Genetic characterization, priming and screening for *Cercospora* leaf spot resistance of different Bambara groundnut (*Vigna subterrannea* [L.] Verdc.) landraces

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Thesis Abstract

The Bambara groundnut (*Vigna subterranea* [L.] Verdc.) is an African plant species which has been cultivated for many years even longer than the groundnut (*Arachis hypogaea* L.). Bambara groundnut is mostly grown by farmers as a subsistence crop. This legume has an admirable balance of carbohydrates (63%), protein (19%) and fats (6.5%) which is beneficial in improving protein deficiencies in cereals. Bambara groundnut has great potential to alleviate food insecurity, especially as the world moves towards a drier future. It may provide a sustainable food source since it is able to adapt well to dry conditions. However, Bambara groundnut is not afforded a lot of attention in scientific research.

The objectives of this study were: 1) to identify the suitable duration of priming Bambara groundnut seeds in order to enhance germination and seedling establishment 2) to assess Bambara groundnut landraces for their reaction to *Cercospora* leaf spot (CLS) with a view to identifying landraces which may be used in breeding programs to develop CLS resistant lines and 3) to genotype 22 Bambara groundnut landraces obtained from various geographical regions in Southern Africa using 20 simple sequence repeat (SSR) primers developed specifically for Bambara groundnut.

Five landraces (Keledi, Mokgalo, SCAM, LMS and MMB) were obtained from the National Genetic Resources Centre (NGRC) in Botswana. Seeds were primed using distilled water (hydropriming) at 24 and 36 hours; and controls (0 hours) were not primed. After priming, seeds were air-dried and planted. The number of days to 50% seedling emergence and the number of seedlings established at 20 days after planting were the two major parameters assessed in this study. Results of this study revealed that priming time had a significant ($p = 0.043$) effect on days to 50% seedling emergence where seeds primed for 36 hours took 15 days to reach the aforementioned level; the least amount of time relative to the other treatments. It was also observed that there was a significant difference ($p < 0.0001$) on seedling establishment as affected by the interaction of landrace and priming time. Landraces SCAM and LMS were the best performers in the group of landraces used.

Nineteen Bambara groundnut landraces were planted at the Ukulinga Research farm on a plot with previous history of *Cercospora* leaf spot infection. Disease was evaluated using a disease rating scale (0 – 4) in order to calculate the disease index; the disease indices were used to calculate area under the disease progress curve (AUDPC) and finally, the apparent

rates of infection were also calculated using Vanderplank's Logistic equation. None of the landraces were resistant to infection by the pathogen. Statistically, there were no significant differences ($p > 0.05$) observed among landraces in final foliar disease percentage and AUDPC. The Bambara groundnut landraces were categorised into reaction groups (resistance) based on the calculated AUDPC values. Eight landraces were considered to be moderately resistant (501 – 1000 units) and 11 were considered to be susceptible (1001 – 1500 units). Landraces KB05 and STN 05 were categorized as moderately susceptible to CLS and a recommendation was made that they could be used in Bambara groundnut breeding programs.

DNA was extracted from 22 Bambara groundnut landraces and was then used in touchdown polymerase chain reaction (PCR) using 20 Bambara groundnut specific SSR primers. The PCR products were subjected to capillary electrophoresis using a genetic analyzer. A phylogenetic tree was constructed using DARwin 5.0 software. After analysis, 110 alleles were detected, with a mean of 5.50 alleles per locus. The mean polymorphic information content (PIC) was 0.62 and the mean expected heterozygosity (H_e) was 0.64; the latter indicating a high gene diversity among the genotypes. The neighbour-joining analysis generated three major genetic groups, where the genotypes were clustered irrespective of their geographic origin. Landraces SCAM and LMS which had improved seedling emergence and seedling establishment did not appear in the same cluster. Similarly, landraces KB05 and STN 05 which were moderately susceptible to *Cercospora* leafspot did not appear in the same cluster.

Poor emergence, disease infection and a lack of improved varieties are three major production constraints of Bambara groundnut that are identified in this study. Concerted efforts in scientific research are necessary in the amelioration of these problems and in the improvement of production of Bambara groundnut.

Declaration

I, Nompumelelo Phumelele Gama, hereby declare that this research project and all of its work was done by the author alone, except where otherwise acknowledged. This work has not been submitted in any other form to another university. Work of other authors has been duly acknowledged in the text.

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Dedication

This thesis is dedicated to my parents, Leo Gama and Lomkhosi Mkhonta-Gama, who have put me through school from the beginning to this point. I am so grateful for everything that they have done for me and I am happy that they have been able to witness all that I have achieved.

Thesis Introduction

All research presented in this thesis was carried out in the School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the joint supervision of Prof. Paramu Mafongoya and Prof. Augustine Gubba.

The Bambara groundnut (*Vigna subterranea* [L.] Verdc.) is an African plant species that is essential for small-scale farmers because it ensures food security and is highly nutritious. Its seed consists on average 63% carbohydrates, 19% protein and 6.5% fats; a commendable nutritional composition. Bambara groundnut has the ability to contribute to the maintenance of soil fertility because it is able to fix nitrogen into the soil. Even though Bambara groundnut has these benefits, it also has major production constraints.

Bambara groundnut is susceptible to a variety of diseases which are caused by fungal and viral pathogens. It is also susceptible to attack by insect pests, nematodes and weeds. Other production constraints are that the crop exhibits slow germination and sporadic growth in the field. The presence of these constraints hinders farmers from producing Bambara groundnut on a large, commercial scale. Furthermore, large scale production is not attractive to farmers due to the lack of improved varieties of Bambara groundnut since very little research has been done in efforts to enhance productivity of this underutilized legume.

The main objective of this research project was to enhance seedling emergence and establishment by priming, screen for resistance to *Cercospora* leaf spot and genetically characterize Bambara groundnut landraces. The information gathered from this work could be used to improve Bambara groundnut production and possibly identify landraces which may be used to develop better varieties of the crop; especially since it already exhibits some potential in ensuring food security. The scope of this research is presented in five chapters:
Chapter 1 provides a review of literature on Bambara groundnut and its importance, priming of Bambara groundnut, genetic diversity and diseases that affect it; highlighting the most common disease *Cercospora* leaf spot.

Chapter 2 covers the investigation of the effect of seed priming (hydropriming) on seedling emergence and establishment of five Bambara groundnut landraces from Botswana.

Chapter 3 depicts the evaluation of Bambara groundnut landraces for reaction to *Cercospora* leaf spot.

Chapter 4 investigates the genetic diversity of Bambara groundnut genotypes revealed by SSR primers developed specifically for Bambara groundnut.

Lastly, Chapter 5 reviews the experimental results, conclusions and gives recommendations for future research.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The Bambara groundnut (*Vigna subterranea* [L.] Verdc.) is an African plant species that has been cultivated for many years, even longer than the groundnut (*Arachis hypogaea* L.) (Hillocks *et al.,* 2012). Bambara groundnut is an essential crop for small-scale farmers because it ensures food security and is high in protein (Hillocks *et al.,* 2012). This legume has been termed a complete food (Murevanhema and Jideani, 2013) whose seed consists on average 63% carbohydrates, 19% protein and 6.5% fats (Bamishaiye *et al.,* 2011). Bambara groundnut is able to contribute to the maintenance of soil fertility because it is able to fix nitrogen into the soil (Hillocks *et al.,* 2012). Unlike other leguminous crops, Bambara groundnut is able to adapt to poor soils and is tolerant to drought (Hillocks *et al.,* 2012). Even though Bambara groundnut has these benefits, it also has major production constraints.

Bambara groundnut is reportedly relatively free from pests and diseases (Bamishaiye *et al.,* 2011). However, this legume has been seen to be commonly infected by *Cercospora* leaf spot (CLS) caused by the pathogen *Cercospora canescens* Ellis and Martin, powdery mildew (*Erysiphe polygoni* DC.) and Fusarium wilt (*Fusarium oxysporum* Schlect.) (Bamishaiye *et al.,* 2011). Secondly, Bambara groundnut exhibits slow germination and sporadic growth in the field (Ogbuehi *et al.,* 2013). These production constraints are viewed as red flags for farmers who may want to grow Bambara groundnut on a vast, commercial scale. Genetic improvement of Bambara groundnut is vital to its productivity in any location; however, very little research has been conducted in efforts to enhance productivity of this underutilized crop (Mohammed, 2014).

Therefore, the overall objective of this study was to explore 3 aspects in Bambara groundnut improvement. This was accomplished by firstly identifying a suitable duration of hydropriming Bambara groundnut seeds in order to enhance germination and seedling establishment. Secondly, an assessment of Bambara groundnut was made for their reaction to CLS, the most common fungal disease that affects Bambara groundnut, with the intention to identify landraces within the study that may be used in breeding programs to develop CLS resistant lines. Lastly, a genotyping study was done with 22 landraces using simple sequence

repeats (SSR) primers developed for Bambara groundnut in order to identify genetic relationships between the various landraces used in the entire study.

1.2 Bambara groundnut: taxonomy, origin and the plant

Bambara groundnut is under the family Leguminosae and the genus *Vigna* (Directorate Plant Production, 2011). Initially, the Bambara groundnut was called various names including Mandubi d'Angola (Mohammed, 2014). In 1763 Linnaeus gave it the name *Plantarum* and later renamed it *Glycine subterranea* (Heller *et al.*, 1997). After a lot of controversy surrounding the taxonomy of the crop, eventually taxonomists named it *Vigna subterranea* (L.) Verdc. (Heller *et al.*, 1997). The crop has numerous names depending on the location it is grown and the tradition of the people who grow it. In Madagascar, it is called the Madagascar groundnut (Heller *et al.,* 1997) and in South Africa it is known as the jugo bean (Swanevelder, 1998). The name of the legume changes according to the different ethnic groups in South Africa which include Phonda (Venda), Ditloo-marapo (Sepedi) and Tindhluwa (Tsonga) (Swanevelder, 1998). Nigerians have numerous names that include Guriya, Ngamgala, Okpa, Epa-kuta and Kwam Bambara groundnut (Mohammed, 2014). These different names change according to the tribes in Nigeria (Mohammed, 2014). The Shona and Ndebele tribes of Zimbabwe call the crop Nyimo and Indlubu respectively (Mohammed, 2014).

Mali is believed to be the center of origin of the Bambara groundnut because it was popular among the Bambara tribe based in the central region of the country (Heller *et al.*, 1997). However, the exact center of origin of the crop in Africa is still unknown because there is no evidence of wild forms of Bambara groundnut in Mali (Heller *et al.*, 1997). In South Africa, it has been postulated that the Bambara groundnut was introduced into Southern KwaZulu-Natal by immigrants from North Africa (Swanevelder, 1998). People of the Venda tribe have claimed to be the first to arrive with Bambara groundnut from Central Africa (Swanevelder, 1998). In Swaziland, Bambara groundnut is an essential legume (Edje and Mavimbela, 2014) and is grown mostly by farmers in the rural areas. Due to modernization of life, the crop is sold as a rare delicacy by small and medium enterprises in the cities (Edje and Mavimbela, 2014). The production of the Bambara groundnut in South Africa is limited mainly to Limpopo, Mpumalanga and KwaZulu-Natal (Directorate Plant Production, 2011). In Mpumalanga they are grown by small-scale farmers both as food and cash crops (Directorate Plant Production, 2011). Bambara groundnut is also found in countries outside of

Africa. These countries include Sri-Lanka, Malaysia, Philippines, India and Brazil (Heller *et al.,* 1997).

Generally, Bambara groundnut is grown on sandy soils either as a single crop or intercropped with maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.) or sorghum (*Sorghum bicolor* [L.] Moench) (Chibudu, 1997). It is perhaps one of the most drought-resistant of the grain legumes (Bamishaiye *et al.,* 2011). It can be grown successfully where rainfall is below 500 mm per year (Bamishaiye *et al.,* 2011). An optimum annual rainfall required for successful growth of the plant is between 900-1000 mm (Bamishaiye *et al.,* 2011). Bambara groundnut pods grow first and reach maturity 30 days after fertilization and the seeds develop in the following 10 days (Chibudu, 1997). It has been reported that fruit development is influenced by photoperiod (Chibudu, 1997). Linnemann *et al.* (1995) observed a high pod growth rate in plants exposed to a 10 hour photoperiod compared to the pod growth rate of plants under a 12 hour photoperiod. Continuous light has been shown to delay flowering by 6-11 days in some plants (Chibudu, 1997).

Bambara groundnut is a herbaceous, annual plant which grows to about 0.3-0.35 m in height, has compound leaves comprised of three leaflets; and pods develop below the ground, similar to the common peanut (Bamishaiye *et al.,* 2011). Generally, the plant leaves take on a bunched pattern above-ground with each branch being made up of internodes (Swanevelder, 1998).

