CHARACTERISING FOUR COWPEA (*Vigna unguiculata* (L.) Walp.) MOZAMBICAN LANDRACES DEPOSITED IN A SEED BANK FOR DROUGHT TOLERANCE

By

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ABSTRACT

Cowpea production is severely affected by environmental stress factors, particularly drought. More drought-tolerant crops are therefore urgently required for future improvement of food production in Mozambique. To increase high productivity and sustainability of cowpea there is a need to establish an active local breeding program, this should also include screening and characterisation of germplasm to select for more drought-tolerant cowpea landraces. This study has been therefore conducted in a temperature-controlled greenhouse at the University of Pretoria and in an open-sided tunnel house in Mozambique. The overall aim of the research work was to identify the most drought-tolerant cowpea landrace currently deposited in the Mozambican gene bank. Results of this study showed that tested Mozambican cowpea landraces have a different degree of drought tolerance with one Mozambican cowpea landrace, Timbawene moteado, better performing under drought conditions. In particular, plants of this landrace had more vigorous growth better overcoming a drought period with fast recovery and re-growth after drought exposure. Plants also maintained high rates of photosynthetic CO_2 assimilation when exposed to drought and used better assimilated carbon to generate biomass than the other tested cowpea landraces. Protein and chlorophyll degradation was further less affected and had only a slight increase in proteolytic activity under drought with the proline content significantly increasing under drought. In contrast, plants of the landrace Massava nhassenje were the most drought-sensitive plants with low water-use efficiency and low CO_2 assimilation as well as the lowest shoot biomass accumulation and high protease activity under drought. This study has overall demonstrated that the Mozambican cowpea germplasm deposited in the seed bank is diverse and contains characteristics that could be useful for a national breeding program. Shoot biomass, were thereby valuable traits which could be easily measured in Mozambique in a tunnel house

experiment. This study might serve as a basis to screen a greater number of landraces to identify a greater number of landraces as useful additions in an active local cowpea breeding program.

THESIS COMPOSITION

Chapter one reviews the current knowledge about characterisation of cowpea landraces from Mozambique deposited in a seed bank. This chapter in particular covers the present knowledge on cowpeas, cowpea breeding in Africa, the potential of cowpeas for Africa, including Mozambique, cowpea growth and constraints, cowpea breeding in Africa and cowpea conservation. The concept of gene banks, selection for superior cowpea lines and the potential of landraces for selection of superior traits are further presented. This chapter also describes how plants respond to drought. Furthermore, a more detailed overview of previous and current research on the different types of plant proteases and protease inhibitors, their activity and relationship in plant drought stress response is provided. **Chapter two** describes the plant genetic research centre, collection, characterisation, regeneration, documentation and the main users of genetic resource in Mozambique. **Chapter three** reports on the genetic similarities and relationships among four Mozambican cowpea landraces. In particular, the chapter deals with detection of genetic differences between the Mozambican landraces using simple sequence repeats. **Chapter four** compares, by measuring a variety of morphological and physiological parameters, plant performance of four cowpea landrace plants under drought conditions to evaluate any mechanisms for drought tolerance. This chapter also reports about studies that have been carried out under temperature-controlled growth conditions in South Africa and in a tunnel house in Mozambique. **Chapter five** describes the performance of cowpea nodules of plants of the four Mozambican cowpea landraces under both well-watered and drought conditions. This chapter also presents the results of measured protein contents and protease activities in nodules and leaves of cowpea landraces under water deficit and well-watered condition. **Chapter six** summarises the new aspects of the study. This chapter specifically focuses on how the study has contributed to

characterisation of cowpea landraces deposited in the Mozambican gene bank. Finally, this chapter also outlines possible future research activities. **Chapter seven** consists of the reference list.

DECLARATION

I, Célia Marília Martins declare that the thesis submitted herewith for the degree Philosophiae doctor in Plant Science at the University of Pretoria, is my own work and has not been submitted for any degree at this or other tertiary institution.

Date.....

Signed……

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DEDICATION

I dedicate this thesis to my mother Isabel Luisa who supported me with her love, care and inspiration throughout my life and being my source of success.

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ABBREVIATIONS AND SYMBOLS

A	Assimilation
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphisms
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
ATP	Adenosine tri-phosphate
BSA	Bovine Serum Albumin
C_2H_2	Ethylene
CIAT	International Centre for Tropical Agriculture
Cm	Centimeter
CO_2	Carbon dioxide
CP	Cysteine protease
Cv.	Cultivar
d	Day
dH_20	Sterile distilled water
DINA/DNDR	Direcção Nacional de Desenvolvimento Rural
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DW	Dry weight
e.g.	Example
E-64	Trans-epoxysuccinyl-L-leucylamino-4-guadine butane
EDTA	Ethylenediamine tetra acetic acid
EUW	Effective use of water
FAO	Food and Agriculture Organisation of United Nations
FAO STAT	Statistics Division of the Food Agriculture of United Nations
FU	Fluorescence Unit
FW	Fresh weight
G	Stomatal conductance
g	Gram
h	Hour
IIAM	Agriculture Research Institute of Mozambique
IITA	International Institute of Tropical Agriculture
IPA	Instituto de Produção Animal
IPGRI	International Plant Genetic Resource Institute
IWUE	Instantaneous water use efficiency
KCl	Potassium chloride
kDa	Kilo Dalton
L	Litre
M	Molar
mA	Milliamperes
mg	Milligrams
min	Minutes
mm	Millimeter
mmol	Millimol
MPa	Mega pascals
N	Nitrogen
NH_3	Ammonia

nm	Nanometer
NPGRC	National Plant Genetic Resources Centre
^{o}C	Degree Celsius
OD	Optical density
PCR	Polymerase Chain Reaction
PI	Protease inhibitor
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
SE	Standard error
Sec	Seconds
SEMOC	Sementes de Moçambique
SNF	Symbiotic nitrogen fixation
SNP	Single nucleotide polymorphisms
SPGRC	SADC Plant Genetic Resources Centre
SSLPs	Simple Sequence Length Polymorphisms
SSRs	Simple Sequence Repeats
SSTRs	Simple Sequence Tandem Repeats
STMS	Sequence tagged microsatellites
STRs	Short tandem repeats
SWC	Soil water content
U	Unit
UNICEF	United Nations International Children's Fund
USD	United States Dollar
USDA	United States Department of Agriculture
UV	Ultra-violet
V	Voltage
v/v	Volume per volume
VNTRs	Variable number of tandem repeats
WUE	Water use efficiency
WW	Well-watered
µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole

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Chapter 1: Introduction

1.1 Introduction

Cowpea is grown in many parts of the world, including Africa and Latin America, but also South-east Asia and the southern part of the United States, for animal fodder, but also as a vegetable, on approximately 14.5 million hectares yielding an annual production of over 4.5 million tons of grain (Singh *et al.*, 2002). Africa has five major cowpea production countries (Nigeria, Niger, Burkina Faso, Senegal and Mali), of which Nigeria is the world's largest producer (22% of global production) (Fery, 2002; FAOSTAT, 2008). In addition, cowpea is also grown in Ethiopia, Kenya, Tanzania, Malawi, Botswana and Zimbabwe (NGICA, 2006).

Cowpea production is also important for Mozambique, where the grain and leaves are the major sources of food for both humans and animals in resource-poor households (Badiane *et al.*, 2004). As a legume, cowpea has a symbiotic relationship with a soil bacterium (*Rhizobium)* in root nodules for fixing atmospheric nitrogen. Through this nitrogen-fixing ability, soil fertility is enhanced and cowpea is therefore used as a rotation crop. Small-scale farmers (almost 40%) grow cowpea on 9% of the total cultivated area, representing 5% of total agricultural production (INIA, 2000). However, cowpea yield is low, at only 300 kg/ha, due to biotic and abiotic stresses, such as drought, greatly contributing to low yields (INIA, 2003).

Drought severely limits cowpea production under rain-fed conditions characterized by low and erratic rainfall, as well as late start or early cessation of rain. The availability of water is, however, crucial for optimal cowpea growth, providing cell turgidity and enabling plant tissues to grow and carry out metabolic functions (Salisbury and Ross, 1992). Plants develop different strategies to overcome drought stress, including escaping and avoiding drought, as

well as being tolerant to drought (Turner *et al.*, 2001; Mitra, 2001) and crop plants can use more than one strategy to survive drought (Chaves *et al*., 2002). Responses to drought stress are complex due to variation in incidence time, duration and intensity of stress and also involvement of several agronomic, climatic and edaphic factors.

Plants use various morphological, physiological, metabolic and molecular stress responses to cope with drought (Hoekstra *et al*., 2001; Chaves *et al*., 2002) and performing during, or recover, from drought (Anyia and Herzog, 2004b). Responses change the phenotype to be better adapted to growth under drought (Bray, 1997). Different drought-tolerant cowpea cultivars have previously been identified including cultivars ceasing growth to conserve water for survival when exposed to drought and also mobilize water from lower leaves. Better understanding of morphological, physiological and biochemical mechanisms involved in these plant responses might ultimately help improving cowpea productivity in dry land areas. However, genetic diversity in responses is vital for any plant improvement program. Such diversity is essential to decrease crop sensitivity to environmental stress ensuring long-term selection gain in genetic improvement (Martin *et al*., 1991; Tesemma *et al*., 1991; Messmer *et al*., 1993; Barrett and Kidwell, 1998).

1.2 Research problem and justification

The southern part of Mozambique is vulnerable to drought affecting the country's food production. More drought-tolerant crops are therefore urgently required for future improvement of food production in Mozambique. Unfortunately, an active breeding program, including screening and using more drought-tolerant cowpea landraces, is yet not very well advanced in the country. This study has therefore provided a first step for future cowpea

screening with the potential for inclusion into a future more active cowpea local breeding program directly addressing the need of resource poor farmers in the country.

The exact scientific basis for drought tolerance in cowpea is, unfortunately, also not very well established. This currently results in a rather slow progress in cowpea breeding for drought tolerance. Two approaches are generally applied to screen for drought tolerance. The first approach is determining grain yield which involves testing segregating material over many years at several locations. This approach has been previously applied in cowpea, but with little success (Cisse *et al*., 1997; Hall and Patel, 1985; Hall *et al*., 1997). A second approach is measuring morphological and physiological parameters under drought conditions impacting final yield. Successes so far reported for Africa include the development of the early maturing cowpea varieties IT84S-2246 and Bambey-21. Both were recently released and widely adopted by farmers, particularly in West Africa (Agbicodo *et al.*, 2009). However, simple transfer of these varieties to other African countries like Mozambique is problematic due to consumer and cowpea grower preference for taste and seed appearance but also growth characteristics. This renders local varieties the preferred choice for both farmers and consumers. Unfortunately, despite the demand, activities to improve local cowpea landraces have so far not been carried out in Mozambique. Understanding the morphological, physiological and biochemical responses of cowpea landraces to typical local conditions and the identification of the mechanisms responsible for plant adaptation/tolerance to stress should be among the actions to be carried out by the research community in Mozambique to contribute to food security. This might possibly be carried out with a combination of the two approaches mentioned above likely facilitating more rapid progress in the development of drought-tolerant varieties (Fussell *et al*., 1991).

Recent research of Chiulele (2010) on cowpea solely focused on the evaluation of a large number of cowpea germplasm for drought stress adaptation under field condition using productivity traits (number of seed per pod, number of pod per plant and seed yield). However, no physiological or biochemical/nutritional traits including nodule characteristics to identify possibly reasons for better field performance have been studied. Since only a small number cowpea accessions have been so far collected locally, which have also not characterized in the greater detail, this study has attempted to initially only characterize four selected locally collected landrace cowpea accessions which can be used as a model. Landraces used are considered to have various degrees of drought tolerance and are currently preferred by local communities. Tools developed in this study should further be generally applicable to screen in the future a much greater number of cowpea accessions.

1.3 Research aim and objectives

In this study a first attempt has been made characterizing four locally collected landraces for their performance under drought. It was hypothesized that Mozambican cowpea landraces might exist that are well adapted to local conditions possessing morphological, physiological and molecular characteristics for better growth under drought conditions. The overall aim of the research work was to identify the most drought-tolerant cowpea landrace currently deposited in the Mozambican gene bank. In particular, phenomics was used characterizing plant growth under drought conditions. The SSRs technology has been further applied determining genetic diversity among the tested landraces. Studies have been carried out in a temperature-controlled greenhouse in South Africa at the University of Pretoria and under open-sided tunnel house conditions in Mozambique to compare growth under different growth conditions and the applicability of biomarkers for plant growth. Specific objectives of the study were:

1) Survey the current collection, characterisation and preservation of cowpea germplasm at the Mozambican Plant Genetic Resource Centre.
2) Determine growth responses of cowpea landraces under drought tolerance and establish possible associations between plant phenotype and tolerance to drought.
3) Apply selected polymorphic SSR markers and determine genetic relationship among four cowpea landraces.
4) Investigate biochemical response with special emphasis on metabolic and proteolytic processes of four selected cowpea landraces and establish possible association between metabolite/protein expression and drought tolerance.
5) Compare drought tolerance responses of fours cowpea landraces when evaluated at two locations using standard growth parameters.

1.4 Literature Review

1.4.1 Cowpea as a crop

1.4.1.1 Cowpea cultivation

Cowpea (*Vigna unguiculata* (L.) Walp) was possibly introduced 2 000 to 3 500 years ago from Africa to the Indian sub-continent (Allen, 1983), with the previous South African Transvaal region being the centre of speciation (Padulosi and Ng, 1997). Cowpea is normally grown in low rainfall, semi-arid regions with a precipitation of 300-600 mm (Fussell *et al.*, 1991).

Figure 1.1 Cowpea plants grown in a field in Namibia. Source: http://www.mawf.gov.na/ Directorates/Research Training/images/cowpea.

1.4.1.2 Nutritional and agricultural value of cowpea

In Mozambique, cowpea seeds are consumed fresh, boiled, dry and dry-roasted. Leaves are also consumed as a vegetable and cowpea seeds are processed into flour, which is used to produce fried products called 'badgias' that are sold by street vendors. Cowpea seed can also be processed into flour used for different purposes, such as 'akara' (fried products prepared from dried peas in Nigeria), snacks and food for children used particularly in the transition period when they are being weaned from breast milk to solid food (Ehlers and Hall, 1997; Taiwo, 1998; Phillips *et al*., 2003).

Cowpea is of value to humans who have limited access to animal protein (Akpapunam and Sefa-Dedeh, 1997), since it has high grain protein content (25% of dry weight). Further, the protein content of cowpea leaves consumed annually in Africa and Asia is equivalent to 5 million tons of dry cowpea seeds, corresponding to about 30% of the total food legume production in lowland tropics (Steele *et al*., 1985). Cowpea is also a source of vitamins and minerals, such as folic acid, vitamins A and B, thiamine, niacin and the water-soluble vitamins riboflavin, pyridoxine and folic acid, as well as minerals such as calcium, zinc, potassium, iron and phosphorous and other trace elements (Aykroyd *et al*., 1982; Walker, 1982; Bressani, 1985; Uzogara and Ofuya, 1992; Singh *et al*., 1997; Nielsen *et al*., 1997; Singh *et al*., 2003). Because of the protein's high lysine content, cowpea is a supplement for cereal-based diets high in the amino acids methionine and cysteine (Ihekoronye and Ngoddy, 1985; Davis *et al*., 1991; Lambot, 2002) (Table 1.1). Cowpea is also low in fat (1.3%) and provides dietetic fibre and carbohydrates (Bressani and Elias, 1984; Bressani, 1985). High medicinal value has been also reported for cowpea reducing the incidence of diseases such as colon cancer and diabetes, as well as coronary disease (Walker, 1982; Uzogara and Ofuya,

1992). Raw leaves are also rich in vitamin C, but 80% of the vitamin's properties are lost when cowpeas are boiled or roasted.

Table 1.1 Chemical composition of cowpea leaves, immature cowpea seed and mature cowpea seed.

Nutrients	Leaves (%)	Immature seed (%)	Mature seed (%)
Carbohydrates	8	7.4	56.8
Proteins	4.7	3.4	22-24
Water	85	86.2	11.0
Fibre	2	1.8	3.9
Lipids	0.3	0.3	1.3-1.5

Source: Adapted from Davis *et al*., 1991; Kay, 1979; Tindall, 1983; Quass, 1995

1.4.2 Cowpea production in Mozambique

In Mozambique, cowpea and peanut are the most important legume food crop (Doto *et al*., 1993) and cowpea is the fourth most essential cultivated crop after maize, cassava and groundnut (INE, 2009). Mozambique is divided into 10 agro-ecological zones, based on altitude, climate (precipitation and temperature) and soil type that influence crop productivity (Maria and Yost, 2006). However, there are two agro-ecological zones (R7 and R8) where most cowpea cultivation occurs (IIAM, 2006) (Figure 1.2). The main areas of production are the Inhambane, Maputo, Gaza, Nampula, Zambézia and Cabo Delgado provinces (INE, 2009). Most of these areas are characterised by low and unpredictable precipitation and low soil fertility.

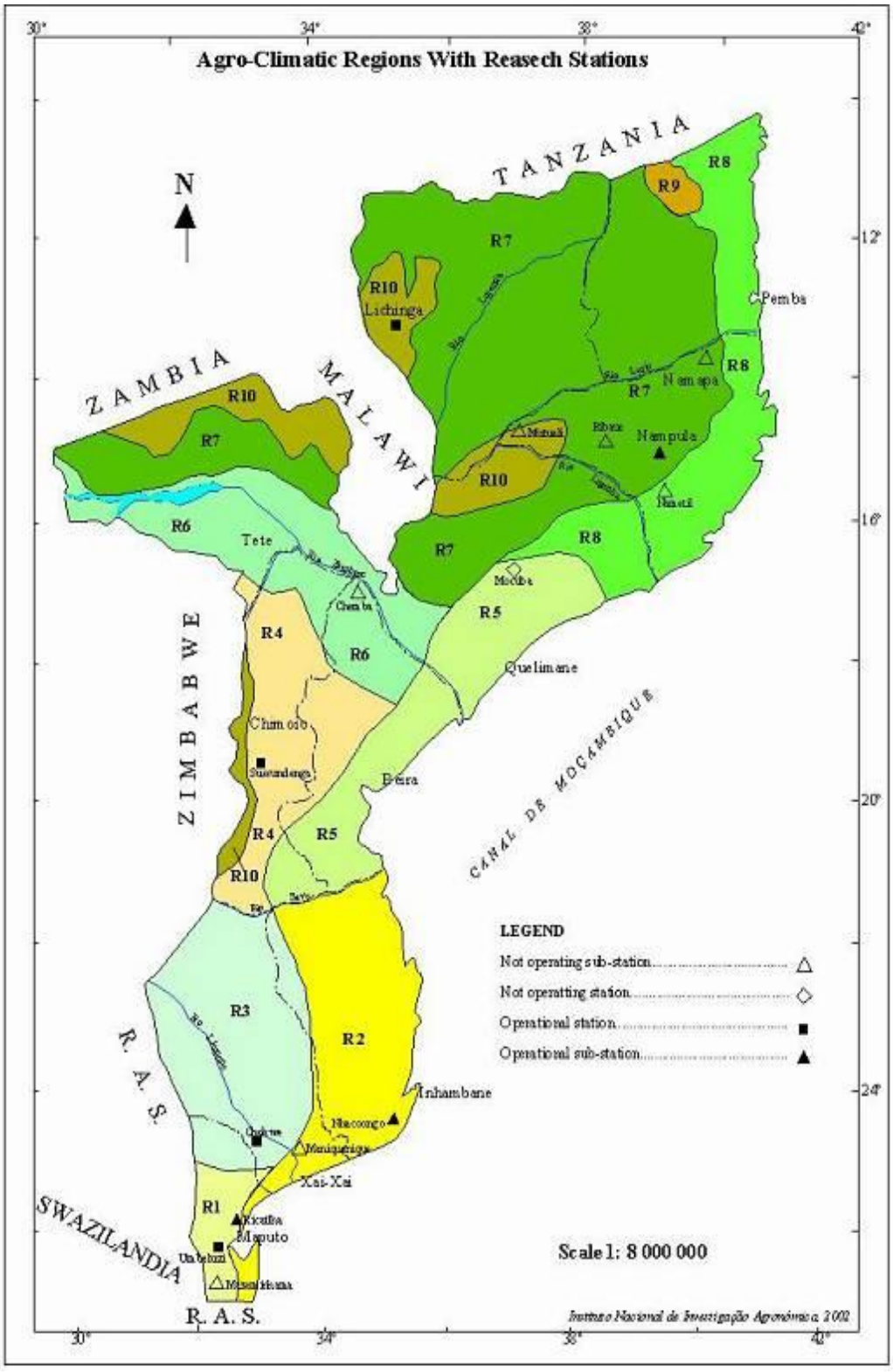

Figure 1.2 Distribution of 10 agro ecological zones of Mozambique. Source: Ministry of Agriculture and Fisheries, 1996.

Cowpea is an annual warm-season legume growing best in humid tropics and temperate areas (Davis *et al*., 1991; Hall, 2001). It also grows in high temperatures and in drought conditions that are intolerable to other legumes (Fery, 2002). Cowpea is moderately drought-tolerant (Peaceful Valley, 1988; Gaiser and Graef, 2001), has a tap-root to access moisture in deeper soil and adapts to temperatures ranging from 20°C to 35°C (Ehlers and Hall, 1996; Valenzuela and Smith, 2002). This crop grows in well-drained sandy loam or sandy soil with a pH range of 5.5 to 6.5 (Davis *et al*., 1991) and tolerates aluminum (McLeod, 1982; Peaceful Valley, 1988).

