]<br>7.26±0.285[b] | - |
| **Leaf width (mm)**<br>L (low)<br>H(high) | - | 52mm-114mm<br>68.9±1.86[a]<br>93.6±1.77[b] | - | 61mm-131mm<br>70.5±3.03[a]<br>103.4±2.37[b] | 12mm-126mm<br>37.7±13.0[a]<br>97.7±2.06[b] | - |
| **Stem length (mm)**<br>S (short)<br>L (long) | 41mm-606mm<br>105.3±27.9[a]<br>401.3±17.7[b] | 44mm-553mm<br>134.5±9.99[a]<br>359.2±16.6[b] | 424mm-5074mm<br>1261±88.9[a]<br>3464±207[b] | 86mm-827mm<br>100.0±9.85[a]<br>422.6±27.9[b] | 50mm-517mm<br>59.7±6.49[a]<br>395.1±13.4[b] | - |
| **Pod number**<br>L (low)<br>H (high) | - | - | 1-182<br>7.46±1.63[a]<br>61.6±21.0[b] | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Flower number**<br>L (low)<br>H (high) | - | - | 1-124<br>8.00±1.38[a]<br>57.7±16.8[b] | - | - | - |
| **Days to seedling emergence**<br>L (low)<br>H (high) | - | 10-125<br><br>23.6±0.831[a]<br>55.3±3.15[b] | - | - | - | - |

[a/b/c] indicate significant difference by statistical analysis in SPSS ($p<0.05$)

The relationships of phenotypic traits of the marama bean were also explored and it was established which traits were more closely related to one another. The results collected are summarized with data from Group 1 and Group 3 presented in Table 3 below.

**Table 3:** Summary of correlation coefficients Group 1(vegetative) and Group 3 (reproductive) plants.

| **Phenotypic characters** | **Correlation Coefficient (r) Group 1** | **Correlation Coefficient (r) Group 3** | **Positive/ Negative** |
|---|---|---|---|
| Internode length vs internode number | -0.110 | 0.163 | +/- (weak) |
| Internode length vs leaf number | 0.061 | 0.239 | + (weak) |
| Internode length vs stem length | 0.386 | 0.208 | + (weak) |
| Internode length vs pod number | - | 0.175 | + (weak) |
| Internode length vs flower number | - | 0.377 | + (weak) |
| Internode number vs leaf number | 0.886 | 0.961 | + (strong) |
| Internode number vs stem length | 0.742 | 0.689 | + (strong) |
| Internode number vs pod number | - | 0.865 | + (strong) |
| Internode number vs flower number | - | 0.793 | + (strong) |
| Leaf number vs stem length | 0.789 | 0.749 | + (strong) |
| Leaf number vs pod number | - | 0.785 | + (strong) |
| Leaf number vs flower number | - | 0.812 | + (strong) |
| Stem length vs pod number | - | 0.544 | + (strong) |
| Stem length vs flower number | - | 0.585 | +(strong) |
| Pod number vs flower number | - | 0.889 | + (strong) |

*SSR screening*

Group 1

The primers MARA 001, MARA 020, MARA 037, MARA 039, MARA 065, MARA 068, MARA 072, MARA 074 and MARA 077 gave polymorphic profiles for the phenotypic traits of Group 1 plants and out of them all, MARA 077 appeared to be linked to internode length as shown by the amplification profile in Figure 29 and the dendogram that resulted in Figure 30 that follows.

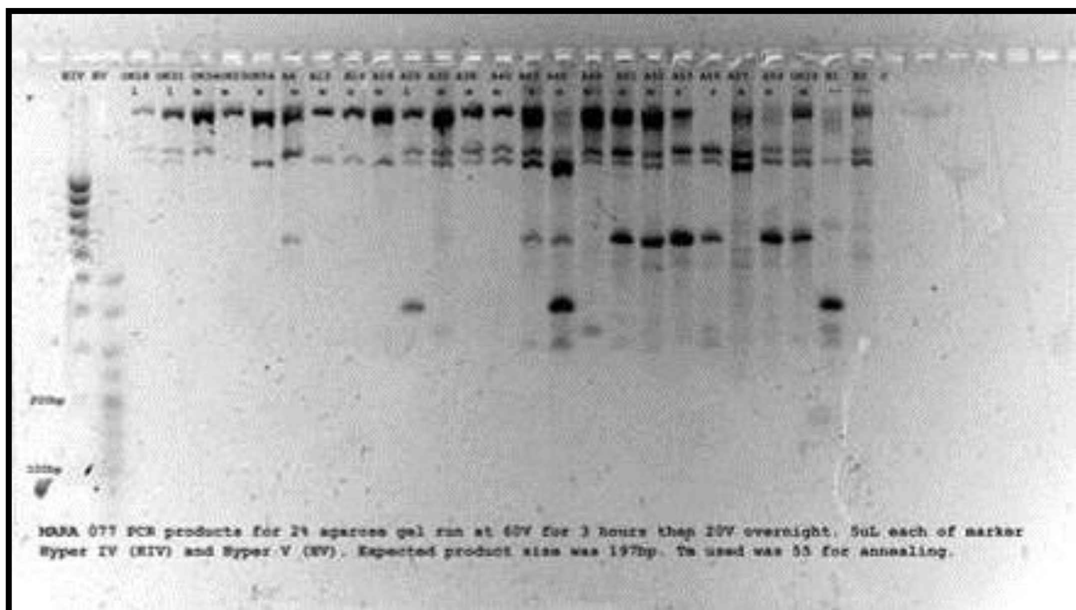


**Figure 29**: Primer MARA 077 amplicons from Group 1 plants genotyped as short(s), medium (m) and long (l) internode lengths. The amplified products were resolved on a 2% agarose gel stained with 1 µg/mL ethidium bromide and run in 1X TBE buffer at 90 V for 1 hour.  HIV is the 100 bp ladder.

The similarity matrix for primer MARA 077 resulted in a dendogram that formed clusters that split the plants in Group 1 into the respective internode lengths as shown in Figure 30 below.

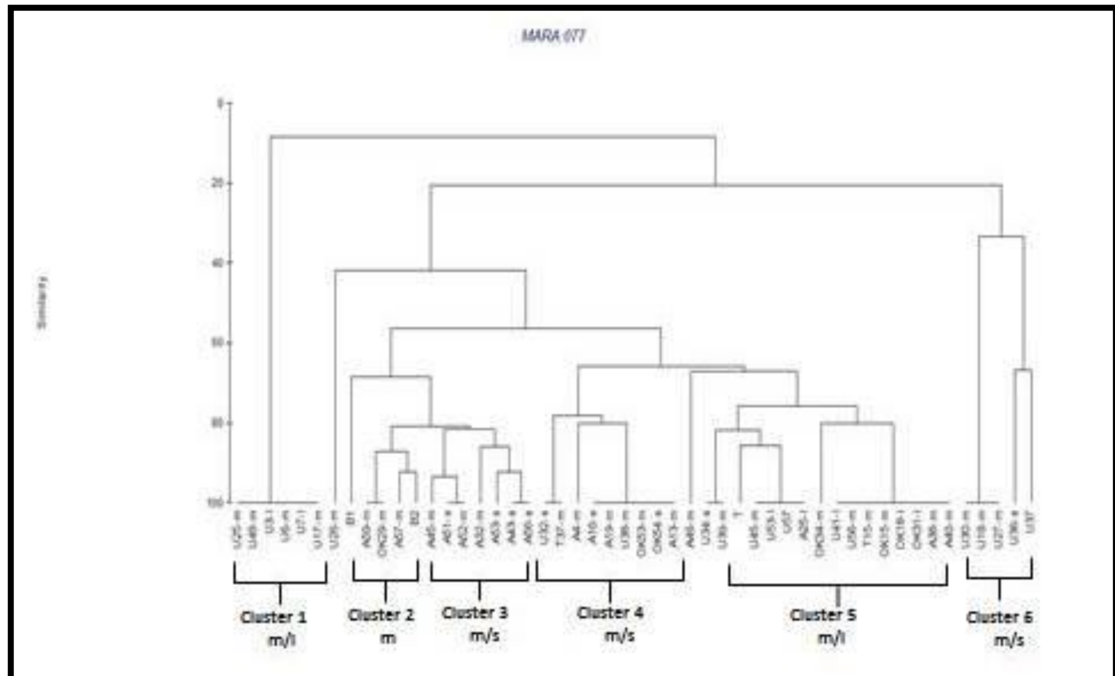


**Figure 30**: The dendogram resulting from the similarity matrix based on primer MARA 077 with Group 1 plants characterized as short(s), medium (m) and long (l) internode lengths.

Similar results were obtained with Group 5 plants and the rest of the similarity matrices and dendograms are presented in the Appendices. The Shannon-Weaver diversity index for each phenotypic character was as follows: internode

length(0.920), internode number (0.300), leaf number (0.614), stem length (0.387), days to seedling emergence (0.690), leaf width (0.680), pod number (0.647), flower number (0.593) and  according to primer MARA 077 (2.48).

***4.1.2 SSR screening for mutation detection***

*(Manuscript submitted to Molecular Biology International)*

*4.1.2.1 Emergence rates and percentage germinations*

When comparing the non-irradiated (control) seeds and the irradiated (50 Gy and 100 Gy) seeds, both relatively higher emergence rates and percentage germinations were observed in irradiated seeds than in the non-irradiated seeds (Figures 31 A and B below, respectively). In terms of the emergence rates, the earliest time of embryonic axis emergence was observed in seeds treated with 100 Gy (3 days), followed by the seeds treated with 50 Gy (5 days) and then the control seeds (10 days). Once again, the highest number of seeds to germinate was observed in seeds treated with 100 Gy (92%), followed by the seeds treated with 50 Gy (76%) and then the control seeds (54%). This observation may signify a dosage-dependent inductive effect of the applied radiation that preferably favoured both enhanced emergence rates and high percentage germinations.

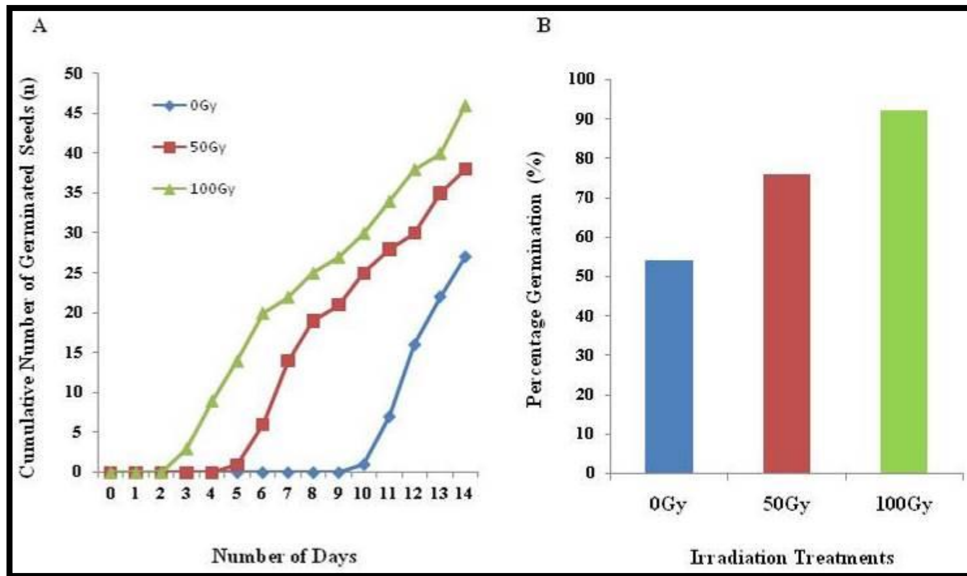


**Figure 31:** Emergence rates and percentage germinations. (A) A comparative analysis of the average time taken for seeds to germinate among three different groups of marama bean seeds. (B) A comparative analysis of the total number of seeds germinated per group (n=50, $p>0.05$).

*4.1.2.2 Polymerase Chain Reaction*

From the PCR work undertaken, the targeted and most desired AGA microsatellite repeat region of the marama bean plant was successfully amplified for both the irradiated and non-irradiated seeds (Figure 32 below). Amplifications were carried out using the MARA039 primer set (L-TCATTAAAGGGCTCCATTGC; R-ATGCCCAAAATCACCAACAT), which in all cases, succeeded to amplify the various amplicon fragments in the approximate and expected size range of 176 bp (Figure 32).

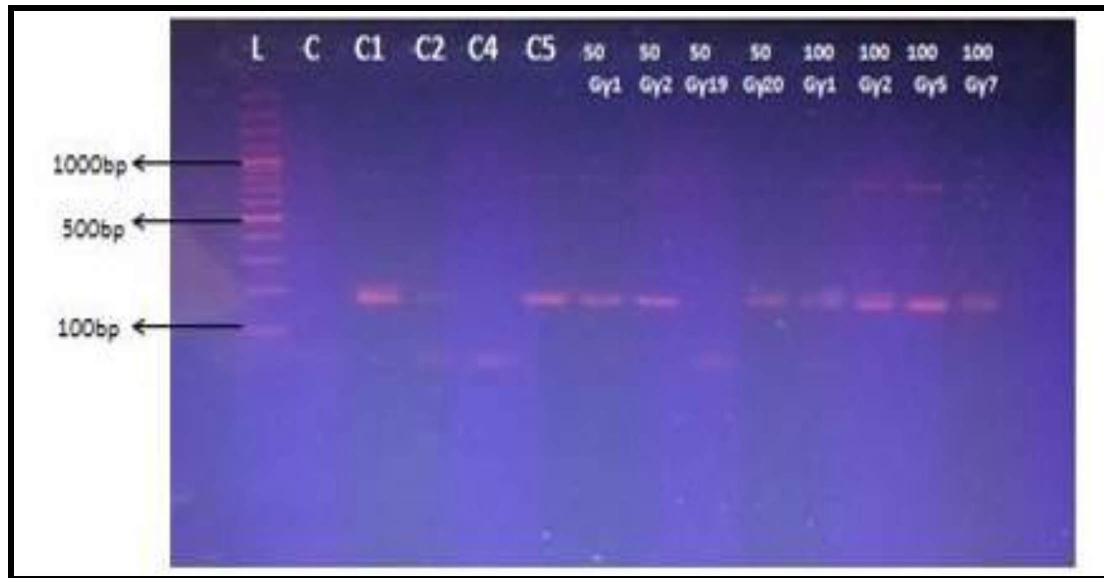


**Figure 32:** Electrophoresis gel of PCR products amplified in the AGA microsatellite repeat region of marama bean by the MARA039 primer set. The amplified products were resolved on a 2% agarose gel stained with 1 µg/mL ethidium bromide and run in 1X TBE buffer at 90 V for 1 hour. L is the 100 bp ladder, C represents the non-irradiated control seeds, 50Gy represents seeds irradiated with 50 grays gamma rays and 100Gy represents seeds irradiated with 100 grays gamma rays.

*4.1.2.3 Sequence alignment*

All obtained amplicons in Figure 32 above were carefully excised and cleaned up for subsequent sequencing at the Inqaba Biotech, Pretoria, South Africa. Sequencing was undertaken in order to specifically determine the exact base pair differences between the various amplicons in the targeted microsatellite repeat region. The exact base pair differences were then determined by aligning the sequenced amplicons against each other in the Cluster X program and as is shown in Figure 33 below. Apparently, the

targeted region of interest displayed several point mutations as well as some differences in the number of the AGA repeat units. All control samples retained their original 5 AGA repeat units and had no point mutations while the irradiated samples either gained or lost one to two more AGA repeat units and had several point mutations. This thus indicated that the irradiation system may have had some mutational effects of either changing the number or type of the AGA repeat units in the targeted microsatellite region.

**Figure 33:** Sequence alignment of the amplified amplicons in the AGA microsatellite repeat region of marama bean. A MARA039 primer set was used to amplify various amplicon fragments of the AGA microsatellite repeat region of the marama bean seeds treated with 0, 50 or 100 grays gamma rays. The amplified fragments were sequenced and then aligned in the Clustal X program in order to determine their specific point or AGA repeat mutations.

*4.1.2.4 BLAST searches*

After amplifying the AGA microsatellite repeat region in each of the irradiated or non-irradiated marama bean seeds and determining its irradiation-associated mutational changes, it was then necessary to attempt and establish the exact identity of this region in the marama bean plant. In order to achieve this, a BLAST search in the NCBI database using one of the non-irradiated amplicons was carried out. The search returned a 75% homologous hit which apparently was the non-catalytic beta sub-unit of a polygalacturonase (PG) enzyme in the *Medicago truncatula* genome (Figure 34). This then proposed that the targeted and amplified AGA microsatellite repeat region in the marama bean could also be the non-catalytic beta sub-unit of a polygalacturonase enzyme of this plant.