Leaves of the Bambara groundnut vary from light green to dark green (Swanevelder, 1998). Flowers are borne on hairy stems on the stem of the plant and are yellow in color (Swanevelder, 1998). Bambara groundnut pods may reach up to 3.7 cm depending on the number of seeds they contain (Stephens, 2012). The pods each contain one or two seeds which are round, smooth and may vary in color from white, cream, dark-brown, red or black and may be speckled or patterned with an amalgamation of these colors (Bamishaiye *et al.,* 2011).

1.3 Economic importance of Bambara groundnut

Although Bambara groundnut is an underutilized crop, it has a number of essential uses. It is predominantly an African crop which is grown mostly by subsistence farmers (Swanevelder, 1998). It is consumed in different forms and at different stages of maturity either as a snack

or a vegetable (Mohammed, 2014). The young seeds may be boiled and eaten as a snack in a similar way as boiled peanuts. As a vegetable, the pods are sometimes harvested at an early stage, boiled and eaten (Swanevelder, 1998). Bambara groundnut can be used for making bread and legume milk (Goli, 1997). They can also be roasted and eaten as candy in the form of flat cakes and biscuits (Hillocks *et al.*, 2012). In Mpumalanga, seeds are pounded into flour to make porridge with maize and groundnuts (Swanevelder, 1998). Bambara groundnut is referred to as a complete food since they are a good source of carbohydrates, protein and fats (Swanevelder, 1998). They have a high lysine content compared to other legumes thus rendering the crop a high quality protein source (Swanevelder, 1998). For this reason, Bambara groundnut is also a good supplement to maize based diets (Hillocks *et al.*, 2012).

Some parts of the Bambara groundnut plant have been proposed to have medicinal properties. In Senegal, the leaves are used to treat infected wounds (Brink and Belay, 2006). To treat epilepsy, the leaf sap is administered through the surface of the eye (Brink and Belay, 2006). The South African community chews raw Bambara groundnut seeds to cure nausea as would be experienced by pregnant women (Heller *et al.*, 1997). Compared to many other legumes, the Bambara groundnut is a cheap source of vitamin B which is critical in the prevention of beriberi (Heller *et al.*, 1997).

Bambara groundnut has always been used as animal feed (Directorate Plant Production, 2011). The seeds have successfully been used to feed chicks (Directorate Plant Production, 2011). Since the leaves are rich in nitrogen and phosphorus, they are suitable for animal grazing (Directorate Plant Production, 2011). The palatable haulms of the crop are suitable as livestock feed during the dry season (Directorate Plant Production, 2011). Bambara groundnut cultivars that are resistant to leaf pathogens would serve a dual function of providing pods for human consumption and fodder for livestock feed.

In agriculture, Bambara groundnut plays a vital role due to the presence of root nodules which aid in fixing nitrogen into the soil (Swanevelder, 1998). In crop rotation systems, Bambara groundnut can be used in rotation with maize (Chibudu, 1997). According to Chibudu (1997), farmers in Chivi, Zimbabwe remained unconvinced of the beneficial effects of Bambara groundnut in crop rotation, even though its potential has been scientifically proven.

1.4 Breeding of Bambara groundnut

Food security relies on a strong seed system which is absent in Bambara groundnut [Food and Agriculture Organization (FAO), 2017]. Even though this legume has great potential to contribute positively towards food security, an informal system of classification, based on provenance and seed coat color (Massawe *et al.,* 2005) is used to identify Bambara groundnut landraces. This leads to one landrace bearing numerous names due to the movement of seeds from one region to another. The lack of improved varieties of Bambara groundnut is detrimental to the production of the crop (Mohammed, 2014). Breeding studies need to be carried out in order to improve the quality of this crop and to introduce farmers to seed innovation (McGuire, 2016).

The use of conventional breeding methods developed both under field and controlled conditions may aid in the identification of new sources of resistance against some of the major pathogens affecting Bambara groundnut (Sillero *et al.,* 2006) and other production constraints. However, relative to the conventional approach, molecular/genetic markers have emerged as the dominant tool in crop improvement (Aliyu *et al.,* 2016; Xu, 2010). There is a lack of knowledge of inheritance and variation in Bambara groundnut which can be obtained from the use of molecular markers.

Molecular markers are biological features that are determined by allelic forms and can be used to track molecular changes in an individual (Xu, 2010). These are the tools mostly used in breeding programs for crop improvement compared to the conventional breeding methods. There are certain criteria of desirable traits that markers have to meet in order for them to be used, which include (i) high polymorphism; (ii) co-dominance; (iii) clear distinct allele features; (iv) even distribution on the whole genome; (v) neutral selection; (vi) easy detection; (vii) cost-effectiveness and (viii) high duplicability (Xu, 2010). There is a wide variety of markers which are available but the most commonly used include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and single sequence repeat (SSR)/ microsatellite markers (Xu, 2010).

The digestion of pure DNA using restriction enzymes leads to the formation of RFLPs (Xu, 2010). The development of these markers aided in the rapid construction of molecular linkage maps for many organisms, improved the accuracy of gene location and reduced the time

required to establish a complete linkage map (Xu, 2010). Most RFLPs meet the criteria of desirable traits of molecular markers, however, RFLP analysis require large amounts of high quality DNA and has low genotyping throughput and is very difficult to automate (Xu, 2010). Another disadvantage of the use of these markers is that there is low duplicability because most genotyping involves radioactive methods which are not available to all laboratories (Xu, 2010).

The use of RAPDs involves a polymerase chain reaction (PCR) on the DNA of the individual under study using a short primer (10 nucleotides) of a random sequence that binds to numerous different loci and amplifies random sequences (Xu, 2010). Similar to RFLPs, RAPDs have desirable traits as well, however, their limitation is that there is low reproducibility of these markers in ongoing genetic and plant breeding programs. Their use is limited to research that does not depend on data sharing or accumulation (Xu, 2010).

AFLP markers are based on the selective PCR amplification of restriction fragments from a total double-digest of genomic DNA (Xu, 2010). AFLP assays can be carried out using small DNA samples and products can be separated in high-resolution electrophoresis systems (Xu, 2010). There are numerous limitations accompanied by this type of genetic markers; limitations are (i) Low polymorphism; (ii) High-quality DNA is needed to ensure complete restriction enzyme digestion; (iii) proprietary technology is needed; (iv) AFLP markers cluster densely in centromeric regions in species with large genomes; (v) Marker development is expensive and (vi) reproducibility is low compared to RFLPs and SSRs (Xu, 2010).

Single sequence repeat (SSR)/ microsatellite markers are short tandem repeats of nucleotides in the genome of prokaryotes and eukaryotes (Vieria *et al.,* 2016). SSRs have been the most commonly used markers for genotyping plants over the past two decades (Vieria *et al.,* 2016) and due to their high level of allelic variation, SSRs are highly informative and may aid greatly in evolutionary studies of wild plant species (Vieria *et al.,* 2016; Xu, 2010). SSRs are developed in such a way that they are species-specific (Xu, 2010). Reportedly, SSRs are more variable than RFLPs and RAPDs and they have the advantage of being readily analyzed by PCR and are easily detected on polyacrylamide gels (Xu, 2010). Additionally, a very small amount of DNA samples are required when using them. The main disadvantages are

that the development process of SSRs is labor-intensive and it is very costly to use automated methods (Xu, 2010).

Marker-assisted breeding is a beneficial way to improve Bambara groundnut production. For the most part, phenotypic descriptors have been used in genetic diversity analyses of Bambara groundnut and very few molecular markers have been used (Aliyu *et al.,* 2016). More genetic diversity studies need to be done on underutilized crops such as Bambara groundnut as much as there are on major crops such as maize (Aliyu *et al.,* 2016). Bambara groundnut exists predominantly as landraces and there is a lack of information on their genetic diversity (Aliyu *et al.,* 2016). Another benefit of marker-assisted breeding is that it can help identify durable sources of resistance to diseases which are faced in legume production (Obagwu, 2003; Sillero *et al*., 2006).

1.5 Priming

Poor germination and seed establishment are both common occurrences in production of any crops (Singh *et al.,* 2015). Reducing the time between planting and emergence is key in crop production (Singh *et al.,* 2015). Priming has been said to be an effective technology to improve germination (Singh *et al.,* 2015). This seed-enhancing technology involves partially hydrating seeds to a point where germination processes begin but radical emergence does not occur (UK Essays, 2013). There are numerous ways of priming which include hydropriming, osmopriming, hormopriming and biopriming (Lutts *et al.,* 2016; Mouradi *et al.,* 2016; Nawaz *et al.,* 2013; UK Essays, 2013).

Hydropriming is the simplest method that involves soaking seed in pure water (Nawaz *et al.,* 2013). It is the most cost effective method since it does not involve the additional use of chemicals. Its main disadvantage is that the seed hydration process is not controlled resulting in non-uniform germination of seeds (Lutts *et al.,* 2016). Drum priming is an improved method of hydropriming involving the gradual hydration of seeds by water vapor in rotating drums (Lutts *et al.,* 2016). This is an appealing alternative to conventional hydropriming (Lutts *et al.,* 2016; Warren and Bennett, 1997). The duration of drum priming is dependent on the absorptive characteristics of the seed species and the desired final seed moisture content (Warren and Bennett, 1997).

Osmopriming is a technique that involves soaking seed in an osmotic solution (Lutts *et al.,* 2016; Mouradi *et al.,* 2016). This method uses a solution of low water potential so as to control the amount of water supply to the seed (Lutts *et al.,* 2016; Mouradi *et al.,* 2016). The most commonly used chemical in osmopriming is polyethylene glycol (PEG) due to its large molecular size that prevents its penetration into seed thus avoiding induction of potential cytotoxicity (Lutts *et al.,* 2016). Other osmotic agents that are used include mannitol, sorbitol, glycerol and inorganic salts such as sodium chloride, potassium chloride and calcium chloride (Lutts *et al.,* 2016).

Hormopriming involves soaking seed in the presence of plant growth regulators such as abscisic acid, auxins, gibberellins, kinetin, ethylene, polyamines and salicyclic acid (Lutts *et al.,* 2016). These regulators have a direct impact on seed metabolism (Lutts *et al.,* 2016) and similarly to other priming techniques promote the growth and development of seedlings (Singh *et al.,* 2015).

Biopriming involves seed imbibition together with bacterial inoculation of seed (Lutts *et al.,* 2016). This method not only improves the rate and uniformity of germination, it additionally protects seed against soil and seed-borne pathogens (Lutts *et al.,* 2016). Reportedly, integrating plant growth-promoting bacteria (PGPB) when biopriming in agricultural practices has shown great promise (Lutts *et al.,* 2016). Niranjan Raj *et al.* (2004) observed an enhancement of plant growth and resistance against downy mildew in Pearl millet (*Pennisetum glaucum* [L.] R. Br.) with *Pseudomonas fluorescens* isolates in biopriming.

Besides pests and diseases, slow germination and sporadic growth of Bambara groundnut are major constraints in the production of the legume. Studies have shown that priming Bambara groundnut results in improvement of germination and establishment (Berchie *et al.,* 2010; Lutts *et al.,* 2016; Mouradi *et al.,* 2016; Ogbuehi *et al.,* 2013;).

1.6 Yield Potential of Bambara groundnut

Both commercial and subsistence farmers grow crops with the objective of obtaining high, favorable yields. A study recorded 2000 kg ha^{-1} in 6 independent trials in Central African countries (Schenkel, 2006). Other records have referred to experimental yields in excess of 3000 kg ha^{-1} (Schenkel, 2006). About one third of world annual production comes from Nigeria followed by Burkina Faso (Swanevelder, 1998). The Bambara groundnut has the

potential of yielding >3000 kg ha^{-1} in both greenhouse and field trials (Hillocks *et al.,* 2012). However, this depends on how well farmers manage the growth of their crop (Mohammed, 2014). Pests and diseases are major yield limiting factors for Bambara groundnut (Mohammed, 2014) as well as the low germination rate of the legume (Ogbuehi *et al.*, 2013).

1.7 Pests and Diseases

Contrary to previous reports (Hillocks *et al.,* 2012; Swanevelder, 1998), Bambara groundnut is vulnerable to infection by pests and diseases. This infection may have a significant economic impact on farmers growing either for subsistence or commercial purposes.

1.7.1 Insect Pests and Nematodes

Aphids and termites have been reported to cause serious damage in some areas of South Africa where Bambara groundnut is grown (Mathews, 2012). It has also been reported that in dry weather, pod attacks by termites in Botswana resulted in the loss of an entire crop (Karikari *et al.,* 1997). In storage, shelled Bambara seeds are extremely susceptible to bruchids (*Callosobruchus maculatus* Fabricius) which require the pod and the seed inside it to complete their life cycle (Karikari *et al.,* 1997). Unshelled seeds have been reported by Karikari *et al.* (1997) to be completely resistant to bruchid attack.