Mozambique is vulnerable to droughts and floods (Figure 1.3) and both these events affect the country's food production (USAID, 2005). New technologies are therefore urgently required in Mozambique to improve food and agricultural production. Among the local crops in demand, cowpea is one of the most drought-tolerant, but it still suffers from frequent and long periods of drought, particularly in the southern part of the country. Since cowpea is grown mainly in the dry areas with no irrigation, any irregular rainfall can be detrimental to crop performance (TIA, 2002).

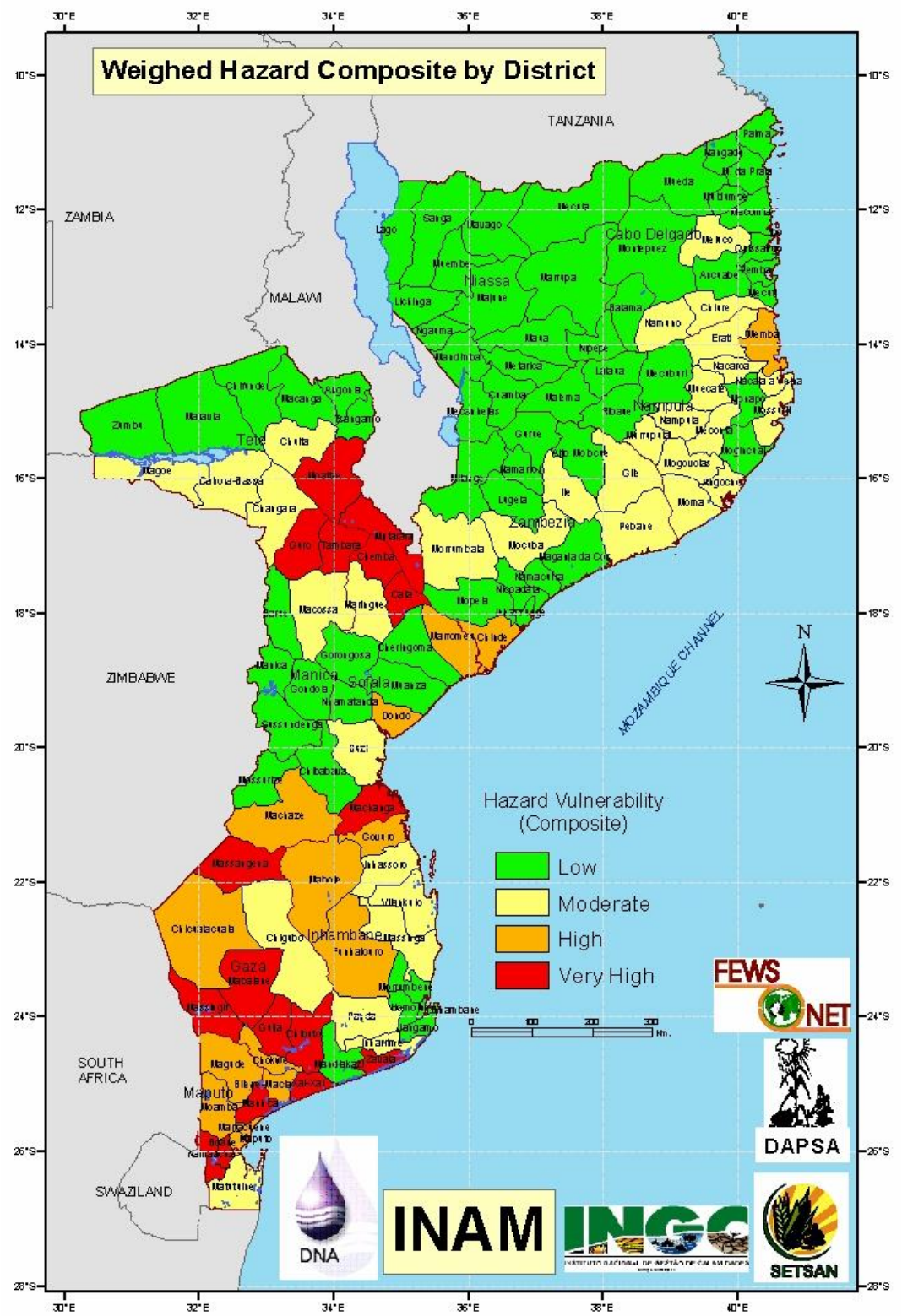

Figure 1.3 Areas of vulnerability resulting from natural risk in Mozambique. Source: USAID, 2005.

1.4.3 Drought and plant growth

Water deficit in plants occurs when transpiration exceeds water uptake, causing reduction in the relative water content (RWC), cell volume and also cell turgor (Lawlor and Cornic, 2002). In legume crops, drought also limits carbon assimilation and symbiotic nitrogen fixation (Serraj *et al*., 1999), thereby reducing crop yield and soil fertility. Water deficit affects particularly plant anthesis, flowering, and the reproductive or seed fill stage, reducing overall yield (Pirdashti *et al.*, 2004; Adejare and Umebese, 2007). It is also referred that it decreases photon accumulation in photosynthesis and a decrease in quantum yield of photo system II (PS II) electron transport (Flexas *et al*., 1999) and photosynthesis inhibition negatively affects adenosine triphosphate (ATP) and ribulose-1, 5-bisphosphate (RuBP) biosynthesis (Tezara *et al*., 1999; Lawlor and Tezara, 2009). In cowpea, water deficit also increases both canopy temperature and proline content resulting in a reduction in starch content, number of pods and seeds per plant (Hamidou *et al*., 2007).

Drought can be intermittent or terminal. Intermittent drought relates to sporadic rainfall, with intervals of drought during the growing season (Schneider *et al*., 1997). In comparison, terminal drought relates to water deficit either during the later plant growth stages or when a crop is planted at the onset of a dry period (Frahm *et al*., 2004). Intermittent drought directly affects biomass accumulation because of a reduction in leaf area and stem elongation (Boyer and McPherson, 1975; Wien *et al*., 1979; Turk and Hall, 1980; Hale and Orcutt, 1987; Maiti *et al*., 1996). In the southern parts of Africa, drought has become more intense and widespread (Fauchereau *et al*., 2003) and is a major yield-limiting factor for production of cowpea as a rain-fed crop.

1.4.3.1 Drought tolerance

Plants develop different mechanisms to survive drought. Drought tolerance can be defined as the capacity of plants to live, grow, and produce adequate crops when subjected to water deficiency or limited availability of soil water (Ashley, 1993). Plants develop different strategies to overcome drought stress, including drought escape, avoidance and tolerance (Turner *et al*., 2001; Mitra, 2001). Crop plants can use more than one strategy to survive drought (Chaves *et al*., 2002). Responses to drought stress are very complex owing to significant variation in time of incidence, duration and intensity of stress, with a range of agronomic, climatic and edaphic factors involved. The complexity of the drought response increases in semi-arid tropic regions because of associated high temperature and solar radiation and poor soil characteristics.

Cowpea has two major types of drought tolerance (Type 1 and Type 2). For Type 1 drought tolerance, plants stop growth after drought stress and maintain uniformity with a decline in turgidity in all tissues, including the uni-foliates and emerging tri-foliates. All plant parts gradually die (Mai-Kodomi *et al*., 1999a). Type 2 drought-tolerant lines remain green for a longer time and tri-foliates continue growing slowly in drought conditions. Under continued drought stress, tri-foliates of tolerant varieties wilt and finally die. The are two possible mechanisms to increase drought tolerance in cowpea: Type 1 mechanism, where the stomata closure to reduce water loss through transpiration and cessation of shoot and leaves growth and Type 2 mechanism, known as osmotic adjustment where there is a continued slow growth (Lawan, 1983; Boyer, 1996).

Plants also use a drought escape strategy to complete their whole life cycle when water is still available (Ludlow, 1989). Early maturing cowpea varieties are therefore useful in dry regions escaping terminal drought (Hall and Patel 1985, Singh 1987; Mortimore *et al*., 1997). These cultivars mature in about 60–70 days. However, they under-perform when exposed to irregular water supply during the vegetative growth phase (Mai-Kodomi *et al*., 1999a) and when drought occurs at the beginning of the reproductive phase (Thiaw *et al*., 1993). Progress has been made in developing such early maturing cowpea cultivars suitable for Africa (Hall and Patel, 1985; Singh and Ntare, 1985). Adoption of these early cultivars, however, has not been rapid and yields are still low (300 kg/ha) (Ehlers and Hall, 1997). This is partly due to an ineffective extension system and lack of high-density cropping and crop husbandry practices required for these modern cultivars to achieve high grain yield (Ehlers and Hall, 1997). For example, the early maturing cowpea varieties IT84S-2246 and Bambey 21 have been released for escaping terminal drought and both have been extensively used by farmers in Africa (Agbicodo *et al*., 2009). However, if exposed to intermittent drought during the vegetative or reproductive stages, these varieties perform poorly. More breeding efforts are therefore required to select varieties better adapted to early, mid- and terminal season drought stress.

1.4.3.1.1 Physiological traits

Physiological traits for drought tolerance selection include water use efficiency (WUE), water potential, relative turgidity, leaf gas exchange, RWC, diffusion pressure deficit, chlorophyll stability index and carbon isotope discrimination (Bates and Hall, 1981; Turk and Hall, 1980; Morgan *et al*., 1991; Hall *et al*. 1997; Anyia and Herzog 2004a; Souza *et al*., 2004). In addition, capacity for abscisic acid (ABA) synthesis, stomatal conductance (Cruz de

Carvalho *et al*., 1998), delayed leaf senescence (Gwathmey *et al*., 1992), stem greenness, recovery of dry weight (Muchero *et al*., 2008), root architecture (Pandey *et al*., 1984; 1986; Itani *et al*., 1992; Silim and Saxena, 1993; Matsui and Singh, 2003), early maturity, starch depletion (Hall and Patel, 1985; Singh, 1994), reduced leaf area and leaf area adjustment, photosensitivity, indeterminacy (Singh and Matsui, 2002), free proline content (Agbicodo *et al*., 2009) and production of reactive oxygen species (Contour-Ansel *et al*., 2006) have been used as traits to be monitored in drought tolerance studies. However, the usefulness of a trait for selection ultimately depends on its correlation with better seed yield in drought conditions (Kumar *et al*., 2008).

Leaf area adjustment is a plant mechanism for adapting to drought. This includes shedding older leaves in response to drought at the reproductive stage and producing new leaves that are smaller in area than the shed leaves (Adejare and Umebese, 2007). Stomatal control with rapid closure of stomata in response to water deficit is a further drought tolerance mechanism (Sarr *et al*., 2001, Ogbonnaya *et al*., 2003). Stomata closure increases WUE, an important drought-tolerant trait (Hall *et al*., 1997). A high negative relationship between stomatal conductance and total root abscisic acid (ABA) has also been reported (Kulkarni *et al*., 2000) and ABA very probably regulates stomatal conductance in drying soil. Stomatal regulation, a strategy in cowpea to avoid dehydration, has also been reported by Hamidou *et al*. (2007). When the water status in a leaf is below a threshold value, the stomata close owing to ABA production rapidly altering ion fluxes in guard cells. Closing stomata is, therefore, one of the most important mechanisms to survive severe water stress (Souza *et al*., 2004). Stomata closure, however, also cuts off access to atmospheric carbon dioxide for the chloroplast, resulting in reduced vegetative growth (Adejare and Umebese, 2007). Doubts have therefore

been expressed about the usefulness of stomata closure in breeding for drought tolerance (Mitra, 2001).

1.4.3.1.2 *Root traits*

Drought tolerance mechanisms in legumes are closely related to the type of root system or root architecture and development (Pandey *et al*., 1984; Itani *et al*., 1992; Silim and Saxena, 1993; Matsui and Singh, 2003). In the cowpea cultivar IT96D-604, better drought tolerance is associated with an increase in root dry matter per leaf area (increased root-shoot ratio) under mild water-stress. A further strategy is a deeper penetration of roots into the soil to access soil moisture in deep soil layers better under more severe water stress. However, screening for root characteristics is often difficult because of the underground distribution of roots. The 'pin-board root-box' (Matsui and Singh, 2003) and herbicidal band screening (Robertson *et al*., 1985), as well as the polyethylene glycol (PEG) method (Badiane *et al*., 2004) have previously been applied to study cowpea root characteristics for drought tolerance. Important varietal differences were identified in cowpea root architecture, with some varieties having a well-spread deep root system while others concentrate roots in the upper soil level.

However, all drought adaptation mechanisms outlined above have disadvantages regarding yield potential. A variety with a shortened life cycle (early maturity variety) usually yields less compared to a later maturity variety with a normal life cycle. The mechanisms which allow drought avoidance by reducing water loss (such as stomatal closure and decreased leaf area) decline carbon assimilation and increase leaf temperature, thus reducing processes that affect negatively yield. Plants may also use the maintenance of the water content by accumulating non-toxic compatible solutes (fructans, trehalose, polyols, glycine-betaine,

proline and polyamines) that do not interfere with plant processes (Yancey *et al*., 1982). However, many ions, concentrated in the cytoplasm owing to water loss, are toxic at high concentration. This leads to a "glassy state" where liquid left in the cell has high viscosity that also increases molecular interactions with proteins which may lead to denaturation of the membranes (Hartung *et al*., 1998). Therefore, the ultimate demand for a crop is to be balanced between escape, avoidance and tolerance, however maintaining satisfactory productivity.

1.4.4 Plant genetic resources

Plant genetic resources (PGR) according to FAO (1984) are defined as the whole generative and vegetative reproductive material of species with economical and/or social value, especially for the agriculture of the present and the future, with special emphasis on nutritional plants. PGR is an important component of agro-biodiversity which includes primitive forms of cultivated plant species and landraces, modern cultivars, obsolete cultivars, breeding lines and genetic stocks, weedy types as well as related wild species (IPGRI, 1993). These resources are maintained in seed or gene banks providing potential genetic material for crop-breeding programs. They therefore contribute to the sustainable development of agriculture and food security (Rao *et al*., 2006). Although germplasm exchange and plant introduction have occurred sporadically for centuries, purposeful efforts only started in the 1920s. There are now about 1 750 gene banks established worldwide, where 130 of them each hold more than 10 000 accessions and the ultimate back up of global crop diversity is been carried out at the recently opened Svalbald Global Seed Vault in Norway (FAO, 2010).

Conservation of crop germplasm diversity includes the establishment of *in situ* and *ex situ* gene banks. Major activities in *ex situ* gene banks are assembling, conserving, characterizing and providing easy access to germplasm for scientists; *ex situ* banks focus on the provision of documented specimens for use in studies to assess, monitor and manage biological diversity across taxonomic levels, trophic levels and ecosystems. Gene banks store material in different forms, such as seeds, herbarium specimens and frozen tissues.

Deoxyribonucleic acid (DNA) banking, to provide DNA for molecular research and phylogenetic analysis, is an emerging technique in genetic resource conservation and utilisation (de Vicente and Andersson, 2006) but not a replacement for conventional germplasm and tissue storage (Callow *et al*., 1997). Therefore, DNA banking is considered as a complementary conservation strategy that together with other conservation methods will lead to an ideal and sustainable use of genetic resource. Germplasm being requested from gene banks in the form of DNA material and the requirement of provision of standardised germplasm being requested from gene banks in the form of DNA material and the requirement of provision of standardised DNA samples are likely in view of more sophisticated genomic research, including functional genome analysis, comparative genomics, transcriptomics and proteomics.

Because of the history of the country, the Mozambican National Gene Bank, located in Maputo, has only recently started any collection of the country's national heritage of plant genetic resources. The gene bank inherited working collections from research stations but mainly material from international trials. Systematic studies and the collection of traditionally used plants, as seeds, have only recently started. Covering all the plants of Mozambique will be an enormous task. The Northern provinces have so far been given first priority, since those

areas are less, if at all, exposed to modern seeds and are rich in traditional landrace materials (Da Silva *et al*., 1996).

Mozambican materials have already been deposited in international gene banks (SINGER database), particularly those of CG-centre's with coordinates that entered from the north of the country. Most germplasm accessions currently maintained in Mozambique are derived from collaborative programs between Institute of Agriculture Research in Mozambique (IIAM), and International Agricultural Research Centres such as International Center for Tropical Agriculture (CIAT), International Maize and Wheat Improvement Center (CYMMYT), International Rice Research Institute (IRRI) and International Institute of Tropical Agriculture (IITA). However, only a small portion of these accessions have been collected locally (Da Silva *et al*., 1996). As a member of the Southern African Development Community (SADC), the Plant Genetic Resources Centre (SPGRC) in Mozambique signed a memorandum of understanding on the management of genetic resources and has the obligation to send duplicates of accessions stored to be maintained as a duplicate at SPGRC at Chalimbana, Zambia (SPGRC, 2011). So far the country has managed to fulfill the requirements to maintain germplasm in SPGRC.

1.4.4.1 Landraces

Landraces are defined as a population of cultivated plants having historical background origin, distinct identity and without any breeding improvement (Camacho Villa *et al*., 2005). Resource-poor farmers in the marginal areas of Africa grow crops under diverse environmental conditions, often characterised by drought and nutrient deficiencies. Farmers, however, prefer using locally adapted legume cultivars with low planting densities and low

yield in traditional intercropping systems with cereal crops. The systematic study of landraces of locally adapted higher yielding varieties is therefore a requirement to address the farmer's preference. Such landraces also provide a valuable source of new variation for the genetic improvement of economically important characteristics such as pods/peduncles, seed index, seed yield and biotic and abiotic stress tolerance and associated with traditional farming systems (Camacho Villa *et al*., 2005; Birrol *et al*., 2009). Local farmers grow such unimproved landraces despite the availability of improved cultivars. This is probably due to the fact that most of the improved cultivars are developed under different conditions for farmers with low capacity to adapt and producing in marginal areas (Keleman *et al*., 2009), further requiring a high quantity of fertilizer (Keleman *et al*., 2009; Bellon and Hellin, 2011) and have a high seed cost when compared to non-improved varieties (Almekinders *et al*., 1994). In many breeding programmes, farmers are also invited very late to participate in varietal development. They are then requested to choose among the already selected lines (Kitch *et al*., 1998), which had been selected predominantly based on high performance in multiple locations and seasons of uniform and favourable environments at a research station.

Improved cultivars consequently do not meet the needs and preferences of farmers and have low adoption rates that have a minimal impact on peoples' livelihood and food security (Singh *et al*., 1997; Inaizumi *et al*., 1999). The lack of suitable varieties to satisfy farmers' needs and conditions, combined with a low rate of adoption of improved varieties, is among the reasons for low productivity (Nkongolo *et al*., 2009). Therefore, current and future breeding programs must be conducted to meet the specific farmers' requirements and preferences, to target specific agricultural practices and production constraints for a specific region and to develop a cultivar with wide adaptability. This will require that farmers be involved in the breeding programs from the beginning to the variety release stage.

1.4.5 Genetic diversity

Identification and characterization of germplasm diversity is an important activity in conservation. This activity is also important to prevent loss of landraces due to the introduction of improved crop varieties. In particular, it helps to identify landraces with the best characteristics for incorporation into crop improvement programmes. Knowledge of genetic diversity also lowers the risk of extinction of wild ancestors with useful characteristics contributing to the genetic pool of our today's major species (Xuebin, 2004; Bruford *et al*., 2003).

Conventional methods, such as determining morphological characteristics, are predominantly carried out in Africa for determination of genetic diversity. These methods include measuring variation in phenotypic or qualitative traits, such as flower color and growth, or quantitative agronomic traits, such as yield potential (Kameswara, 2004). However, in this approach expression of quantitative traits is subject to strong environmental influence. DNA markers are therefore more powerful to differentiate among genotypes at species and also sub-species level (Kumar, 1999). Molecular techniques, such as DNA markers, have greatly improved the ability to characterise genetic diversity. DNA-based assays are also more robust and information is obtained from any phase of plant development without any change under environmental conditions.

There are many different types of DNA-based assays, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites and single nucleotide polymorphisms (SNPs). RAPDs are DNA fragments, which are polymorphic in size, generated by

polymerase chain reaction (PCR) using one or two randomly selected primers. Characterisation of a DNA sample by RAPD analysis is often referred to as DNA 'fingerprinting'. RAPD analysis is possibly the simplest DNA-based test for cultivar identification. The technique consists on the production of multiple copies of DNA segments from plant DNA and several million-fold amplification of the segment in a PCR. The RAPD technique allows differentiation via change in banding patterns between individuals of the same species (Corniquel and Mercier, 1994).

RFLP, as a further technology, is based on the analysis of patterns derived from a DNA sequence digested with known restriction enzymes. Differences are evident when the length of fragments is different with restriction enzymes digesting DNA at different sites or locations. RFLP occurs as a result of a point mutation creating or destroying a restriction site or insertions/deletions altering the size of a given restriction fragment (Mburu and Hanotte, 2005). AFLP analysis is a combination of RFLP and RAPD markers as a result of restriction site polymorphisms detected by amplification with specific adapters (Vos *et al*., 1995). The method allows selective amplification of restriction fragments, giving rise to large numbers of DNA fragments. AFLP markers have been widely used in the construction of genetic maps in cowpea (Quédraogo *et al*., 2001, 2002; Boukar *et al*., 2004).