Download GenPept Graphics

Polygalacturonase-1 non-catalytic subunit beta [Medicago truncatula]

Sequence ID: ref|XP_003613234.1| Length: 630 Number of Matches: 1

See 1 more title(s)

Polygalacturonase-1 non-catalytic subunit beta [Medicago truncatula]

Sequence ID: gb|AES96192.1|

Range 1: 30 to 131 GenPept Graphics Next Match Previous Match

| Score | Expect | Method | Identities | Positives | Gaps | Frame |
|---|---|---|---|---|---|---|
| 166 bits(420) | 6e-44 | Compositional matrix adjust. | 76/102(75%) | 87/102(85%) | 0/102(0%) | -2 |

```
Query  438   EKNPFTPKAFLLRYWDKEIRNNLPKPGFLLSKASPLSAVDSAAFAKLAASNTLSTQLPEF  259
             +KNPFTPKAFL RYWDKEIRN LPKP FL SKASPLS V++A FAKLA+ N LST+LPEF
Sbjct  30    DKNPFTPKAFLNRYWDKEIRNGLPKPSFLFSKASPLSTVEAATFAKLASGNVLSTRLPEF  89

Query  258   CSSAGLLCISQLGPSLEKHSKDVNFAVYQDKNFTNYGTDRLG  133
             CS+A LLC+ ++  SLEKH  DVNFA+Y+DKNFTNYGTDR G
Sbjct  90    CSAANLLCLPEVAQSLEKHGDDVNFAIYRDKNFTNYGTDRPG  131
```

**Figure 34:** Sequence identity of the AGA microsatellite repeat region in the marama bean. A control amplicon from the non-irradiated marama seeds was used as a search motif to BLAST the NCBI database and it returned an identity that was the non-catalytic beta sub-unit of a *Medicago truncatula* polygalacturonase (PG).

### 4.2 Karyotyping

*(Published: Takundwa et al., (2012). A chromosome count in Marama bean (Tylosema esculentum) by Feulgen staining using garden pea (Pisum sativum L.) as a standard. Research Journal of Biology 2(6) pp.177-181)*

Root tips from *Tylosema esculentum* and *Pisum sativum* L. were used to obtain dividing cells from the meristematic region. Somatic chromosome analysis from more than 100 metaphase plates revealed that the mitotic chromosomes in marama were small, they ranged in length from 2.56 µm to 6.38 µm in mitotic pro-

metaphase. In pea, the chromosomes were found to be generally larger and longer than those in marama bean the shortest being found to be 7.89 µm in length and the longest 23.7 µm. The mitotic metaphase chromosomes are presented in *Fig. 35* to *Fig. 40*.

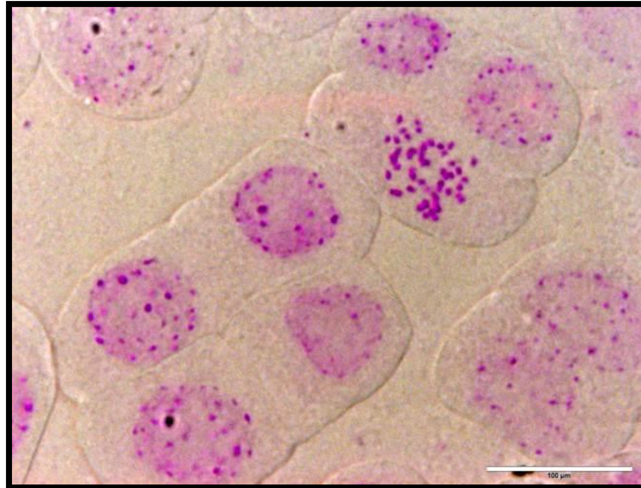

**Figure 35**: Somatic metaphase plate from a plant of *T. esculentum* showing 2n = 44 chromosomes. Bar = 100µM.

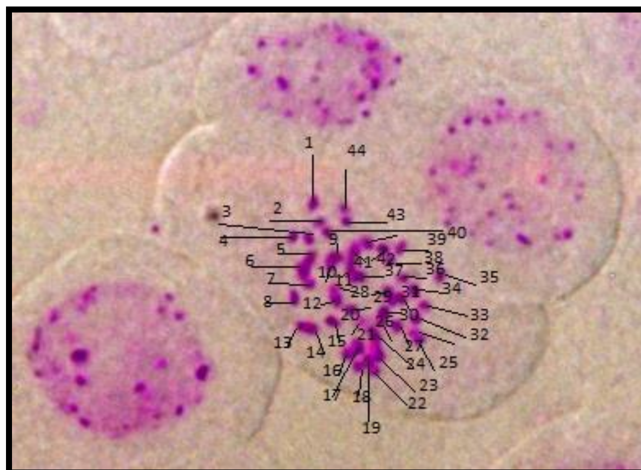


**Figure 36**: Somatic metaphase plate from a plant of *T. esculentum* showing 2n = 44 chromosomes.

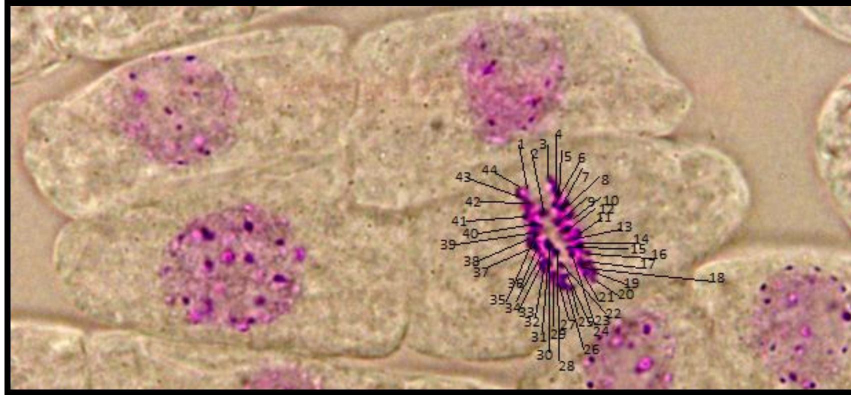

**Figure 37**: Somatic metaphase plate from a plant of *T. esculentum* showing 2n = 44 chromosomes.

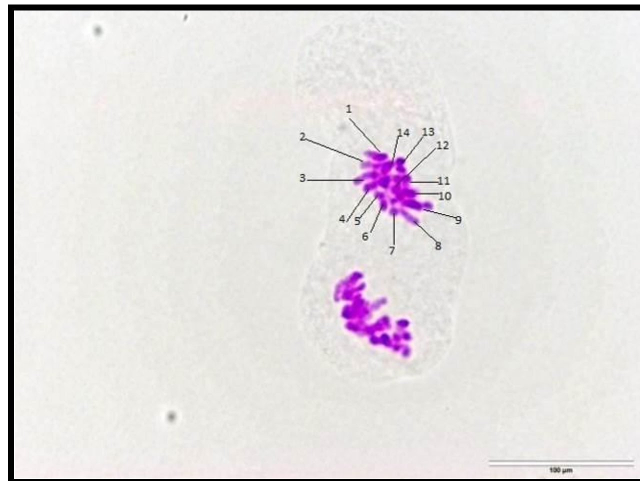

**Figure 38**: Somatic metaphase plate from a plant of *Pisum sativum L.* showing 2n = 14 chromosomes. Bar = 100µM.

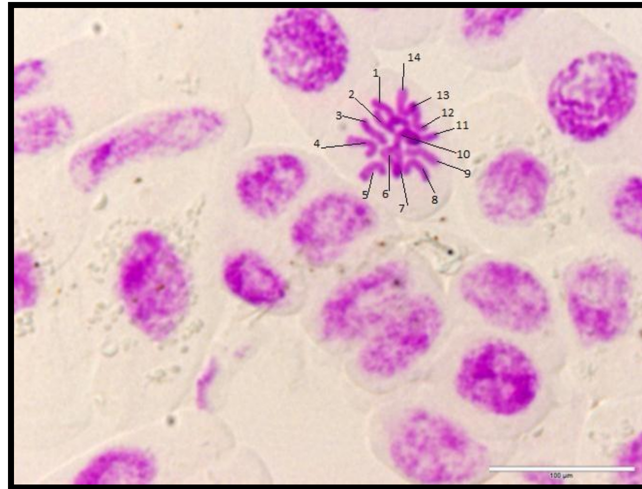


**Figure 39**: Somatic metaphase plate from a plant of *Pisum sativum* L. showing 2n = 14 chromosomes. Bar = 100μM.

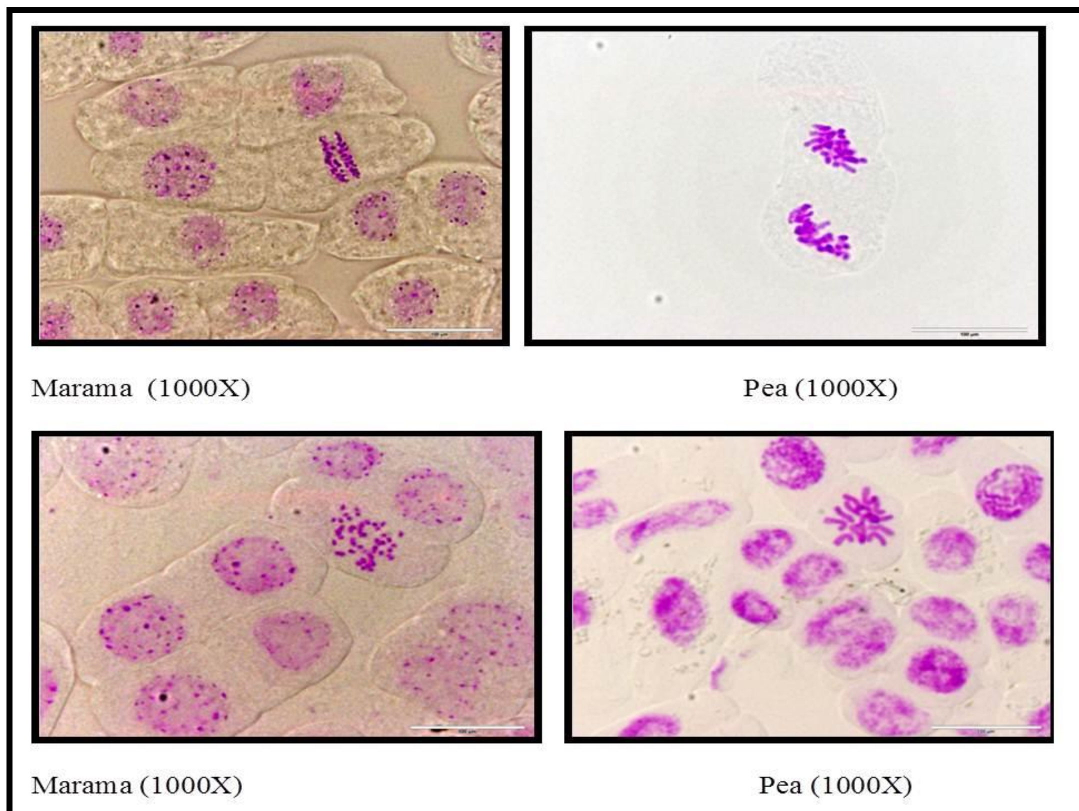


**Figure 40**: Somatic metaphase plate from a plant of *T. esculentum* showing 2n = 44 chromosomes. Bar = 100μM.

It appears there are 22 haploid chromosomes in the marama genome (2n=44). What can be seen from the spreads is that the genome or nuclear material of marama is about half that of pea and the chromosomes are smaller and more numerous. It also appears that the heterochromatic knobs in the interphase nucleus are distinct and not dispersed as in the pea spreads. An intriguing and perhaps significant observation is that the chromosomes in marama appear to have more heterochromatic regions in comparison to the pea chromosomes. The positions of dark staining are heterochromatic while light staining regions are referred to as euchromatic.

### 4.3 Regeneration- Grafting

Attempts to graft marama bean were unsuccessful in 2 trials as the plant resprouted from a different point on the stem from the point of the graft. Figure 41 below shows how this was observed.

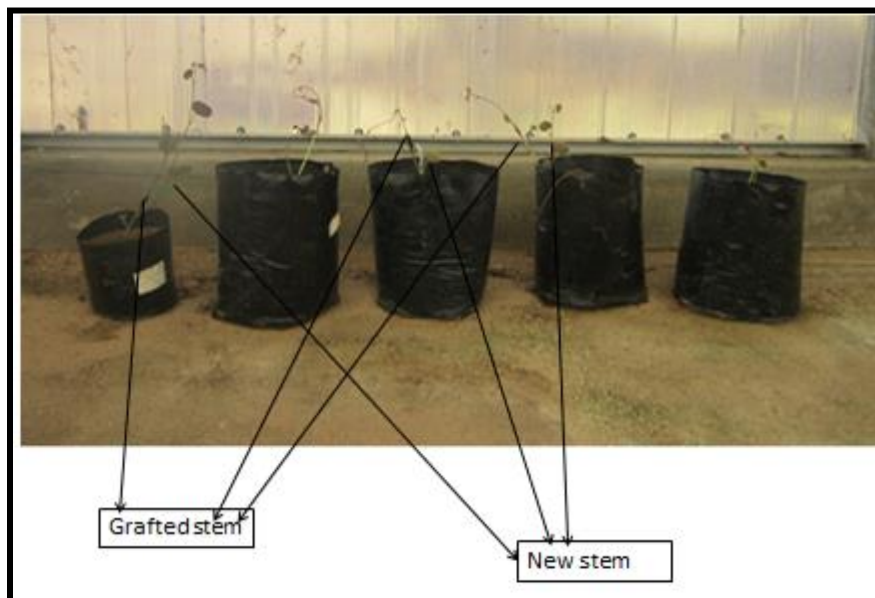


**Figure 41**: Resprouting in grafted marama bean plants.

### 4.4 Germination and transplantation of marama bean under greenhouse setting

A method for generation of marama seedlings that established a protocol that could be easily adopted by farmers in rural areas of Namibia to raise seedlings before transplanting into cultivation plots was determined. The root choking observed in Group 1 and Group 2 plants led to the use of black plastic bags for Group 4A and 4B. Trials to cultivate root tips for chromosome counts showed that incubating marama seeds at 30°C induced germination of root tips by day 5- day 10 as shown in Figure 42 and Figure 43 below. This method could be used to germinate seedlings before transferring them to sandy potting soils for greenhouse experiments.

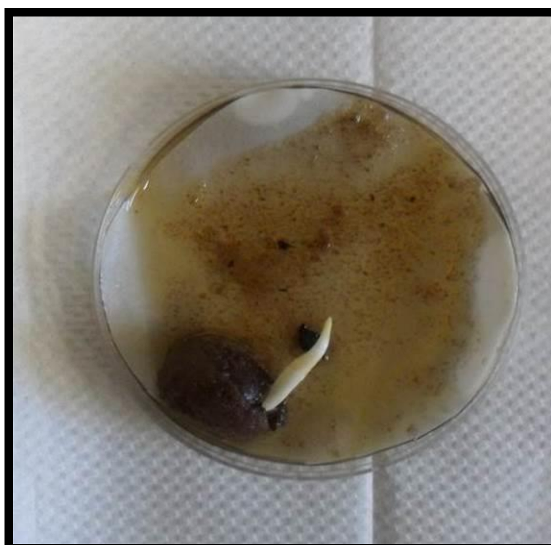

**Figure 42**: Marama root shoot emerging from seed on moistened filter paper at 30°C in an incubator.

The seedlings were transferred into bigger containers and grew further in the incubator before being transferred to potting soil. Within a 3 week period healthy seedlings were growing in the greenhouse.

**Figure 43**: Marama seedlings growing on moistened filter paper at 30°C in an incubator.

**Figure 44**: Marama seedlings transferred to sterile potting soil.

This method was adopted in Namibia without the extensive use of incubators. The seedlings are taken from petri-dishes to soil in seedling trays or bags providing an inexpensive and rapid method for resource poor farmers to be able to generate viable seedling without wasting land planting seeds that will fail to germinate.