Parasitic nematodes of the Bambara groundnut include *Meloidogyne incognita* Kofoid and White and *M. javanica* Treub (Mathews, 2012). These have been reported in Botswana, Kenya, Zimbabwe and South Africa (Mathews, 2012). Root knot nematodes are a problem in light soils (Mathews, 2012). These nematodes cause yield reduction directly and also promote infection by *Fusarium* (Mathews, 2012).

1.7.2 Viruses

Several viral symptoms have been observed on Bambara groundnut (Mathews, 2012). The productivity of Bambara groundnut is severely affected by viral infection (Drabo *et al.*, 1997). Drabo *et al.* (1997) reported loss of an entire germplasm collection due to foliar viruses in Burkina Faso. Viruses of Bambara include *Cowpea aphid-borne mosaic virus*, *Black-eye cowpea mosaic virus*, *Peanut mottle Potyvirus*, *Cowpea mottle comovirus* and *Cowpea mosaic comovirus* (Hillocks *et al.,* 2012). The Bambara groundnut belongs to the same genus as cowpea (*Vigna unguiculata* [L.] Walp. hence the virulence of the aforementioned viruses to the crop (Hillocks *et al.*, 2012).

1.7.3 Fungi

Fusarium wilt is an important disease of Bambara groundnut and it has been reported in Kenya (Ngugi, 1997). It has also been stated that most farmers in South Africa experience wilting problems in their fields (Masindeni, 2006). Rust and leaf blight caused by *Puccinia* and *Colletotrichum spp.* respectively have been identified under conditions of high temperature and humidity in the Nigerian Guinea Savannah (Mohammed, 2014). Other diseases which affect Bambara include leaf spot (*Cercospora canescens* Ellis and Martin), leaf blotch (*Phomopsis sp.*), powdery mildew (*Erysiphe polygoni* DC.) and *Sclerotium rolfsii* Sacc. (Swanevelder, 1998). The crop is also susceptible to seed borne diseases caused by *Fusarium oxysporum f. sp. voandzeia* (Schlent.), *Fusarium solani* (Mart.) Sacc. Michelia, *Aspergillus niger* (van Tiegh) and *Aspergillus flavus* (Link) (Swanevelder, 1998).

Abiotic stresses such as temperature, water, soil conditions and drought are limiting factors which cause unstable yield (Masindeni, 2006). Biotic stresses such as those already mentioned cause yield reduction (Masindeni, 2006). This study only focuses on *Cercospora canescens.*

1.7.3.1 Cercospora leaf spot (*Cercospora canescens*)

*Cercospora canescens (*Ellis and Martin) is the major causal organism for leaf spotting and defoliation in several legumes; causing losses of up to 40% (Adegbite and Amusa, 2008). This fungus is an Ascomycete, therefore, species in this genus have no known sexual stage. Only a few species of *Cercospora* have been identified to have a sexual stage called *Mycosphaerella* (Adegbite and Amusa, 2008).

Symptoms of Cercospora leaf spot on Bambara groundnut include sub-circular to broadly irregular spots which will eventually coalesce (Brink and Belay, 2006). Generally the spots are brown with a pale tan center surrounded by a dark brown or reddish margin (Brink and Belay, 2006). Spots appear on both the upper and lower surface of the leaf; as well as on the stem, cotyledons and drying pods (Brink and Belay, 2006). *Cercospora canescens* produces a toxin called cercosporin (Wongpiyasatid *et al.,* 1999). Due to its red pigment, this toxin is responsible for the red margin around leaf spots in the early stage of infection (Daub and Ehrenshaft, 2000; Wongpiyasatid *et al.,* 1999). Cercosporin degrades plant cell membranes by membrane lipid peroxidation, resulting in the easy access of the pathogen in the plant (Daub *et al.,* 1998). The toxicity of cercosporin is activated by light and its effect does not

manifest in the dark or under low light conditions (Daub and Chung, 2007) therefore, shading can be used to reduce the toxicity of cercosporin.

In order to prevent the pathogen from developing, the amount of leaf wetness should be minimized by avoiding excessive irrigation (Sheku *et al.,* 2013). *Cercospora* infection usually occurs during overhead irrigation schemes (Masindeni, 2006). Drip irrigation is a better option because it prevents water-splash onto the leaves (Masindeni, 2006). The fungus survives on fallen diseased leaves which remain on the ground, therefore, the removal of diseased leaves may help prevent the spread of the infection onto other plants (Sheku *et al.,* 2013).

Once infection has occurred, the leaves can be treated with chemicals such as Mancozeb, chlorothalonil and tebuconazole (Kimber, 2011; Sheku *et al.,* 2013). Treatment with such chemicals keeps disease severity significantly low and reduces defoliation and pod infection (Kimber, 2011). Application of fungicides should be done when the crops begin to flower and when pods are starting to develop (Boa, 2014). A maximum of 2-3 applications per planting season is recommended (Boa, 2014).

1.8 Conclusion

The Bambara groundnut is an underutilized crop, in spite of its popular growth and use throughout Sub-Saharan Africa. Seeds of the crop are consumed at various stages of growth and provide a good source of protein (Mohammed, 2014). Additionally, Bambara groundnut has the potential of being a component of food security in Africa because of its high nutritional value and adaptability to dry areas. Subsistence farmers rely on this crop and it is a rare occurrence for Bambara groundnut to be grown for commercial use in these communities.

Bambara groundnut is one of the crops that has been neglected by science over the years and is not the most popular commercially (Hillocks *et al.,* 2012). The low germination rate and sporadic growth of Bambara groundnut are two problems that need to be tackled if the crop is to be grown on a large scale commercially. Bambara groundnut has also been known to be relatively free from pests and diseases. This belief has gone to the extent of some farmers not believing that pests and diseases are a threat to the crop; which is not true. It is necessary for farmers to know the most important pests and diseases that infect this legume in order for

them to produce economic yields. Furthermore, research needs to be done with Bambara groundnut, especially developing varieties resistant to pests and diseases. Further studies may go on to identify resistance genes that may be used in breeding programs with efforts to produce the best Bambara groundnut variety (super crop) that would be best for commercialization. Numerous gaps exist in Bambara groundnut research; therefore science has a duty to identify areas which are lacking in the crop's overall improvement and which Bambara groundnut landraces will be sustainable due to their resistance to various diseases. The lack of improved varieties has led to farmers growing the crop below its potential (Mohammed, 2014) and research can aid in alleviating this problem.

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CHAPTER 2

EFFECT OF SEED PRIMING ON SEEDLING EMERGENCE AND ESTABLISHMENT OF FIVE BAMBARA GROUNDNUT (*VIGNA SUBTERANNEA* [L.] VERDC.) LANDRACES

Abstract

There is a high demand for good quality seed in the agricultural market which requires a substantial amount of research to accomplish. Priming of seeds has proven to be an effective way to improve seed quality. The Bambara groundnut (*Vigna subterranea* L. Verdc.) is an African legume which is adapted to dry areas and is mostly grown by subsistence farmers. Bambara groundnut seed can take a very long time to germinate and shows sporadic growth in the field. This study was conducted to determine the suitable duration of priming Bambara groundnut seeds in order to enhance germination and seedling establishment. Five landraces; Keledi, MMB, LMS, Mokgalo and SCAM were soaked in closed Petri dishes using 25ml distilled water for 24 and 36 hours. The controls were not soaked in water (0 hours). Ten seeds for each landrace were planted in each pot and treatments were arranged in a Randomized Complete Block Design (RCBD) with three replicates of each treatment. The days to 50% emergence and number of seedlings established after 20 days were recorded. Results of this study revealed that priming time had a significant effect on days to 50% seedling emergence where seeds primed for 36 took 15 days to reach 50% emergence; the shortest amount of time relative to the other treatments. It was also observed that there was a significant difference ($p < 0.0001$) in the interaction of landrace and time on seedling establishment. The highest seedling stand was observed at 36 hours priming time for all landraces except MMB which had its highest at 24 hours priming time. This result indicates that seed priming was dependent on the landrace. This study showed that hydropriming improved seedling emergence and establishment. There is need to expand this study by testing more soaking times for a wide range of landraces in order to generate data on priming that can be useful to farmers.

2.1 Introduction

There is a high demand for good quality seed in the agricultural market (Paparella *et al.*, 2015). Crop growers are always seeking ways to grow their crops in the best way possible in order to achieve favorable yields. Seedling germination is an important factor in crop

production and is essential in producing good quality seed. Usually, slow germination rates expose plants to adverse environmental conditions and soil-borne diseases (Paparella *et al.,* 2015). Achieving rapid and uniform seedling emergence is an essential aspect for crop performance (Paparella *et al.,* 2015).

Priming of seeds has proven to be an effective way to improve seed quality (Paparella *et al.,* 2015). Primed seeds have shown increased germination rates that result in high levels of biotic and abiotic stress resistance and high crop yields (Paparella *et al.,* 2015). Priming is a water-based technique that allows controlled seed rehydration to trigger pre-germinative metabolism but preventing the seed transition towards full germination (Paparella *et al.,* 2015). There are different priming methods that are used in efforts to reducing emergence time in seedlings (Nawaz *et al.,* 2013). These include hydropriming, osmopriming, hormopriming and biopriming (Lutts *et al*., 2016; Mouradi *et al*., 2016; Nawaz *et al*., 2013; UK Essays, 2013).

Hydropriming is the simplest and most cost-effective method that involves soaking seed in pure water (Nawaz *et al.,* 2013). Even with those benefits, this method has a disadvantage; the seed hydration process is not controlled and this leads to uneven germination of seed (Lutts *et al*., 2016). Drum priming is an alternative to the conventional hydropriming where hydration is controlled using rotating drums and water vapor (Lutts *et al*., 2016; Warren and Bennett, 1997).

Osmopriming is a technique that relies on the use of an osmotic solution with lower water potential when soaking seed (Lutts *et al*., 2016; Mouradi *et al*., 2016). This method controls the water supply to the seed in order to achieve uniform germination (Lutts *et al*., 2016; Mouradi *et al*., 2016). Polyethylene glycol (PEG) is the most commonly used chemical in this method (Lutts *et al*., 2016). Previous studies have reported that osmopriming results in greater germination compared to hydropriming seed (Li *et al.,* 2009; Singh *et al.,* 2012; Singh *et al.,* 2014).

Hormopriming involves soaking seed in the presence of plant growth regulators which aid in the development of seedlings during the germination phase (Singh *et al.,* 2015). Plant growth regulators are also known as phytohormones and include auxins, gibberellins, cytokinins, abscisic acid and ethylene (Lopez-Lauri, 2016; Lutts *et al.,* 2016). Hormopriming has been

reported to improve photosynthetic properties and enhance salt tolerance in a number of crops (Lutts *et al.*, 2016).

Biopriming involves imbibition together with bacterial inoculation of seed in order to, not only improve rate and uniformity of germination, but also to protect seeds against soil and seed-borne pathogens (Lutts *et al.*, 2016). This is a beneficial method which is essential to integrate in all seed priming techniques that lack a protective measure against soil-borne pathogens once the seeds are planted. Niranjan Raj *et al.* (2004) reported improved growth of Pearl millet and induced resistance against downy mildew (*Sclerospora graminicola* [Sacc.] J. Schroet.) after using *Pseudomonas fluorescens* isolates in biopriming.

The Bambara groundnut is an African legume which is adapted to dry areas and is grown mostly by subsistence farmers (Ogbuehi *et al.*, 2013). It is known that Bambara groundnut has a long germination time since seedlings of this crop may take up to 21 days after sowing to emerge (Ogbuehi *et al.*, 2013). In addition to slow germination, Bambara groundnut grows sporadically in the field (Ogbuehi *et al.*, 2013; University of Nottingham *et al.*, 1997). Priming may be a solution to the crop's slow germination since this delay in seedling emergence and establishment of the crop may have detrimental effects on its development. The effect of priming depends on the duration of seed soaking (Ogbuehi *et al.*, 2013).

The aim of this study was to identify a suitable duration of priming Bambara groundnut seeds in order to enhance germination and seedling establishment using the hydropriming method.

2.2 Materials and Methods

2.2.1 Plant Material

This study was conducted at the University of KwaZulu-Natal (UKZN), Pietermaritzburg (PMB) campus in the institution's Controlled Environment Facility (CEF). The facility is located on a Latitude 29º 37'S, Longitude 30º 24'E. Five Bambara groundnut landraces were obtained from the National Genetic Resources Centre (NGRC) in Botswana and were labeled; Keledi, Mokgalo, SCAM, LMS and MMB (Fig. 2.1). The seeds differed in seed coat color and eye color.