Microsatellites are short DNA sequences (usually 1-5 bp long) tandemly repeated and flanked by a unique sequence (Scribner and Pearce, 2000; Mburu and Hanotte, 2005). They are also called simple sequence repeats (SSRs), short tandem repeats (STRs), simple sequence tandem repeats (SSTRs), variable number of tandem repeats (VNTRs), simple sequence length polymorphisms (SSLPs) or sequence tagged microsatellites (STMS) (Mburu and Hanotte, 2005). Microsatellites are used in 90% of all diversity studies (Baumung *et al*., 2004). They

are possibly the most informative polymorphic marker (Tautz and Renz, 1984; Powell *et al*., 1996; Rafalski *et al*., 1996), further co-dominant and detectable using the PCR technique (Bruford and Wayne 1993; Queller *et al*., 1993; Dallas *et al*., 1995). The advantages, disadvantages and applications of DNA marker systems are shown on Table 1.2.

There is growing interest in using DNA microsatellites for the selection of superior cowpea material and knowledge of genetic variation and relationships among cowpea genotypes will ultimately assist breeders to develop appropriate breeding strategies to solve cowpea production constraints by providing more information of parental lines used in breeding programs. Several microsatellites have been identified in cowpea to study in particular cowpea relationships (Li *et al*., 2001; Diouf and Hilu, 2005; Asare *et al*., 2010). Sequencing and analyzing the gene-rich, hypo-methylated portion of the cowpea genome resulted in identifiable simple-sequence repeat in approximately 12% of all gene space sequence reads in cowpea, providing a dataset for microsatellite design (Timko *et al*., 2008). A total of 1071 microsatellites were further identified by screening 15740 cowpea unigene sequences (Gupta and Gopalakrishna, 2010). However, information on any association of a microsatellite to a specific cowpea characteristic is still very limited, despite a considerable number of microsatellites having been isolated from cowpea. One cowpea microsatellite marker (SSR-1) has recently been reported to co-segregate with resistance to *Striga gesnerioides* (striga) race 3 (SG3) (Li and Timko, 2009). In a recent study by Afiukwa *et al.* (2011) microsatellites significantly differentiated cowpea accessions but did not allow clustering for seed protein content; however, one primer pair amplified a (AG)12 repeats exclusively from a late flowering accession not found within early flowering accessions.

Table 1.2 Comparison of molecular marker techniques regarding their advantages, disadvantages and applications.

Marker	Advantage	Disadvantage	Application
RFLP	High genomic abundance Co-dominant marker Highly reproducible and stable Need for map-based cloning	Large amount of good quality DNA Laborious (compared to RAPD) Cloning and characterization of probe are required	Diversity, phylogenetic, gene-mapping, hybridization and introgression studies.
RAPD	High genomic abundance Lower amount of DNA Relatively faster and simple No sequence information	No probe or primer information. Dominant markers Can be used across species	Applied from studies at the individual level (e.g. genetic identity) to studies involving closely related species Gene-mapping studies
AFLP	High genomic abundance High polymorphism No need for sequence information Can be used across species	Very complicated owing to changes in patterns with respect to materials used. Cannot get consistent map (not reproducible) Need of very good primers	Studies involving genetic identity, parentage and identification of clones and cultivar. Phylogenetic studies of closely related species, gene-mapping studies and genetic diversity studies.
SSR	High genomic abundance Highly reproducible High polymorphism Easy to automate	Cannot be used across species. Need sequence information	Population genetics studies, ranging from the individual level (e.g. clone and strain identification) to closely related species. Gene-mapping studies Assessment of genetic variation in germplasm collections

*RFLP, RAPD, AFLP and SSR are restriction fragment length polymorphisms, random amplified polymorphic DNA, amplified fragment length polymorphisms and simple sequence repeats microsatellites respectively (adapted from Budak *et al.*, 2004 and Kumar *et al.*, 2009).

Chapter 2: Collection, characterisation and preservation of cowpea germplasm at the Mozambican Plant Genetic Resource Centre

2.1 Abstract

Mozambique holds an important reservoir of indigenous plant genetic resources including wild relatives of crops, landraces, medicinal plants, pasture species and forest genetic resources. The objective of this chapter was to provide an overview about the current collection, characterisation and preservation of plant germplasm, and in particular of cowpea germplasm, at the Mozambican Plant Genetic Resource Centre. The study was carried out by searching for information on the world-wide web, accessing journal articles via the internet and library, or contacting directly the Plant Genetic Resource Centre for available information. National conservation activities in Mozambique are aimed at conserving, characterizing and promoting the sustainable utilization of the country's plant genetic resources. Currently activities are focused on adopting complementary strategies by integrating *ex situ* and *in situ* methods. Seed conservation is carried out at the Mozambique National Plant Genetic Resources Centre (NPGRC), housed and managed by the Agriculture Research Institute of Mozambique (IIAM). Besides cowpea, the NPGRC currently holds a total of 2823 germplasm accessions of maize, rice, sorghum, pigeon pea, bambara groundnut, sunflower, soybean and triticum.

2.2 Plant germplasm collection

2.2.1 Germplasm collection as a genetic resource

Crop germplasm provides basic material for selection and improvement of crops in breeding programs. Trait-specific genetically diverse parents for trait enhancement are the primary needs of the plant breeder. Agronomically superior, or similar, lines are preferred by breeders to maintain the agronomic performance of breeding lines while improving the trait. Another dimension of breeders' requirements is agronomic desirable characteristics of the germplasm lines (Upadhyaya *et al*., 2008). This helps them to maintain or even improve the agronomic performance of breeding lines while enhancing the trait's expression.

Since the availability of germplasm is basic to crop improvement programs for sustainable agriculture, this will ultimately ensure food security for the world's rapidly rising population. Conservation and utilization of this germplasm at a PGR are therefore important components of *ex situ* collections maintaining agro-biodiversity of crops with superior characteristics (Upadhyaya *et al*., 2008). It will ultimately also contribute to the Millennium Development Goals to achieve food security, poverty alleviation, environmental protection and sustainable development.

In general, germplasm collections include primitive forms of cultivated plant species, landraces, modern cultivars, obsolete cultivars, breeding lines and genetic stocks and weedy types of related wild species (IPGRI, 1993). Although germplasm exchange and plant introduction have occurred for centuries, purposeful efforts to collect such material as genetic resources started only in the 1920s (Upadhyaya *et al*., 2005). Since then crop germplasm as a

genetic resource has been deposited into collections in many countries. The number of accessions conserved in about 1750 collections now exceeds 7.6 million (FAO, 2010).

Conservation of crop germplasm diversity is currently carried out by deposition of material into *in situ* and *ex situ* gene-banks (Acquahh, 2012). However, the purpose of the conservation to maintain genetic diversity of crop plants is not only storage and maintenance, but also sustainable utilisation. Activities of *ex situ* gene-banks include not only the assembly, conservation, and characterization of germplasm, but also provision for easy access to collected germplasm for scientists to carry out genetic diversity studies in plant taxonomy.

2.2.2 Germplasm characterization

Plant breeders, geneticists and botanists are the main users of gene-bank materials, using either local or introduced materials to produce improved varieties that have been locally adapted and have high potential. Traditionally, gene-banks store the plant germplasm as seeds, but also as dried herbarium specimens and recently also as DNA. However, to maximize the usefulness of a gene-bank, the germplasm has to be adequately characterized for its agronomical and morphological traits to be used, for example, in breeding programs. In general, in germplasm characterization distinctly identifiable and heritable characteristics are documented (Upadhayaya *et al*., 2008). For crops, this also includes recording agronomic traits considered to be important for crop improvement.

Objectives of germplasm characterization are to describe accessions, establish their diagnostic characteristics and identify duplicates, classify groups of accessions using sound criteria, identify accessions with desired agronomic traits, select entries for more precise evaluation, develop interrelationships between or among traits and between geographic groups of

cultivars, and estimate the extent of variation in the collection through intensive field and laboratory screening and purification to identify a wide range of sources for desirable traits. Characterization in Mozambique is done to generate information on the morphological characteristics of each accession. For example, in the 2003/04 crop season the PGRP carried out germplasm characterization of rice (39 accessions) at Chókwe Research Station (Gaza) (http://spgrc.org.zm/index).

2.2.3 Regeneration of germplasm

Even under good storage conditions a collected and stored seed will lose its viability and genetic integrity (Harrison, 1996; Ross, 1984). It is therefore vital to regenerate stored seeds periodically (Richards *et al*., 2010). However, regeneration frequency depends on the initial viability of the seed, the rate of loss of viability and the regeneration potential. The general aim of regeneration is to increase the quantity of seed of any collected accession when either the number of seeds available is low or maximum seed viability has to be restored. Regeneration of seed accessions is carried out in gene-banks when the percentage of germination, determined in a representative sample, is lower than 85% (Rao *et al*., 2006; ISTA, 2008), although for wild species lower than 70% is used in some gene-banks. Regeneration priority is mainly given to landrace accessions of important crops and the original accessions and all regenerated material from these accessions are stored separately to avoid mixing up ‘fresh’ and ‘old’ material. Regeneration of germplasm is, therefore, one of the most crucial processes in gene-bank management (Upadhyaya *et al*., 2008). However, the regeneration process, which also depends on the crop species (e.g. in- or out-breeding) is often costly in terms of required resources and time (Breese, 1989) and the process also involves the risk of changing genetic integrity. The purpose of conservation of germplasm as seeds is to maintain the integrity of the material conserved to the highest standard over

prolonged periods of time. It is necessary to set standards based on current scientific knowledge and available technologies for the proper handling and storage of seeds in gene-banks that will ensure their conservation over the longest possible time, without the need for frequent costly regeneration (Ehsan and Engles, 2003). Four accessions of bambara groundnut, 80 accessions of cowpea (*Vigna unguiculata*) and 11 accessions of maize were multiplied at Umbelúzi Research Station (Maputo) during the 2003/04 growing season (Figure 2.1) (http://spgrc.org.zm/index.php? option=com_content...id).

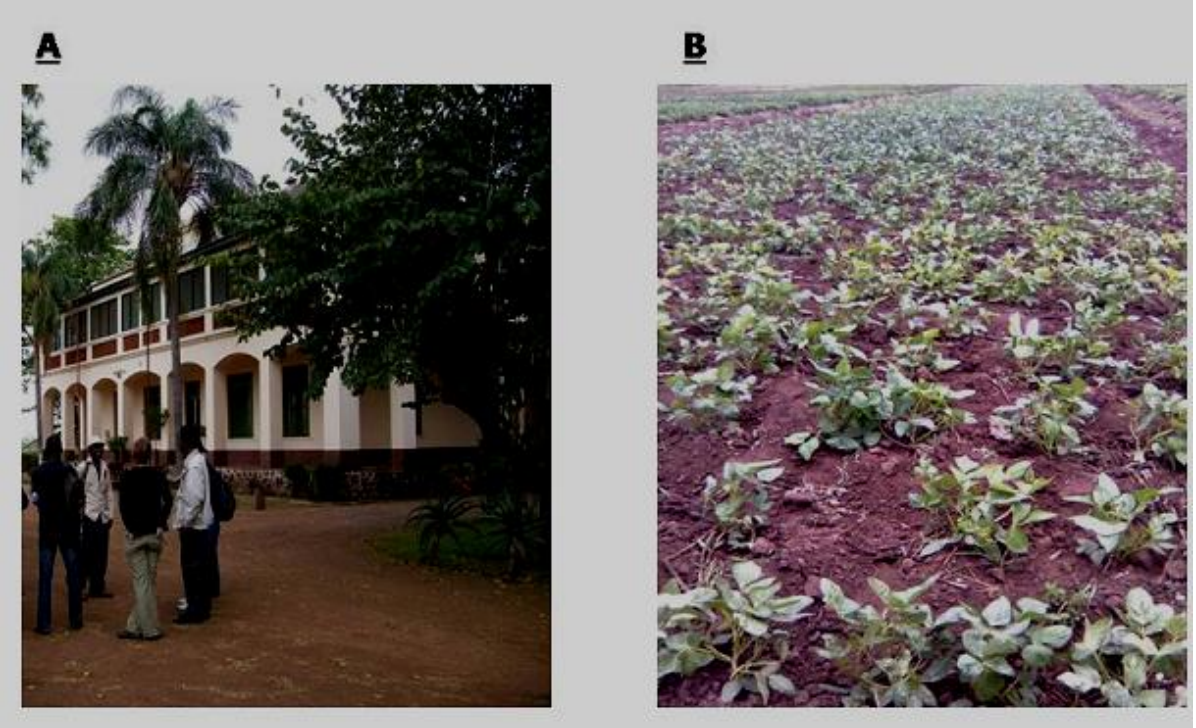

Figure 2.1 (A) Umbelúzi Agricultural Research Station where germplasm regeneration is mainly carried out and (B) field with cowpea multiplication and characterization.

DNA banking is also currently used as a new technology for germplasm conservation. However, DNA banking is not a replacement of conventional germplasm storage but will provide DNA samples for molecular and phylogenetic analysis (de Vicente and Andersson, 2006). The need for standardised DNA samples is likely to increase in future in response to

the advances in genomic research and provision of quality standards for DNA samples and this will be a major future task for DNA banks.

2.2.4 Documentation

Improving gene-bank utilization requires an adequate program for characterization, evaluation and documentation of the germplasm. Documentation is in particular important to allow efficient and effective use of the germplasm. Any characterization and evaluation data are meaningless when not adequately documented and deposited into an information system facilitating easy access to stored data. Especially computerized documentation systems allow rapid dissemination of information to users of gene-banks and assist the curator of a gene-bank to manage the collections efficiently. An overview of the activities of a plant genetic resource centre is shown in the figure below (Figure 2.2).

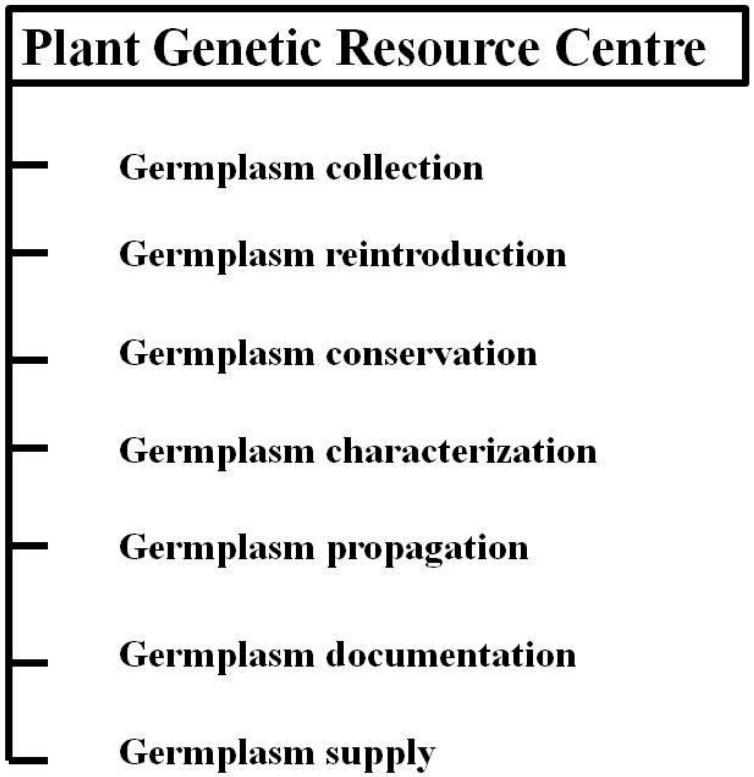

Figure 2.2 Main activities of a Plant Genetic Resource Centre

2.3 Plant germplasm collection in Mozambique

Plant germplasm collections have an extremely important natural value for any country, including Mozambique, but any collection has to be properly and carefully maintained. Mozambique belongs to the vegetation class of the Zambeziaca endemic region (Wild and Fernandes, 1968**).** The main vegetation types are the following: Miombo woodland, Mopane woodland, grassland, tropical dune forest and mangroves. The country is rich in botanical resources with approximately 6000 species of higher plants, many of them endemic (MICOA, 1997). As a result of the 15-year-long war in Mozambique, many accessions have been lost and re-collection of germplasm occurred only recently (Da Silva *et al*., 1996).

Only 5500 species are currently taxonomically classified and these are kept in the LMA Herbarium at IIAM. The National Centre of Plant Resources (NCPR), located at IIAM, is the main institution in Mozambique promoting the protection of plant genetic resources. The Centre conserves the genetic resources of crop species, especially those of importance to agriculture and food security in the country (da Silva *et al*., 1996). The Centre was established in 1989 and funding was provided by the SADC SPGRC and the government of Mozambique (Figure 2.3). Storage facilities used to conserve germplasm at IIAM headquarters in Maputo include a cold room, deep freezers, a drying machine, sealing machine, alumina containers and an electronic balance; seed storage conditions comply with international standards as described by Dulloo and Engles (2003).

Figure 2.3 (A) Plant Genetic Resource Centre building and (B) deep freezers used to conserve germplasm at PGRC in Mozambique.

The main commercial crops in Mozambique are maize, rice, groundnuts, vegetables and beans. Most varieties released are selections of local varieties or selections of materials introduced into the national collections from different areas of the country. A few foreign materials have been used in the recent past, especially in emergency situations, when large amounts of seeds were donated by foreign countries. Although a resource centre exists in Mozambique, local farmers still select their own seed and the reasons why local farmers maintain landraces vary (http:/www.fao.org/fileadmin/templates/agphome/documents/.../MOZAMBIQ). These include specific quality characteristics, such as taste, agronomic adaptation to the environment in Mozambique as well resistance to pests, diseases and also drought. This is particularly evident in traditional crops, such as cassava (*Manihot esculenta*), sorghum and cowpea (*Vigna unguiculata*). Several wild relatives of domesticated plants are also native to Mozambique. Unfortunately, there is only limited information on these plant species because of the lack of any coordinated actions on survey, collection and documentation.

Modern DNA banking is not applied in Mozambique owing to lack of resources. Establishing and managing DNA banks need several kinds of professionals, such as a genetic resource manager, taxonomist, molecular biologist, bio-informaticist, data analyst, web manager and policy expert (de Vicente and Andersson, 2006). Harnessing and integrating all the expertise available in the region to develop a common framework for DNA banking will benefit all the countries in the region. Specific capacity building plans need to be developed, including student training, staff and student exchange programmes, workshops and training courses.

Most germplasm accessions currently maintained in Mozambique have been obtained through collaborative programs between IIAM and international agricultural research centres (CIAT,

CYMMYT, IRRI and IITA) and only a small portion of these accessions have been collected locally. As a member of the SADC SPGRC, Mozambique signed a memorandum of understanding on the management of genetic resources and has the obligation to send duplicates of accessions stored to be maintained as duplicates at the SPGRC at Chalimbana, Zambia.

Material collected by the NCPR in Mozambique is evaluated in a first step for specific traits. Seed material not used for multi-locational trials is kept at the NCPR. During the period 2006 – 2008, major germplasm collections for important crops in Mozambique were carried out in the Gaza, Manica, Sofala and Cabo Delegado provinces and 326 samples were collected. The NCPR in Mozambique currently stores germplasm of 2823 accessions of maize, rice, sorghum, cowpea, pigeon pea, bambara groundnut, kidney bean, sunflower, soybeans and triticum (Figure 2.4). Cereals represent 53% of the collection, 36% are leguminous plants, 9% are vegetables and 2% are other cultures. Thirty seed samples have also been deposited at SPGRC for safe duplication (SPGRC, 2011).

In Mozambique, germplasm accessions are also kept in the field as field gene-banks. Examples are clones of banana kept at the Umbelúzi Agricultural Research Station and also 576 clones of cashew nut that are kept country-wide in Ricathla, Nhacoongo and Nampula. Unfortunately, most of these collections are not properly maintained or documented and have not been evaluated. Lack of financial resources and trained personnel and poor management capacity are the main constraints to adequate management of these field gene-banks. The Root and Tuber Crops Sector of IIAM is further managing a tissue culture laboratory for rapid multiplication, maintenance and distribution of cassava and sweet potato germplasm.

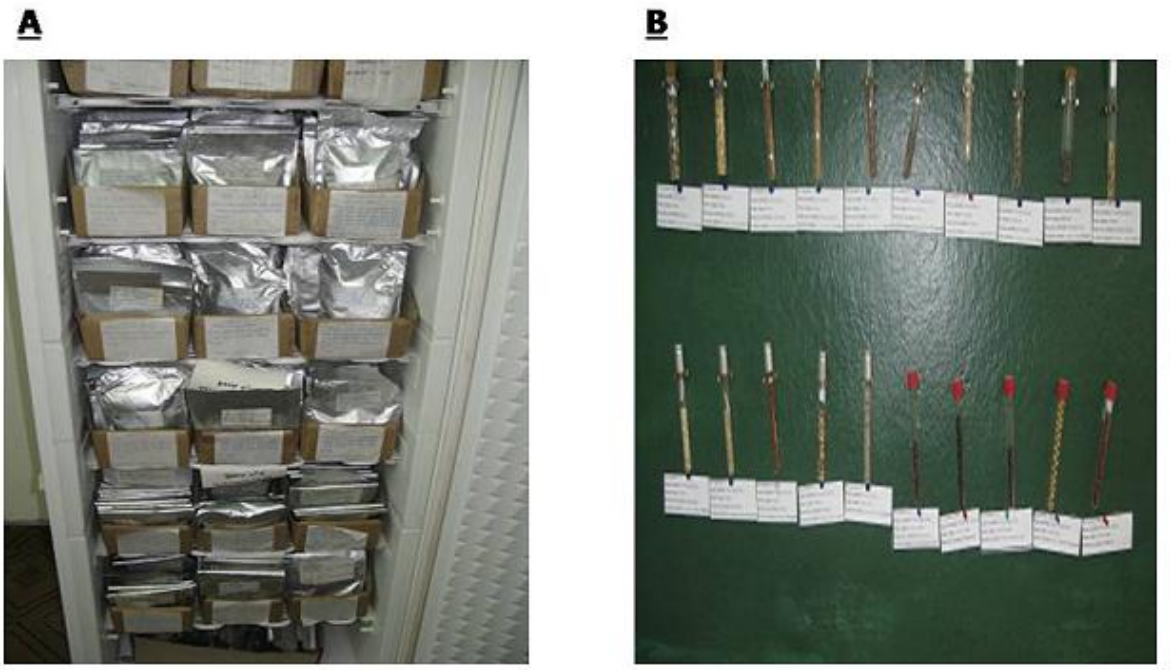

Figure 2.4 Germplasm collected and kept at the plant genetic resource centre in Mozambique (A and B).