## 4.5. Investigating growth promoting factors

The change in number of leaves, stem length and internode length were noted and compared to the initial in the variable before treatment to determine the effect of high water (HW), fertiliser (F) and gibberellins (G) on the vegetative growth of marama in comparison to controls. The results obtained from One way analysis of variance (ANOVA) showed no significant difference in internode length ($p=0.362>0.05$, accept $H_o$), stem length ($p=0.256>0.05$, accept $H_o$) and number of leaves ($p=0.466>0.05$, accept $H_o$) for plants in the 4 groups, Figure 45 below shows the trend in internode length.

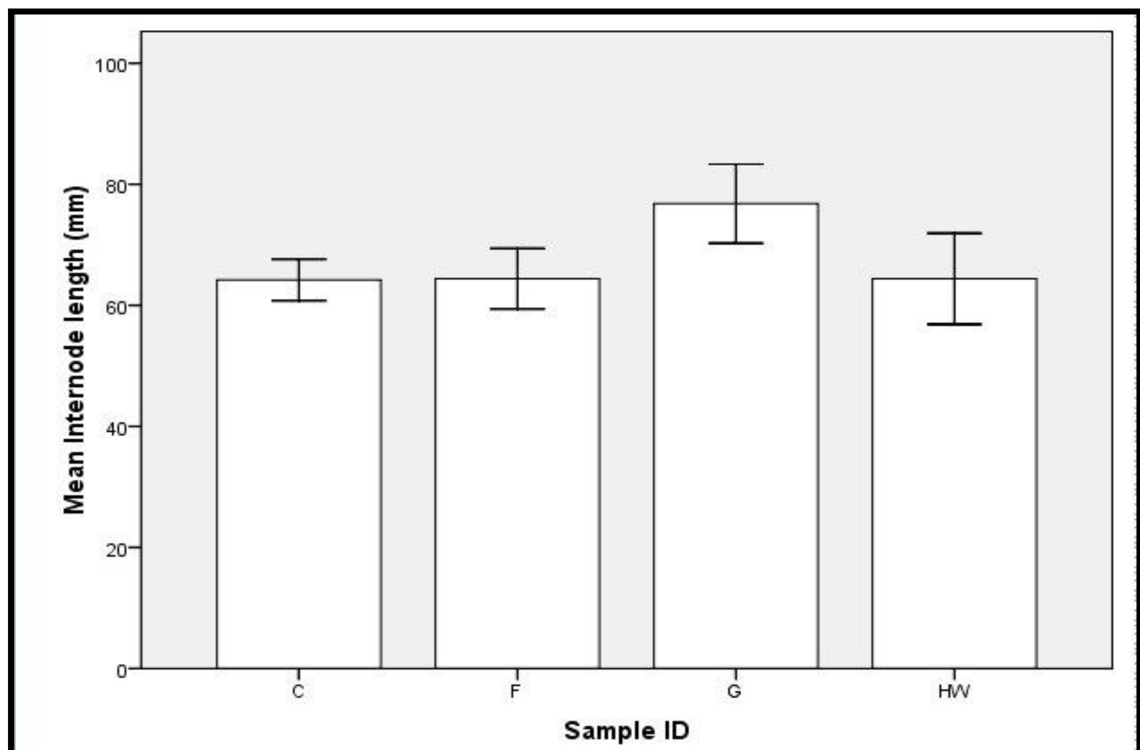


**Fig 45**: Mean internode length of control plants (C), fertilizer treated (F), gibberellin treated (G) and high water treated plants. There was no significant difference between treatments as revealed by one-way ANOVA ($p= 0.362>0.05$).

The Post Hoc Turkey and Bonferroni methods were used to identify which mean differed from the other as the difference sought was between the control and the several treatments applied. The results are presented in Table 4, 5 and 6 below. Where 1 =HW, 2=G, 3=C, 4=F.

**Table 4**: Post Hoc analysis of mean internode lengths, control vs high water, gibberellins and fertilizer treatments.

**Multiple Comparisons**

Dependent Variable:Internode length (mm)

| | (I) Sample ID | (J) Sample ID | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 1 | 2 | -12.400 | 8.249 | .459 | -36.00 | 11.20 |
| | | 3 | .200 | 8.249 | 1.000 | -23.40 | 23.80 |
| | | 4 | .000 | 8.249 | 1.000 | -23.60 | 23.60 |
| | 2 | 1 | 12.400 | 8.249 | .459 | -11.20 | 36.00 |
| | | 3 | 12.600 | 8.249 | .445 | -11.00 | 36.20 |
| | | 4 | 12.400 | 8.249 | .459 | -11.20 | 36.00 |
| | 3 | 1 | -.200 | 8.249 | 1.000 | -23.80 | 23.40 |
| | | 2 | -12.600 | 8.249 | .445 | -36.20 | 11.00 |
| | | 4 | -.200 | 8.249 | 1.000 | -23.80 | 23.40 |
| | 4 | 1 | .000 | 8.249 | 1.000 | -23.60 | 23.60 |
| | | 2 | -12.400 | 8.249 | .459 | -36.00 | 11.20 |
| | | 3 | .200 | 8.249 | 1.000 | -23.40 | 23.80 |
| Bonferroni | 1 | 2 | -12.400 | 8.249 | .914 | -37.22 | 12.42 |
| | | 3 | .200 | 8.249 | 1.000 | -24.62 | 25.02 |
| | | 4 | .000 | 8.249 | 1.000 | -24.82 | 24.82 |
| | 2 | 1 | 12.400 | 8.249 | .914 | -12.42 | 37.22 |
| | | 3 | 12.600 | 8.249 | .877 | -12.22 | 37.42 |
| | | 4 | 12.400 | 8.249 | .914 | -12.42 | 37.22 |
| | 3 | 1 | -.200 | 8.249 | 1.000 | -25.02 | 24.62 |
| | | 2 | -12.600 | 8.249 | .877 | -37.42 | 12.22 |
| | | 4 | -.200 | 8.249 | 1.000 | -25.02 | 24.62 |
| | 4 | 1 | .000 | 8.249 | 1.000 | -24.82 | 24.82 |
| | | 2 | -12.400 | 8.249 | .914 | -37.22 | 12.42 |
| | | 3 | .200 | 8.249 | 1.000 | -24.62 | 25.02 |

**Table 5**: Post Hoc analysis of mean leaf number, control vs high water, gibberellins and fertilizer treatments.

**Multiple Comparisons**

Dependent Variable:Leaf number

| | (I) Sample ID | (J) Sample ID | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 1 | 2 | 2.600 | 3.604 | .887 | -7.71 | 12.91 |
| | | 3 | 4.600 | 3.604 | .590 | -5.71 | 14.91 |
| | | 4 | 5.400 | 3.604 | .461 | -4.91 | 15.71 |
| | 2 | 1 | -2.600 | 3.604 | .887 | -12.91 | 7.71 |
| | | 3 | 2.000 | 3.604 | .944 | -8.31 | 12.31 |
| | | 4 | 2.800 | 3.604 | .864 | -7.51 | 13.11 |
| | 3 | 1 | -4.600 | 3.604 | .590 | -14.91 | 5.71 |
| | | 2 | -2.000 | 3.604 | .944 | -12.31 | 8.31 |
| | | 4 | .800 | 3.604 | .996 | -9.51 | 11.11 |
| | 4 | 1 | -5.400 | 3.604 | .461 | -15.71 | 4.91 |
| | | 2 | -2.800 | 3.604 | .864 | -13.11 | 7.51 |
| | | 3 | -.800 | 3.604 | .996 | -11.11 | 9.51 |
| Bonferroni | 1 | 2 | 2.600 | 3.604 | 1.000 | -8.24 | 13.44 |
| | | 3 | 4.600 | 3.604 | 1.000 | -6.24 | 15.44 |
| | | 4 | 5.400 | 3.604 | .921 | -5.44 | 16.24 |
| | 2 | 1 | -2.600 | 3.604 | 1.000 | -13.44 | 8.24 |
| | | 3 | 2.000 | 3.604 | 1.000 | -8.84 | 12.84 |
| | | 4 | 2.800 | 3.604 | 1.000 | -8.04 | 13.64 |
| | 3 | 1 | -4.600 | 3.604 | 1.000 | -15.44 | 6.24 |
| | | 2 | -2.000 | 3.604 | 1.000 | -12.84 | 8.84 |
| | | 4 | .800 | 3.604 | 1.000 | -10.04 | 11.64 |
| | 4 | 1 | -5.400 | 3.604 | .921 | -16.24 | 5.44 |
| | | 2 | -2.800 | 3.604 | 1.000 | -13.64 | 8.04 |
| | | 3 | -.800 | 3.604 | 1.000 | -11.64 | 10.04 |

**Table 6**: Post Hoc analysis of mean stem length, control vs high water, gibberellins and fertilizer treatments.

**Multiple Comparisons**

Dependent Variable:Stem length (mm)

| | (I) Sample ID | (J) Sample ID | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 1 | 2 | 360.600 | 237.455 | .450 | -318.76 | 1039.96 |
| | | 3 | 344.000 | 237.455 | .489 | -335.36 | 1023.36 |
| | | 4 | 474.200 | 237.455 | .230 | -205.16 | 1153.56 |
| | 2 | 1 | -360.600 | 237.455 | .450 | -1039.96 | 318.76 |
| | | 3 | -16.600 | 237.455 | 1.000 | -695.96 | 662.76 |
| | | 4 | 113.600 | 237.455 | .963 | -565.76 | 792.96 |
| | 3 | 1 | -344.000 | 237.455 | .489 | -1023.36 | 335.36 |
| | | 2 | 16.600 | 237.455 | 1.000 | -662.76 | 695.96 |
| | | 4 | 130.200 | 237.455 | .946 | -549.16 | 809.56 |
| | 4 | 1 | -474.200 | 237.455 | .230 | -1153.56 | 205.16 |
| | | 2 | -113.600 | 237.455 | .963 | -792.96 | 565.76 |
| | | 3 | -130.200 | 237.455 | .946 | -809.56 | 549.16 |
| Bonferroni | 1 | 2 | 360.600 | 237.455 | .890 | -353.75 | 1074.95 |
| | | 3 | 344.000 | 237.455 | 1.000 | -370.35 | 1058.35 |
| | | 4 | 474.200 | 237.455 | .379 | -240.15 | 1188.55 |
| | 2 | 1 | -360.600 | 237.455 | .890 | -1074.95 | 353.75 |
| | | 3 | -16.600 | 237.455 | 1.000 | -730.95 | 697.75 |
| | | 4 | 113.600 | 237.455 | 1.000 | -600.75 | 827.95 |
| | 3 | 1 | -344.000 | 237.455 | 1.000 | -1058.35 | 370.35 |
| | | 2 | 16.600 | 237.455 | 1.000 | -697.75 | 730.95 |
| | | 4 | 130.200 | 237.455 | 1.000 | -584.15 | 844.55 |
| | 4 | 1 | -474.200 | 237.455 | .379 | -1188.55 | 240.15 |
| | | 2 | -113.600 | 237.455 | 1.000 | -827.95 | 600.75 |
| | | 3 | -130.200 | 237.455 | 1.000 | -844.55 | 584.15 |

The post-hoc analysis confirmed there was no significant difference in the means between the controls and treatments for all 3 phenotypic characteristics (internode length, leaf number, stem length).

## 4.6. Investigating the causal agent(s) of leaf spotting and necrosis

### *4.6.1 Isolation of pure fungal cultures*

From all the infected leaf tissues, a total of 8 morphologically distinct fungal isolates were isolated and cultured as is shown in *Figure 46* below. Conceivably, a moderately wide range of diverse fungi were found to be growing on the marama leaf tissue.

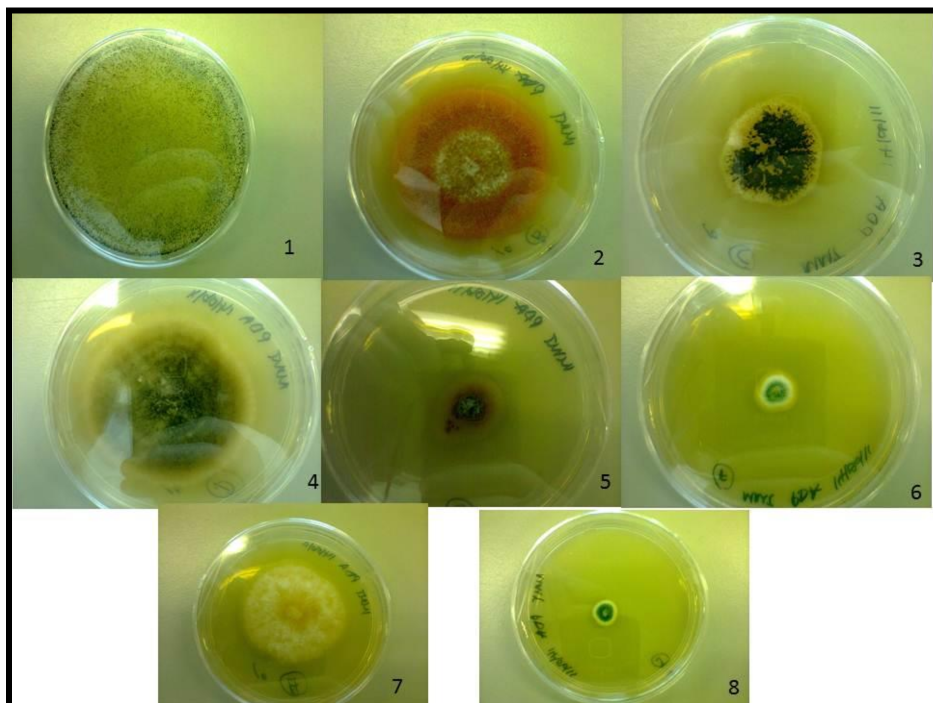


**Figure 46:** Pure cultures of the 8 morphologically distinct fungi isolated from the infected marama leaf tissue**.**

Infected marama seedling leaves were first surface-sterilized and then used in the form of leaf discs (2 mm X 2 mm), to inoculate PDA (potato dextrose agar) followed by an incubation at room temperature (25°C) for 5 days under white fluorescent light. For all isolates, pure cultures were then obtained by repeatedly sub-culturing hyphal edges onto new and fresh PDA at 25°C up until the desired pure cultures were obtained.

*4.6.2 Polymerase Chain Reaction*

From the PCR work undertaken, fungal ITS regions were successfully amplified for primer set ITS 1+4 (500-800 bp product) and primer set ITS 4+5 (750-830 bp product) and their associated nucleotide sequence data successfully obtained from Inqaba Biotechnology, Pretoria, South Africa. The electrophoretic results of the amplified ITS regions are shown in *Figure 47* below while the associated illustrative sequence information for the amplified ITS regions is presented in *Figure 47* below.

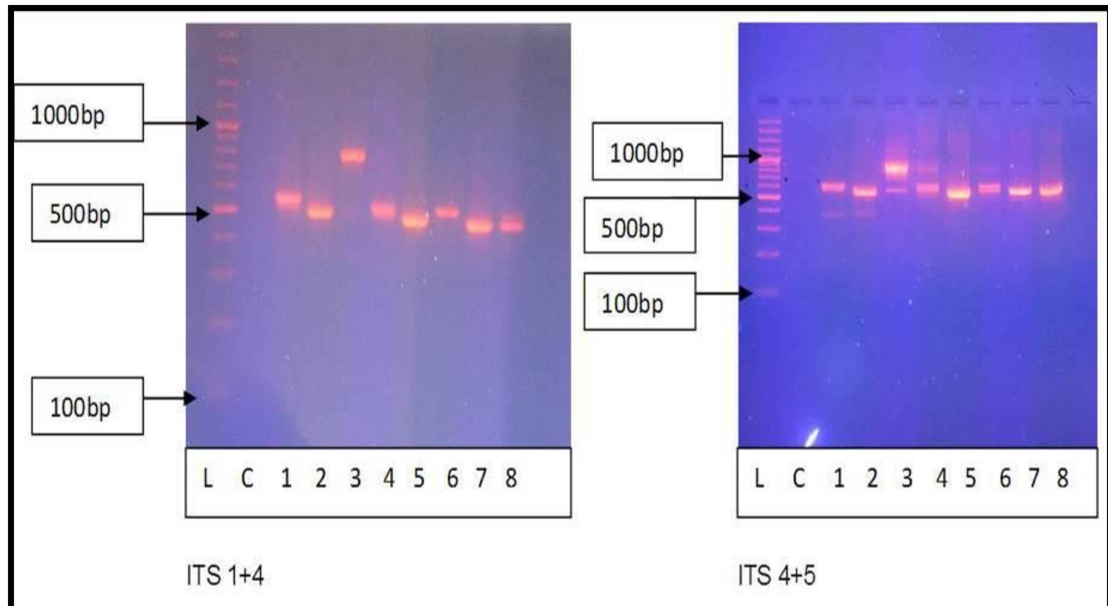


**Figure 47:** Electrophoresis gels of PCR amplification products of the ITS regions of fungal isolates found in marama leaf tissues**.**

Gels were resolved on 2% agarose stained with 1µg/mL ethidium bromide and run in 1X TBE buffer at 90 V for 1 hour.