2.2.2 Priming

Before priming, the seeds were surface sterilized using 10% Jik (Sodium hypochlorite) for 30 s, rinsed twice using distilled water in beakers and then air-dried. Ten seeds per landrace, with three replicates (30 in total), were used for each priming period. Seeds were soaked in 25ml of distilled water in closed Petri dishes for 24 and 36 hours. There was also a closed Petri dish which kept the seeds which would not be primed to represent 0 hours of priming. Treated seeds were air-dried at ambient temperature for 2 hours after their respective priming durations.

Figure 2.1: The five landraces obtained from the NGRC in Botswana whose differences are easily discerned by coat colour, eye shape and colour and hilum. They are labeled as follows; A=Mokgalo, B=SCAM, C=Keledi, D=LMS and E=MMB

2.2.3 Planting

A total of ten seeds per landrace were planted in 30cm diameter pots filled with composted pine bark growing media for the various priming times. There were 3 replicates for each treatment. Pots were placed in a glasshouse that was exposed to 50% humidity and 12 hours of day light. The temperature in the glasshouse was 25 ^{o}C. Pots were watered everyday using a watering can and uniformity was kept when doing so. No fertilizer was added into the irrigation water.

2.2.4 Assessment

The number of days to 50% seedling emergence was observed and recorded. Emergence of seedlings was considered to be when the first true leaf breaks from the soil and is visible. At 20 days after planting, the number of seedlings which had established themselves was recorded.

2.2.5 Statistical Analysis

A general analysis of variance (ANOVA) was used in order to compare means using the Genstat, 18th edition statistical software (VSN International, 2015). The two aforementioned parameters assessed were analyzed. Means were separated by least significant differences (LSD) at a 95% confidence level. The experiment was not repeated due to a lack of Bambara groundnut seed.

2.3 Results

There was a significant difference ($p = 0.043$) observed between mean days to 50% emergence as affected by priming time (Fig 2.2). The interaction of landrace and priming time significantly affected ($p < 0.0001$) the number of seedlings present at 20 days after planting (Table 2.1).

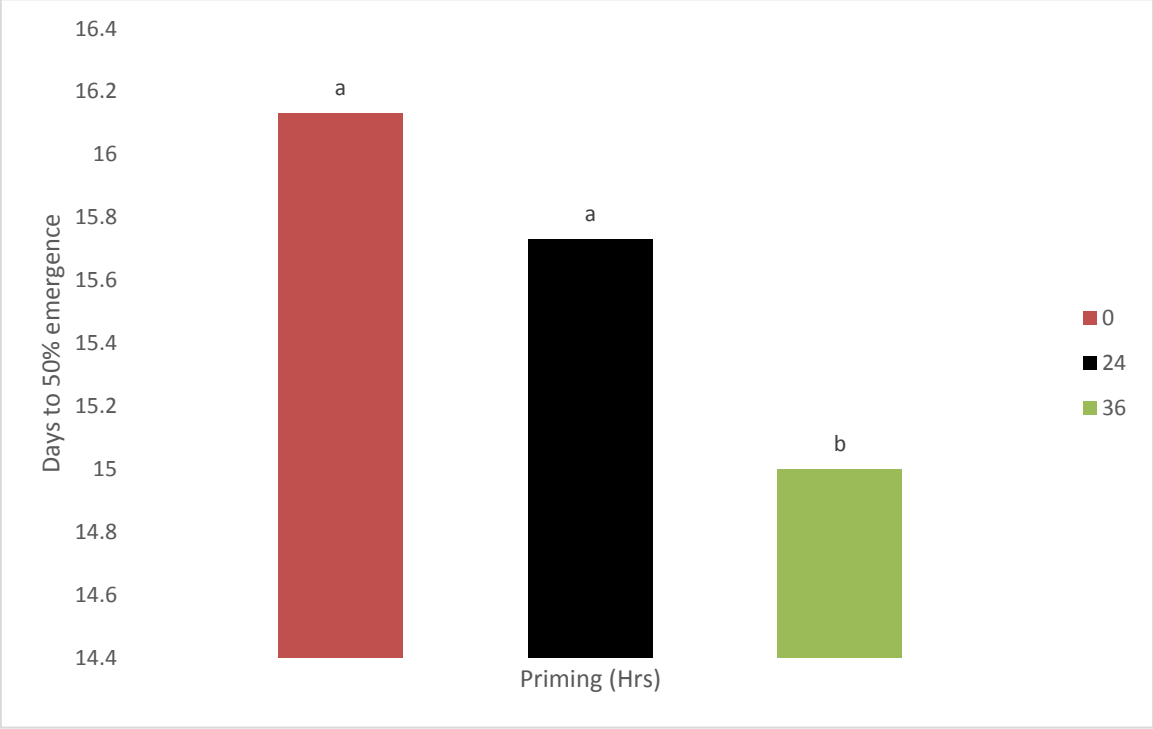

Figure 2.2: Differences between mean days to 50% emergence of Bambara groundnut as affected by priming time.

Bars labeled with a different letter (a, b) are statistically different. Figure 2.2 shows that at 0 hours it took Bambara groundnut a mean of 16.13 days to achieve 50% emergence, which was the most number of days. It took Bambara groundnut a mean of 15 days to achieve 50% emergence after being primed for 36 hours, which was the least number of days.

Table 2.1: Effect of landrace x time on number of seedlings present at 20 days after planting.

TIME	LANDRACE				
	Mokgalo	Keledi	SCAM	LMS	MMB
0	9.33^{aA}	8.67^{abB}	9.67^{aA}	9.67^{aA}	9.33^{aA}
24	8.00^{bA}	8.33^{aA}	10.00^{aB}	10.00^{aB}	10.00^{bB}
36	10.00^{cA}	9.00^{bB}	10.00^{aA}	10.00^{aA}	9.00^{aB}
LSD 0.05			0.61		
P-VALUE			<0.0001		
CV%			3.88		

Means within columns followed by the same letters (lowercase) are not significantly different and means within rows followed by the same letters (uppercase) are not significantly different

At 0 hours, Mokgalo, SCAM, LMS and MMB had the highest seedling stand and Keledi had the lowest. At 24 hours, SCAM, LMS and MMB had the highest seedling stand compared to Mokgalo and Keledi. Lastly, Mokgalo, SCAM and LMS had the highest seedling stand at 36 hours. Under all priming durations, SCAM and LMS appeared to be the best performers of the five landraces.

2.4 Discussion

Priming is important because it has beneficial effects on crop production. This technology has the ability to reduce germination time, it enables crops to compete more effectively with weeds and may also reduce disease incidence in seeds (Hasegawa, 2016; Trueman, 2017). Priming improves crop uniformity in the field, increases plant vigor and yield potential (Hasegawa, 2016). Priming may also allow seeds to germinate under adverse conditions (Hasegawa, 2016). Even though this technique has its benefits, it is essential to know that primed seeds have a shortened life span and once they have been primed, the seeds need to be used within a set time period or else they lose viability (Stark Ayres, 2017).

Results in this study revealed that priming time had a significant effect on days to 50% seedling emergence. At 0 and 24 hours priming it took a mean of 16.13 and 15.73 days respectively to reach 50% emergence (Fig. 2.2). There was no significant difference between these two priming times. Priming to 36 hours stood out significantly from the others since the seeds primed at this duration reached 50% emergence in the least amount of time (15 days). It

is evident from Figure 2.2 that the control seeds (0 hours) did not perform well in this parameter. This is in accordance with previous studies which revealed that priming seeds enhances their development compared to those which are not primed (Ogbuehi *et al.,* 2013; Rajpar *et al.,* 2006). Ogbuehi *et al.* (2013) observed that 36 hours was the least effective treatment in hydropriming of Bambara groundnut and 24 hours priming time resulted in the best seedling emergence. In a similar study, the highest seedling emergence of Bambara groundnut was observed under 24 and 48 hours hydropriming relative to the control (Berchie *et al.,* 2010). All of these studies do not agree with the results evident in this study. Furthermore, contrary to what is observed in this study using five Botswana landraces, Legwaila *et al.* (2013) reported that 24 hours was the best duration for the Botswana landraces they used in their priming study. However, the origin of the landraces has no bearing on the fact that two different priming times were considered to be the best in each study. This is because it cannot be confirmed whether or not the landraces used in both experiments were the same.

There was a significant difference ($p < 0.0001$) observed on the landrace and time interaction on the number of seedlings present at 20 days after planting (Table 2.2). It may be inferred that priming the two best performers (SCAM and LMS) at either 24 or 36 hours does not matter; both durations are favorable. Moreover, Table 2.2 shows the highest seedling stand to be at 36 hours priming time for all landraces except MMB which has its highest at 24 hours. This result indicates that seed priming was dependent on the landrace; a result similar to that by the University of Nottingham *et al.* (1997). Seed soaking for 24 to 72 hours promoted initial germination by 2 to 3 days and this depended on the landrace (University of Nottingham *et al.,* 1997). Hydropriming seed improves germination and emergence and further ensures satisfactory stand establishment (Singh *et al.,* 2015). This is evident from the data presented in this study.

It has been reported that seedling emergence of Bambara groundnut is sensitive to moisture which is attributed to the restrictive uptake of water by the legume due to its hard seed coat (Berchie *et al.,* 2010; Sinefu, 2011). The differences observed in the interaction of landrace and duration of priming in this study may have been attributed to this fact. Additionally, the seed coat color of the landraces may also have had an effect on their differing permeability. Reportedly, the seed coat color has an effect on seed quality and its ability to take up water (Sinefu, 2011). An investigation of the effect of water uptake and seed morphology of

Bambara groundnut on hydropriming duration would increase the existing knowledge and subsequently add more value to this technique.

In order to utilize this cost-effective technique to improve seed germination, plant species, priming media concentration and priming duration are factors which need to be considered (Singh *et al.,* 2015). Nawaz *et al.* (2013) reported that hydropriming is not suitable for some plant species because the rates of hydration of seeds differ. Hydropriming may cause rapid hydration which leads to the escape of vital nutrients out of the seed, resulting in damage (Nawaz *et al.,* 2013). Consequently, this leads to non-uniform germination (Nawaz *et al.,* 2013). Results obtained by Eskandari and Kazemi (2011) showed that hydropriming of cowpea (*Vigna sinesis* L.) was comparatively superior in laboratory tests compared to halo priming and the same was observed in the field. However, Iqbal (2015) found that priming cowpea with 2% and 5% concentrations of Moringa leaf extract resulted in higher final germination than hydropriming, on-farm priming (tap water) and halo priming. Therefore, a similar study may need to be conducted on Bambara groundnut in order to see whether hydropriming is the best technique to use in the improvement of germination of this legume.

From this study, 36 hours of hydropriming seed was the best duration for the improvement of Bambara groundnut emergence and establishment. Literature shows that the best duration in hydropriming varies from study to study (Berchie *et al.,* 2010; Ogbuehi *et al.,* 2013; Rajpar *et al.,* 2006; University of Nottingham *et al.,* 1997). It is therefore recommended that this study be expanded by either using a wider range of treatments (priming duration) or by keeping the treatments the same in order to get consistency. In addition, it may be of significant importance to investigate whether hydropriming is the best technique to use for Bambara groundnut by comparing the aforementioned technique with other seed priming techniques. This may result in an optimal combination of priming duration and technique best suited for Bambara groundnut improvement. Soaking seed is cost-effective and environmentally friendly and is a good strategy which farmers may use in Bambara groundnut production.

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CHAPTER 3

EVALUATION OF BAMBARA GROUNDNUT (*VIGNA SUBTERRANEA* [L.] VERDC.) LANDRACES FOR REACTION TO *CERCOSPORA* LEAF SPOT

Abstract

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is an African legume commonly grown for subsistence purposes. It has been reported that Bambara groundnut is free from pests and diseases; which is not true since the crop has shown susceptibility to a variety of diseases. The aim of this study was to evaluate Bambara groundnut landraces for their reaction to *Cercospora* leaf spot; the most common disease of the legume; with the aim to identify landraces which may be used in breeding programs to develop resistant varieties. Nineteen Bambara groundnut landraces were evaluated for their reaction to *Cercospora* leaf spot caused by *Cercospora canescens* on a plot with previous history of *Cercospora* infection. A visual assessment was used in order to calculate disease incidence (DI) using a scale of 0-4 where 0= no infected leaves; 1= 1-25% infection; 2= 26-50% infection; 3= 51-75% infection; 4= 76-100% infection. The DI values at different intervals were used to calculate the Area Under the Disease Progress Curve (AUDPC). The apparent rate of infection (r) was calculated for each line using Vanderplank's Logistic equation. The results of this experiment revealed that all of the Bambara groundnut landraces evaluated were susceptible to *Cercospora* leaf spot infection. There was no significant difference ($p > 0.05$) between the landraces based on both the AUDPC and final disease indices. However, differences were seen after categorizing the landraces into different resistance groups (moderately resistant and susceptible) based on AUDPC units. Landraces KB05 and STN 05 show promise in that they may be used in future breeding programs since they were both moderately resistant. In this study, it was recommended that the trial be repeated for two more seasons in order to get more conclusive results and to observe whether the landraces would react consistently. Furthermore, Bambara groundnut landraces could also be screened for a resistant gene to cercosporin since this toxin is a prime factor that influences disease severity.