The information is systematized in the SADC Documentation and Information System (SDIS). This research programmer is used at regional level, allowing the management of information about the material stored. The material of major species that is kept in the centre is shown in Table 2.1. For example, a total of 386 germplasm accessions (maize, cowpea and beans) have been regenerated/ multiplied since 1991. Using a regional documentation system from SADC (SDIS), the Mozambique National Plant Genetic Centre does the manual registration and computerization of all material collected. To date, the NPGRC has manually registered 2823 accessions (SPGRC, 2011) and 2314 accessions have been computerized using the SDIS software.

The four cultivars were selected on the basis of already available information from National Plant Genetic Resource Centre regarding their drought tolerance to serve as models for the identification of easily measurable physiological, biochemical and molecular markers for

drought tolerance. The four evaluated cowpea landraces are the most commonly grown landraces in different parts of Mozambique characterized by low and unpredictable precipitation and low soil fertility. Screening of a much larger number of cultivars for each evaluated characteristic could not be carried out with the limited space available for greenhouse and tunnel house experiments in South Africa and Mozambique, respectively, and also the overall limited financial support of the PhD project.

Table 2.1 Accessions of major plant species held at NPGRC in Mozambique

Species	Common name	Number of accessions
Arachys hipogea	Peanut	41
Cajanus cajan	Red gram	19
Citrullus lanatus	Watermelon	130
Cucumis sativus	Cucumber	10
Cucurbita maxima	Gourd	27
Discorea sp	Inhame	2
Eleusine coracana	Finger millet	12
Glycine max	Soybean	72
Helianthus annus	Sunflower	21
Macadamia sp	Macadam	18
Oryza longistaminata	Wild rice	26
Oryza sativa	Rice	344
Pennisetum glaucum	Pearl millet	29
Phaseolus vulgaris	Common bean	234
Sesamum sp	Sesame	9
Sorghum bicolor	Sorghum	281
Triticale + Triticum	Wheat	115
Vigna sp	Wild bean	1
Vigna subterranean	Jugo bean	106
Vigna unguiculata	Cowpea	144
Zea mays	Maize	334

Source: IIAM, 2008. The table is organized in alphabetic order of species kept in the Mozambican gene-bank.

2.4 Cowpea germplasm collection

2.4.1 International Institute of Tropical Agriculture

In Africa, IITA's genetic resources centre maintains the largest collection of cowpea germplasm. Of the 28,000 accessions of different crops maintained, about 50% are cowpea accessions collected from 89 countries (http://r4dreview.org/2010/09/to-conserve-or-not-to-conserve/). Since 1985, IITA has distributed this cowpea germplasm to various institutions in the world for research on genetic improvement or agronomy and biotechnology research aimed at developing new cultivars or varieties, in particular for rural farmers. The focus of the research for which collected germplasm has been used is mainly on obtaining a higher yield, pest resistance, a particular seed color and size, better nutritional value, early flowering and storability, as well as drought resistance to cross with other accessions.

According to the IITA website 2010, $72 has been spent annually on each cowpea accession to conserve and manage cowpea and only about half of that on wild *Vigna*. Cowpea germplasm is regenerated in the screen-house to produce high-quality germplasm, taking into account purity and sanitation, and a large part of the expenses went into the regeneration of the maintained 2228 accessions of cowpea, at an average cost of about $12.81 per accession. Other high costs were for seed health testing ($13.94/accession) and distribution ($22.63/accession). To reduce this cost there is a need to increase the number of accessions, thus lowering the unit cost.

2.4.2 Cowpea germplasm collection in Mozambique

Cowpea germplasm collection is currently done at NPGRC in Mozambique to address the need for adequate characterization of agronomic and morphological traits in order to facilitate proper utilization of the maintained germplasm. During the period 2006 – 2008 four missions of germplasm collection of the main crops took place in the provinces of Gaza, Manica, Sofala and Cabo Delegado, and 326 samples of different crops were collected and kept at NPGRC in Mozambique. The NPGRC conducted three multi-crop collection missions in 2009. Two collection missions were carried out in the northern provinces of the country, namely, Cabo Delgado and Niassa provinces, during which a total of 50 seed samples were collected in the Cabo Delgado province and 65 in the Niassa province. The third collection mission was conducted in four districts of the Gaza province (southern region of the country) and a total of 84 seed samples were collected and conserved. The duplicates, with sufficient amounts of seeds of all three missions, were sent to SPGRC. Although the NPGRC in Mozambique collects the germplasm all over the country, no proper characterization of this material has been done so far.

Current morpho-agronomic characterization is based on the International Plant Genetic Resources Institute (IPGRI) descriptor lists (http://www.bioversityinternational.org/publications/search.html). For example, 66 cowpea accessions have been characterized in the 1992/93 cropping season (Table 2.2). The morpho-agronomic characterization of the collected cowpea accessions were assessed by measuring variation in phenotypic traits such as flower color, growth habit or quantitative agronomic traits, such as yield potential, which are of direct interest to users. This approach has certain limitations, because it is well known that genetic information provided by morphological characters is often limited and expression of quantitative traits is subject to strong environmental influence. Other characterizations of cowpea were conducted in 2006,

also according to the descriptor's list and mostly based on morphological traits. Most of the varieties used in 1992/93 were included again (Afonso, 2006 personal communication) and this was done mainly to re-evaluate and regenerate the material already deposited at the NPGRC. Recently Chiulele (2010) characterized under field conditions part of this cowpea germplasm collections for drought tolerance where he found a wide genotypic variability among the germplasm. The germplasm could be grouped into four categories such as high yielding-drought tolerant, high yielding-drought susceptible, low yielding-drought tolerant and low yielding-drought susceptible. However, no further and proper characterization has been done under controlled conditions and published on cowpea at NPGRC in Mozambique.

Currently, proper characterization is also severely affected by the availability of skilled scientists and technicians in government-funded institutions, with rather low salaries, lack of adequate infrastructure and scarcity of research funds exacerbated by qualified research personel leaving the institution and a lack of reliable transport to the field plots or collection target sites (SPGRC, 2011). The main users of the available plant genetic resources in Mozambique are researchers involved in breeding, agronomy, botany and other research areas from different organizations in the country and overseas (Figure 2.5). Field extension workers and people dealing with seed multiplication and distribution are also important users of genetic resources. For example, in 2011 a total of 66 maize seed samples were processed and distributed to various end-users. In general, the requesters are students studying for MSc degrees at national and international universities. Closer collaboration with regional and international institutions would be of help to enhance the characterization of currently maintained germplasm. There are around 144 cowpea accessions that have been collect in 31 districts across Mozambique (Table 2.1). Four of the landraces were used in this study and they are all prostrate in habit and particular characteristics are shown in Table 2.2. Namarua, Massava nhassenje and Timbawene moteado are the most common grown cowpea landraces

in southern Mozambique and Tete-2 is grown in semi-arid areas, which are prone to drought, and the landrace has been classified as high yielding and drought tolerant (Chiulele, 2010). However, the large number of accessions kept in Mozambican gene banks shows an urgent need for proper characterization to also avoid having duplicates of samples.

Table 2.2 Cowpea accessions collected and kept at the Plant Genetic Resource Centre in Mozambique

Accession Number	Variety/local name	Accession number	Variety/local name
375	*INIA 36*	417	IT84D-460
376	*INIA 70*	418	IT845-2246-4
377	*INIA 62*	419	IT85F-2205
378	*INIA 46*	420	IT86D-472
379	*INIA 72*	421	IT86D-534
380	*INIA 42*	422	IT86D-641
381	*INIA 39*	423	IT82D-699
382	*INIA 1*	424	IT845-2118
383	*INIA 59*	425	IT86D-537
384	*INIA 37*	426	IT835-702-2
385	*INIA 51*	427	IT85D-901
386	*INIA 40*	428	IT86D-1057
387	*INIA 34*	429	IT835-990
388	*INIA 41*	430	IT86D-1056
389	*INIA 32*	431	IT86D-1035
390	*INIA 50*	432	IT86D-1038
391	*INIA 16*	433	IT82D-812
392	*INIA 78*	434	IT82D-885
393	*INIA 76*	435	*INIA-15*
394	*INIA 67*	436	*INIA 120*
395	*INIA 3*	437	*INIA 152*
396	*INIA 30*	1066	*INIA 35*
398	IT-85-5-33577	1067	*Timbabwene violeta*
399	IT-86 D-314	1068	IT85F-2020
400	IT85F-867	1069	IT82D-889
401	IT85F-2120	1070	IT82E-16
402	IT845-2140	1071	IT-505
403	IT83D-338-1	1072	*Tete-1*
404	IT82F-18	1073	***Tete-2***
405	IT86D-104	1074	*Massava-5*
406	IT86D-612	1075	*Massava -14*
407	IT86D-396	1076	*Maputo-1*
408	IT845-2163	1077	*Maputo-2*
409	IT835-742-2	1078	*Maputo-3*
410	IT85F-2805	1079	*Massava*
411	IT85F-867-5	1080	*Maputo-4*
412	IT82E-32	1081	*INIA-11*
413	IT85F-1517	1082	*INIA-73*
414	IT845-2085	1083	*Namuesse*
415	IT81D-1137	1084	IT82D-875
416	IT840-449	1085	K80
		2459	Thulandzelo
1086	Mhuti	2472	Chinhanguelane
1087	Frisland	2495	Litamba
1088	IT85-5-3577	2524	Namathapuata
1089	INIA-31	2525	Kaiacaha

1090	INIA-81	2632	Sacana
1123	INIA 54	2632	TinhawaTatikulo
1124	INIA 14		
1125	INIA 207		
1126	INIA 211		
1127	INIA 212		
1128	INIA 216		
1254	***Timbawene moteado***		
1255	Massava-11		
1256	Massava-2		
1257	Timbawene crème		
1266	***Namarua***		
1286	Muinana Iawe		
1287	Muikuha		
1300	Tkolo		
1302	Pathire		
1303	Ecute		
1505	Mutabela		
1510	Makulo		
1682	Khobwe		
1674	Canhembanhemba		
1685	Ngogodu		
1737	Khabalabala		
1760	Kambossa		
1763	Nhemba		
1880	Nhemba makulo		
1917	Mazololo		
1946	Chidoco		
1948	Bwadala		
2082	Nkululo		
2094	Timbawene		
2132	Chinhawane		
2106	Xigjaiandlala		
2115	Chicongondzo		
2176	Nandwa		
2249	Txabala		
2251	Mawacha		
2272	Thomana		
2279	Tyhulu		
2294	Thomhuane		
2225	Tchengane		
2339	Tathikulo		
2361	Huradjua		
2416	Tinhemba tahombe		
2428	***Massava Nhassenje***		

*Accessions that have been multiplied and characterised at the NPGRC are in *italics* and those used in this study are in *italics* and underlined.

Table 2.3 Characteristics of Mozambican cowpea landraces

Landraces	Average time to maturity (days)	Areas of origin in Mozambique
Namurua	76	Centre and north (Zambézia, Nampula, and Cabo Delgado)
Massava nhassenje	74	North and south (Inhambane)
Tete 2	89	Centre (semi-arid areas and very warm)
Timbawene moteado	95	South (Maputo, Gaza)

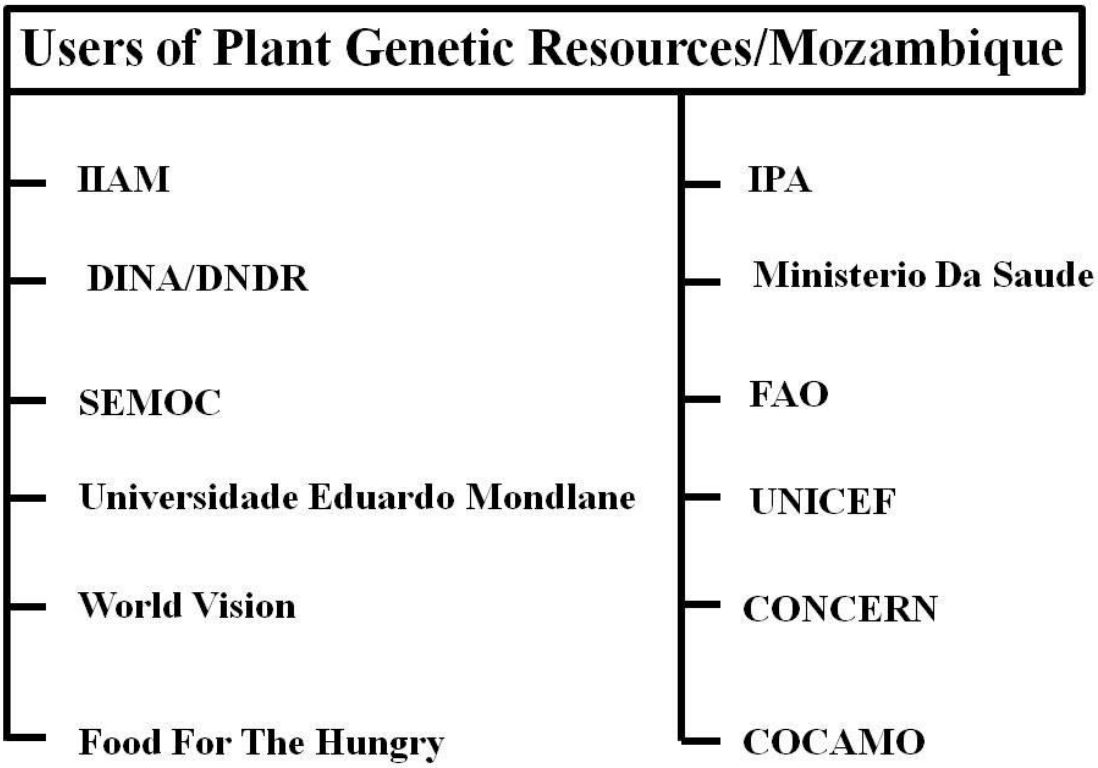

Figure 2.5 Main users of the plant genetic resources in Mozambique.

IIAM: Instituto de Investigação Agronómica de Moçambique; DINA/DNDR: Direcção Nacional de Desenvolvimento Rural; IPA: Instituto de Produção Animal, UNICEF (United Nations (International) Children's Fund); FAO: Food and Agriculture Organisation (United Nations); SEMOC: Sementes de Moçambique and COCAMO: Cooperation Canada Mozambique.

The next chapter reports on the genetic similarities and relationships among four Mozambican cowpea landraces, Tete 2, Massava nhassenje, Namarua and Timbawene moteado. In particular, the chapter deals with the detection of morphological and genetic differences between the Mozambican varieties using simple sequence repeats.

Chapter 3: Genetic relationship among Mozambican cowpea landraces as revealed by microsatellites

3.1 Abstract

Eleven primer pairs were used to amplify by PCR single sequence repeats (SSR) from isolated genomic DNA in an attempt to differentiate plants of cowpea landraces Tete 2, Massava nhassenje, Namarua and Timbawene moteado. Seven primer pairs tested amplified a single DNA band and the existence of SSR in these amplified products was confirmed by sequence analysis. SSRs amplified with primer pairs VM68 and VM70 showed a high level of polymorphism between the four landraces and these primers could differentiate between these landraces using SSR sequencing. Selected morphological, physiological and biochemical characteristics were less suitable for differentiation. Seed coat color varied from red in Massava nhassenje and Namarua to light brown in Tete-2 and to dark brown in Timbawene moteado. One-hundred seeds' weight varied between 12 and 22.8 g, with Massava nhassenje and Namarua having the highest, identical seed weight and Namarua the lowest weight. Only slight differences were found between landraces in total soluble seed protein content, ranging from 22.5 to 24.3%, as well as amino acid content. Overall, results have shown that seeds of the four landraces can be differentiated by application of phenotypic and genetic tools.

3.2 Introduction

Characterization of genetic variation within natural populations and among breeding lines is crucial for effective conservation and exploitation of genetic resources for crop improvement programs. Genetic variation in cultivated cowpea has therefore been assessed on the basis of morphological and physiological markers (Ehlers and Hall, 1997) but also by molecular techniques. These include RFLP (Fatokun *et al*., 1993), RAPD (Mignouna *et al*., 1998; Fall *et al*., 2003), DNA amplification fingerprinting (DAF; Spencer *et al*., 2000), AFLP (Fatokun *et al*., 1997; Tosti and Negri, 2002; Coulibaly *et al*., 2002), and SSR or microsatellites (Li *et al*., 2001). In particular, SSRs have been found to be useful as DNA markers in many plant species (Roder *et al*., 1995; Varshney *et al*., 2005; Wang *et al*., 2008; Ogunkanmi *et al*., 2008; Uma *et al*., 2009, Xu *et al*., 2010; Asare *et al*., 2010). SSRs currently also hold the best promise to characterize and identify cowpea accessions on the DNA level present in seed banks.

SSRs are short sequences of nucleotides one to five base pairs in length. They are tandemly repeated and flanked by unique DNA sequences (Scribner and Pearce, 2000). Produced by errors during DNA replication, SSRs are randomly distributed throughout the plant and animal genome (Tautz and Renz, 1984). High levels of polymorphism, high reproducibility, their hyper-variability and co-dominance, as well as their rapid and simple detection through the PCR, are their main advantages when compared to other types of DNA markers (Dib *et al*., 1996; Powell *et al*., 1996). Furthermore, SSRs have become a popular tool for genetic mapping and genome analysis (Chen *et al*., 1997; Li *et al*., 2001) and also genotype identification and are used in protection of variety (Senior *et al*., 1998) and seed purity evaluation, as well as in germplasm conservation (Brown *et al*., 1996), diversity studies (Xiao *et al*., 1996) and marker-assisted selection in breeding programs (Weising *et al*., 1998).

A number of SSRs have been isolated, identified and applied for characterisation of cowpea breeding lines and local varieties (Li *et al.*, 2001; Diouf and Hilu, 2005; Asare *et al*., 2010). In general, the SSR approach is also useful for cowpea because a large number of primers to amplify SSRs are already available. Timko *et al*. (2008) could differentiate 97% of 141 cowpea accessions by using a set of 25 primer combinations pre-selected by their ability to amplify SSRs by PCR in cowpea germplasm. In addition, Gupta and Gopalakrishna (2010) recently identified a total of 1071 SSRs by screening 15 740 cowpea unigene sequences.

The aim of this part of the study was to evaluate if already existing SSRs, which have been used in cowpea genetic diversity studies, are applicable to differentiate four Mozambican cowpea landraces that have not been previously characterised on the DNA level. The potential of the SSR technique was further compared to morphological, physiological and biochemical characteristics, which might also be applied for differentiation, such as seed coat color, 100 seeds' weight and protein and amino acid content.

3.3 Materials and methods

3.3.1 Plant material

Seed of four cowpea landraces (*Vigna unguiculata* (L.) Walp.) Massava nhassenje, Timbawene moteado, Namarua and Tete-2 with possibly various degrees of drought tolerance observed in the field was obtained from the National Germplasm Collection Bank at the IIAM, Mozambique.

3.3.2 Determination of seed weight and seed coat color

Seed weight was determined by weighing 100 seeds of each cowpea landrace investigated. The seed coat color was determined visually. Although the main focus of the study was on a physiological, biochemical and molecular characterization of the four cultivars, agronomic traits, such as yield, number of pods per plant, number of seeds per pod/ plant, flowering date, maturity date, delayed leaf senescence have not been investigated in the study. This would have required a more field-based study which was beyond the aim and resources for the PhD study. Therefore, only simple phenotypic characteristics, such as seed color and 100 seed weight, have been measured in the study.

3.3.3 Total seed protein and amino acid content

For total protein determination the seed coat was first removed manually and seeds were then ground into fine powder with a mortar and pestle. The powder was defatted with 40–60˚C petroleum ether and the excess solvent was removed in a vacuum evaporator at 50˚C. The total protein content of the four Mozambican cowpea landraces was estimated by determining the total nitrogen content by the Micro-Kjeldhal method (AOAC, 2000).

The amino acid content was determined using AccQ·Tag-ultra ultra-performance liquid chromatography (UPLC). For that, 40 mg cowpea seed flour was hydrolysed under vacuum in 6N HCl for 24 hr at 110^{o}C. For derivation, 60 µl borate buffer was added to the hydrolysed sample, followed by the addition of 10 µl of 1N NaOH solution and 20 µl of AccQTag reagent. The reaction mixture was transferred to a heating block and incubated at 55°C for 10 min. After cooling, 1 µl of the reaction mixture was injected for UPLC analysis. UPLC was performed on an Acquity System (Waters, Milford, MA, USA), equipped with a fluorescence

detector. The column used for amino acid separation was a BEH C18 100 mm × 2.1 mm, 1.7 µm column (Waters, UK). The flow rate was 0.7 ml min^{-1} and the column temperature was kept at 55°C. The excitation (λ_{ex}) and emission (λem) wavelengths for amino acid detection were set at 266 and 473 nm, respectively.