*4.6.3 Illustrative sequence alignment*

The figure below (*Figure 48*) illustrates the molecular identification of fungal isolates isolated from the infected marama leaf tissue using the BLAST search tool in the NCBI database. Sequences presented in the figure are for fungal isolate 2.

*Epicoccum sorghi* isolate ALF60 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|KC106698.1|Length: 534Number of Matches: 3

Range 1: 1 to 534 GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 965 bits(522) | 0.0 | 531/536(99%) | 2/536(0%) | Plus/Plus |

```
Query  501   TCCGTAGGGTGAACCTGCGGAAGGATCATTACCTAGTAGYTGTAGGCTTTGCCTGCTATC  560
             |||||| ||||||||||||||||||||||||||||| || ||||||||||||||||||||
Sbjct  1     TCCGTA-GGTGAACCTGCGGAAGGATCATTACCTAG-AGTTGTAGGCTTTGCCTGCTATC  58

Query  561   TCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGTGGGTTCGCCCACCGATTGGAC  620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  59    TCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGTGGGTTCGCCCACCGATTGGAC  118

Query  621   AAACTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTC  680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  119   AAACTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTC  178

Query  681   AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTG  740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  179   AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTG  238

Query  741   TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATT  800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239   TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATT  298
```

**Figure 48:** Sequence identity of fungal isolate 2 from the infected marama leaf tissue and as is determined by the BLAST search tool in NCBI database. The identified isolate has a 531/536 similarity level to *Epicoccum sorghi*.

The summarized results for the molecular identification of all the fungal isolates studied in this work are shown in Table 7 below. Identification was done using the BLAST search tool in the NCBI database and sequence analysis by the CLUSTAL X tool.

**Table 7.** Concerted identities of the various fungi isolated from the infected marama leaf tissues.

| Test isolate | Plant part | Accession no. | Closest species [a] | Similarity (%, x/y) | Reference |
|---|---|---|---|---|---|
| 1 | Leaf | AM948959.1 | *Penicillium brevicompactum* | 94 % (506/540) | Kasana and Salwan (2008, unpublished) |
| 2 | Leaf | KC106698.1 | *Epicoccum sorghi* | 99% (531/536) | Goncalves et al. (2012, unpublished) |
| 3 | Leaf | JQ606829.1 | *Rhizopus stolonifer* | 100% (129/129) | Wicklow (2012, unpublished) |
| 4 | Leaf | JF491193.1 | *Alternaria solani* | 96% (504/526) | Chowdappa et al. (2011, unpublished) |
| 5 | Leaf | JQ690085.1 | *Fusarium equiseti* | 98% (543/553) | Zhao (2012, unpublished) |
| 6 | Leaf | AY373925.1 | *Penicillium olsonii* | 98% (573/583) | Haugland et al. (2004) |
| 7 | Leaf | EU520242.1 | *Fusarium chlamydosporum* | 98% (543/552) | Yu (2008, unpublished) |
| 8 | Leaf | FN597588.1 | *Fusarium incarnatum* | 99% (519/522) | Santori et al. (2009, unpublished) |

[a] Closest species was determined by comparing the ITS gene sequence of the test isolate with sequences in the NCBI database and then establishing the exact level of similarity. (x/y) = (Isolate sequence size/NCBI sequence size).

# CHAPTER 5: DISCUSSION

## 5.1 SSR screening for Molecular Marker Assisted Selection

The study sought to determine genetic diversity in phenotypically characterized marama bean plants and the usefulness of previously developed microsatellite markers in determination of phenotypic traits. The establishment of phenotypic characterization of marama bean plants was achieved and the linking of traits to microsatellite markers will allow progression to mapping efforts. The success of any breeding or genetic conservation programme is dependent on an understanding of the amount and distribution of the genetic variation present in the gene pool.

Traditionally, a combination of morphological and agronomic traits has been used to measure genetic diversity. The best known phenotypic data are those given by Mendel for the garden pea, *Pisum sativum* (Weir, 1996). Mendel observed seven characters (seed shape, cotyledon colour, seed coat colour, pod shape, unripe pod colour, flower position and stem length) in a series of crosses and gave the results of offspring of the resulting hybrid plants (Weir, 1996).

However, heterogeneous plants have many overlapping morphological, biochemical and physiological attributes (Purseglove, 1968; Wickremasinghe, 1979). In addition, most vegetative characteristics are influenced by environmental factors and show a continuous variation and a high degree of plasticity, this makes it difficult to identify discrete taxonomic groups (Wickramaratne 1981; Paul, Wachira, Powell & Waugh,

1997). Furthermore, those which are identified may not reflect true genetic similarities. In an attempt to overcome these problems, for example in tea, molecular techniques were used in combination with agronomic traits to monitor genetic variability (Wachira, Waugh, Hackett & Powell, 1995) and this was the approach adopted for marama bean.

The data presented herein support the link between previously developed SSR markers and two particular traits of interest in marama bean. Internode length was linked to Primer MARA 077 as discussed hereon and rate of emergence was linked to Primer MARA 039 as discussed later in this section. This does not mean these are the only loci involved in these traits as most traits are polygenic and controlled by several loci. Open pollinated populations of outbreeding crops such as corn, rye, the brassicas and many forage species have until recently changed little in general genetic structure following to their domestication. The reason is that nearly all individuals in such populations have a different multilocus genotype; moreover, in each generation, nearly all individuals are likely to intercross with several other individuals and the resulting ongoing extensive segregation and recombination breaks up favourable multilocus combinations of alleles. Segregation and recombination are also likely to insert large numbers of novel, untried genotypes into such populations each generation (Allard, 1999).

The locus MARA 077 was found to be multiallelic with 9 alleles being amplified for Group 1 plants. When the original single read sequence from which this primer was designed around the $(AAG)_5$ repeat was put through a BLAST search at NCBI it was

revealed that this locus was 100% similar to the *Arabidopsis thaliana* auxin efflux carrier family protein mRNA. Local accumulation of the plant growth regulator auxin mediates pattern formation in *Arabidopsis* roots and influences outgrowth and development of lateral root- and shoot-derived primordia (Blilou et al., 2005).

Auxin was the first plant hormone discovered by plant scientists. Phototropism where uni-directional light altered the growth of plant coleoptiles in grass seedlings was one of the first by botanists including Charles Darwin (Darwin & Darwin, 1881). A number of other researchers then showed that these effects could be induced by plant extracts which were subsequently shown to contain the chemical indole-3-acetic acid (IAA) (Hartmann et al., 2011). Primary sites for auxin biosynthesis include root and shoot meristems, young leaf primordia, vascular tissue and reproductive organs including developing seeds. It is no surprise therefore that this locus was found to be closely related to internode length in marama bean. This finding should however be taken with caution and does not suggest this could be the only gene involved for this trait as further characterization would be required considering there are likely to be more than 30 000 genes in marama and internode length being a continuous trait is likely to be multifactorial.

The 6 clusters formed in the dendogram either had short internode length plants (s) with medium internode length plants (m) or long internode length plants (l) with medium length internode plants (m) (Figure 30). This clearly distinguished the short and long internode classes and led to the association of locus MARA 077 with the trait. The diversity index for internode length was H’= 0.920, showing a high

phenotypic diversity for this trait. The weak correlation between internode length and internode number leads to the quest for the locus related to internode number which could not be identified with the primers screened here. Internode number was revealed to be the trait requiring further investigation as it is positively and strongly correlated to yield traits like leaf number, pod number and flower number. The more internodes present in a marama bean plant the more productive the plant is likely to be. Knowledge of correlations among characters is useful in determining the success of indirect selection of one trait for the other in an improvement programme for any crop (Falconer & Mackay, 1996).

Improvement of open-pollinated outcrossing populations depends on increasing the frequency of favourable alleles and favourable multilocus genotypes and at the same time, maintaining past gains. Genetic uniformity in mass-selected outbreeding populations is difficult and trueness-to-type for adaptation and high performance are features of the population as a whole and rarely characteristics of individual plants. In the late decades of the nineteenth century, corn breeders recognized that their populations were responding hardly at all to conventional mass selection procedures. This observation stimulated interest in alternative methods of population improvement, particularly in the development of F1 hybrid varieties. Since then, the methods that have been adopted with outbreeding crops have often featured the development of monogenotypic F1 hybrid varieties (Allard, 1999).

The study further described how the process of gamma irradiation was practically used as a method to induce detectable mutations in seeds of the marama bean (*T. esculentum*). After exposing the seeds to the various dosages of gamma rays (0, 50 and 100 Gy), the mutational effects of the applied radiation onto the exposed seeds were then studied by analysing the molecular features of an AGA microsatellite repeat region that naturally exists in one specific area of the total genomic DNA of these seeds. The region amplified by primer MARA 039 appears to be part of a polygalactorunase (PG).

PG activities have been shown to be associated with a wide range of plant developmental programs such as seed germination, organ abscission, pod and anther dehiscence, pollen grain maturation, fruit softening and decay, xylem cell formation, and pollen tube growth illustrating divergent roles for members of this gene family (Kim *et al*., 2006). A focus was placed upon the germination aspect for the discussion here as the irradiated seeds were seen to germinate faster than the controls. Germination is a process that begins with water uptake, stimulating enzyme synthesis or activating enzymes already present. We infer that the mutations induced in the 50Gy and 100Gy seeds could have led to protein changes leading to the faster germination rates observed in the irradiated seeds. The proteins involved may have led to increased transcription of germination genes.

When the MARA039 primer set was used to target and amplify the desired AGA microsatellite repeat region in the total genomic DNA of both the irradiated (50 Gy and 100 Gy) and non-irradiated (control) seeds, a number of amplicons were

successfully amplified and all in the expected size range of between 100 and 200 bp (~176 bp) (Figure 32). A subsequent sequence alignment of those amplified fragments using Cluster X revealed that while DNA from all control seeds had maintained its original 5-AGA repeat, DNA from the irradiated seeds had either gained or lost one to two more AGA repeats (Figure 33), an aspect which typically suggested that the used irradiation system was practically capable of inducing some marginal genetic mutations into the exposed seeds.

Furthermore, besides just altering the number of AGA repeat units, the irradiation system also could induce several point mutations in the exposed seeds (Figure 33). Apparently, when a single amplicon from the control seeds was used as a motif to BLAST search the NCBI database, it managed to return a single hit to which it had a 75% homologous similarity level, and this hit happened to be the non-catalytic beta sub-unit of a polygalacturonase (PG) enzyme from *Medicago truncatula* (Figure 34). This then thus proposed that the targeted and amplified AGA microsatellite repeat region in the marama bean could also be the non-catalytic beta sub-unit of a polygalacturonase enzyme of this plant.

In the plant family, polygalacturonases (PGs) are a group of enzymes which chiefly degrade cell wall pectin compounds (Swain, Kay & Ogawa, 2011) by primarily hydrolysing the α-1,4 glycoside bonds that naturally exist between two residues of galacturonic acid in the pectin chain (Swain et al., 2011; Ghiani et al., 2011). PGs are always strongly associated with the various and very important plant developmental processes such as seed germination (Kanai, Nishimura & Ayhashi, 2010), pollen tube

growth (Clarke & Gleeson, 1981; Mu, Lee & Kao, 1994), fruit maturation (Ghiani et al., 2011), lateral root outgrowth (Peretto et al., 1992), organ abscission (Taylor et al., 1990; Bonghi, Rascio, Ramina & Casadaro, 1992), seed pod dehiscence (Jenkins et al., 1996), and the intrusive growth of non-articulated laticifers (Wilson, Nessler & Mahlberg, 1976). In the process of seed germination, the hydration of a seed by water usually results in increased metabolic activities, which either stimulate the synthesis of new enzymes or literally enhance the activation of pre-existing ones, with the mobilization of reserves and digestion of the cell wall, weakening it and causing a consequent rupture of the integument by the radicle (Baskin & Baskin, 1998).

In their study Sitrit et al., (1999) reported that radicle protrusion from tomato seeds to complete germination requires weakening of the endosperm tissue opposite the radicle tip. In common with other cell wall disassembly processes in plants, polygalacturonases (PGs) may be involved and suggesting that PG is involved in cell wall loosening of the endosperm necessary for radicle protrusion from tomato seeds and in subsequent embryo and seedling growth. Konno, Yamasaki & Katoh (1983) suggested that exo-PGs act coordinately with other cell wall-modifying enzymes to promote remodelling and/or loosening of the plant cell wall, permitting growth from their work with thalloid liverwort (*Marchantia polymorpha*). The expression of LeXPG1 in a conspicuous band of cells in the tomato radicle tip and later throughout the expanding embryo was found to be consistent with a role for PGs in potentiating the cell elongation necessary for growth (Sitrit et al., 1999). The observations made in tomato and thalloid liverwort fall in line with the suggestion that gamma radiation

alters the polygalactorunase region in marama bean causing radicle protrusion to increase germination in irradiated seeds.

Now considering the proposed possible link between the AGA microsatellite repeat region and the non-catalytic beta sub-unit of the polygalacturonase gene in the marama bean, it was therefore not unusual to speculate for some possible phenotypic changes in the germination profiles of those seeds whose microsatellite regions had been altered by exposure to the applied gamma irradiation. Not surprisingly, all irradiated seeds somewhat showed some relatively high emergence rates (Figure 31A) as well as some relatively enhanced germination percentages (Figures 31B) when compared to their non-irradiated counterparts, and thus suggesting to us for a possible induction of some relatively positive and somewhat favourable mutational effects onto the exposed seeds by the applied gamma irradiation, which eventually improved their emergence and germination efficiencies. In this regard, it appears as though that the generated and acquired mutational changes (both the repeat unit changes and point mutations) in the microsatellite repeat region of the marama bean seeds could have had favoured some conformational and structural changes in the non-catalytic domain of the polygalacturonase gene that then eventually enhanced the catalytic activity of this enzyme and finally resulting in improved emergence and germination profiles of the irradiated seeds.

By summing up all these findings, it is conceivable to state that the process of gamma irradiation can be potentially used as a practical method in both the marker-assisted and mutational breeding programs of the marama bean for its ultimate

improvement as an indigenous legume of the Kalahari regions of Africa. Notably, the fact that the observed induced mutations tended to target a microsatellite region that seems to be part of an enzyme with central roles in various important plant developmental processes, typically makes this suggestion an imminent possibility.

Apparently and in some previous domestication efforts of the marama bean, researchers have frantically been trying to search for ways to reduce the seed cycle of this legume, which apparently appears to be not less than 18 months (unpublished data) but to no success. However and based onto the findings here, the approach of mutational breeding may be a possible option to attempt and reduce this lag period by producing new mutants with early and improved germination efficiencies. Furthermore, the fact that the PG enzyme is also primarily involved in earlier fruit maturation means that its potential modification to favour both the early seed germination and early fruit maturation would strongly augment the practical shortening of its seed cycle in the developed marama bean mutants and probably making this wild legume an ideal candidate for possible domestication and becoming one of the mainstream crops for the -Kalahari regions of Africa.

## 5.2 Karyotyping

The chromosomes of *Tylosema esculentum* were successfully stained and compared with those of *Pisum sativum* L. The haploid number of chromosomes in pea was reconfirmed as 7 (2n=14) as was found by Murtaza, Ahmed & Majid, 2005 in their karyotype analysis of *Pisum sativum* L. That of marama bean was 22 haploid

chromosomes in the genome (2n=44). The marama bean genome is probably double that of common bean whose chromosome number were found to be 2n=22 on all Phaseolus cultivars (Cimpeanu, Cimpeanu, Capraru & Soiman, 2005).

A tetraploid ploidy level has been reported for *Tylosema fassoglense* (Goldblatt & Davidse, 1977). It is possible *Tylosema esculentum* also has a tetraploid genome structure, however flow cytometry and counting would need to be performed to confirm this. The difference in size of pea and marama chromosomes further demonstrates that chromosomes differ greatly in size and number. Between organisms, the size difference can be over 100-fold while with a species, some chromosomes are often 10 times as large as others (Siva, 2011).