3.1 Introduction

Bambara groundnut (*Vigna subterranea* [L.] Verdc.) is an African legume commonly grown by poor farmers for subsistence purposes (Department of Agriculture, Forestry and Fisheries (DAFF), 2016). In South Africa, it is cultivated as an intercrop with maize, cowpeas and

melons (*Cucumis melo* var. *cantalupensis* Naudin) (DAFF, 2016). It is not grown on a large scale commercially due to the neglect by national research institutes in the past (DAFF, 2016). Internationally, Bambara groundnut seed is difficult to obtain because it is grown for subsistence purposes (DAFF, 2016). Nigeria is the leading Bambara groundnut producer in Africa with 100,000 tons per year (DAFF, 2016).

Legumes such as Bambara groundnut have a significant role to play in addressing food insecurity. Therefore, it is essential that the production of these crops, especially those considered as underutilized e.g. Bambara groundnut, is increased. For the most part, Bambara groundnut is used for human consumption (Gqaleni, 2014). It provides an important source of proteins (19%), carbohydrates (63%) and fats (6.5%) (Bamishaiye *et al.,* 2011; Gqaleni, 2014). The protein component of Bambara groundnut can also be beneficial when used in supplementing maize-based animal diets (Gqaleni, 2014) such as in diets of herbivorous animals in order for them to get as much protein as they can get. Like other legumes, Bambara groundnut has the ability to introduce or replenish nitrogen in the soil (Chivenge *et al.,* 2015). This may be beneficial to resource-poor farmers who may be unable to afford nitrogen fertilizers (Chivenge *et al.,* 2015).

Bambara groundnut has the ability to grow in dry areas where there are short periods of rainfall (Akpalu *et al.,* 2013). Day temperatures of 20^{o}C – 28^{o}C are suitable for the growth of this crop (Akpalu *et al.,* 2013). Bambara groundnut is also well adapted to a wide variety of soils such as light or sandy loam and has the advantage of being able to grow on poor soils where other crops fail (Akpalu *et al.,* 2013). Pests and diseases are one of the major constraints in any crop production system. Earlier research of Bambara groundnut has stated that the legume is free from pests and diseases (Hillocks *et al.,* 2012; Swanevelder, 1998). Contrary to this, it has been observed that the leaves of this crop are easily infected by pathogens such as *Cercospora* and *Erysiphe* species (Heller *et al.*, 1997; Obagwu, 2003). Bambara groundnut are also susceptible to viruses, insect pests and nematodes (Hillocks *et al.,* 2012; Karikari *et al.,* 1997).

The most common disease that affects Bambara groundnut is *Cercospora* leaf spot (CLS) which is caused by the pathogen *Cercospora canescens* (*C. canescens*) (Adegbite and Amusa, 2008). Symptoms of CLS are brown irregular-shaped spots which eventually coalesce to form spots resembling those of blight (Brink and Belay, 2006). The spots are

usually brown with a reddish margin (Brink and Belay, 2006). CLS may lead to defoliation and a severe infection may lead to lesions spreading to the pods and the stems as well (Booker and Umaharan, 2007). CLS has been reported to cause up to 40-100% yield losses in legumes (Adegbite and Amusa, 2008; Gaikpa *et al.*, 2015).

It is essential to develop effective strategies that can be used to manage important disease such as CLS. The use of chemicals in controlling crop diseases is no longer considered to be the best approach in this regard. It is essential to obtain more resistant varieties of crop species in order to feed the world (Walter, 2016) and tackle food insecurity. Thus, the aim of this study was to assess Bambara groundnut landraces for their reaction to CLS under field conditions with a view to identifying landraces which may be used in breeding programs to develop CLS resistant lines.

3.2 Materials and Methods

3.2.1 Plant Material and Planting Site

This study was conducted at the Ukulinga Research farm, at the University of KwaZulu-Natal in Pietermaritzburg. The farm is situated on a Latitude 29º 39'S and Longitude 30º 24'E. A total of 19 landraces of Bambara groundnut seeds obtained from various origins (Table 3.1) were evaluated for their reaction to natural CLS infection and they differed in coat and eye color. Some landraces with no labels were given labels based on their area of origin whilst the landraces with labels were maintained.

The plot on which the landraces were planted had a previous history of *Cercospora* leaf spot infection. Ten seeds per landrace were planted at an inter-row spacing of 30 cm and an intra-row spacing of 40 cm. The planting was done on the 2nd of December 2016 and the study was completed by June of the following year. A randomized complete block design was done and replicated twice. Weeds were controlled by hand weeding once a week and sprinkler irrigation, with no fertilizer added was done every afternoon.

3.2.2 Disease assessment: Disease Index, Area under the disease progress curve and apparent rate of infection

Disease index was assessed as soon as *Cercospora* symptoms were visible. A visual assessment was used in order to rate the reaction of Bambara groundnut landraces to the *Cercospora* leaf spot using a scale of 0-4 (Wongpiyasatid *et al.,* 1999) where: 0= no infected

leaves; 1= 1-25% infection; 2= 26-50% infection; 3= 51-75% infection; 4= 76-100% infection. A total of 3 disease ratings were done for each plant in both replicates at a 15 day interval between the first and second rating days and at an 11 day interval between the second and third rating days. The disease index (DI) was calculated for each line per replicate using the acquired infection scores using the standard DI formula (Wongpiyasatid *et al.*, 1999).

The DI values at different intervals were used to calculate the area under the disease progress curve (AUDPC) by using the formula obtained from (Simko and Piepho, 2012). The apparent rate of infection (r) was calculated for each landrace using Vanderplank's Logistic equation obtained from (Kumar *et al.*, 2011). Calculations for all the aforementioned assessments are as follows:

Disease Index

DI = [(0 x a) + (1 x b) + (2 x c) + (3 x d) + (4 x e)] / (a + b + c + d + e) x 100/4

where a, b, c, d and e are the number of Bambara groundnut plants with the levels of infection equal 0, 1, 2, 3 and 4 respectively.

Area Under the Disease Progress Curve

AUDPC = $\sum_{i-1}^{n-1} \left[\frac{Yi+1+Yi}{2}\right] [ti + 1 - ti]$

where Y_i = assessment of disease (%) at the i^{th} observation

t_i = time (days) at the i^{th} observation

n = total number of observations

Apparent Rate of Infection

$r = 1/t (\ln x_f /1-x_f) – (\ln x_i /1-x_i)$

where $t = t_{final} - t_{initial}$

x_f = final disease % x_i = initial disease % (i.e. 30% = 0.3)

3.2.3 Statistical Analysis

Data was statistically analyzed using Genstat, 18th Edition software by running a general analysis of variance (ANOVA). Treatment means of AUDPC and final foliar disease percentage were separated by the least significant differences (LSD) at 95% confidence level.

Table 3.1: List of Bambara groundnut landraces evaluated for reaction to *Cercospora* leaf spot and their origin.

LANDRACE	COUNTRY OF ORIGIN	LANDRACE	COUNTRY OF ORIGIN
SWZ 01	Swaziland	**STN 07**	Stanger, South Africa
SWZ 02	Swaziland	**STN 08**	Stanger, South Africa
SWZ 03	Swaziland	**STN 09**	Stanger, South Africa
SWZ 04	Swaziland	**II 42-1**	Zimbabwe
STN 01	Stanger, South Africa	**211-90**	CAPS, South Africa
STN 02	Stanger, South Africa	**011-7**	Pietermaritzburg, South Africa
STN 03	Stanger, South Africa	**M09-3**	Zimbabwe
STN 04	Stanger, South Africa	**KB05**	ARC, South Africa
STN 05	Stanger, South Africa	**211-2-2**	CAPS, South Africa
STN 06	Stanger, South Africa		

Legend for seed sources: Swaziland= Manzini market from vendors who obtain seeds from farmers throughout the country; **Stanger=** Umvoti Industries, Umvoti Beans; **Zimbabwe=** Department of Research and Specialist Services; **CAPS=** Capstone Seed Company, South Africa; **Pietermaritzburg=** Farmer collection; **ARC**= Agricultural Research Council, South Africa

3.3 Results

The results in this experiment revealed that all of the Bambara groundnut landraces evaluated were susceptible to CLS infection. All the landraces showed the characteristic symptoms of the disease (Table 3.2 and Fig. 3.1). There was no significant difference ($p > 0.05$) between the landraces based on both the AUDPC and final disease percentage (Table 3.2). P-values were 0.133 and 0.056 for final foliar disease percentage and AUDPC respectively. The apparent rates of infection are also shown in the aforementioned table. Landrace SWZ 02 had the highest infection rate of 0.075 per unit per day over the course of the rating period, whereas landrace STN 05 had the lowest of 0.012 per unit per day. This is in accordance with results on Table 3.3 since SWZ 02 was categorized as susceptible and STN 05 was categorized as moderately resistant. The groupings in Table 3.3 were made using AUDPC values.

Table 3.2: Final foliar disease percentage, AUDPC and calculated apparent rate of infection of Bambara groundnut landraces

LANDRACE	FINAL FOLIAR DISEASE[1] (%)	AUDPC[2]	APPARENT RATE OF INFECTION[3] (R)
SWZ 01	52.32[a]	1092[ab]	0.032
SWZ 02	70.23[a]	1224[ab]	0.075
SWZ 03	72.91[a]	1483[b]	0.059
SWZ 04	70.00[a]	1379[ab]	0.056
STN 01	56.66[a]	1119[ab]	0.040
STN 02	52.50[a]	1148[ab]	0.021
STN 03	43.76[a]	875[ab]	0.033
STN 04	48.75[a]	959[ab]	0.040
STN 05	34.03[a]	820[ab]	0.012
STN 06	54.86[a]	1082[ab]	0.047
STN 07	49.30[a]	953[ab]	0.038
STN 08	58.75[a]	1134[ab]	0.037
STN 09	56.25[a]	1039[ab]	0.047
KB05	41.66[a]	695[a]	0.043
211-2-2	54.16[a]	1095[ab]	0.049
211-90	45.23[a]	952[ab]	0.032
II 42-1	40.28[a]	915[ab]	0.027
011-7	47.42[a]	934[ab]	0.032
M09-3	61.43[a]	1117[ab]	0.058
F RATIO	1.69	2.13	
P-VALUE	0.133	0.056	
CV%	21.4	17.2	

1=Final disease percentage is the amount of disease on the final day of rating, 2= Area under the disease progress curve was calculated over a 26 day period at various intervals, 3=Apparent rate of infection calculated using Vanderplank's Logistic equation with initial and final disease percentage. Means within a column not followed by the same symbol (a, b) are significantly different.

Figure 3.1: Characteristic symptoms of *Cercospora* leaf spot viewed in the field at Ukulinga Research Farm.

Picture A: Infected leaves with black irregular spots. Picture B: A severely infected Bambara groundnut plant that appears to be nearing defoliation.

Table 3.3: Grouping of Bambara groundnut landraces based on AUDPC using scale adapted by Kumar *et al.* (2011).

Category	No. of lines	AUDPC	Bambara groundnut landrace
Resistant	0	1-500	-
Moderately resistant	8	501-1000	KB05, STN 03, STN 04, STN 05, II 42-1, 211-90, STN 07, 011-7
Susceptible	11	1001-1500	SWZ 02, SWZ 03, 211-2-2, SWZ 01, STN 02, STN 01, STN 09, SWZ 04, STN 06, STN 08, M09-3
Highly susceptible	0	>1500	-

3.4 Discussion

In this study, none of the landraces evaluated were immune to infection by CLS. This is in accordance with reports by Obagwu (2003) but is contrary to past reports that Bambara groundnut is free of diseases (Haq, 1983; Swanevelder, 1999). The results of this study showed no significant difference between the landraces in terms of AUDPC units (Table 3.2). However, differences were seen after categorizing the landraces into different resistance

groups. Landraces KB05 and STN 05 show promise in that they may be used in future breeding programs since they were both moderately resistant (Table 3.3).These two landraces had the lowest AUDPC values and the lowest apparent rate of infection respectively. The apparent rate of infection was calculated using the last and first observations of disease progress which were recorded during disease rating. According to Van der Plank (1963), the higher the infection rate, the higher the percentage of disease when the field is ripe. The same is the same when the rate of infection is low, as seen with landrace STN 05. The AUDPC values calculated are the most appropriate measures of disease since they provide a summary of disease intensity over time (Kumar *et al.,* 2011). All other landraces considered to be moderately resistant, such as KB05 and STN 05 may also be considered when developing improved Bambara groundnut varieties.