3.3.4 DNA extraction, purification and amplification

Plant genomic DNA was extracted from cowpea seed powder using the Zymo Research Plant/Seed DNA Extraction Kit^{Tm} according to the manufacturer's instructions. DNA concentration was determined with a Nanodrop spectrophotometer (Fermentas, Canada). The quality of purified DNA was determined by electrophoresis on a 1% agarose gel. For DNA amplification, a PCR reaction was carried out with an MJ Mini^{Tm} BioRad Thermal cycler (Bio-Rad, Germany). The reaction mixture (25 µl) contained 50 ng of genomic DNA, 2.5 µl of 1x PCR buffer, 0.3 µl of Taq DNA polymerase (Fermentas, Canada), 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTP mix, 0.5 µl of 10 mM of each primer made up to a total volume of 25 µl with nuclease-free water (Fermentas, Canada.). All primers used for amplification of SSRs were synthesised and supplied by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

The PCR reaction was performed by denaturing DNA at 94^{o}C for 3 min, which was followed by 35 cycles each consisting of 94^{o}C for 30 sec, primer annealing at 55^{o}C for 30 sec and DNA extension at 72^{o}C for 2 min and a final DNA extension time after 35 cycles at 72^{o}C for 10 min. After amplification, each PCR product (2 µl) was analysed on a 2% agarose gel for amplification of a single DNA band. PCR-amplified DNA was purified using a QIAquick® PCR Purification Kit (QIAGEN, ICI Americas Inc., USA), following the manufacturer's

instructions. After purification, DNA was quantified using a Nanodrop® spectrophotometer and the DNA was then sequenced to determine the presence and size of an SSR.

3.3.5 DNA sequencing

DNA sequencing was performed using the BigDye® Terminator Cycle Sequencing FS Ready Reaction Kit, v3.1 (Perkin Elmer, Applied Biosystems, USA) on an ABI PRISM® 3100 automatic DNA sequencer (Applied Biosytems, USA). For that, a PCR reaction was carried out with 20 ng of purified PCR product, 0.5 µl of Big Dye, 2.1 µl of a 5 x PCR buffer, 1 µl of either a forward or reverse primer (10 mM) made up to a final volume of 10 µl with sterilised, distilled water. The PCR reaction was carried out at 96^{o}C for 10 sec, which was followed by 35 cycles each consisting of 55^{o}C for 5 sec and 60^{o}C for 4 min, with a final extension at 72^{o}C for 10 min. The PCR product was cleaned using the NucleoSEQ kit (Macherey-Nagel, Germany) containing 650 µl of a Sephadex solution (Sephadex® G-50, Sigma) into a NucleoSEQ column and centrifuged at 2 800 rpm for 2 min. The column was transferred into a 1.5 ml micro-centrifuge tube. The sequencing DNA reaction was loaded into the centre of the column and centrifuged at 2 800 rpm for 2 min at room temperature. The DNA was transferred into a 0.5 ml microcentrifuge tube and dried at 45^{o}C for 20-25 min using a vacuum drier (Eppendorf Concentrator 5301, Eppendorf, Germany). After drying, the samples were submitted for sequencing.

3.3.6 Statistical analysis

Data were analysed using the JMP 5.0 Statistical Package (SAS Institute Inc., Cary, NC, USA). The significance level was set at 1% or 5% using analysis of variance (ANOVA). The repeat number and number of alleles were determined based on the DNA sequences and the

polymorphism information content (PIC) of each microsatellite was determined using the formula described by Weir (1996).

3.4 Results

3.4.1 Seed coat color, seed weight determination and protein content

The seed coat color of the four landraces varied from dark red in Massava and Namarua (Figures 2.1A and C) to light brown in Tete-2 (Figure 2.1D) and dark brown in Timbawene moteado (Figure 3.1B). When the weight of 100 seeds was determined, it varied from 12 to 22.8 g, with Tete-2 and Massava nhassenje having the highest seed weight and Namarua the lowest (Table 3.1). The protein content of seeds ranged from 22.5 to 24.38% with an average of 23.45% in the four Mozambican landraces (Table 3.1). However, no significant differences ($P<0.01$) in protein content was found between the seeds of the four landraces.

Figure 3.1 Cowpea seeds of four Mozambican cowpea landraces. Massava nhassenje (A), Timbawene moteado (B), Namarua (C) and Tete-2 (D).

Table 3.1 Total protein content seed weight, and seed coat color of cowpea landraces

Landraces	Total protein content (%)	100-seed weight (g)	Seed coat color
Massava	23.23±0.45ab	22.8±0.037a	Red
Timbawene	24.38±0.32a	17.5±0.026b	Brown
Namarua	22.50±0.45b	12.0±0.02c	Red
Tete 2	23.69±0.35ab	22.8±0.019a	Brown

3.4.2 Amino acid content

The amino acid content of seeds of the four cowpea landraces is shown in Table 3.2. All landraces showed a similar amino acid content pattern, with the essential amino acid content for histidine, cysteine, methionine, iso-leucine, leucine, threonine and lysine varying from 2.49-2.64, 0.28-0.30, 0.72-0.74, 1.17-1.22, 09.98-1.05 and 1.25-1.32 g/100 g protein, respectively. For the non-essential amino acid arginine, the content only varied from 4.30 to 4.56% and the tyrosine content was similar for all four landraces. Seven amino acids (threonine, methionine, lysine, glutamic acid, serine, valine and phenylalanine) showed significantly differences between the four landraces ($P \leq 0.05$).

Table 3.2 Amino acid content of cowpea landraces

Amino acids	Landraces				
	Massava	Timbawene	Namarua	Tete-2	P value
Histidine	2.53	2.64	2.49	2.62	0.221
Threonine	0.86	0.91	0.87	0.90	0.024
Methionine	0.72	0.78	0.72	0.74	0.042
Cysteine	0.28	0.30	0.28	0.30	0.185
Isoleucine	1.17	1.22	1.14	1.20	0.312
Leucine*	1.00	1.05	0.98	1.01	0.318
Lysine	1.30	1.37	1.25	1.32	0.0136
Aspartic acid	0.71	0.76	0.70	0.75	0.118
Glutamic acid	1.12	1.19	1.11	1.15	0.1908
Serine	1.49	1.62	1.52	1.62	0.0056
Glycine	1.02	1.06	0.98	0.99	0.48
Arginine	4.45	4.52	4.30	4.56	0.108
Alanine	0.99	1.04	0.99	1.02	0.500
Proline	0.92	0.98	0.91	0.96	0.002
Tyrosine	0.02	0.02	0.02	0.02	0.540
Valine	1.52	1.57	1.48	1.58	<0.001
Phenylalanine	1.74	1.83	1.70	1.79	0.004

*Essential amino acids. Amino acid content expressed as percentage (g/100g protein)

3.4.3 Determination of SSR polymorphism

SSRs with predicted sizes and primers used for DNA amplification and sequencing analysis are presented in Table 3.3. The microsatellites used were selected according to those published for cowpea (Li *et al*., 2001) based on their high polymorphism information content (PIC) ranging from 0.55-0.73.

Of 11 primer pairs tested, four primers pairs, VM5, VM12, VM19, VM23, did not amplify any bands from genomic DNAs of the four landraces. Seven primer pairs, VM31, VM35, VM36, VM 39, VM68, VM 70 and VM71, amplified a single DNA band from all genomic DNAs tested. The amplification products obtained with primer VM31, VM39 and VM68 where a single band product was found, which was always identical on a gel, are shown in Figure 3.2 and it was only possible to differentiate these after sequencing.

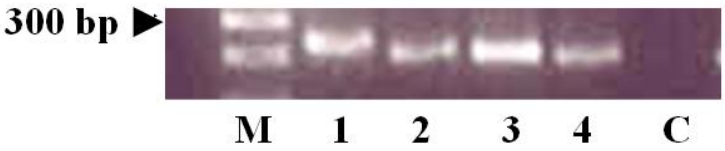

Figure 3.2 PCR amplification product showing a single band obtained by primer pairs VM31, VM39, VM68 isolated from genomic DNA of four Mozambican cowpea landraces.
M represents a 100 bp DNA ladder, lane 1 represents Massava nhassenje; lane 2 Timbawene moteado; lane 3 Namarua and lane 4 Tete 2. C represents a negative control without addition of DNA. Lanes 1-4 amplification obtained by primer VM31, lanes 5-8 amplification by VM 39 and lanes 9-12 by VM 71.

When the single amplified DNA bands from the seven primer pairs were analysed by sequencing, polymorphic SSRs were found and the types and numbers of repeats detected are shown in Table 3.4. For the seven microsatellite primers used for sequencing, a total of 20 alleles were detected from the four cowpea landraces (Table 3.4). The number of alleles per primer varied from two to four. Primer pairs VM36, VM39 and VM71 amplified only two alleles, while pairs VM68 and VM70 amplified the maximum number of four alleles. SSRs amplified with primer pairs VM68 and VM70 showed a high level of polymorphism between the four landraces and these primers could differentiate between these landraces, which was

not possible to do with the other parameters analysed. The polymorphism information content varied from 0.375 to 0.871, with an average of 0.482.

Table 3.3 SSR primer pairs for SSR amplification, type of SSR, expected fragment size and polymorphism information content (PIC)

Primer	Sequence	Repeat	Size (bp)	PIC
VM5	5'-AGC GAC GGC AAC AAC GAT-3' 5'-TTC CCT GCA ACA AAA ATA CA-3'	(AG)	188	0.66
VM12	5'-TTG TCA GCG AAA TAA GCA GAG A-3' 5'-CAA CAG ACG CAG CCC AAC T-3'	(AG)	157	0.66
VM19	5'-TAT TCA TGC GCC GTG ACA CTA-3' 5'-TCG TGG CAC CCC CTA TC-3'	(AC).(AC)	241	0.65
VM23	5'-AGA CAT GTC GGC GCA TCT G-3' 5'- AGA CGC GTG CCC ATG TT -3'	(CT)	174	0.60
VM 31	5'-CGC TCT TCG TTG ATG GTT ATG-3' 5'-GTG TTC TAG AGG GTG TGA TGG TA-3 '	(CT)	200	0.59
VM 35	5'-GGT CAA TAG AAT AAT GGA AAG TGT-3' 5'-ATG GCT GAA ATA GGT GTC TGA-3'	(AG).(T)	127	0.55
VM 36	5'-ACT TTC TGT TTT ACT CGA CAA CTC-3' 5'-GTC GCT GGG GGT GGC TTA TT-3'	(CT)	160	0.56
VM 39	5'-GAT GGT TGT AAT GGG AGA GTC-3' 5'-AAA AGG ATG AAA TTA GGA GAG CA-3'	(AC).(AT) (TACA)	212	0.69
VM 68	5'-CAA GGC ATG GAA AGA AGT AAG AT-3' 5'-TCG AAG CAA CAA ATG GTC ACA C-3'	(GA)	254	0.73
VM 70	5'-AAA ATC GGG GAA GGA AAC C-3' 5'-GAA GGC AAA ATA CAT GGA GTC AC-3'	(AG)	186	0.70
VM 71	5'-TCG TGG CAG AGA ATC AAA GAC AC-3' 5'-TGG GTG GAG GCA AAA ACA AAA C-3'	(AG) (AAAG)	225	0.55

* Primer pairs (forward and reverse) according to Li *et al*. (2001)

Table 3.4 Number of microsatellite repeats, number of alleles and polymorphic information content (PIC) amplified with different primer pairs in cowpea landraces

Landraces	VM31 (CT)	VM35 (AG)	VM36 (CT)	VM39 (AC)	VM68 (GA)	VM70 (AG)	VM71 (AG)
Massava	18	12	14	12	12	18	9
Timbawene	20	23	9	13	13	19	8
Namarua	19	12	14	12	8	23	8
Tete-2	18	10	14	13	15	20	8
Alleles	3	3	2	2	4	4	2
PIC	0.688	0.688	0.375	0.375	0.871	0.871	0.375

3.5 Discussion

In this part of the study, characteristics such as seed color and 100 seeds' weight, total protein and amino acid content of seeds, as well as the presence of SSRs in genomic DNA, were investigated. In general, knowledge of such characteristics is vital knowledge extensions for the National Germplasm Bank regarding characterization of Mozambican cowpea landraces. This adds to traditional characteristics used by Mozambican farmers to date, such as pod or seed size or general plant productivity. Only a fraction of the conserved germplasm is actually being characterised in greater detail in Mozambique. In addition, landraces have been named after people or localities, which is an artificial and problematic system of classification and nomenclature. Varieties with identical morphological characteristics might therefore have different names only determined by locality or an ethnic group. This clearly hampers correct classification as a variety in the National Germplasm Bank and the effectiveness of breeding programmes in the country. Cowpea landraces used in this study were further collected in the country from farmers' fields and conserved in the national gene bank in Mozambique with the

aim of tracking local diversity and facilitating breeding for improved varieties in Mozambique in the face of changing biotic and abiotic factors affecting production of the crop. Traditionally, subsistence farmers save seed and rely on their own experience to select and improve their varieties and during this process there is a possibility that farmers can mix up when eeds look almost identical.

Clear differences between landraces were found in this study in seed coat color and also 100 seeds' weight. However, both characteristics could not greatly differentiate all four landraces. The small differences in total seed protein content, determined to be between 22.5 and 24.4%, which is in agreement with the values reported by Horax *et al*. (2004) and also recently by Gupta *et al*. (2010), were not well-suited as characteristics for differentiation of the four landraces. Furthermore, the essential amino acid content varied in this study from 0.28-0.30 for cysteine to 2.49-2.64 for histidine. These values are lower when compared to the values previously reported for cowpea (Oluwatosin, 1997; Hussain and Basahy, 1998; Gupta *et al*., 2010), soybean (Leverton, 1967), and pea (Wang and Daun, 2004). However, the differences in amino acid content of seeds were also too small to use these characteristics as an indicator for differentiation.

Although seed color and weight allowed differentiation of the four landraces, these characteristics are determined by both genetic and environmental conditions, which can change rapidly. In contrast, genetic characteristics, such as SSRs, are more stable and have been recognised as excellent tools for assessment of genetic diversity in many legume food crops, including cowpea (Li *et al*. 2001, Diouf and Hilu, 2005, Asare *et al*., 2010). In particular the application of the SSR marker technology has recently attracted much attention to develop a molecular marker for a specific characteristic applicable in germplasm screening (Segal and Yadav, 2010). In this PhD study, 11 primers selected from Li *et al*. (2001) based

on the high polymorphism information content, were originally tested for their potential to differentiate the four cowpea landraces; four primer pairs, VM5,VM12, VM19, VM23, did not amplify any bands from genomic DNAs of the four landraces. The lack of amplification could be due to either sequence errors or to problems of primer synthesis (Guyomarch *et al*., 2002).

When the PCR product was visualised on an agarose gel, only a single band was detected which was always identical on a gel. Therefore differences were only found after sequence analysis. The agarose gel electrophoresis method is appropriate to distinguish the bands when the alleles are long enough, which is more than 200-300 base pairs, and the differences among alleles are also great enough (i.e. more than 10-20 bp). In this study, the fragment size ranged from 127 to 254 bp and the difference between the alleles in most of the primers was too short. The seven primers that amplify a single-locus were further used for sequencing analysis. Of the seven primers, primers VM68 and VM70 could distinguish four cowpea landraces, which could not be done with the other parameters analysed. Li *et al*. (2001) found that of a total of 46 microsatellites evaluated, 27 amplified single-locus and only five polymorphic primers could distinguish 88 of 90 cowpea lines. These results suggest that microsatellites are useful molecular markers in the classification of the Mozambican seed bank of cowpea. The results also show the potential use of microsatellite markers in the breeding programs of this important legume crop. A low degree of genetic polymorphisms in cowpea, also found in this study, appears to be inherent in cultivated cowpea and might result from both the domestication process and inherent self-pollination mechanism (Asare *et al*., 2010). However, the degree of polymorphism, with two to four alleles per primer, is rather low when compared with that found by other authors (Li *et al*., 2001). This can be an indication of a need to evaluate more microsatellites to distinguish Mozambican cowpea landraces at DNA level.

Further, in comparison to this study, Li *et al.* (2001) found lower PIC values for cowpea derived from an IITA breeding programme with a relatively narrow genetic base. Higher PIC values might be due that cowpea, which is currently grown in Mozambique, largely consists of landraces selected for local conditions. These landraces are therefore more diverse and not genetically uniform. However, the PIC value obtained in this study ranged from 0.375 to 0.817 with an average of 0.6, which is in agreement with most other plant SSR studies (Zhao *et al*., 2010; Sharma *et al*., 2009)

In conclusion, many gene-banks in Africa, particular in Mozambique, hold collections that have only been characterised by morphological markers. This study contributed to the characterisation in more detail of four Mozambican cowpea landraces available in the IIAM Mozambican gene-bank. It also confirmed that seeds of the four landraces can be differentiated by application of phenotypic and genetic characteristics, such as SSRs, but that genetic characterisation is superior to phenotypic characterisation. Development of knowledge of the SSR profile can therefore be an advantage in breeding, if a combination of genetic, morphological and biochemical markers for characterisation of germplasm present at gene banks is used. In the past SSRs were expensive to develop and only were applied to the major commercial crops (Scott *et al*., 2000). Until recently, developing SSR markers for new species was a laborious and costly exercise, thus limiting their potential application. However, the SSR approach is cost-effective for cowpea because of the large number of SSR primers already available (Li *et al*., 2001). This study has overall demonstrated the utility of SSR markers for analysis of the currently available cowpea germplasm in Mozambique. However, more primers should be tested in the future and SSRs added as a characteristic to complement characterization of collections present in the Mozambican gene bank.

In the next chapter the morphological and physiological characterisation of cowpea landraces under well-watered and drought conditions is reported.

Chapter 4: Morphological and physiological characterization of Mozambican cowpea landraces under drought conditions

4.1 Abstract

The growth of four Mozambican cowpea landraces (*Vigna unguiculata* (L.) Walp) was investigated under drought stress using a range of plant growth and physiological parameters for plant performance measurement. Drought stress was induced in either a temperature-controlled greenhouse or in a tunnel house experiment by withholding water supply, while control plants were maintained under well-watered conditions. Growth under drought conditions decreased in plants of all four landraces photosynthesis and stomatal conductance, as well as leaf and stem biomass accumulation. Root biomass increased in plants of all landraces in response to drought treatment, possibly to access more water under drought conditions. Plants of the landrace Timbawene moteado always performed best under both growing conditions, with Massava nhassenje plants performing worst. Data of this study have contributed to characterise in greater detail existing cowpea accessions in the NPGRC and also to establish the tools for future characterisation of cowpea accessions in Mozambique.

4.2 Introduction

Drought is one of the most important environmental factors limiting plant growth and productivity (Boyer, 1982; Baker, 1989). In general, plants respond to stress at morphological, physiological, biochemical and molecular levels (Chaves *et al*., 2003). The magnitude of the response varies among species and among varieties within a crop species (Kramer, 1980). Adaptation strategies of plants to drought stress include drought escape, drought avoidance and drought tolerance (Levitt, 1980; Turner *et al*., 2001). Plants with an escape strategy can survive dry conditions and have the ability to complete their entire life cycle within a short time period during a rainfall season (Ludlow, 1989). Drought avoidance involves maintenance of plant water status in the presence of drought stress, while drought tolerance is the ability of the plant to endure or withstand a dry period by maintaining a favourable internal water balance under drought conditions (Kramer, 1980). Different plants can use mechanisms of both tolerance and avoidance to cope with drought (Chaves *et al*., 2002). Response to drought stress may also involve metabolic and structural changes that improve plant functioning under stress (Bohnert and Sheveleva, 1998), including changes in the root-to-shoot biomass ratio, with increased root biomass (Munns and Cramer, 1996). Drought stress further reduces plant productivity by inhibition, or modification, of photosynthesis and stomatal conductance (Chaves *et al*., 2002; Lawlor, 2002; Lawlor and Cornic, 2002; Lawlor and Tezara, 2009).

Cowpea is generally regarded as more drought-tolerant than other legumes such as common bean and soybean (Ehlers and Hall, 1997). This is due to its inherent capacity to survive drought. Many studies have shown that water deficit during critical growth stages can result in substantial yield reduction (Turk and Hall, 1980; Watanabe *et al*., 1997). Research has further demonstrated that significant differences exist among cowpea genotypes to perform under

drought (Watanabe *et al*., 1997; Mai-Kodomi *et al.*, 1999b). Selection of cowpea genotypes under drought conditions using both phenological and morphological traits, such as date of flowering and delayed leaf senescence, have been used successfully in cowpea-breeding programmes (Hall *et al*., 1997; Anya and Herzog, 2004a; Souza *et al*., 2004). Physiological, biochemical and agro-morphological processes under drought conditions have been investigated in several Sub-Saharan cowpea varieties and results have shown that stomatal closure is a common strategy used by cowpea to avoid dehydration (Zombré *et al*., 1994; Nwalozie *et al*., 1996; Pimentel *et al*., 1999; Diallo *et al*., 2001; Sarr *et al*., 2001; Ogbannaya *et al*., 2003; Anya and Herzog, 2004a; Hamidou *et al*., 2007). Such investigations have not been carried out yet with Mozambican cowpea landraces grown in Mozambique, neither have any physiological parameters been applied in Mozambique to select plants for better drought tolerance.