A larger chromosome number means the organisms can code for more and have more room for duplicates (backups) in case something goes wrong. In general, species having lower chromosome number have longer chromosomes than those having higher chromosome numbers. This was demonstrated clearly in the comparison between marama bean and garden pea (Figure 40). In plants, dicots in general have a higher number of chromosomes than monocots. Chromosomes are longer in monocots than in dicots. Genome size varies widely among legumes, with pea having a genome size 10 times that of some related diploid genera (Choi *et al*., 2004).

### 5.3 Regeneration- Grafting

This study explored the possibility of grafting as a method of side stepping juvenility. *In vitro* grafting experiments conducted to determine their potential for improving pulse crop breeding techniques for pea, faba bean and lentil had been reported to be successful (Gurusamy et al., 2012). In our case however we were not able to graft marama bean *in vivo* with a minimum of 5 replicates in each case.

Tuberous root plants like marama bean have special swollen root systems attached to the crown in these herbaceous perennials. They are usually biennials and produce in one season after which they are dormant and the herbaceous shoots die. The tuberous roots serve as storage organs to survive dormant periods. In the spring, the buds produce new shoots, which utilize the food materials from the old root during their initial growth (Hartmann et al., 2011). This mechanism was thought to be the reason why grafting failed to be successful in marama.

The root stock of the marama bean plants regenerated a new stem from the tuber as they would normally do if the season were changing from dormancy to spring. Instead of growing into the scion, new shoots emerged from the root. Alternatives to grafting like tissue culture are the next step following the failed grafting attempts and the revelation that due to the anatomy of marama bean grafting may not be the best option for shortening juvenility. Vegetative propagation is often more satisfactory in these types of plants with stem, leaf or leaf bud cuttings. An *in vitro* approach as shown in other protein legumes to shorten generation cycles may yield better results

(Ochatt et al., 2002; Gurusamy et al., 2012). The rationale for development of grafting tools in genetic improvement strategies for large-seeded annual pulse crops like marama bean relates to overcoming problems of seed multiplication caused by the relatively low numbers of seeds produced per pollination.

### 5.4 Germination of marama bean under greenhouse setting

A reliable and robust method of growing *T. esculentum* under greenhouse conditions was sought. The low germination rates observed with Group 1 plants led to the investigation of a method to obtain more plants to be able to carry out further investigations in subsequent groups. A working protocol was established to generate marama bean plants in a greenhouse setting in Namibia.

It was necessary to imbibe seeds in water for at least 24 hours to induce germination in petri dishes before transferring emerging seedlings to potting soil in black bags. Where possible the time to induce germination was reduced even further by the use of incubators set at 30°C to imbibe seeds. The results presented here with improved germination rates after a pre-soaking treatment concur with the findings of Travlos et al., (2007) who found no physiological dormancy in marama bean seeds and improved germination rates with scarification with sand paper, pre-soaking in water and also sulphuric acid. Growing the plants as seedlings before transferring them to cultivation fields will enable farmers to overcome the problem of low germination rates in some ecotypes of marama bean.

It was observed that larger seeds of *T. esculentum* took a shorter time to germinate than smaller seeds. This is probably due to the establishment of a mature embryo in larger seeds than smaller ones where the embryo may have been aborted and also that larger seeds have more food reserves stored in them for the new embryo. By definition, germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis (Bewley & Black, 1994). The visible sign that germination is complete is usually the penetration of the structures surrounding the embryo by the radicle as was shown in Figure 42, the result is often called visible germination (Bewley, 1997).

**5.5. Investigating growth promoting factors**

The effect of nutrients, high moisture and hormone treatment on the growth habit of marama bean was investigated to see if these treatments could be used to improve plant growth. Marama naturally occurs in very dry (low water holding capacity) soils which are nutritionally poor and low in organic matter, including nitrogen (Dakora, et al., 1999; Mitchell, et al., 2005) and thus the question was whether or not growth promoting factors could be used to improve growth and therefore yield and productivity of marama bean. There was no significant difference found between controls and marama bean plants treated with high water, fertilizer or gibberellic acid.

Prehistoric farmers may have started fertilizing their fields after noticing that grass grew faster and greener where animals had defecated. The Romans used to fertilize

the crops and Native Americans buried fish along with seeds when they planted corn. Today, most farmers use commercially produced fertilizer containing minerals mined or prepared by industrial processes. Fertilizers are usually enriched with nitrogen, phosphorus and potassium (NPK), the three elements that are most commonly deficient in farm soils (Reece et al., 2011). Nitrogen is a component of proteins, nucleic acids, chlorophyll and certain coenzymes whilst potassium is involved in osmotic and ionic balance, opening and closing of stomata and activation of over 40 enzymes. Phosphorus functions in nucleic acids, phospholipids and energy transfer via ATP (adenosine tri-phosphate).

Human activities generate or aggravate soil problems including nutrient mineral depletion, soil erosion and accumulation of salt. It was on this background that the study explored the effect of enriching soil with NPK fertilizer on the growth of marama bean. Commercial inorganic fertilizers as the one we used are manufactured from chemical compounds and their composition is known (Solomon, Berg & Martin, 2002). Because they are soluble, they are immediately available to plants. These fertilizers are available for only a short period compared to organic fertilizers because they quickly leach away and this may be a reason why no significant difference could be observed between control plants and those treated with fertilizer in this study.

It is well known that limiting water availability causes plants to switch into reproductive mode in order to survive by investing into seed production and ensuring they will continue into future generations. The observations we have made in the

wild however are from plants that are already mature and have been growing for several seasons. The investigations in this instance required us to start with understanding the growth habit from a seed to seedling stage through to the time of flowering and producing seed. We sought to answer some basic questions on the vegetative growth as well in line with the main objective which is to shorten the juvenility period, we also needed to confirm if indeed it takes 2 seasons to reach flowering. The hypothesis was that increasing biomass rapidly by increasing water and nutrient availability would lead to acceleration of triggering the biological signal for the plant to switch from vegetative to the reproductive phase through accelerating vegetative maturity if the critical plant size was reached in a shorter time. Short vegetative phase was thought would lead to a shorter time to reach the reproductive phase.

The fact that marama already occurs in a low nutrient, low moisture environment is what led to the exploration of improving water availability in efforts to accelerate the transition from juvenile seedling to adult vegetative and to adult reproductive as it is normally only during the adult vegetative phase that plants are capable of forming reproductive organs, and day length-dependent plants can be induced to flower by photoperiodic induction during this phase (Huijser & Schmid, 2011). Although water stress is known to trigger early flowering in adult plants the approach here was in attempting to get the plant to the adult vegetative phase faster which would in turn shorten the time taken to reach to adult reproductive phase. Travlos and Karamanos experiment of 2008 found that water shortage significantly reduced leaf and stem growth of marama in comparison with well-watered controls.

The results presented here are in contrast with their finding as the well watered plants in our case had no significant difference in stem length, internode length and leaf number with the controls (Figure 45). An alternative approach for future work would be to investigate the effect on drought stress on the vegetative growth but more particularly the reproductive growth of plants of *T. esculentum* as Travlos & Karamanos' study noted a reduction in stem length and leaf number. Another point of view maybe that there is no need for further investigation of the water relations as high water inputs would not be suitable for the targeted farming communities that would typically grow marama bean as a crop.

Stem elongation and flowering usually occur simultaneously in long day plants under the influence of gibberellic acid (Cleland & Zeevaart, 1970; Blázquez, Trénor & Weigel, 2002). In a natural setting, light and specifically photoperiod is also known to regulate the biosynthesis of GAs. As shown in this work, an increase in $GA_3$ had no significant effect on stem length, internode length and leaf number in marama bean (Table 4, 5 & 6) in contrast for example to Ouzounidou et al., (2010) who found that at 100µM $GA_3$ among several plant growth regulators (PGRs) was effective in promoting flowering and better for vegetative characteristics in peppers (*Capsicum annuum*). The results therefore suggest that treatment of marama bean with gibberellic acid may not be necessary to side step juvenility in the vegetative plants. Information regarding the effectiveness of PGRs on growth and other physiological parameters of *Tylosema esculentum* is still scarce.

### 5.6. Investigating the causal agent(s) of leaf spotting and necrosis

To our best knowledge, this is the first report of identifying fungal species that can affect marama leaf tissue and probably having a role as disease-causing pathogens and/or plant growth promoting endophytes. In this case, a moderately wide range of fungi (8 isolates) were isolated (Figure 46) and identified (Table 7) and therefore, the exact and specific disease-causing pathogen in this plant and at this point, could not be easily and clearly spelt out. Hence some further investigations using approaches like the Koch's postulates or other methods would need to be undertaken in order to exactly determine which of these fungal isolates is and/or are really pathogenic to the marama plant. Indeed there is surely a possibility that one or more of these 8 isolates is and/or are the actual causal agent(s) of diseases in the marama plant while the other candidates could just be opportunistic or mutualistic partners that simply take advantage of the already established leaf infection.

Vega et al. (2006) in their previous study, found *Penicillium brevicompactum* to be present as an endophyte in coffee leaves collected in Colombia, Hawaii and at a local plant nursery in Maryland (Vega et al., 2006). Based on this premise, it is also highly possible that the Penicillium species isolated here could also have been present in the marama leaves as an endophyte. *Epicoccum sp*. and *Fusarium sp.* are the causal agents of sooty mold and leaf blight respectively in broad bean (French, 2006).

The presence of *Epicoccum sorghi*, *Fusarium equiseti*, *Fusarium chlamydosporum* and *Fusarium incarnatum* in the infected marama plant could primarily have contributed to the necrosis and leaf wilting observed in the growing seedlings. *Alternaria solani* is the causal agent of early blight disease in tomato and is responsible for significant economic losses sustained by tomato producers each year (Spletzer & Enyedi, 1999). It is also the causal agent of early blight disease of not only commercially-produced tomatoes but also potatoes and eggplants too. Early blight may affect the foliage, stems, and fruits of infected plants (Jones, Jones, Stall & Zitter, 1991). Therefore, the presence of *Alternaria solani* among the fungal species isolated from the infected marama leaves is thus not surprising as foliage was affected at the seedling stage similar to what happens in tomato, potato and eggplant seedlings.

*Penicillium olsonii* is among the rarest clearly distinct species of the genus *Penicillium* which are often associated with post-harvest diseases or are pathogenic to intact growing plants (Wagner, Kusserow & Schäfer, 2000) while *Rhizopus stolonifer* is found as a pathogen to vegetables, fruits and ornamentals (Kwon, Kang, Kim & Park, 2001). Thus these two can also possibly be potential pathogens to the vulnerable marama. A previous report of fungi found in marama bean was that of Uzabakiriho, Shikongo and Chimwamurombe (2013) where two isolates associated with necrotic Marama pods were identified as *Alternaria tenuissima* and *Phoma* spp.

Overall, there were 2 *Penicillium* species and 3 *Fusarium* species identified in this study, as well as a single isolate each of the genus *Epicoccum*, *Alternari* and

*Rhizopus*. These fungi are commonly known to be involved in plant diseases of some common legumes and other agronomically important crop plants. Conceivably, the presence and involvement of these fungal species in marama leaf decay should be noted with great concern and interest as marama bean has since been identified and earmarked as a potential leguminous crop for possible domestication. The fungal species identified here can probably be potential pathogens that could adversely affect yields in marama bean in the case of this plant being adopted for both domestication and farming efforts.

The *Penicillium*, *Fusarium*, *Epicoccum*, *Alternari* and *Rhizopus* species identified here if found to have a disease causing effect on marama will lead to a need to search for genotypes that may display resistance to any disease caused by their presence in marama bean. The identification of disease resistant genotypes will ensure the risk of loss of crops due to fungal disease is reduced.

# CHAPTER 6: RECOMMENDATIONS

For scientific studies of finding association between molecular markers and quantitative trait variability, large populations are required. Future studies should select one ecotype and go into depth in characterizing more plants for phenotypic class rather than collecting seeds from many ecotypes. Parents of a mapping population must have sufficient variation for a trait of interest at the level of both DNA sequence and phenotype. The magnitude of phenotypic and marker variation is of utmost importance, especially when the goal of genetic mapping is to find genes controlling specific traits. When the parents represent extreme phenotypes, it is likely that genetic variation exists between the two phenotypes, provided that these differences are not totally environmentally based.

Plant breeding has evolved and is no longer limited to simple selection, but is also concerned with the creation, selection, evaluation and multiplication of desirable genotypes. The process of mutation breeding can also be shortened by new techniques such as *in vitro* culture, molecular markers as well as the use of mutagenesis to increase the genetic variability of plants. The mutation breeding technique has led to the recent significant increase in cultivars released and applying it to emerging crops like the marama bean will eventually benefit its production. Thus based on findings of this work, the use of gamma irradiation in the marama bean for the induction of various favourable mutations seems a very feasible approach that will result in the generation and establishment of mutants with very

important and most desirable agronomic traits such as early seed germination and early fruit maturation.

The high levels of genetic and phenotypic diversity and minimal differentiations between populations could be a result of the tetraploid ploidy level observed in *T. esculentum* as increased heterozygosity is associated with autotetraploidy. Flow cytometry experiments will be needed to sort the chromosomes by size and confirm the suggestions made here about the ploidy level. Next Generation Sequencing will also be useful in order to conclude on the estimated genome size.

Vegetative propagation methods from stem and tuber cuttings will need to be explored to side stepping juvenility as grafting was found not to be feasible for marama bean. The use of intensive inputs for agricultural cultivation of this legume was found to be unnecessary as the use of high water and growth promoting factors like fertilizer and hormones were found to have no significant effect on the growth of the marama bean. This should be good news for farmers as the low-input nature of this plant has been confirmed and this should make it an attractive option for domestication despite the challenge of the long juvenility phase.

The fungi identified on marama leaf tissue could have had some endophytic and/or pathogenic roles in the infection process of the affected plant of which whatever role(s) each species may or might play in this process still needs to be properly established through further investigation. This area has been less explored to date and the next steps would be to test Koch postulates and survey marama seeds and leaf

tissue from different regions for potential pathogens as these are the vital organs related to yield. Resistance genotypes would also be identified through these surveys.

# CHAPTER 7: CONCLUSION

The utility of microsatellites to be profitably utilized in marama bean not only for detecting polymorphism and trait association but also genotype identification and for estimation of genetic diversity was demonstrated with MARA 039 and MARA 077 being linked to germination and internode length respectively. It is evident that microsatellites can be successfully used in linking traits of marama bean as markers if explored further. The availability of the marama bean genome sequence and establishment of high throughput genotyping platforms will be expected to further accelerate Molecular Marker Assisted selection in marama bean improvement.

The chromosome number was confirmed for marama bean and found to be 2n=44 and that of garden pea was reconfirmed as 2n=14. Chromosomes in marama bean were smaller than those in pea though more numerous suggesting that the genome size of *Tylosema esculentum* is likely to be smaller than that of *Pisum sativum* L. The results suggested marama maybe an autotetraploid as all plates used for the chromosome counts were from the same species.

The use of high cost agricultural inputs such as high water and growth promoting factors like fertilizer (NPK) and hormones were found to have no significant effect on the growth of the marama bean. This is good news for farmers as the marama crop will be a low input cost option for cultivation. The method established for growing plants in greenhouses to pre-screen viable seeds for planting will also help farmers make effective use of land when cultivating marama bean.

The early identification of potential pathogens to marama bean led to a relatively wide community of the fungal organisms being associated with the leaf decay symptoms of young marama bean seedlings. The fungal species were isolated and identified using molecular techniques whereby the ITS DNA region of the isolated fungi were amplified and sequenced followed by a comparison of the obtained sequences with the GenBank. From this attempt, the presence of a complex of fungal strains with 8 known species was revealed: *Penicillium brevicompactum, Epicoccum sorghi, Rhizopus stolonifer, Alternari solani, Fusarium equiseti, Penicillium olsonii, Fusarium chlamydosporum* and *Fusarium incarnatum.* Mitigation strategies to defend marama plants from the potential diseases caused by these fungi can already be investigated.

Overall, the present study managed to answer the basic questions that were unclear about marama at the initiation of the study but at the same time many more questions were uncovered. The domestication efforts for the species *Tylosema esculentum* need to be augmented with intensive government aided programs and the establishment of dedicated research laboratories or research stations in the areas where the species is found occurring.