Not much research has been done on Bambara groundnut regarding breeding for resistance to CLS. However, breeding research has been done on other legumes such as mungbean; a legume in the same genus as Bambara groundnut. It has been demonstrated that a single major gene is responsible for CLS resistance in *Vigna* species (Booker and Umaharan, 2007). Results of a particular study found a quantitative trait locus for *Cercospora* resistance by crossing F_1 and BC_1F_1 populations which were developed from crosses between CLS-resistant and CLS-susceptible mungbean cultivars (Souframanien and Dhanasekar, 2014). Segregation analysis confirmed that CLS resistance was controlled by a single dominant gene. By extension, landraces from this study; KB05 (moderately resistant) and STN 09 (susceptible) may be used in a trial similar to that described by Souframanien and Dhanasekar (2014).

Another approach which can be taken in addressing this problem is that of understanding the mode of action of the pathogen. In the present study, all Bambara groundnut landraces which were assessed were exposed to a lot of sunlight throughout the duration of the trial. Red margins around the lesions were visible in the beginning of the infection process. The severity of the leaf spots on Bambara groundnut caused by *C. canescens* substantially depends on the production of cercosporin by the pathogen (Wongpiyasatid *et al.,* 1999) which results in a red pigment due to the presence of the toxin (Daub and Ehrenshaft, 2000). Cercosporin destroys plant cells by peroxidation of the membrane lipids resulting in membrane degradation (Daub *et al.,* 1998). The damage to the plant membrane gives the

pathogen access to nutrients which are in the leaves and for this reason, the pathogen is able to grow and establish itself successfully in the plant (Daub *et al.,* 1998).

The toxicity of cercosporin is activated by light and its effect does not manifest in the dark or under low light conditions (Daub and Chung, 2007). Previous studies have shown that there is a correlation between disease severity and light exposure when assessing *Cercospora* diseases of coffee (*coffea* L.), sugar beet (*Beta vulgaris* L.) and banana (*Musa acuminata* L.) (Daub and Ehrenshaft, 2000). Shading has been shown to reduce penetration of coffee leaves by *C. beticola* leading to a reduction of lesions (Daub and Chung, 2007).

In conclusion, the findings in this study showed that the Bambara groundnut landraces evaluated were all susceptible to CLS infection. Landraces KB05 and STN 05 showed potential and may be considered in being used in future breeding programs. However, more conclusive results may be obtained by repeating the trial for two more seasons using a wider range of landraces in order to determine whether the landraces would react consistently to the disease. Furthermore, Bambara groundnut landraces could also be screened for a resistant gene to cercosporin since this toxin is a prime factor that influences disease severity.

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CHAPTER 4

GENETIC DIVERSITY OF BAMBARA GROUNDNUT (*VIGNA SUBTERRANEA* [L.] VERDC.) GENOTYPES REVEALED BY SSR PRIMERS

Abstract

Bambara groundnut (*Vigna subterranea* [L.] Verdc.) is an African legume mostly grown as a subsistence crop by small-scale farmers. It exists as landraces rather than improved varieties and this threatens food security which relies on a strong foundation of plant genetic resources. Simple sequence repeat (SSR) markers are reported to be sufficient in genetic diversity studies of Bambara groundnut landraces and may further be used in efforts to improve this crop. The aim of this study was to genotype 22 Bambara groundnut landraces obtained from various geographical regions in Southern Africa using SSR primers developed for Bambara groundnut with the objective of determining any genetic differences that can be exploited to improve the landraces that were used in this study. DNA was extracted from 22 landraces and was then used in touchdown PCR using 20 Bambara groundnut specific primers. The PCR products were subjected to capillary electrophoresis using a genetic analyzer. A phylogenetic tree was constructed using DARwin 5.0 software. After analysis, 110 alleles were detected, with a mean of 5.50 alleles per locus. The mean polymorphic information content (PIC) was 0.62 and the mean expected heterozygosity (H_e) was 0.62; the latter indicating a high gene diversity among the genotypes. The neighbor-joining analysis generated three major genetic groups, where the genotypes were clustered irrespective of their geographic origin. Landraces SCAM and LMS which had improved seedling emergence and seedling establishment did not appear in the same cluster. Landraces KB05 and STN 05 which were moderately susceptible to *Cercospora* leafspot also did not appear in the same cluster. The findings in this study confirmed the strength SSR markers possess in genetic diversity studies of Bambara groundnut genotypes.

4.1 Introduction

Bambara groundnut (*Vigna subterranea* [L.] Verdc.) is an African crop which is grown mostly for its edible and nutritious seeds, which may be eaten raw, roasted or boiled (Abu and Jeduah, 2011). The crop is a member of the Leguminosae family; an essential legume taxon (Odongo *et al.,* 2015). Bambara groundnut follows peanut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* [L.] Walp.) in importance (Odongo *et al.,* 2015). Bambara

groundnut has great potential in terms of its high nutrient content and its tolerance to grow in drought-stricken areas (Murevanhema and Jideani, 2013). Bambara groundnut is a very popular, yet underutilized grain legume the scientific research community seems to neglect (Mohammed, 2014).

Food security relies on a strong foundation of plant genetic resources in order to support the sustenance of every person on Earth (Food and Agriculture Organisation (FAO), 2017). It is essential to strengthen seed systems in order to have a thorough understanding of them (FAO, 2017). Formal seed systems are present but are rarely used by farmers as a channel to obtain seed (McGuire, 2016). Currently, Bambara groundnut exists as landraces rather than improved varieties. This may be attributed to the distribution of seed via informal seed systems. An informal method of classification, based on provenance and seed coat color (Massawe *et al.,* 2005), has been used to identify Bambara groundnut. This leads to one landrace bearing numerous names due to the movement of seeds from one region to another.

Farmers' main objective is to obtain high yields under all conditions (Mabhaudhi, 2012). The same objective exists in breeding programs as well. A major production constraint to large scale production of Bambara groundnut is low yield (Mohammed, 2014).Most farmers in Ghana grow Bambara groundnut landraces which produce low yields which are attributed to poor germination and poor crop establishment (Abu and Jeduah, 2011). Mabhaudhi (2012) has also reported low yields in South African Bambara groundnut landraces and attributed it to a variability within the crop under drought stress. Reports suggest that farmers in Botswana obtain low Bambara groundnut yields due to low rainfall conditions in that country and resort to additional irrigation in order to increase their yields (Ngwako *et al.,* 2013). The lack of improved varieties and poor production technologies has a negative effect on the large-scale production of the crop (Mohammed, 2014).

The improvement of crops in most breeding programs is marker-assisted. Random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers have all been reported to be sufficient in genetic diversity studies of Bambara groundnut landraces and may further be used in efforts to improve this crop (Amadou, *et al.,* 2001; Ho *et al.,* 2016; Massawe *et al.*, 2002).

The use of RAPDs involves a polymerase chain reaction (PCR) on the DNA of the individual under study using a short primer (10 nucleotides) of a random sequence that binds to numerous different loci and amplifies random sequences (Xu, 2010). With regards to Bambara groundnut, Amadou *et al.* (2001) reported the use of RAPDs for germplasm classification and consequently improving the crop.

AFLP markers are based on the selective PCR amplification of restriction fragments from a total double-digest of genomic DNA (Xu, 2010). Results by Massawe *et al.* (2002) stated that the AFLPs used in their study were highly polymorphic and it was easy to determine the amount of genetic diversity and to establish genetic relationships among Bambara groundnut landraces. These results will help in the formulation of marker-assisted breeding in Bambara groundnut.

Microsatellite markers such as SSRs are short tandem repeats of nucleotides in the genome of prokaryotes and eukaryotes (Vieria *et al.,* 2016). SSRs have been the most commonly used markers for genotyping plants over the past two decades (Vieria *et al.,* 2016). This is because they are highly informative and may aid greatly in evolutionary studies of wild plant species (Vieria *et al.,* 2016). A major constraint in molecular breeding of Bambara groundnut is the shortage of locus-specific co-dominant markers in this crop (Beena *et al.,* 2012) such as SSR markers. Whole genome sequencing of Bambara groundnut has begun, based on a 2015 progress report (Timberlake *et al.,* 2015) by African Orphan Crops Consortium (AOCC). The Bambara groundnut is one of the 101 crops that are the focus of the AOCC and efforts are being made to breed a Bambara groundnut cultivar that matures in 90-100 days rather than 140 days (Timberlake *et al.,* 2015).

Genetic improvement of Bambara groundnut is vital to its productivity in any location (Mohammed, 2014). Genetic diversity analyses are conducted for major crops in breeding programs for the selection of parental lines (Aliyu *et al.,* 2016). This type of information may be important in underutilized crops such as Bambara groundnut, which predominantly exists as landraces which lack information about their genetic diversity (Aliyu *et al.,* 2016). For the most part, phenotypic descriptors have been used in genetic diversity analyses of Bambara groundnut (Aliyu *et al.,* 2016). Very few molecular marker technologies have been reported relative to the aforementioned technology (Aliyu *et al.,* 2016). Relative to the traditional

approach, molecular markers have emerged as the most dominant tool in the evaluation of genetic variability (Aliyu *et al.*, 2016).

The aim of this study was to genotype 22 Bambara groundnut landraces obtained from various geographical regions in Southern Africa using 20 simple sequence repeat (SSR) primers developed for Bambara groundnut (Ho *et al.*, 2016). This was done with the objective to determine if the findings of the previous two chapters (reaction to *Cercospora* leaf spot and emergence/establishment after hydropriming) of this thesis were due to the genetic differences/similarities among landraces.

4.2 Materials and Methods

4.2.1 Plant Material

A total of 22 Bambara groundnut landraces (Table 4.1) were planted in seedling trays (two seeds per landrace). Most of the seeds used in this study were also used in the two previous experimental chapters.

4.2.2 DNA Extraction and Genotyping

Leaf material from each landrace was sampled from 2-week old plants. DNA was extracted from frozen material using a protocol from the GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific, USA). DNA purity was checked and quantified using a NanoDrop2000 (Thermo Scientfic, USA) using a volume of 1µl per sample. The genotyping was performed at SciCorp, Pietermaritzburg where DNA was amplified by PCR with the 20 primer sets (Table 4.2) and the reactions for each marker were labelled with one of four fluorescent dyes. Amplification was carried out under touchdown PCR cycling conditions with annealing temperatures from 60 – 52 °C. The PCR products were pooled in sets of four, and subjected to capillary electrophoresis using an Applied Biosystems 3130xl Genetic Analyzer.

4.2.3 Data Analysis

The genetic structure and diversity among the genotypes were analyzed using two methods. In the first method, the presence or absence of polymorphisms were viewed using binary data. This data was used to obtain a dissimilarity matrix using the Jaccard index. The matrix was used to run a cluster analysis based on Neighbor-joining employing the software DARwin 5.0 (Perrier and Jacquemoud-Collet, 2006). However, to determine the genetic

structure within and among genotypes, a second approach based on the co-dominant nature of the marker was adopted using GenAlex version 6.5 (Peakall and Smouse, 2012).

Genetic diversity parameters, such as number of alleles per locus (Na), number of effective alleles per locus (Ne), observed (Ho) and expected (H_e) heterozygosity, were calculated using GenAlex version 6.5 (Peakall and Smouse, 2012) according to the protocol described by Nei and Li (1979). Polymorphic information content (PIC) was calculated using the formula: PIC =1 - ΣPij2, where Pij is the frequency of jth allele of the ith locus. Nei's unbiased genetic distance was also estimated to determine the degree of population differentiation among the study material.