The aim of this part of this study was therefore to investigate the morphological and physiological response of cowpea landraces measuring a variety of plant performance parameters. In particular, four Mozambican cowpea landraces currently used in the country by local farmers were exposed to water deficiency. The study was carried out in South Africa using vermiculite instead of soil in a greenhouse under temperature-controlled conditions and also in Mozambique in a tunnel house using local soil and naturally environmental growing conditions.

4.3 Materials and methods

4.3.1 Plant material and growing conditions

For cowpea growth in a temperature-controlled greenhouse, cowpea (*Vigna unguiculata* (L.)

Walp) seeds of four Mozambican landraces (Massava nhassenje, Timbawene moteado, Namarua and Tete-2) provided by IIAM in Mozambique were grown in pots (17.5 cm x 20 cm) containing fine grade vermiculite (Mandoval PC, South Africa). Fine grade vermiculite has a particle size of 0.5-3 mm and a loose bulk density of 100 kg/m^3. Vermiculite was used instead of soil to avoid interference of soil nitrogen with nodule development and to ease harvesting of intact nodules for analysis. The seed was placed into a small hole containing 0.5 g *Bradyrhizobium* powder (Stimuplant CC, South Africa). Experiments were carried out in an environmentally controlled greenhouse at a light intensity of 600 mmol $m^{-2}s^{-1}$ with a 13 h photoperiod. Additional artificial light was provided for 3 h in the evening by metal-halide lamps to increase the day length to 13 h and a day/night temperature of 27^{o}C/17^{o}C and 60% of relative humidity were maintained. Plants were watered daily with distilled water and nitrogen-free Hoagland nutrient solution was supplied twice per week.

Figure 4.1 Cowpea plants grown for 14 days in a greenhouse under either well-watered conditions or drought conditions induced by withholding water for 14 days.

For cowpea growth in a tunnel house in Maputo, Mozambique, plants of the four Mozambican cowpea landraces Massava nhassenje, Timbawene moteado, Namarua and Tete-2 were grown for eight weeks in 17.5 cm x 20 cm diameter plastic pots, filled with soil obtained in Maputo, Mozambique (25^{o} 28'S and 32^{o} 36'E) under water-controlled conditions. The tunnel house was covered with a plastic roof to exclude rain (Figure 4.2). The soil used was collected from the experimental farm of the Faculty of Agronomy and Forestry Engineering of the Eduardo Mondlane University in Maputo, Mozambique. The soil characteristics were determined using the Micro-Kjeldhal method (AOAC, 1984; Table 4.1). Plants were established from pre-germinated seeds incubated for 72 hrs on filter paper moistened with distilled water. Pre-germinated seeds with a radical of 10 mm length were planted, with one plant per pot. The plants were then grown in a tunnel house in Maputo, Mozambique (25^{o} 28'S and 32^{o} 36'E) under well-watered conditions.

Figure 4.2 Cowpea plants growing in an open-sided tunnel house covered with a plastic roof to shelter against rain (A) and cowpea plants exposed to drought conditions (B).

Table 4.1. Chemical and physical soil characteristics

Characteristics	Values
Sand (%)	84.50
Clay (%)	1.00
Silt	13.20
pH	6.80
Carbon (%)	0.07
Organic matter (%)	0.12
Total N (%)	0.08
Total P (mg kg^{-1})	188.00

4.3.2 Drought treatment

Cowpea plants were grown to the third foliar stage before exposure to drought stress. Experimental plants (40 plants) were maintained under well-watered conditions in a temperature-controlled greenhouse and 40 plants were subjected to drought stress by withholding the supply of water and nutrient solution for 14 consecutive days. When plants were not watered for longer than 2 weeks severe wilting occurred. Pots containing well-watered and drought-stressed plants were randomly distributed throughout the greenhouse and plants were harvested on day 0, 7 and 14 after exposure to drought stress for analysis.

Drought experiments in Mozambique were carried out in a tunnel house (open-sided, covered with a plastic roof to provide shelter from rain). The minimum and maximum air temperature during the growth period was 24.5^{o}C and 41.2^{o}C. The relative humidity of the air ranged from 29.8 to 78.1%. Natural light was used and measured with a quantum sensor (SK P215, Skye Llandrindod Wells, UK); the light had an average photon flux density at the canopy level of 345± 55 µmol.$m^{-2}s^{-1}$.

During the first four weeks, plants were irrigated in the tunnel house to field capacity with normal tap water. Drought stress was induced after four weeks based on a gravimetric method. For that, half of the experimental plants were left without watering until plants showed symptoms of wilting (10 days), while the remaining half were maintained at 100% field capacity. The amount of water that evaporated was monitored daily by weighing unplanted pots placed randomly between planted pots in both stressed and non-stressed treatments in each block. Pots were watered with the amount of water equivalent to the loss of weight. This was done to bring them to the pre-determined level of moisture whenever the weight of pots fell below the lower limit established for the treatment (25% for drought and 100% for non-drought treatments) until the end of the experiment. For analysis, four plants per treatment were harvested four weeks after drought treatment. At harvest, plants were separated into leaves, stems, and roots. The leaves were divided equally; one half was used for dry biomass and leaf area measurement and the other half was used for chlorophyll and protein determination.

4.3.3 Measurement of growth

Growth parameters measured included the fresh and dry mass of roots, stems and leaves, which was determined by weighting on a balance. The dry biomass of leaves, stem and roots was determined after exposure of plant parts in a drying oven (Type U 40, Mommert Germany) to a temperature of 80°C for 48 h. Leaf area, as a morphological parameter, was determined by using a leaf area meter (Li-3000A, LI-COR, Inc. Lincoln, USA). The number of nodules was counted by visual examination of plants.

4.3.4 Measurement of physiological and biochemical parameters

Photosynthetic gas exchange parameters were measured on attached leaves of greenhouse-grown plants with an irradiance of 700 µmol photons $m^{-2}s^{-1}$ and a CO_2 concentration of 350µmol^{-1}. All measurements were performed on the fourth leaf counted from the shoot apex at day 1, 7 and 14 after drought exposure. The instantaneous water use efficiency (IWUE) values were calculated as the ratio between CO_2 assimilation rate and the stomatal conductance value as described by Soares-Cordeiro *et al*. (2009). The leaf water potential (LWP) was measured from the same central leaf part as described for gas exchange with a pressure bomb model 3005 (ICT International, Australia) (Valenzuela-Vazquez *et al.*, 1997). For determination of vermiculite water content, a vermiculite sample was taken by using a cylindrical cork borer (1.4 cm diameter and 11cm length) on day 1, 7 and 14. The 11 cm deep sections were representative of water content more than halfway down the pots. The fresh weight of the sample was measured immediately by using a balance. Vermiculite samples were then placed into a drying oven (Type U 40, Mommert, Germany) at a temperature of 80^0C for 24 h. Water mass was calculated as the difference between the weight of the wet and oven-dried samples and soil water content (SWC) was calculated as dry weight as percentage of fresh weight.

Total protein concentration in leaves was determined using a commercially available Bradford protein determination assay reagent (Bio-Rad, UK) with Bovine Serum Albumin (BSA) from Sigma (South Africa) as a standard. For measurement, cowpea leaves were ground into powder in liquid nitrogen with a mortar and pestle for protein isolation. Proteins were dissolved by the addition of 1 ml of 50 mM Tris-HCl buffer, pH 8.0, to the powder. The extract was centrifuged at 13 000 rpm for 10 min at 4^oC in an Eppendorf centrifuge. The resulting clear protein-containing supernatant was used for protein determination. The

absorbance was further measured in a total volume of 1 mL at 595 nm with a spectrophotometer (Macince UK). All the measurements (samples and standard) were done in duplicate.

The chlorophyll content of leaves from four different plants per treatment was measured. For determination of chlorophyll *a* and *b* content, the absorption of an 80% acetone extract containing the chlorophyll was measured at 663 and 645 nm in a spectrophotometer (Pharmacia LKB, Ultrospec III, UK) and the chlorophyll content was determined using absorption coefficients, according to MacKinney (1941).

The free proline content in leaves was determined according to Bates *et al*. (1973). For determination, 0.5 g of cowpea leaves were homogenized in 10 ml of 3% aqueous sulpho-salicylic acid and the homogenate was centrifuged at 2000 g for 5 min. Two milliliters of the extract was incubated with 2 ml of glacial acetic acid and 2 ml of acid-ninhydrine reagent for 1 h at 100^{o}C. The reaction mixture was treated with 4 ml toluene, the solution containing toluene was separated and the absorbance was read on a spectrophotometer at 520 nm. The free proline content was determined using a standard curve.

4.3.5 Statistical analysis

The experimental design was a completely randomized block with four landraces and two watering regimes (well-watered and drought treatment) resulting in eight treatments for both the greenhouse and tunnel house experiment, respectively. Data were analysed using the JMP 5.0 Statistical Package (SAS Institute Inc., Cary, NC, USA). The significance level was set at 1% or 5% using ANOVA. Differences between parameters, measured under well-watered and drought conditions, were determined using the Student's t-test.

4.4 Results

4.4.1 Greenhouse experiments

4.4.1.1 Plant growth changes

At the beginning of the drought experiment plants of all landraces had a similar biomass (leaf, stem, root and total biomass; Figures 4.3A, B, C, D). Leaf and stem dry biomass as well as total biomass production increased significantly ($P \leq 0.05$) in all landraces over 14 days treatment under both drought and well-watered conditions. However, the increase was much higher under well-watered conditions than under drought conditions. Timbawene moteado, Namarua and Tete 2 plants had greater increases in leaf, stem and total biomass under well-watered conditions when compared to Massava nhassenje plants, which had the lowest increase (Figures 4.3A, B, D). When plants of all four landraces were exposed to drought stress, leaf, stem and total biomass was much lower for all plants when compared to biomass under well-watered conditions. Under drought conditions, all four landraces greatly increased their root dry biomass, possibly to collect more water. However, Timbawene moteado root dry biomass was significantly higher ($P \leq 0.05$) than that of the three other landraces tested (Figure 4.3C). Timbawene moteado was always superior to the other landraces in drought with the highest leaf, stem and total dry biomass ($P \leq 0.05$) (Figure 4.3A, B, D).

When the leaf area under well-watered conditions was measured, Timbawene moteado and Tete 2 plants both had the largest leaf area (Figure 4.4A), with Massava nhassenje having the smallest leaf area. Exposure to drought had no great effect on leaf area in plants of all four landraces when compared to plants grown under well-watered conditions. Watering of plants for 14 days increased the leaf area in Timbawene moteado and Tete 2 when compared to

drought treatment, but there was little change in leaf area for Massava nhassenje and Namarua (Figure 4.4A). The shoot-to-root ratio also increased much more in drought when compared to well-watered conditions in all plants, indicating that landraces increase their root biomass as a consequence of drought treatment. The highest increase was in Namarua and Timbawene moteado plants and the lowest in Massava nhassenje plants (Figure 4 4.4B). This indicates that Massava nhassenje plants are much more drought-sensitive than plants of the other landraces.

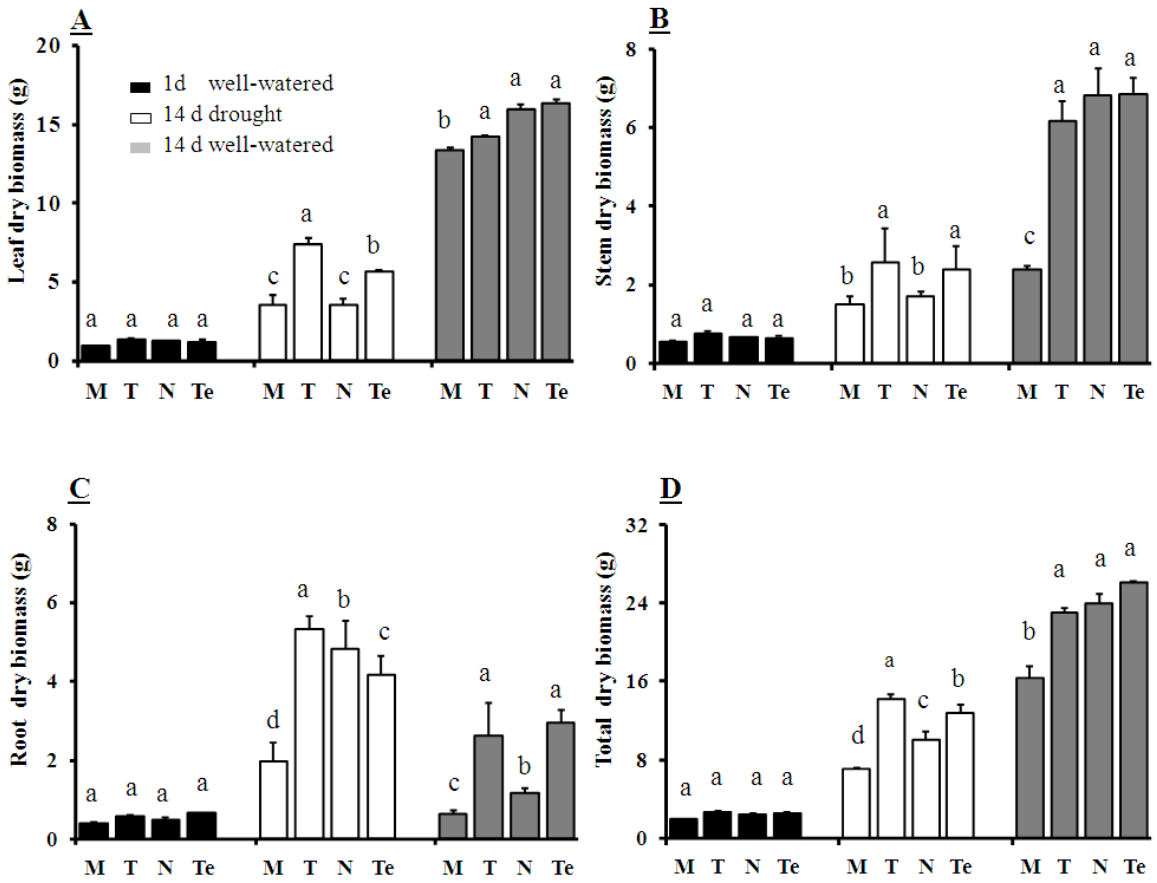

Figure 4.3 Effect of drought stress on (A) leaf (A), (B) stem, (C) root and (D) total plant biomass in plants of landraces Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) under well-watered conditions after 1 day (black closed bars) and after 14 days either under drought conditions (open bars) or well-watered conditions (grey closed bars).

Bars represent the mean biomass ± SE of four individual plants. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

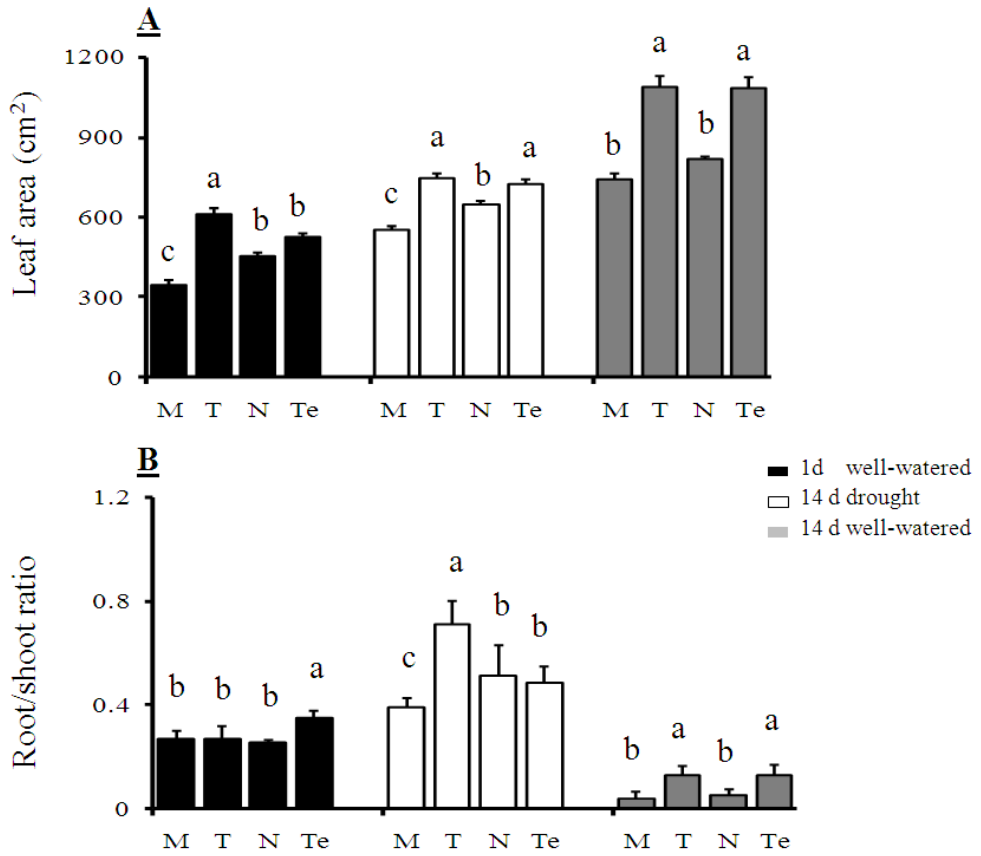

Figure 4.4 Leaf area (A) and root-to-shoot ratio (B) expressed on a dry weight basis in plants of landraces Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) after 1 day (black closed bars) and after 14 days under drought conditions (open bars) or under well-watered conditions (grey closed bars).

Bars represent the mean shoot-to-root ratio ± SE of four individual plants. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

4.4.1.2 Physiological changes

Photosynthetic gas exchange parameters were measured on the third foliar leaves of well-watered and drought-treated plants (Table 4.1). A significant difference in photosynthesis assimilation was observed between the four landraces under well-watered conditions, where Timbawene moteado had the highest photosynthesis assimilation and Namarua the lowest ($P<0.05$). Photosynthesis assimilation rates decreased as a result of drought stress in all four landraces. After 14 days of drought treatment, photosynthetic CO_2 assimilation was, however, highest in Timbawene moteado, with significantly lower assimilation ($P \leq 0.05$) in Massava nhassenje and Namarua (Table 4.2).

A similar pattern was observed between the landraces for stomatal conductance and transpiration, with values decreasing in all the landraces when plants were exposed to drought conditions. However, Timbawene moteado plants had a significantly higher value ($P \leq 0.05$) in drought than any of the other landraces (Table 4.2). In contrast, water use efficiency, measured as instantaneous water use efficiency (IWUE), was similar in all four landraces under both well-watered and drought conditions, but much higher for all landraces in drought when compared to well-watered conditions (Table 4.2).

The leaf water potential (LWP) was similar in all four landraces under well-watered conditions. Leaf water potential was significantly ($P \leq 0.05$) lower in Timbawene moteado plants than in plants of the other three landraces (Table 4.3). Drought treatment decreased the soil water content for all four landraces dramatically. Lower soil water content was found in Timbawene moteado, possibly due to more water uptake by plants (Table 4.2). When the chlorophyll content of plants was measured, drought treatment did not greatly reduce the chlorophyll content on a fresh weight basis in any of the plants of the different landraces

(Figure 4.5). However, Namarua plants had a significantly ($P \leq 0.05$) lower chlorophyll a content compared to plants of the other three landraces.

Table 4.2. Photosynthetic assimilation, stomatal conductance, instantaneous water use efficiency and transpiration cowpea landraces

Landrace	Photosynthesis ($\mu mol\ m^{-2}s^{-1}$)	Stomatal conductance ($mmol\ m^{-2}s^{-1}$)	IWUE ($mol\ m^{-2}s^{-1}$)	Transpiration ($mmol\ H_2O\ m^{-2}s^{-1}$)
Well-watered				
Massava	16.06±1.1b	0.24±0.03a	65.50±3.68b	6.03±0.42a
Timbawene	18.88±0.6a	0.27±0.01a	70.36±3.76[a]	6.59±0.13a
Namarua	15.49±0.5b	0.25±0.02a	63.50±5.27b	6.34±0.42a
Tete 2	16.1±0.47b	0.24±0.03a	65.37±2.99b	6.70±0.44a
Drought				
Massava	3.99±0.42b	0.021±0.001b	190.0±10.74b	0.81±0.6a
Timbawene	6.10±0.53a	0.034±0.001a	179.4±16.86a	1.23±0.03a
Namarua	4.2±0.009ab	0.023±0.004b	183.6±4.96b	0.94±0.18a
Tete 2	4.82±0.3ab	0.022±0.06b	219.0±12.43b	0.91±0.25a

Cowpea landraces were grown for 14 days under well-watered and drought conditions. Means followed by the same letter within a column are not significantly different, as determined by a Tuckey HSD test ($P \leq 0.05$). Data are the means of four individual plants ± SE.

Table 4.3. The effect of drought on soil water content and on leaf water potential in cowpea landraces

Local variety	Leaf water potential (Mpa)		Soil water content (%)	
	WW	D	WW	D
Massava	-0.502±0.038a	-1.35±0.046ab	79.70±0.59a	12.2±1.15ab
Timbawene	-0.505±0.034a	-1.27±0.035b	80.37±0.20a	9.87±0.70b
Namarua	-0.532±0.024a	-1.42±0.032a	79.72±0.68a	14.17±2.12a
Tete 2	-0.515±0.018a	-1.36±0.008ab	79.72±0.58a	10.04±0.48ab

Means followed by the same letter within a column are not significantly different as determined by a Tuckey HSD test ($P \leq 0.05$). Data are the means of four plants ± SE.