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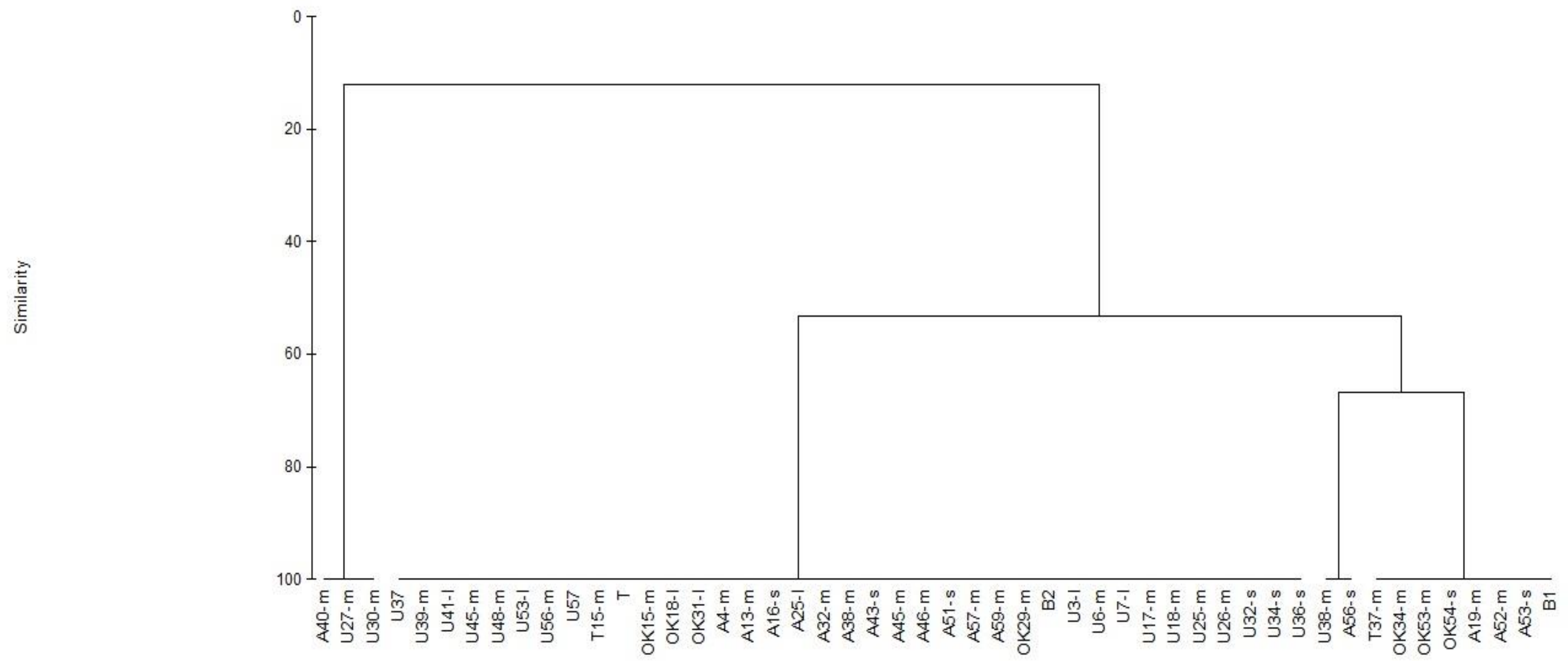
# APPENDIX A

Group 1 Dendograms



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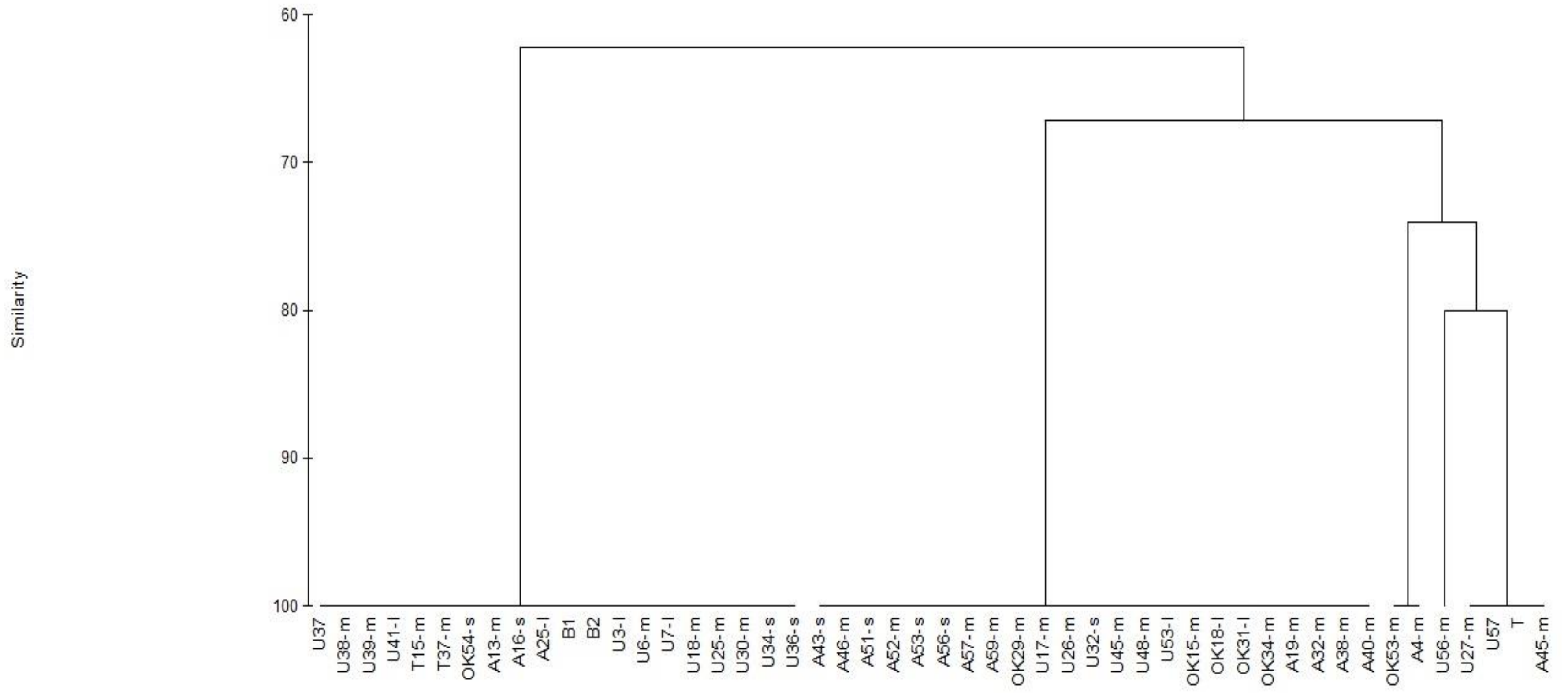

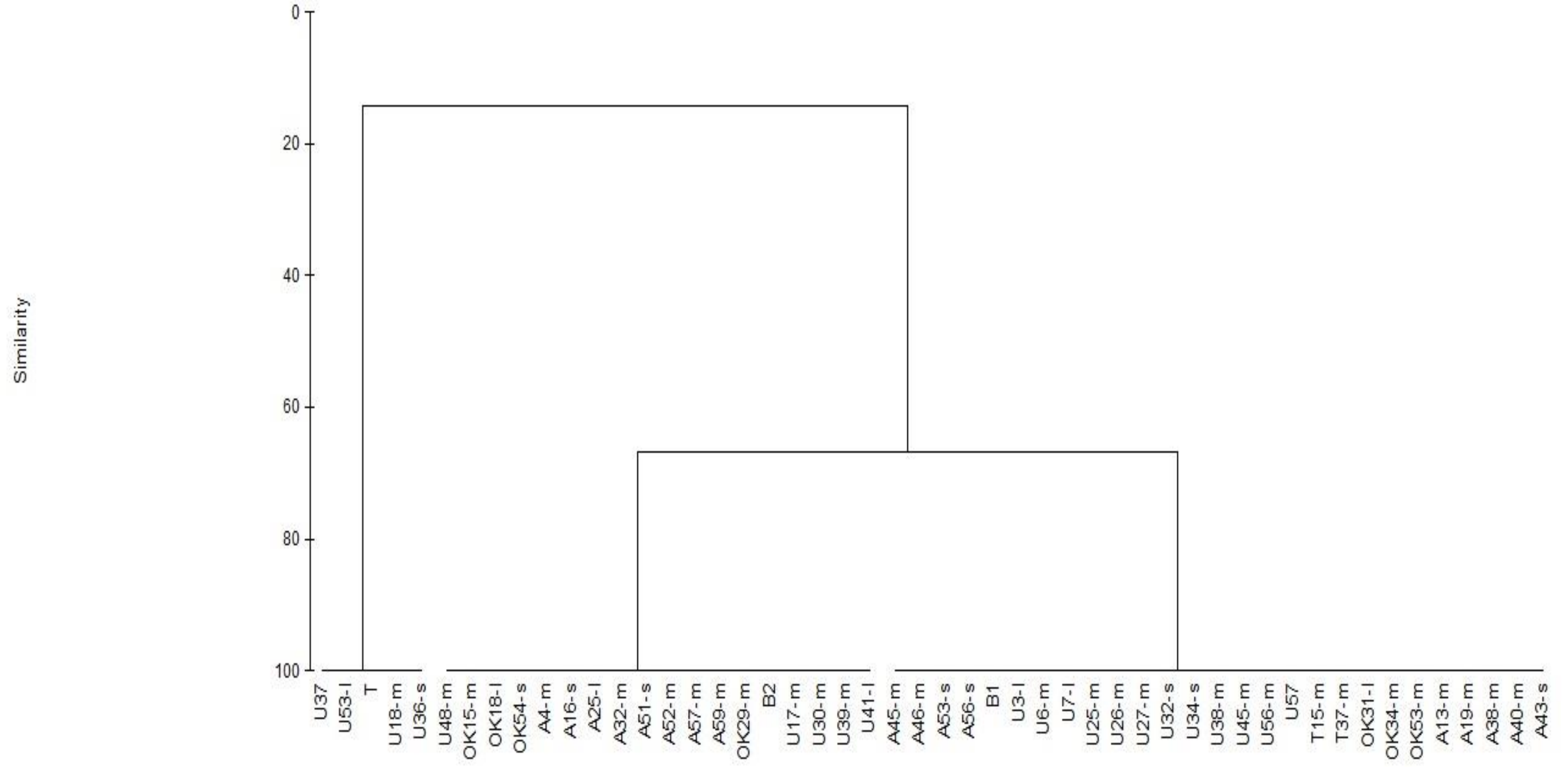
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100
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U53-l
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U18-m
U36-s
U48-m
OK15-m
OK18-l
OK54-s
A4-m
A16-s
A25-l
A32-m
A51-s
A52-m
A57-m
A59-m
OK29-m
B2
U17-m
U30-m
U39-m
U41-l
A45-m
A46-m
A53-s
A56-s
B1
U3-l
U6-m
U7-l
U25-m
U26-m
U27-m
U32-s
U34-s
U38-m
U45-m
U56-m
U57
T15-m
T37-m
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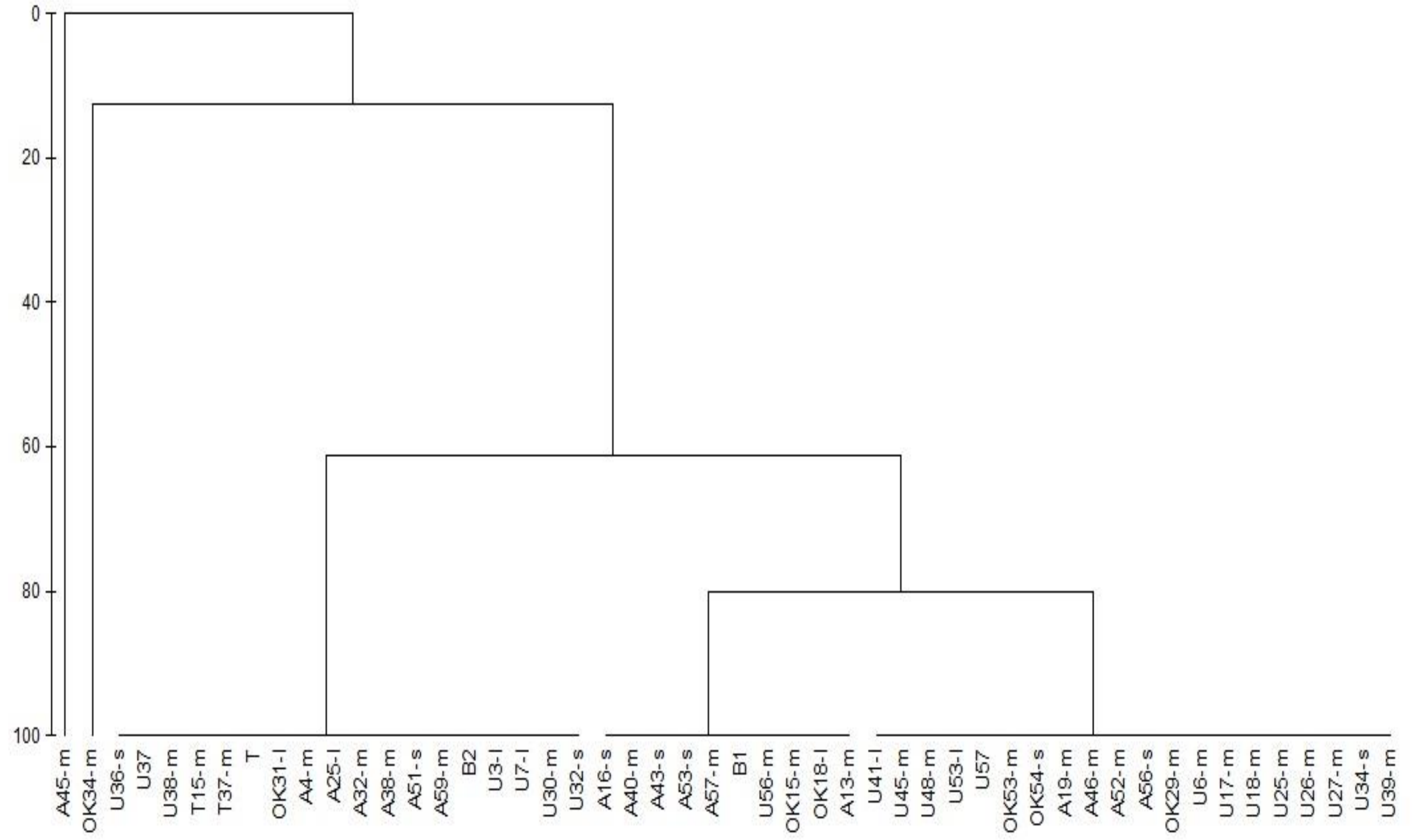
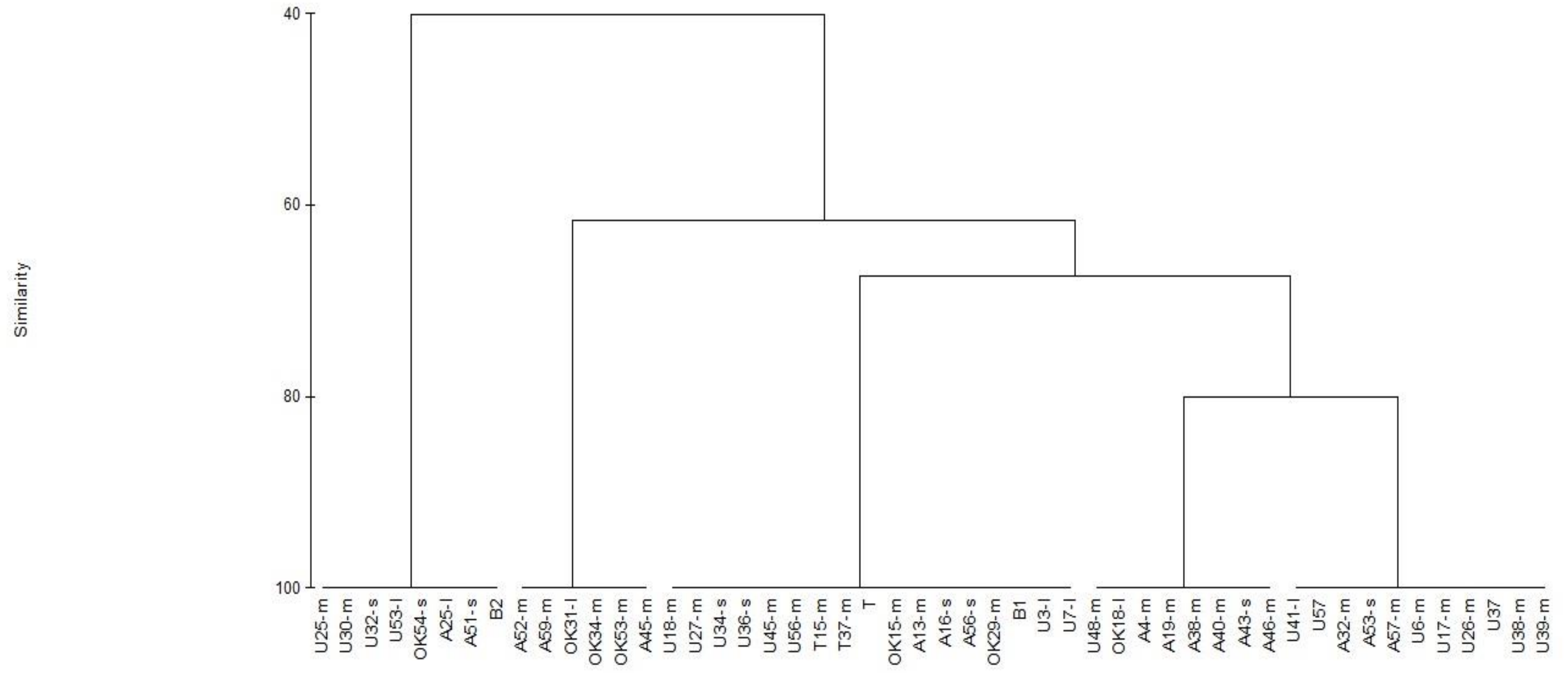
OK34-m
OK53-m
A13-m
A19-m
A38-m
A40-m
A43-s