Table 4.1: List of Bambara groundnut landraces and their origin used in this study

Landrace	Origin	Landrace	Origin
STN 01	Stanger	**SWZ 04**	Swaziland
STN 02	Stanger	**Mokgalo**	Botswana
STN 03	Stanger	**MMB**	Botswana
STN 04	Stanger	**Keledi**	Botswana
STN 05	Stanger	**SCAM**	Botswana
STN 06	Stanger	**LMS**	Botswana
STN 08	Stanger	**M09-3**	Zimbabwe
STN 09	Stanger	**011-7**	Pietermaritzburg, South Africa
SWZ 01	Swaziland	**211-90**	CAPS, South Africa
SWZ 02	Swaziland	**II42-1**	Zimbabwe
SWZ 03	Swaziland	**KB05**	ARC, South Africa

Legend for seed sources: Botswana= National Genetic Resources Centre (NGRC); **Swaziland=** Manzini market from vendors who obtain seeds from farmers throughout the country; **Stanger=** Umvoti Industries, Umvoti Beans; **Zimbabwe=** Department of Research and Specialist Services; **CAPS=** Capstone Seed Company, South Africa; **Pietermaritzburg=** Farmer collection; **ARC**= Agricultural Research Council, South Africa

Table 4.2: List of SSR primers used in this study (Ho *et al.*, 2016)

SSR Primer	Sequence 5'-3'	Annealing temperature (°C)
P1	F: AACTTGCCATACGTGGAAGG R:ACACGCTGCATAATTCACCA	59
P7	F: GTAGGCCCAACACCACAGTT R: GGAGGTTGATCGATGGAAAA	55
P10	F: TCAGTGCTTCAACCATCAGC R: GACCAAACCATTGCCAAACT	55
P15	F: AGGAGCAGAAGCTGAAGCAG R: CCAATGCTTTTGAACCAACA	55
P16	F: CCGGAACAGAAAACAACAAC R: CGTCGATGACAAAGAGCTTG	57
P19	F: AGGCAAAAACGTTTCAGTTC R: TTCATGAAGGTTGAGTTTGTCA	55
P21	F: CAAACTCCACTCCACAAGCA R: CCAACGACTTGTAAGCCTCA	57
P23	F:CAGTAGCCATAATTTGCTATGAACA R: CGAATCACCATTCAATACGC	55
P30	F: AATGCAAGATTTTGGCTTGG R: CCCACTCAAACCATACACCA	59
P31	F: GCTAAGGTGGAGTGGTGGAA R: CAATCATCTTTTGCGCTTCA	57
P32	F: TTCACCTGAACCCCTTAACC R: AGGCTTCACTCACGGGTATG	57
P33	F: ACGCTTCTTCCCTCATCAGA R: TATGAATCCAGTGCGTGTGA	57
P37	F: CCGATGGACGGGTAGATATG R: GCAACCCTCTTTTTCTGCAC	55
P44	F: TGTGGGCGAAAATACACAAA R: TCGTCGAATACCTGACTCATTG	59
Pco	F:GAGTCCAATAACTGCTCCCGTTTG R:ACGGCAAGCCCTAACTCTTCATTT	59
D8	F:GCATCTTTACAGCAAGAGTTTCAA R:TGGATCTTCCTCATTGCAGTATAA	59
D11	F: GAGGAAATAACCAAACAAACC R:CTTACGCTCATTTTAACCAGACCT	59
D14	F:GAACGAAGCCAGGATAATGATAGT R:CGAAAGCGACAACTCACTACTAAA	59
D15	F: TGACGGAGGCTTAATAGATTTTTC R:GACTAGACACTTCAACAGCCAATG	59
E7	F: CATGATTTGTTGTGATGATGAT R:AACAACAAATGTACCAAAGAATCG	51

Forward primers all had an M13 sequence attached on the 5' end.

4.3 Results

Table 4.3 presents the genetic parameters calculated using 20 SSR markers on 22 Bambara groundnut genotypes. The 20 SSR markers detected a total of 110 alleles with different fragment size and allele frequencies. The number of alleles detected ranged from 1 (D8) to 13 (D14), with a mean of 5.50 per locus. The polymorphic information content (PIC) observed in this study ranged from 0.00 to 0.90, with a mean of 0.62, as revealed by D8 and D14 respectively. The allelic diversity, as explained by expected heterozygosity (H_e), varied between 0.00 and 0.92 for D8 and D14 markers respectively. In the set of genotypes presented, 30% of the markers showed an F_{IS} value of 1 and two markers, P44 and D11 showed very low F_{IS} values of -0.07 and -0.06 respectively. A total of 40% of the loci (highlighted) had negative F_{IS} values.

The genetic distance among the 22 Bambara groundnut genotypes from the various geographical locations are presented in Table 4.4, with a minimum of 0.28 to a maximum of 0.74 among two pairs of genotypes (highlighted). The smallest genetic distance was observed between STN 08 and SWZ 04. These two landraces both share a brown color; the difference being that STN 08 has a speckled coat. Landrace Keledi and II42-1 had the maximum genetic distance between them which is further confirmed by their two different coat colors; Keledi having a cream coat with a butterfly eye and II42-1 having a dark brown coat (Pictures not provided).

The levels of similarities and divergence among the 22 Bambara groundnut genotypes are presented in Figure 4.1 and Table 4.5 where genotypes were grouped into three clusters. The clusters were composed of seeds with different phenotypic characteristics, specifically coat color. Therefore, there was no link between genotypic parameters and phenotypic characteristics of Bambara groundnut. Additionally, landraces SCAM and LMS which were the best performers in Chapter 2 did not appear in the same cluster. Similarly, the best performers in Chapter 3, landraces KB05 and STN 05 did not appear in the same cluster.

Table 4.3: Genetic diversity parameters generated by 20 SSR markers among 22 Bambara groundnut genotypes.

Loci	N_a	N_e	H_o	H_e	F_{IS}	PIC
P1	4	3.04	1.00	0.69	-0.49	0.67
P7	3	1.91	0.00	0.49	1.00	0.48
P10	9	5.29	0.40	0.84	0.51	0.81
P15	6	4.19	0.05	0.78	0.94	0.76
P16	6	5.83	1.00	0.85	-0.21	0.83
P19	7	5.04	0.00	0.82	1.00	0.80
P21	2	1.10	0.00	0.09	1.00	0.09
P23	5	2.29	1.00	0.58	-0.77	0.56
P30	4	2.80	1.00	0.66	-0.56	0.64
P31	3	2.09	1.00	0.53	-0.92	0.52
P32	11	9.05	0.05	0.91	0.95	0.89
P33	4	1.85	0.00	0.47	1.00	0.46
P37	6	4.52	1.00	0.80	-0.28	0.78
P44	7	5.26	0.86	0.83	-0.07	0.81
Pco	6	3.84	0.00	0.76	1.00	0.74
D8	1	1.00	0.00	0.00	-	0.00
D11	5	2.47	0.00	0.61	1.00	0.60
D14	13	9.98	0.95	0.92	-0.06	0.90
D15	6	3.36	0.64	0.72	0.09	0.70
E7	2	1.71	0.05	0.43	0.89	0.42
Total	110	76.6	9	12.8	6.4	12.4
Mean	5.50	3.83	0.45	0.64	0.32	0.62
SE	0.67	0.54	0.10	0.06	0.16	0.05

N_a= Number of alleles per locus; N_e = number of effective alleles per locus; H_o= observed heterozygosity, H_e = expected heterozygosity; F_{IS} = Inbreeding coefficient; PIC = polymorphic information content, SE= Standard error

Table 4.4: Genetic distance among 22 Bambara groundnut genotypes estimated using Jaccard genetic distance index using 20 SSR markers.

Genotypes	STN 01	STN 02	STN 03	STN 04	STN 05	STN 06	STN 08	STN 09	SWZ 01	SWZ 02	SWZ 03	SWZ 04	Mokgalo	MMB	Keledi	SCAM	LMS	M09-3	011-7	211-90	II42-1	KB05
STN 01																						
STN 02	0.64																					
STN 03	0.66	0.63																				
STN 04	0.62	0.57	0.62																			
STN 05	0.64	0.61	0.57	0.60																		
STN 06	0.68	0.66	0.65	0.64	0.62																	
STN 08	0.60	0.58	0.53	0.56	0.41	0.59																
STN 09	0.62	0.59	0.58	0.58	0.56	0.60	0.53															
SWZ 01	0.66	0.64	0.56	0.62	0.57	0.65	0.54	0.59														
SWZ 02	0.62	0.53	0.62	0.55	0.60	0.64	0.56	0.58	0.62													
SWZ 03	0.65	0.62	0.61	0.61	0.59	0.52	0.56	0.57	0.62	0.61												
SWZ 04	0.64	0.61	0.57	0.60	0.44	0.62	0.28	0.56	0.57	0.60	0.59											
Mokgalo	0.65	0.62	0.54	0.61	0.56	0.63	0.52	0.57	0.51	0.61	0.60	0.56										
MMB	0.66	0.63	0.55	0.62	0.56	0.64	0.53	0.58	0.49	0.62	0.61	0.56	0.51									
Keledi	0.71	0.68	0.61	0.67	0.62	0.70	0.58	0.63	0.56	0.67	0.66	0.62	0.56	0.55								
SCAM	0.56	0.58	0.60	0.57	0.58	0.62	0.55	0.56	0.60	0.57	0.59	0.58	0.59	0.60	0.65							
LMS	0.69	0.66	0.66	0.65	0.63	0.58	0.60	0.61	0.66	0.65	0.55	0.63	0.64	0.65	0.70	0.63						
M09-3	0.63	0.54	0.62	0.56	0.60	0.64	0.57	0.58	0.62	0.49	0.61	0.60	0.61	0.62	0.67	0.57	0.65					
011-7	0.61	0.58	0.50	0.57	0.51	0.59	0.48	0.53	0.44	0.57	0.56	0.51	0.46	0.35	0.50	0.55	0.60	0.57				
211-90	0.61	0.58	0.54	0.57	0.42	0.59	0.39	0.53	0.54	0.57	0.56	0.42	0.53	0.53	0.59	0.55	0.60	0.57	0.48			
II42-1	0.72	0.70	0.69	0.68	0.66	0.70	0.63	0.60	0.69	0.68	0.67	0.66	0.67	0.68	0.74	0.67	0.71	0.69	0.63	0.63		
KB05	0.67	0.58	0.67	0.60	0.64	0.69	0.61	0.63	0.67	0.43	0.66	0.64	0.65	0.66	0.72	0.61	0.70	0.54	0.61	0.61	0.73	

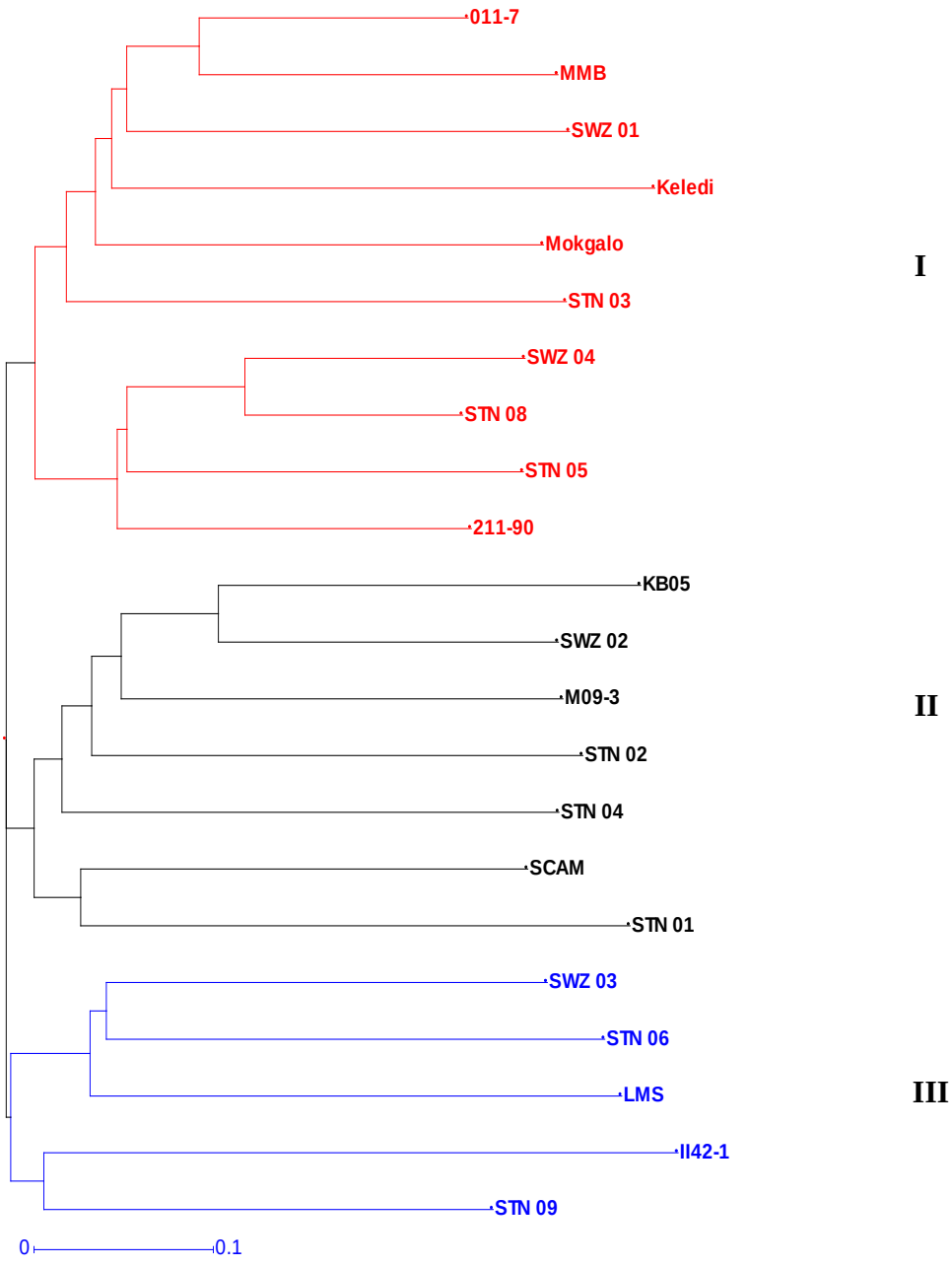

Figure 4.1: Dendrogram using neighbor-joining algorithm using the unweighted pair group method (UPGMA) revealing genetic relationships among 22 Bambara groundnut genotypes based on 20 SSR markers.