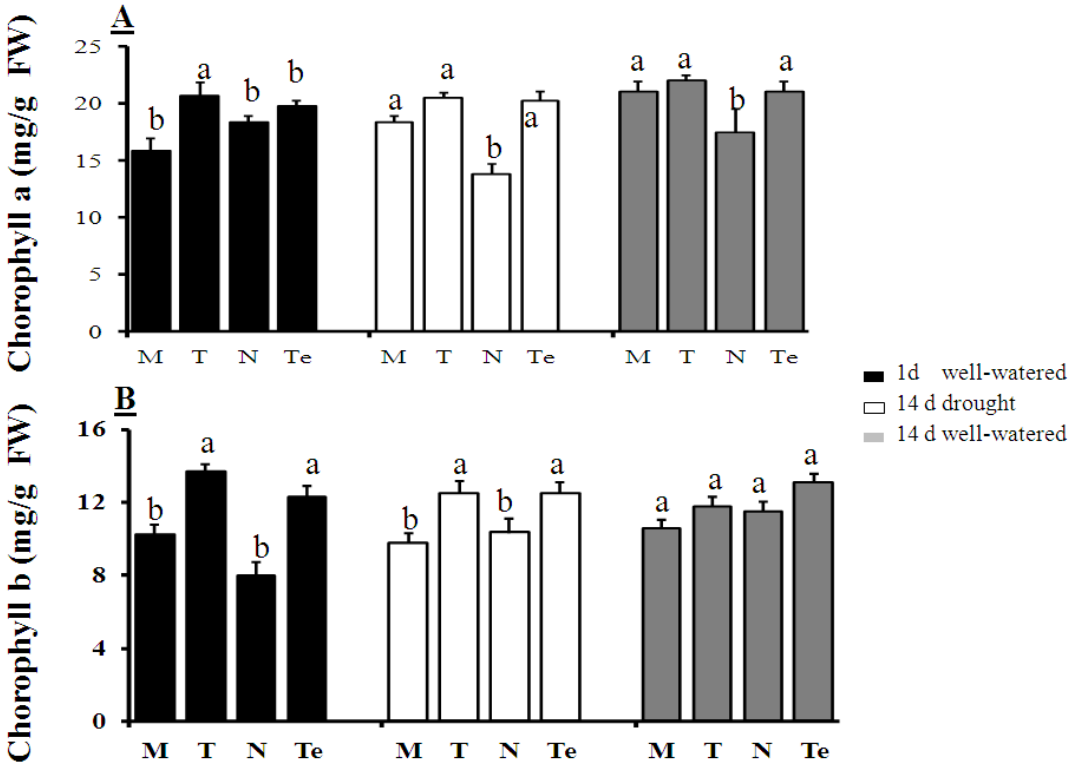

Figure 4.5 Effect of drought stress on chlorophyll a (A) and chlorophyll b (B) content in landraces Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) under well-watered conditions after 1 day (black closed bars) and for 14 days under drought conditions (open bars) or under well-watered conditions (grey closed bars).

Bars represent the mean ± SE from four individual plants. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

4.4.2 Tunnel experiments

4.4.2.1 Plant growth changes

Under well-watered conditions plants of all landraces had a similar biomass (leaf, stem and total dry biomass; Figures 4.6A, B, D). Exposure to drought generally decreased dry biomass of all investigated plant parts (leaf, stem, total biomass), with the most significant ($P \leq 0.05$) decrease in Massava nhassenje plants. Plants of this landrace always had the lowest biomass. The highest biomass of all four landraces was found for Timbawene moteado, which always had a significantly higher biomass compared to the other three landraces tested (Figure 4.6). Root dry biomass was significantly higher in plants of landraces Timbawene moteado and Tete 2 ($P \leq 0.05$) under well-watered conditions than in plants of the other two landraces (Figure 4.6C). As already found in the greenhouse study, drought increased the root biomass in all four landraces, with the highest root biomass found in Timbawene moteado. Plants of this landrace had a significantly ($P \leq 0.05$) higher root biomass than plants of the three other landraces (Figure 4.6C).

In general, exposure to drought decreased the leaf area in plants of all four landraces when compared to plants grown under well-watered conditions (Figure 4.7). Under well-watered conditions, plants of Timbawene moteado again had the highest leaf area, with Massava nhassenje and Namarua the lowest under both growing conditions. Tete 2 plants had the largest leaf area under both well-watered and drought conditions and Massava nhassenje the smallest leaf area under both conditions (Figure 4.7A). Under well-watered conditions, plants of landraces Timbawene moteado and Tete 2 also had a significantly higher ($P \leq 0.05$) root-to-shoot ratio than Massava nhassenje and Namarua (Figure 4.7B). As found in the greenhouse study, in drought the root-to-shoot ratio increased in plants of all landraces, with

the highest ratio in Timbawene moteado, which was significantly ($P \leq 0.05$) different from the ratios found in plants of all other landraces.

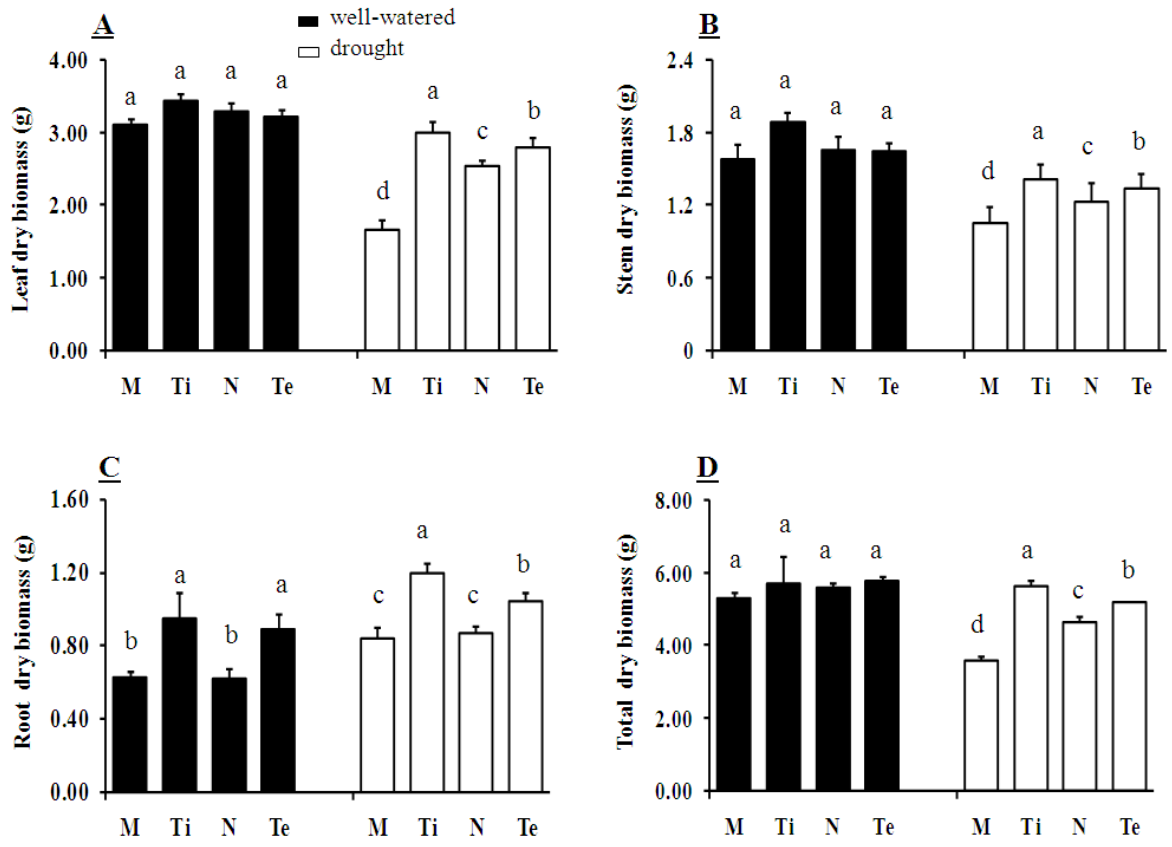

Figure 4.6 Effects of drought stress on (A) leaf, (B) stem, (C) root and (D) total plant biomass in plants of landraces Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) after four weeks' growth under either well-watered or drought conditions, grown in a tunnel house.

Bars represent the mean of four individual plants ± SE. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

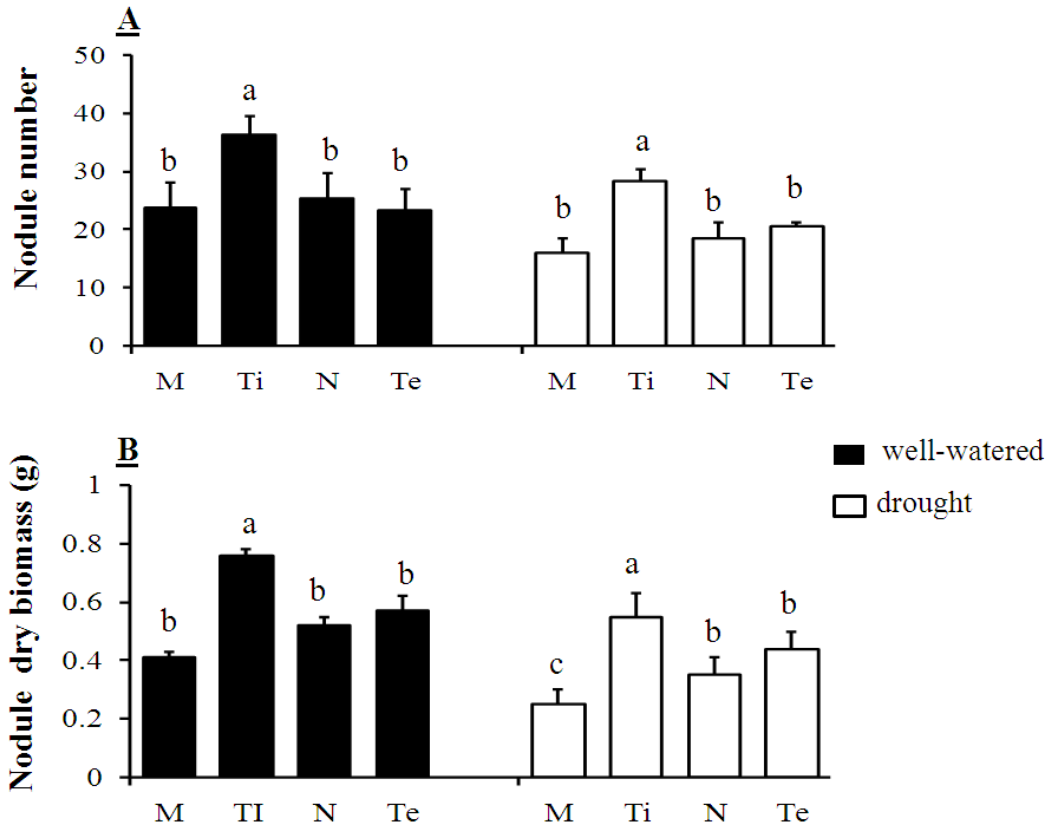

Figure 4.7 Leaf area (A) and root-to-shoot ratio (B) expressed on a dry weight basis in plants of landraces Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) grown for four weeks under well-watered (closed bars) or drought conditions (open bars). Bars represent the mean of four individual plants ± SE. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

4.4.2.2 Biochemical changes

Under well-watered conditions plants of Timbawene moteado and Tete 2 had a higher chlorophyll a and b content based on fresh weight compared to plants of Massava nhassenje and Namarua. The chlorophyll content based on fresh weight decreased as a result of drought exposure in all four landraces, with Massava nhassenje plants having a significantly ($P \leq 0.05$) lower content than plants of the other three landraces (Figure 4.8A).

Drought also decreased the protein content based on the fresh weight of leaves in plants of all four landraces. Timbawene moteado had a higher protein content than leaves of the other three landraces, with Massava nhassenje having the lowest protein content ($P \leq 0.05$) under-well watered conditions (Figure 4.9A). In contrast, drought greatly increased the free proline content based on fresh leaf weight, with Timbawene moteado having the highest and Massava nhassenje the lowest free proline content after drought exposure ($P \leq 0.05$) (Figure 4.9B). Since measurement of fresh weight does not take into consideration water loss due to drought, the proline-to-protein ratio was also calculated. Massava nhassenje, Namarua and Tete 2 had a ratio of 1.7, whereas Timbawene moteado had a lower ratio of 1.3, indicating that Timbawene moteado was less responsive in proline production compared to the other three landraces.

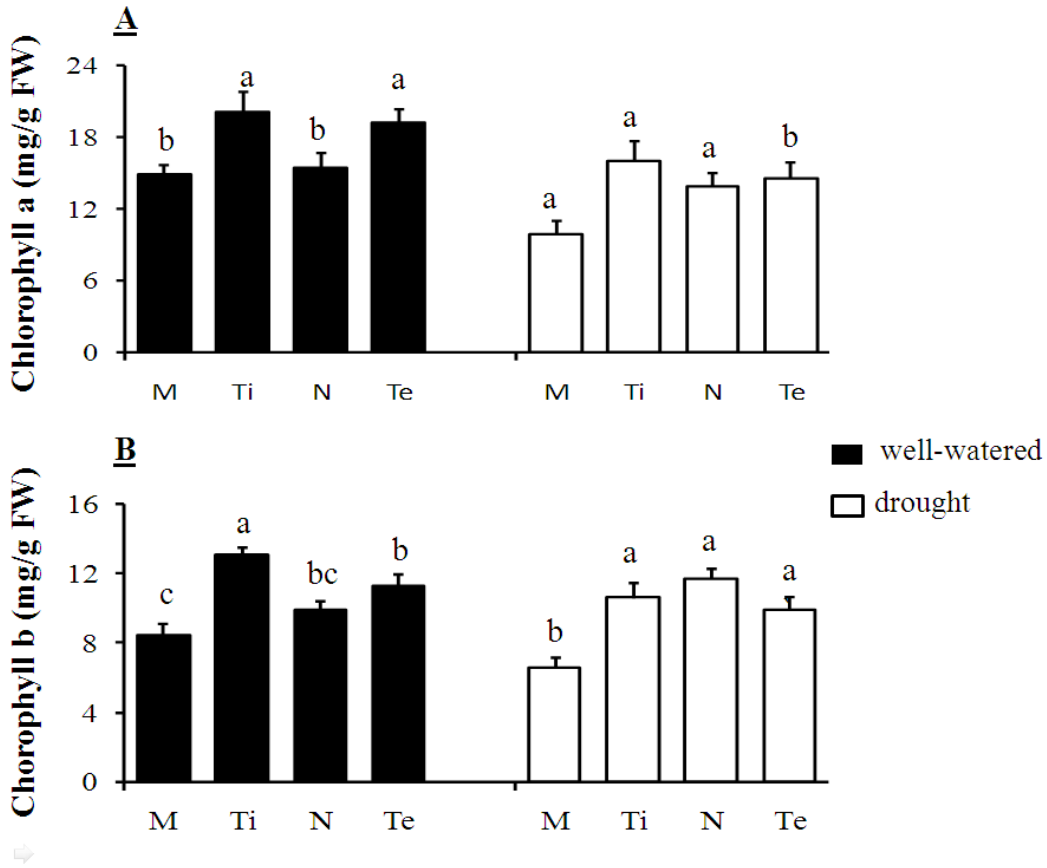

Figure 4.8 Chlorophyll a (A) and chlorophyll b (B) in plants of landraces Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) grown for four weeks in a tunnel house under well-watered (closed bars) or drought conditions (open bars).

Bars represent the mean of four individual plants ± SE. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

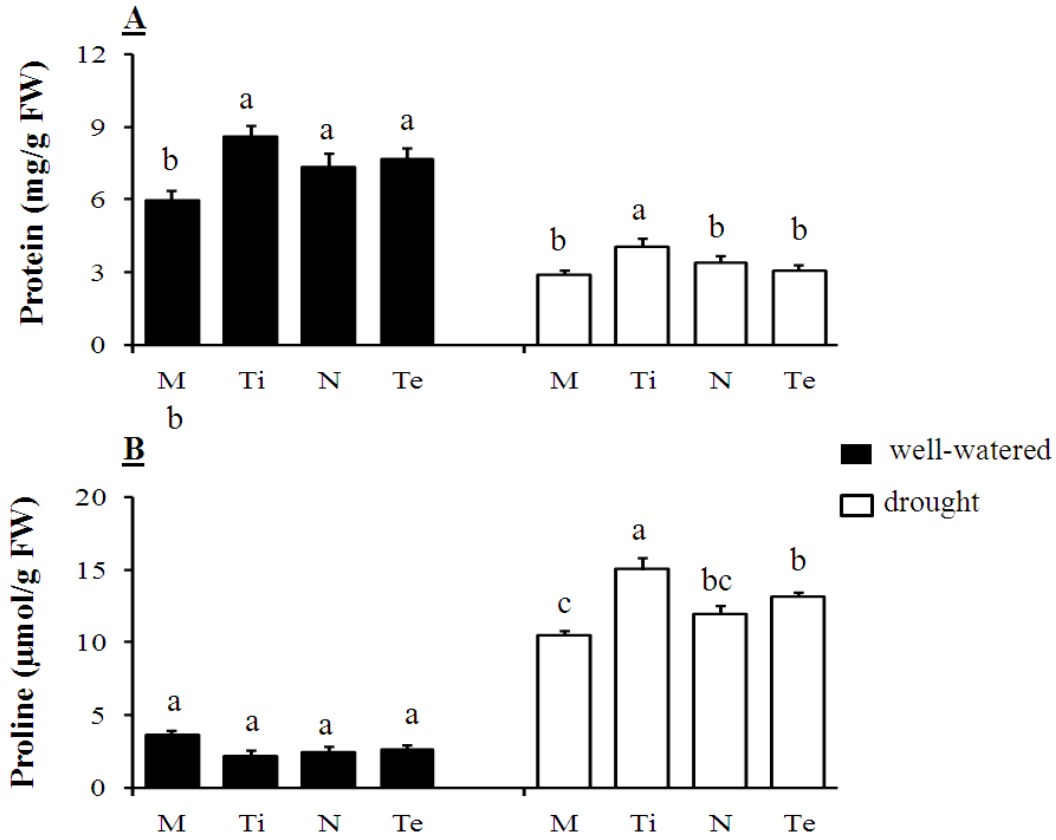

Figure 4.9 Protein content of leaves (A) and free proline in leaves (B) in plants of landraces Massava (M), Timbawene (T), Namarua (N) and Tete 2 (Te) grown for four weeks under well-watered (closed bars) or drought conditions (open bars).

Bars represent the mean of four individual plants ± SE. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

4.5 Discussion

Drought exposure, as discussed in this part of this study, reduced whole plant biomass (leaf, stem and total biomass) in plants of all four tested cowpea landraces. These effects on plant growth after drought exposure were found in both greenhouse and tunnel house studies carried out in South Africa and Mozambique, respectively, demonstrating that tunnel house and green house experiments yielded comparable results. The effects of drought stress on growth and productivity have previously been well-documented in cowpea (Craufurd and Peacock, 1993; Savin and Nicolas, 1996; Jiang and Huang, 2001; Rizhsky *et al*., 2002; Anyia and Herzog, 2004b). Reduction in growth is one of the best known consequences of drought stress, mainly caused by inhibition of leaf and stem elongation when the water potential decreases below a certain threshold. Decline in leaf area was also found in this study after drought exposure in cowpea plants of all four landraces, very probably due to cessation of the initiation of new leaves and a decrease in the expansion and growth of individual leaves.

Massava nhassenje plants had the highest decline in leaf area after drought and also had the highest decline in leaf and stem biomass. It is very likely that leaf area reduction affects plant biomass production because of less photosynthesis. Photosynthesis assimilation was significantly reduced by drought, confirming previous results by Anyia and Herzog (2004a) that drought significantly reduces the photosynthetic rates of cowpea. Reduction of plant biomass by drought is largely caused by a change in the balance between photosynthesis and respiration affecting the whole-plant carbon status (Flexas *et al*., 2006a). Timbawene moteado plants also showed much more vigorous growth and accumulated more biomass in drought compared to Massava nhassenje plants. Timbawene moteado might therefore not only be able to maintain high rates of photosynthetic CO_2 assimilation when experiencing drought, but is also able to use assimilated carbon better to generate biomass in comparison to Massava

nhassenje. After drought exposure, Timbawene moteado plants were also able to maintain high rates of CO_2 assimilation. Timbawene moteado plants might therefore be able to protect the photosynthetic processes more effectively than Massava nhassenje plants, with lower CO_2 assimilation under drought. This also suggests that Massava nhassenje plants are much more susceptible to drought than Timbawene moteado plants.

A decline in available soil water limits water uptake by roots, which is associated with reduced nutrient uptake (Poorter and Nagel, 2000; Marschner, 1995), affecting overall plant growth. The Leaf water potential (LWP) decreased in plants of all four landraces after drought, but less in Timbawene moteado plants. Drought might induce rapid leaf senescence and abscission in plants, with leaf age further contributing to a decrease in LWP. An indication of leaf senescence was also provided in this study, since the protein content, possibly due to increased proteolytic activity (Demirevska *et al*., 2008), as well as the chlorophyll content, a marker for senescence (Balazadeh *et al*., 2008), decreased in all landraces in response to long-term drought conditions in the tunnel house experiment. This result is also in agreement with the work of Upadhayaya *et al*. (2007), where a reduction in chlorophyll a concentration was found in rice under drought conditions. The decrease in chlorophyll content under drought has been considered a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation (Anjum *et al*., 2011).