*MARA 039*

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OK54-s
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B2
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A59-m
OK31-l
OK34-m
OK53-m
A45-m
U18-m
U27-m
U34-s
U36-s
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U56-m
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T
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A13-m
A16-s
A56-s
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B1
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U7-l
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U37
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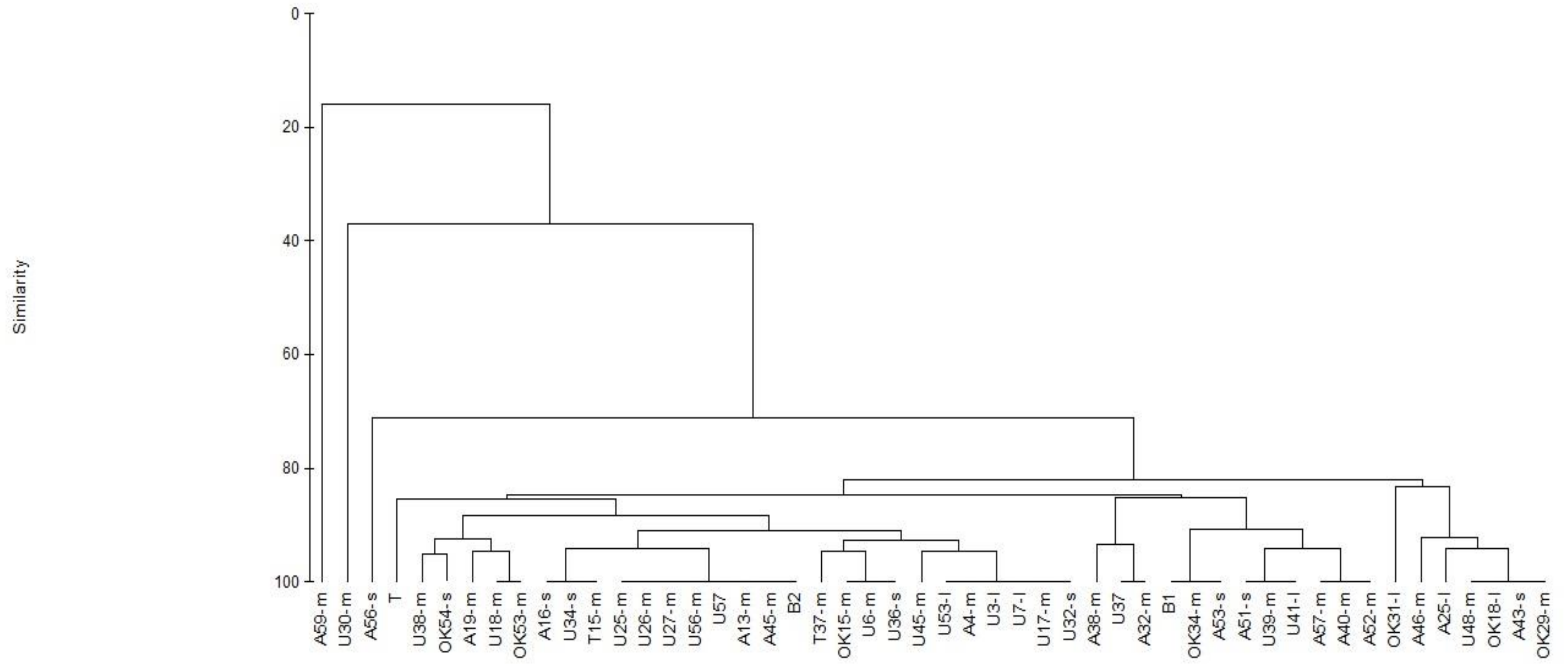
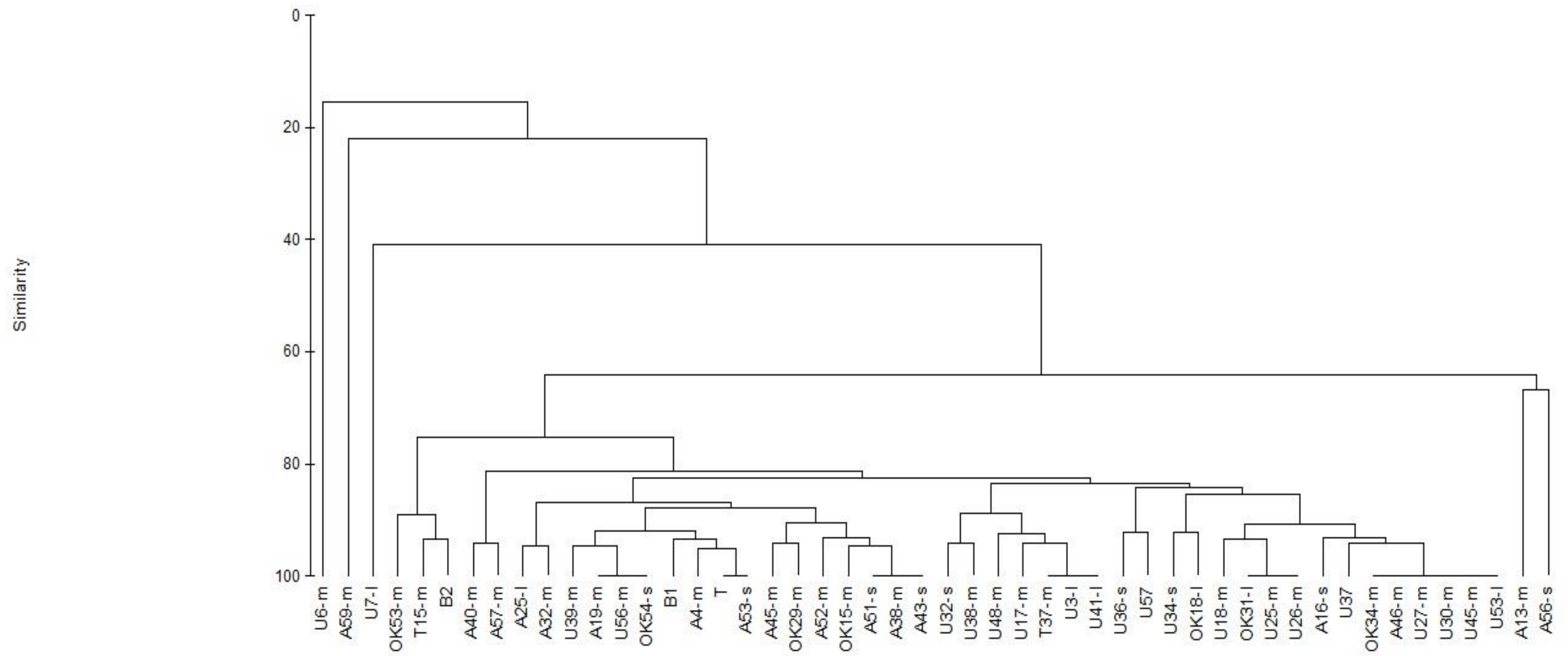
U39-m

*MARA 068*

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B2
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A57-m
A25-l
A32-m
U39-m
A19-m
U56-m
OK54-s
B1
A4-m
T
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OK29-m
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OK15-m
A51-s
A38-m
A43-s
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U38-m
U48-m
U17-m
T37-m
U3-l
U41-l
U36-s
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OK31-l
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U26-m
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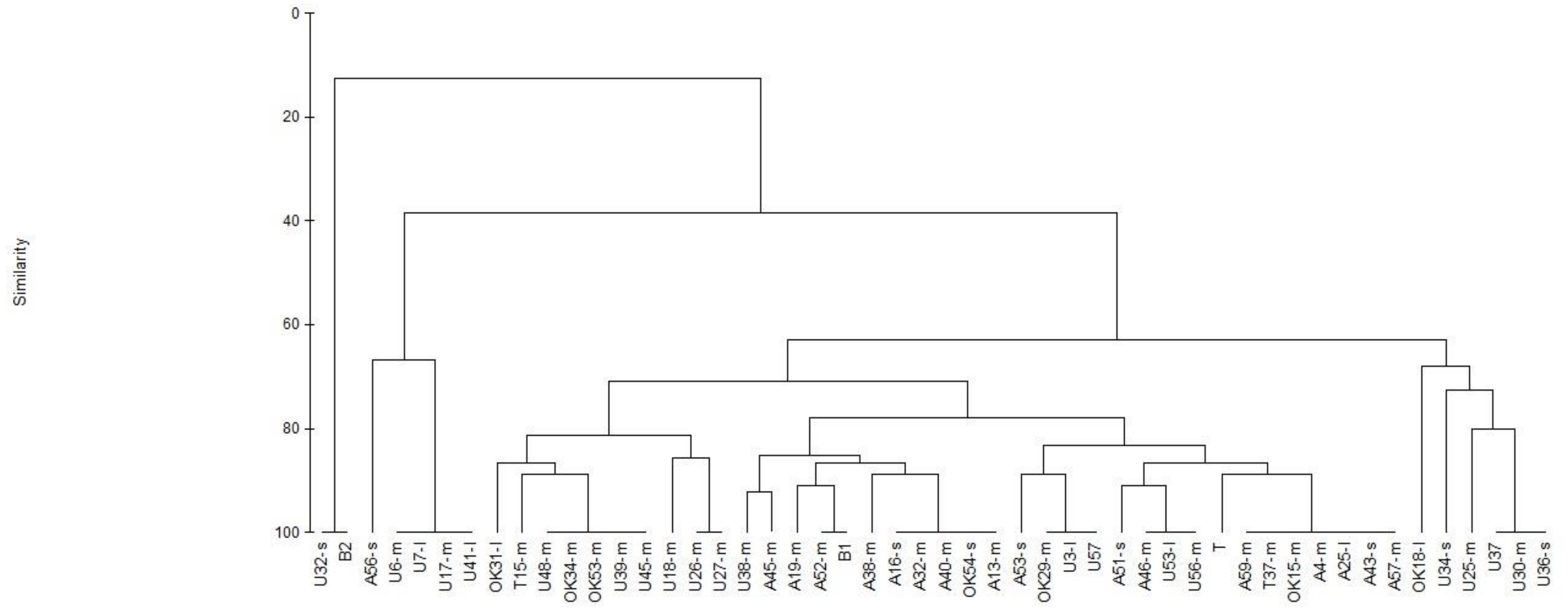
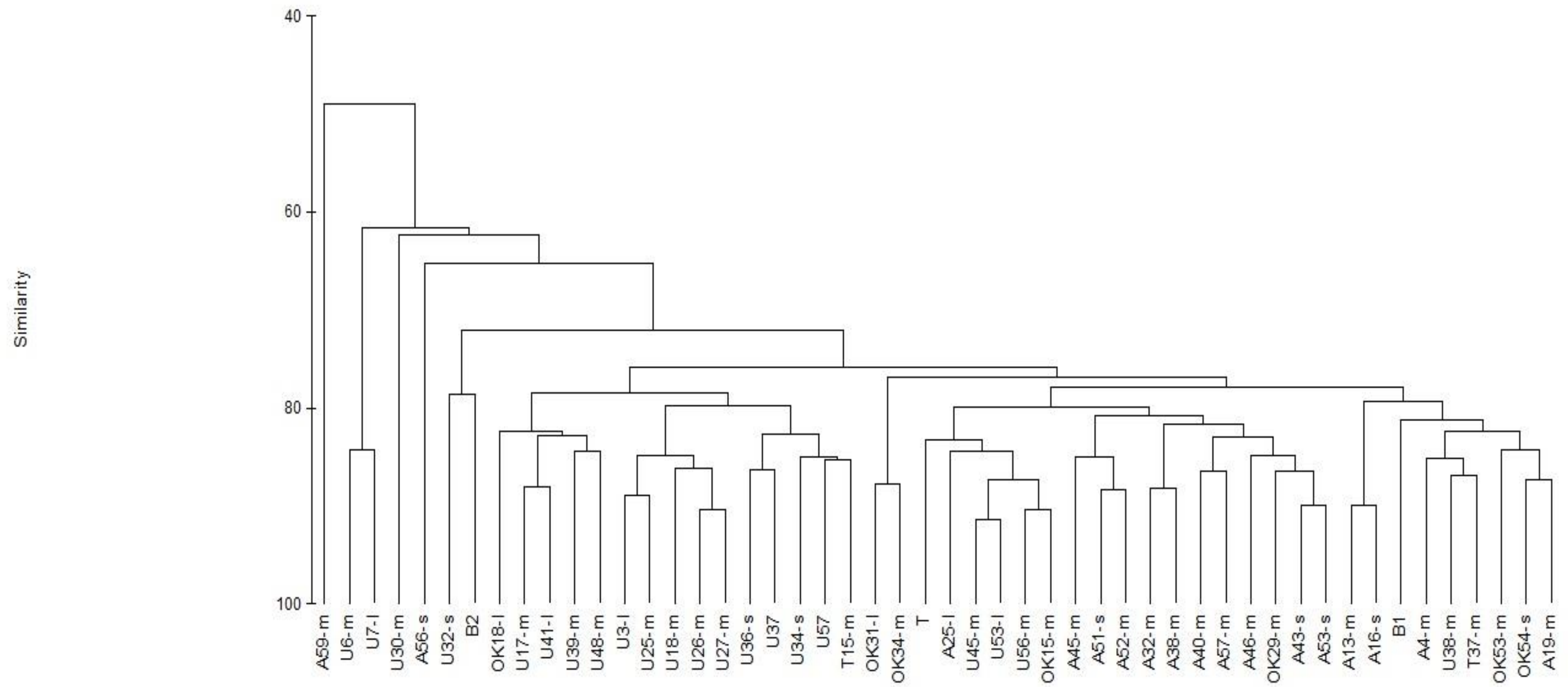
A56-s