Table 4.5: Cluster grouping of the 22 genotypes and their origin.

Cluster	Genotype	Origin	Mean GD
I	011-7	Pietermaritzburg, SA	0.52
	SWZ 01, SWZ 04	Swaziland	
	MMB, Keledi, Mokgalo	Botswana	
	STN 03, STN 08, STN 05	Stanger, SA	
	211-90	Capstone seed, SA	
II	KB05	ARC, SA	0.57
	SWZ 02	Swaziland	
	M09-3	Zimbabwe	
	STN 01, STN 02, STN 04	Stanger, SA	
	SCAM	Botswana	
III	SWZ 03	Swaziland	0.61
	STN 06, STN 09	Stanger, SA	
	LMS	Botswana	
	II42-1	Zimbabwe	

Legend for seed sources: Botswana= National Genetic Resources Centre (NGRC); **Swaziland=** Manzini market from vendors who obtain seeds from farmers throughout the country; **Stanger=** Umvoti Industries, Umvoti Beans; **Zimbabwe=** Department of Research and Specialist Services; **CAPS=** Capstone Seed Company, South Africa; **Pietermaritzburg=** Farmer collection; **ARC**= Agricultural Research Council, South Africa; **GD**= Mean genetic distance

4.4 Discussion

This study determined the genetic diversity among 22 Bambara groundnut genotypes using 20 SSR markers. These 20 markers amplified a total of 110 alleles, with an average number of 5.50 alleles per locus. The mean alleles per locus (5.50) observed in this study was higher than that observed by Odongo (2015) whose mean was 2.00 where 11 of the markers employed in this study were used. However, Odongo (2015) studied Bambara groundnut landraces which originated from Kenya only. This may indicate that there was low genetic diversity from the crops because they had a close origin. Mohammed (2014) observed a higher mean of alleles per locus (10.6) compared to this study. This may be due to the fact that a more diverse group of Bambara groundnut was used whereas in the present study, Bambara groundnut only originated from Southern Africa.

The PIC describes how effective markers are in genetic diversity studies of crops (Mohammed, 2014), in this case, in Bambara groundnut genotypes. This tool is useful when selecting genetically distinct parents when used in the genetic enhancement of the crop (Amadou *et al.,* 2001; Massawe *et al.,* 2002; Mohammed, 2014). In this study, marker D8 had the lowest PIC (0.00) which indicates that it is monomorphic and is not a good marker for genetic diversity studies (Massawe *et al.,* 2002) whereas the best marker (high polymorphism) was D14 with a PIC of 0.90. Overall, it was observed that a total of 55% of the markers used in this study were above the mean PIC (0.62) and this indicates that the markers used were of good strength and quality. The high PIC values observed in this study suggests that most of the SSR markers could be used in any Bambara groundnut genetic diversity study and genetic map construction.

Heterozygosity is an essential measurement of gene diversity (Wang *et al.,* 2014). Reportedly, the number of loci and populations incorporated in studies might have an effect on the estimates of gene diversity (H_e) (Wang *et al.,* 2014). The mean expected heterozygosity (H_e) observed in this study was 0.64 which is a relatively high value. This is not in agreement with Mohammed (2014) who observed an H_e of 0.79 in a study using SSR markers on Bambara groundnut. Mohammed (2014) observed greater heterozygosity in the genotypes used compared to those used in this study. However, this difference may have been because 50 genotypes of Bambara groundnut were used (Mohammed, 2014). A low H_o value is expected for self-pollinated crops such as Bambara groundnut (Mohammed, 2014) and is observed (0.45) in this study.

The inbreeding coefficient (F_{IS}) indicates the level of heterozygosity within and among populations (Assefa, 2012). A high F_{IS} value indicates that landraces are homozygous (Assefa, 2012). In this set of 22 Bambara groundnut genotypes, 30% of the markers showed an F_{IS} value of 1 suggesting that the alleles at these loci are fixed i.e. reached 100% homozygosity. Conversely, negative F_{IS} values indicate an excess of heterozygosity (Al-Faifi *et al.,* 2016) which was observed for markers P44 and D11 with F_{IS} values of -0.07 and -0.06 respectively. In this study 40% of the loci had negative F_{IS} values suggesting all the genotypes at these loci had an excess of heterozygotes. For example, for locus P1, 69% of the genotypes are expected to be heterozygous at the specific locus under random mating conditions; however, 100% of the genotypes at this locus were heterozygotes. It may be due to high out-crossing or mutation at the specific loci.

According to Nei (1987), the genetic distance is the measure of the extent of genetic differences that exist between individuals, populations or species which is measured by some numerical quantity and can be described by allelic variation. The genetic distances calculated based on Nei (1987) using the 20 SSR markers among the 22 Bambara groundnut genotypes from the various geographical locations ranged from 0.28 to 0.74. The lowest observed genetic distance of 0.28 was between landraces STN 08 and SWZ 04. The highest genetic distance of 0.74 was between Keledi and II42-1. Genetic distance ranges from 0 to 1 where 0 means complete sharing of genetic material and 1 means no sharing (Mohammadi and Prasanna, 2003). The findings in the current study revealed that Bambara groundnut genotype STN 08 from Stanger, South Africa and SWZ 04 from Swaziland share the most genetic material compared to the other genotype pairs. This reflects geographical proximity of the two genotypes. Since none of the genotype pairs had a genetic distance of 0.00, it can be confidently deduced that none of the genotypes are identical but are rather diverse as observed with previous parameters in this chapter.

Similarities and divergences among the 22 Bambara groundnut genotypes are presented (Fig.4.1) using the neighbor-joining algorithm using the unweighted pair group method. Genotypes were grouped into three clusters (Fig. 4.1 and Table 4.5). Similar to this study, Mohammed (2014) also demonstrated the ability of SSR markers to section the genotypes of Bambara groundnut into close groups. Previous genetic diversity studies of Bambara groundnut using AFLP and RAPD markers have shown the grouping of the crop into fewer

clusters than SSR markers after sequencing (Amadou *et al.,* 2001; Massawe *et al.,* 2002). This indicates the high polymorphism of SSR markers compared to other markers.

The largest among the three clusters was Cluster I which consisted of 10 genotypes originating from 5 geographic sources. One genotype originated from Pietermaritzburg, two from Swaziland, three from Botswana, three from Stanger and one from Capstone seed company. In this cluster, two pairs of genotypes (011-7 and MMB; and SWZ 04 and STN 08) were closely positioned. Mohammed (2014) used genotype 011-7 in a similar study and found it to be an outlier in the cluster it was found in. However, in the current study genotype 011-7, from a famer's collection in Pietermaritzburg is closely positioned to MMB from Botswana. This suggests that they possess similar genes. Cluster I was followed by Cluster II with seven genotypes, three from Umvoti Industries, Stanger and one from Swaziland, ARC, Zimbabwe and Botswana each.

Capstone Seed Company is a seed company in South Africa that buys and sells Bambara groundnut seeds composed of a mixture of morpho-types. Hence there is a possibility that CAPS may have secured Bambara groundnut seed landraces from Botswana and other neighboring countries hence the grouping of 011-7 and MMB closely together. The farmer may have bought seed (011-7) from CAPS which was earlier sourced from a different country.

Cluster III was the smallest with five genotypes. Genotypes SWZ 03 and STN 06 were the most closely positioned in this cluster but varied in coat color: while SWZ 03 was cream and had a black eye, STN 06 had a cream coat and a brown eye. Even though II42-1 and STN 09 were in the same clade, they had a high genetic distance between them. Furthermore, the coat colors of the seeds of the two genotypes were extremely different: STN 09 was cream with a black, speckled eye whereas II42-1 was red with no eye. Even with this difference, the positioning of the two genotypes in the same clade suggests a small degree of similarity in their genotypes.

In Chapter 2, landraces SCAM and LMS were the best performers of the five Botswana landraces with improved emergence and seedling establishment at 36 hours and 24 hours priming time. However, these two landraces did not appear in the same cluster after sequencing despite the fact that they were sourced from the same origin. Similarly, the two

landraces KB05 and STN 05 in Chapter 3 which were observed to have shown the most promise in *Cercospora* leaf spot resistance also did not appear in the same cluster. However, landraces SWZ 04 and STN 08 with the lowest genetic distance of 0.28 between them were both grouped under the same category (susceptible) in Chapter 3.

In conclusion, the findings in this study confirmed the strength SSR markers possess in genetic diversity studies of Bambara groundnut genotypes. This study has also, to a certain degree, shown that the similarities in reaction of Bambara groundnut landraces to disease or seed priming exposure does not necessarily mean they will be genetically similar.

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CHAPTER 5
THESIS OVERVIEW

5.1 Major Findings

This study sought to gather information which may be used to enhance Bambara groundnut production and possibly identify landraces which may be used to develop improved varieties of the crop. A priming experiment was conducted using durations of 0, 24 and 36 hours in order to view the improvement of seedling emergence and seedling establishment of Bambara groundnut. Secondly, the reaction of Bambara groundnut to *Cercospora* leaf spot, a major disease of the crop, was evaluated. Lastly, a genetic diversity study was conducted on the 22 Bambara groundnut landraces using 20 SSR primers developed specifically for the crop.

In Chapter 2 it was revealed that 36 hours of priming Bambara groundnut seed resulted in the best seedling emergence and establishment for all the landraces studied, except MMB whose best priming duration was 24 hours. It was also observed that landraces LMS and SCAM were the best performers with regard to the two parameters measured.

Results reported in Chapter 3 highlighted landraces KB05 and STN 05 as showing the best resistance to *Cercospora* leaf spot. These two landraces were both moderately resistant. These two landraces may be used in breeding programs where the goal is to develop varieties resistant to the disease.

The findings in Chapter 4 revealed that the 22 Bambara groundnut landraces were highly diverse as reflected by an average H_e value of 0.62. This study confirmed the strength SSR markers possess in genetic diversity studies of Bambara groundnut genotypes. Additionally, their strength was also revealed by the relatively high average PIC value of 0.62. Results of this study showed that the similarities in reaction of Bambara groundnut landraces to disease or seed priming exposure does not necessarily indicate that they will be genetically similar.

5.2 Implications of Study

Since Bambara groundnut is an underutilized crop (Swanevelder, 1998) and is neglected in the scientific research world compared to other crops, it becomes necessary to compile

information that could help in its improvement. This study focused on the production constraints of Bambara groundnut. Determining the optimum seed priming duration was essential since this knowledge may be used by farmers in order to improve their crop stand at planting. Furthermore, priming has also been reported to additionally equip crops in overcoming biotic and abiotic stress (Lutts *et al.,* 2016; Ogbuehi *et al.,* 2013). Identifying landraces which have some degree of resistance to major diseases such as *Cercospora* leaf spot is also beneficial in that it opens up doors to genetically improve the crop by developing resistant varieties which are not currently present in Bambara groundnut. The genetic diversity of Bambara groundnut is an area of research which is still being explored. The genetic diversity study conducted in this research was essential since it showed the high diversity of the landraces and the usefulness of the SSR primers used.

5.3 Way Forward

There are a variety of seed hydropriming durations best suited for Bambara groundnut improvement. This normally depends on the range of times used in each individual study and the priming technique used as well. There is a need to expand this study by focusing on both priming duration and priming technique to develop a protocol that is suited for Bambara groundnut production on a commercial scale. However, soaking seed in water is a cost-effective method for farmers who are only interested in the crop for subsistence purposes.

In order to obtain more conclusive results, it is necessary to screen the Bambara groundnut for resistance to *Cercospora* leaf spot for an additional two seasons. Since this disease is caused by the toxin cercosporin produced by the pathogen, Bambara groundnut landraces can also be screened for a resistance gene to this toxin since it is a prime factor that influences disease severity.

Future research should focus on genetic diversity analyses of Bambara groundnut; similar to those conducted for major crops such as maize. The information gathered from that research may be important in underutilized crops such as Bambara groundnut which exist mainly as landraces with little information about their genetic diversity.

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