*MARA 074*

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U7-l
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A56-s
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B2
OK18-l
U17-m
U41-l
U39-m
U48-m
U3-l
U25-m
U18-m
U26-m
U27-m
U36-s
U37
U34-s
U57
T15-m
OK31-l
OK34-m
T
A25-l
U45-m
U53-l
U56-m
OK15-m
A45-m
A51-s
A52-m
A32-m
A38-m
A40-m
A57-m
A46-m
OK29-m
A43-s
A53-s
A13-m
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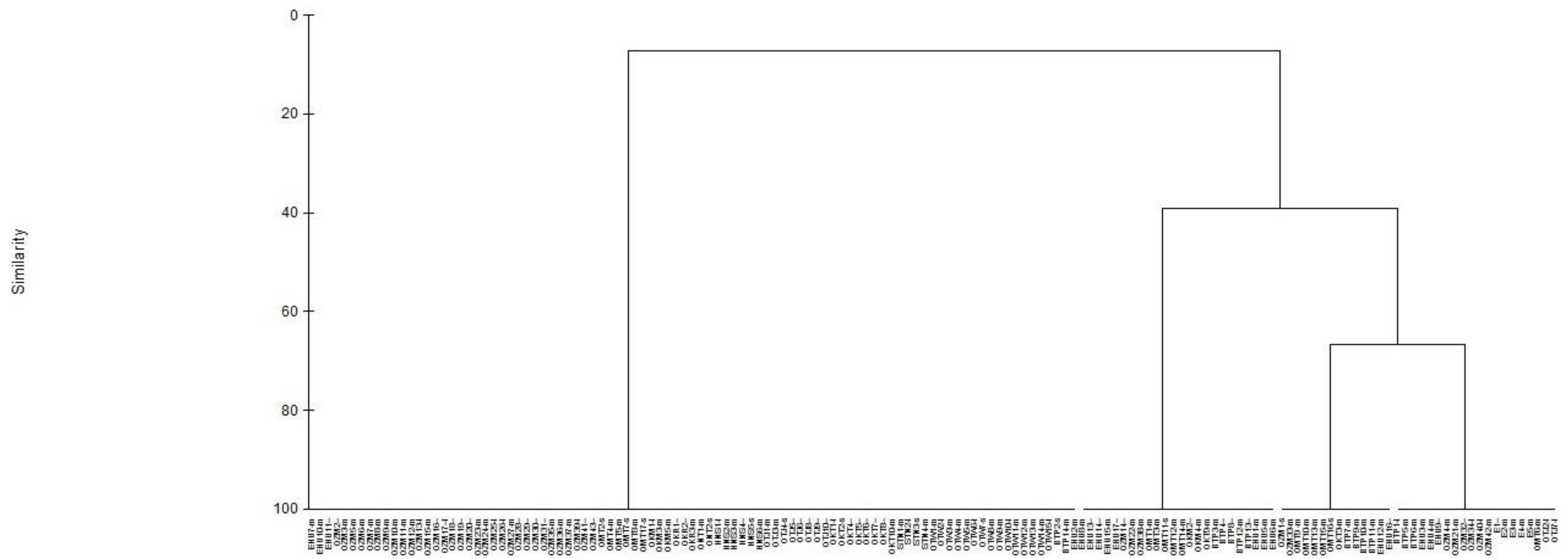
OK54-s
A19-m

# APPENDIX B

Group 5 Dendograms



*MARA 037 internodes*

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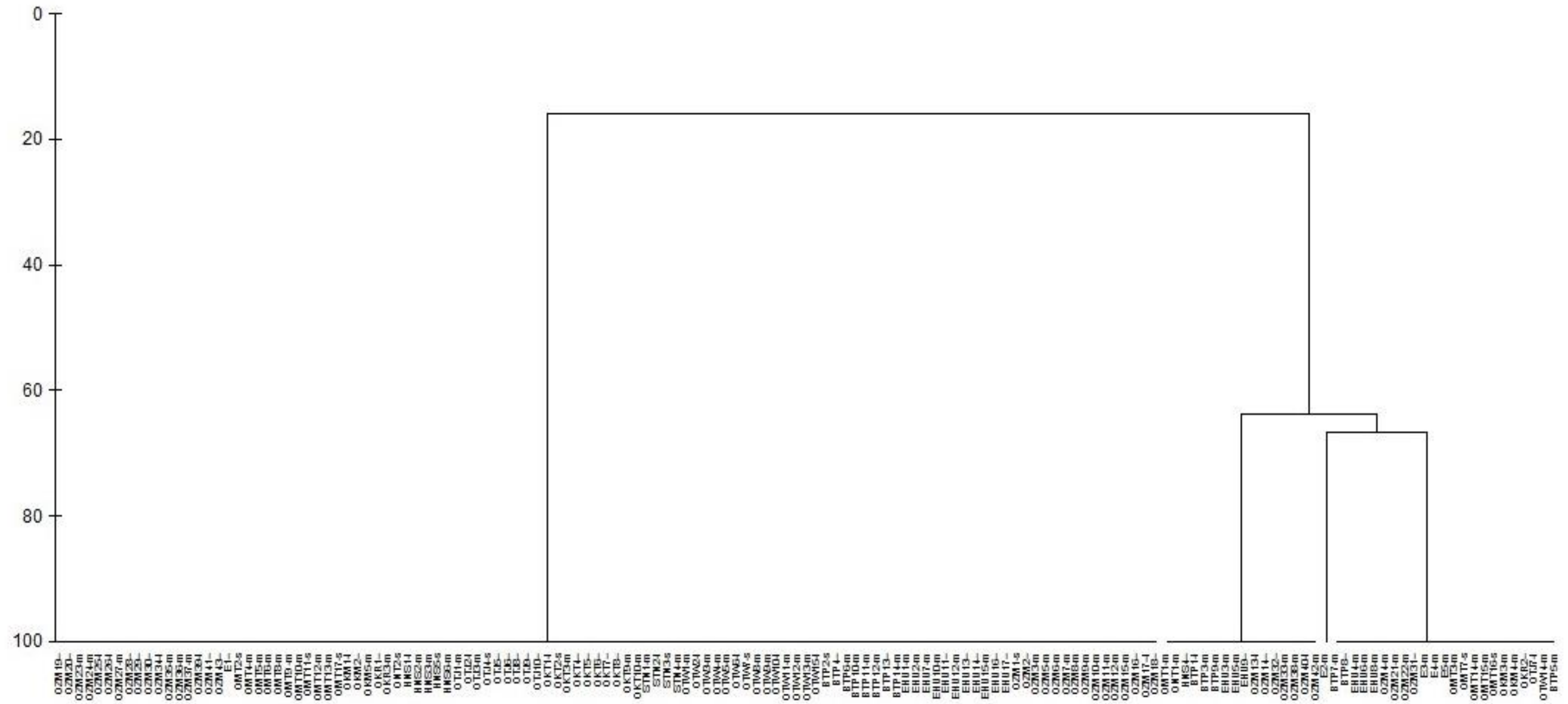

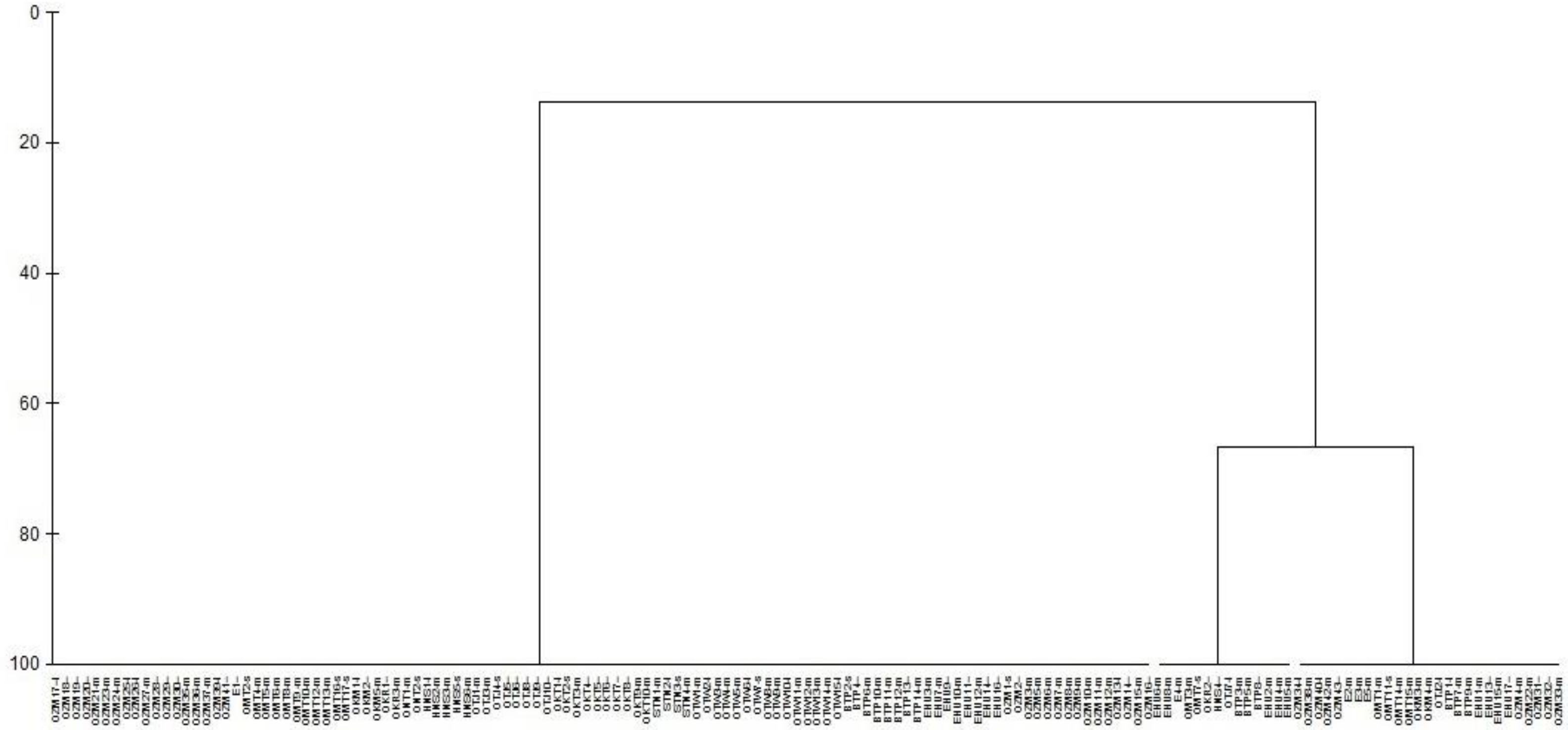
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Similarity
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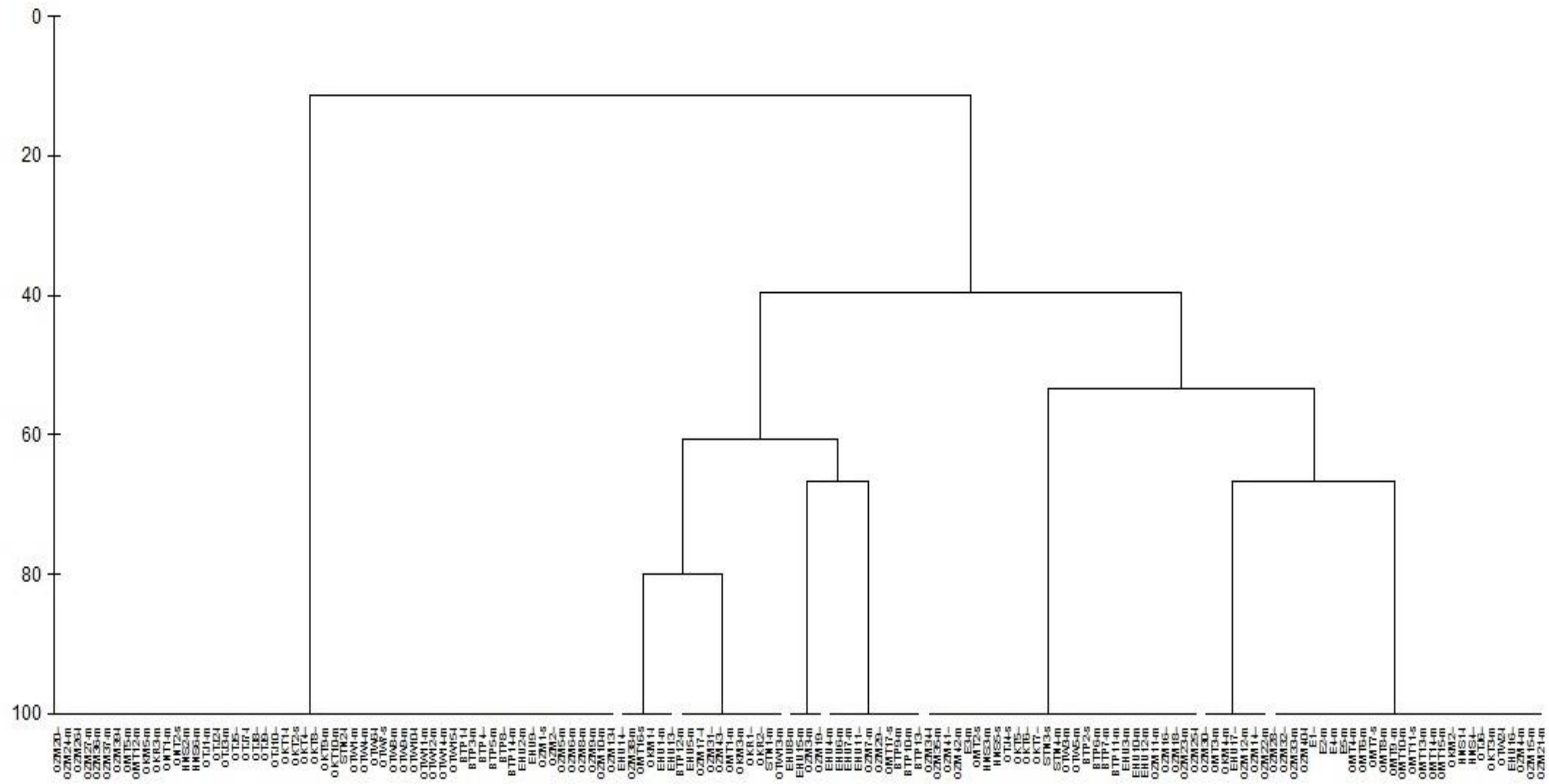
*MARA 068*

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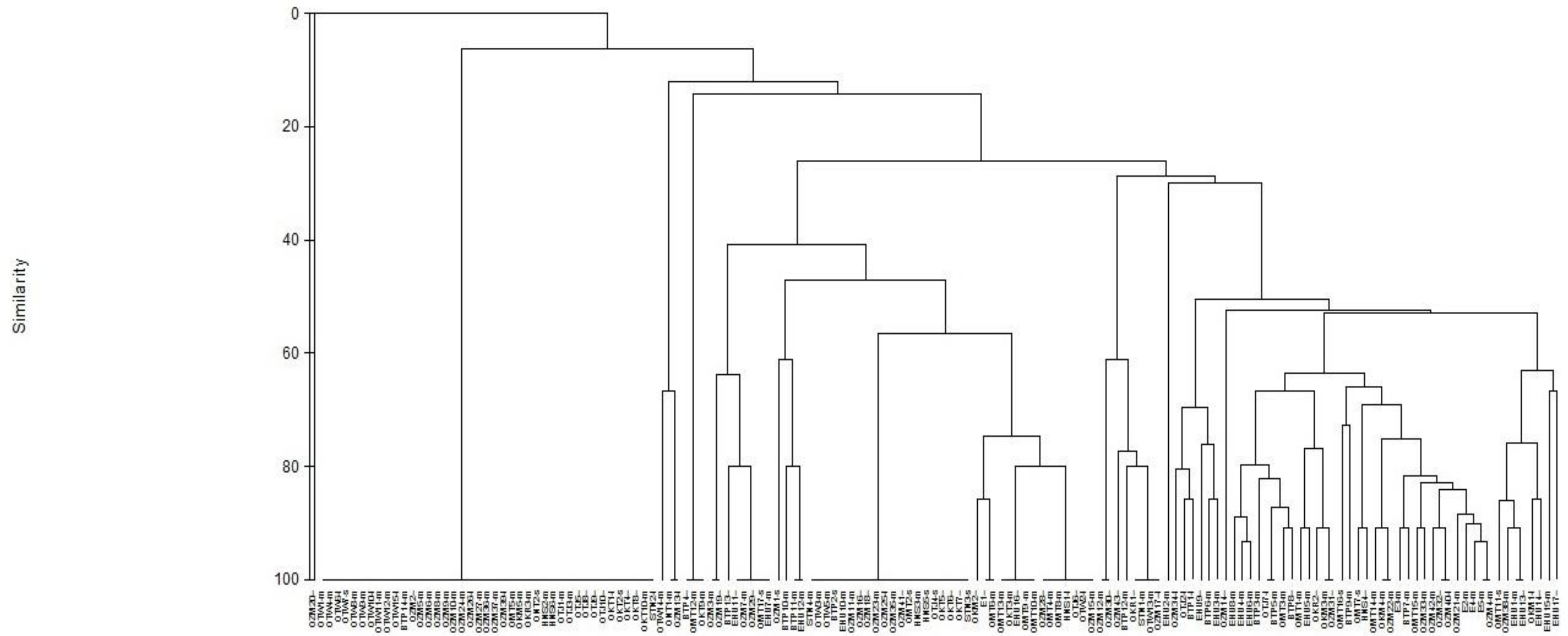
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100