Proof that the effects of drought can be successfully avoided by changing carbon allocation patterns, because of the development of a better root system, was also found in this study. Drought treatment induced an increase in the root-to-shoot ratio in plants of all landraces associated with a reduction in whole plant biomass under drought. However, plants of the landrace Timbawene moteado always outperformed plants of the other tested landraces, with

plants of Massava nhassenje being the worst performing plants. Timbawene moteado plants were able to maintain a high root biomass and had the highest shoot biomass during a long period of drought. This result also suggests that there is considerable genotypic variation in the control of the root-to-shoot ratio in response to drought and considerable flexibility in the genome-environment interaction.

Besides the root-to-shoot ratio, the content of free proline also changed in plants of all four landraces in response to drought treatment. Proline has been reported to accumulate in a range of plant species in response to water stress (Bates *et al*., 1973; Naidu *et al*., 2001) because of a reduction in water potential (Handa *et al*., 1986; Ober and Sharp 1994; Bussis and Heinekee, 1998). Such an increase could be also confirmed in this study. Proline accumulation seems to aid drought tolerance, providing energy and nitrogen after stress, stabilising membranes, reducing enzyme denaturation and acting as a neutral osmoticum (Gardner *et al*., 1985; Ain-Lhout *et al*., 2001). Lawlor (1979) reported that proline accumulation may indeed be an effective indicator during the initial stages of stress development. Since the proline-to-protein ratio was lowest in plants of the landrace Timbawene moteado after exposure to drought and plants possibly suffered less stress, proline measurement in cowpea might be a good indicator when selecting for drought-tolerant cowpea.

In the next chapter the performance of cowpea nodules of plants of the four Mozambican cowpea landraces under both well-watered and drought conditions is reported.

Chapter 5: Evaluation of nodule performance of four Mozambican cowpea landraces under drought conditions

5.1 Abstract

The responses of nodules of four Mozambican cowpea landraces (*Vigna unguiculata* (L.) Walp) to drought stress were evaluated. Symbiotic nitrogen fixation, the number of nodules and biomass greatly decreased in plants of all landraces after drought treatment. Plants of Timbawene moteado always performed best in drought and those of Massava nhassenje worst. Drought treatment increased protease activity in nodules. Nodules of Timbawene moteado displayed lower cysteine protease activity in drought, which was associated with higher protein content in Timbawene moteado, whereas Massava nhassenje had higher cyteine protease activity and low protein content. Protease activity can possibly be used as a biochemical marker for drought stress tolerance.

5.2 Introduction

Legume nodules harbour symbiotic rhizobia and this symbiotic relationship is vital in providing biological N_2 fixation (Serraj *et al*., 1998). However, this process, involving the enzyme nitrogenase, is highly sensitive to drought, severely affecting yield. In legumes, nodule viability is therefore critical for survival, growth and productivity of the plant (Mhadhbi *et al*., 2004) and any stress might lead to cessation of nitrogen supply (Echevarría-Zomeño *et al*., 2009). Drought stress causes in particular a marked decrease in the number, size and biomass of nodules, thus reducing the nitrogen fixation capacity of plants (Elowad and Hall; Serraj *et al*., 1999; Fenta *et al*., 2012). Premature senescence due to stress is further associated with an increase in proteolytic activity (Palma *et al*., 2002; Simova-Stoilova *et al*., 2010). The nature and variety of proteases expressed in nodules during senescence have, however, not been studied yet in greater detail. In rice and wheat, lower proteolytic activity, specifically low expression of cysteine proteases genes under water deficit, has been found to be related to drought tolerance (Salekdeh *et al*., 2002; Simova-Stoilova *et al*., 2010).

The aim of this part of the study was to investigate if nodule performance and proteolytic activity change in plants of the different landraces and to determine which landrace might be superior in nitrogen fixation with less proteolytic activity under drought conditions.

5.3 Materials and methods

5.3.1 Plant material and growth

Cowpea *(Vigna unguiculata* (L.) Walp*)* plants were grown from seeds in a temperature-controlled greenhouse or a tunnel house as outlined in the previous chapter 4.

5.3.2 Drought stress treatment

Cowpea plants grown to the third foliar stage were used to induce drought stress. Experimental plants (40 plants) were maintained under well-watered conditions and 40 plants were subjected to drought stress by withholding the supply of water and nutrient solution for 14 consecutive days in the greenhouse and for six weeks in the tunnel house experiment. Pots containing well-watered and drought-stressed plants were randomly distributed throughout the greenhouse and plants were harvested for analysis on day 0, 7 and 14 after exposure to drought stress.

5.3.3 Nodule activity and fresh weight

Cowpea nodules were counted and the mass fresh weight was determined by weighing nodules on a scale (Fel-20005, Adam Equipment, UK). The nodules were frozen in liquid nitrogen and stored at -80°C for further analysis.

For determination of nitrogenase activity, plants were removed from pots and all crown and lateral nodules were carefully harvested. The nodules were incubated for 10 min in an air-tight 43 ml flask in the presence of 1% (v/v) acetylene for determination of nitrogenase activity in a gas chromatograph (Varian, USA) using the acetylene reduction assay according to Turner and Gibson (1980). Before the analysis of samples, calibration was made with different amounts of ethylene and a standard curve was set up for calculation of nitrogenase activity (acetylene reduction assay).

5.3.4 Protein extraction and determination

Crown nodules or leaves were ground into powder in liquid nitrogen with a mortar and pestle for protein isolation. Proteins were dissolved by the addition of 1 ml of 50 mM Tris-HCl buffer, pH 8.0, to the powder. The extract was centrifuged at 13000 rpm for 10 min at 4^{o}C in an Eppendorf centrifuge. The resulting clear protein-containing supernatants were stored for further analysis at -80^{o}C. Total protein concentration from the crown nodule was determined using a commercially available Bradford protein determination assay reagent (Bio-Rad, UK) with BSA from Sigma (South Africa) as a standard. Absorbance of the protein-assay mixture was measured in a total volume of 1 ml at 595 nm with a spectrophotometer (Macince, UK). All the measurements (samples and standard) were done in duplicate.

5.3.5 Cysteine protease activity

The cysteine protease activity of samples was determined using a Fluostar Galaxy Fluorimeter (BMG, Offenburg, Germany), with excitation and emission wavelengths of 355 and 460 nm, respectively. Total protein extracts from leaves and nodules were used for the assay. Extracts were prepared by crushing the material in liquid nitrogen and a 100 mM sodium phosphate buffer at pH 6.5 was added in a ratio of 1:2 (100 µg extract: 200 µl buffer). The solution was incubated for 30 min on ice before being centrifuged at 13000 rpm for 10 min at 4^{o}C. The supernatant was removed and the total protein concentration was determined by the Bradford method (1976), as described in 4.5.2. The measurement of cysteine proteases activity was performed using blank plates with 96 wells from Nunc (UK). Each well contained a total reaction volume of 100 µl. For the measurement, 8 µM of the100 µM stock Z-Phe-Arg-MCA (cathepsin L-like substrate from Sigma-Aldrich) dissolved in DMSO (Sigma-Aldrich) was used as the synthetic fluorescence substrate and an identical concentration of protein was added to the assay. Hydrolysis reactions were performed at 25^{o}C in sodium phosphate buffer (100 mM, 10mM L-cysteine, and pH 7.0).

5.3.6 Statistical analysis

Data were analysed using the JMP 5.0 Statistical Package (SAS Institute Inc., Cary, NC, USA). The significance level was set at 1% or 5% using ANOVA. Correlation analyses were carried out to determine the significance of relationships between the physiological and morphological parameters measured.

5.4 Results

5.4.1 Nodule performance and symbiotic nitrogen fixation (SNF)

Drought greatly decreased nodules' biomass, the number of nodules as well as symbiotic nitrogen fixation in plants of all four landraces when plants were grown in a temperature-controlled greenhouse. However, plants of the landrace Timbawene moteado had a significantly ($p \leq 0.05$) higher nodule biomass and number of nodules, as well as SNF, under both well-watered and drought conditions than all other plants. Massava nhassenje plants always had the lowest nodule biomass and number and SNF under both growing conditions when compared to all other plants ($p \leq 0.05$) (Table 5.1).

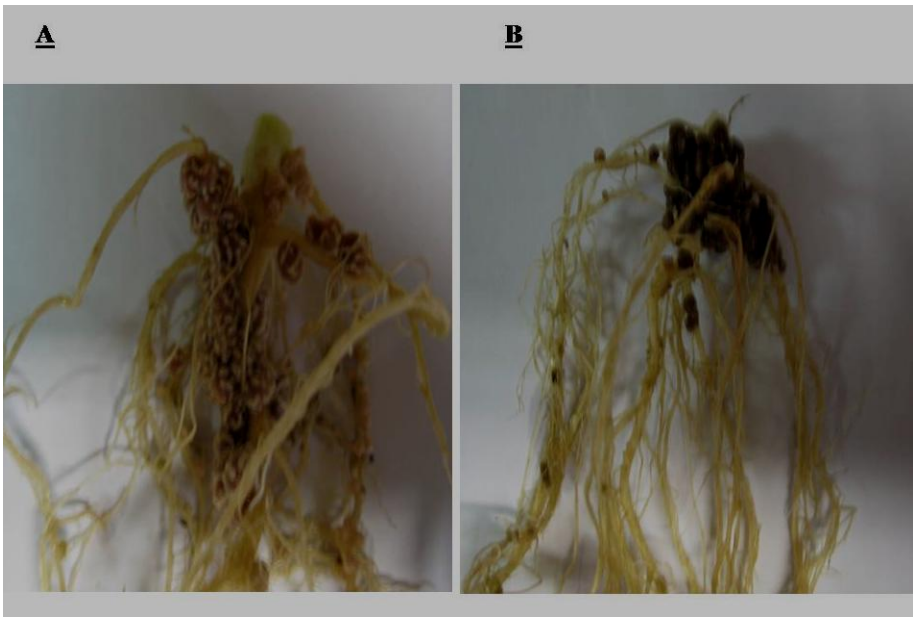

Figure 5.1 Nodules of cowpea plants under well watered conditions (A) and drought stress (B)

When plants were grown in a tunnel house in Mozambique in soil and exposed for six weeks to drought conditions, nodule number and biomass decreased in response to drought exposure. Again, plants of Timbawene moteado had the highest ($p \leq 0.05$) and Massava nhassenje the

lowest nodule number as well as biomass when compared to plants of the other landraces (Figure 5.1).

5.4.2 Protein content and protease activity

Growth under drought conditions for 14 days in a greenhouse decreased protein production in nodules, with the greatest decrease in nodules of the landrace Massava nhassenje (Figure 5.2). In contrast, after 14 days of drought, Timbawene moteado nodules had the highest protein content (Figure 5.2).

Proteolytic activity, measured as cysteine protease activity, greatly increased in nodules after exposure to drought and, for comparison, also in leaves (Figure 5.3). However, nodules of Timbawene moteado had the lowest proteolytic activity, almost identical to well-watered nodules, which was significantly ($p \leq 0.05$) lower than for all other three landraces tested. In contrast, proteolytic activity greatly increased in response to exposure to drought in nodules of the landrace Massava nhassenje, and nodules of this landrace displayed the highest proteolytic activity under drought conditions.

Table 5.1 Comparison of nodule fresh biomass, nodule number and symbiotic nitrogen fixation (SNF) in cowpea landraces

Landrace	Biomass (g)		Number		SNF (µmol h^{-1} per plant)	
	WW	D	WW	D	WW	D
Massava	1.83±0.25	0.67±0.20	32.75±1.4	15.25±1.4	0.99±0.14	0.06±0.04
Timbawene	2.75±0.21	1.02±0.17	45.50±4.5	27.00±1.7	1.39±0.14	0.22±0.03
Namarua	2.01±0.13	0.99±0.22	43.25±1.9	25.50±2.9	1.08±0.08	0.06±0.02
Tete 2	2.33±0.35	0.78±0.15	42.75±5.2	25.25±1.7	1.18±0.03	0.10±0.07
Significance	*ns*	*ns*	*0.02**	*0.0058**	*0.0103**	*0.0093**

Cowpeas were grown for 14 days under well-watered (WW) and drought (D) conditions in a temperature-controlled greenhouse. Means followed by the same letter within a column are not significantly different, as determined by a Tuckey HSD test ($P \leq 0.05$). Data are the means of four plants ± SE.

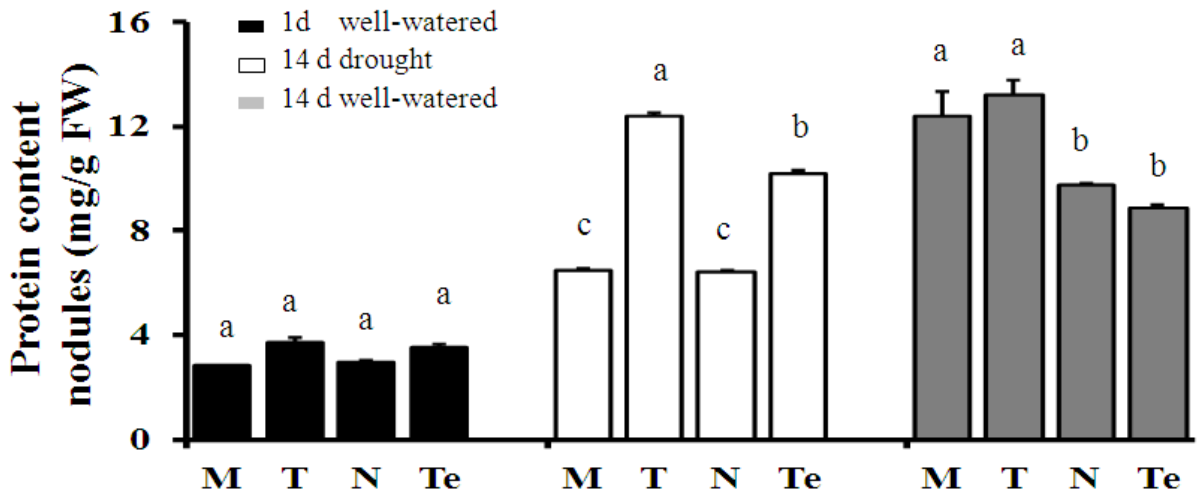

Figure 5.2 Effect of drought stress on protein content of nodules in Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) grown in a temperature-controlled greenhouse under well-watered conditions for 1 day (black bars) and 14 days under drought conditions (open bars) or well-watered conditions (grey bars).

Bars represent the mean ± SE from four individual plants. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

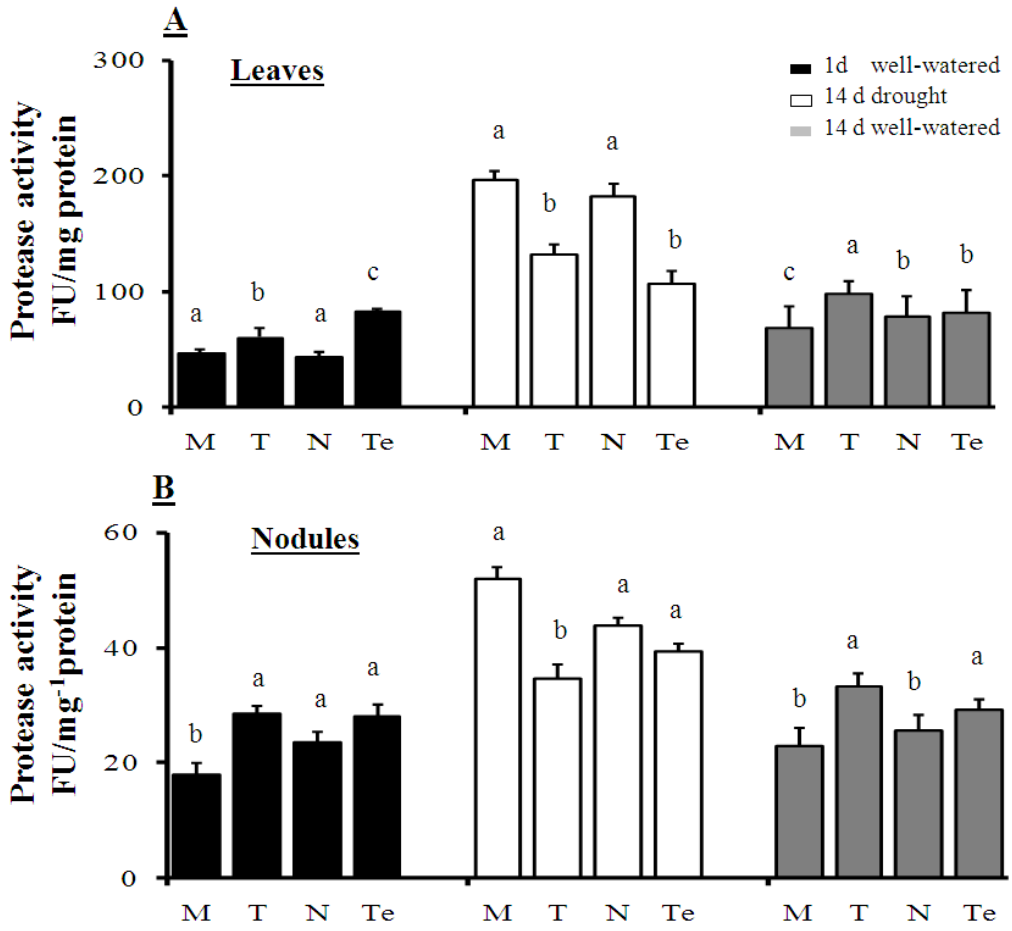

Figure 5.3 Total proteolytic activity of leaves (A) and nodules (B) of Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) grown in an environmentally controlled greenhouse under well-watered conditions for 1 day (black bars) and for 14 days under drought conditions (open bars) or well-watered conditions (grey bars).

Cat L-like; Z-Phe-Arg-MCA was used as substrate and 1.25 µg protein was added to each protease assay. Bars represent the mean ± SE from four individual plants. Different letters at a particular growing period denote values that differed significantly at $P \leq 0.05$.

When nodule performance was investigated in a tunnel house, long-term exposure to drought decreased the number and biomass of nodules in plants of all four landraces (Figures 5.4). Timbawene moteado plants had again the highest number of nodules, as well as the highest

nodule biomass under both growing conditions ($P \leq 0.05$), when compared to all other plants. In contrast, Massava nhassenje plants always had the lowest number of nodules and biomass, whatever condition applied (Figures 5.4).

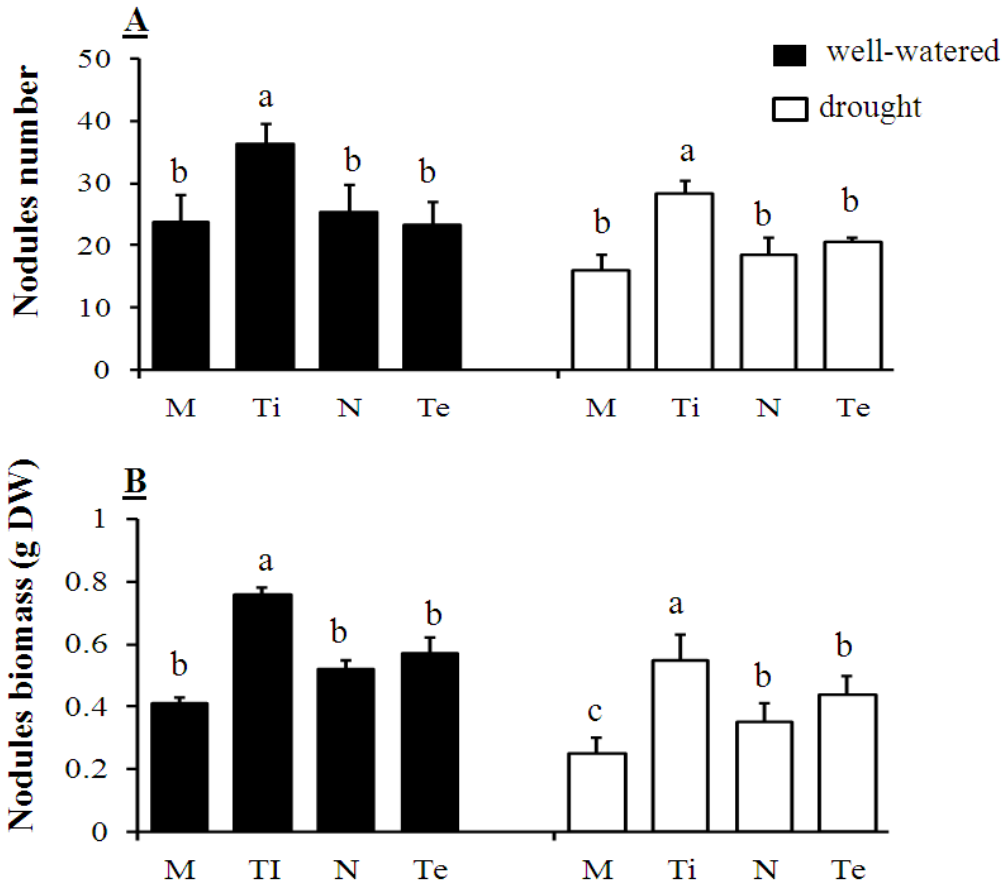

Figure 5.4 Nodule number (A) and nodule biomass (B) expressed on a dry weight basis in plants of landraces Massava nhassenje (M), Timbawene moteado (T), Namarua (N) and Tete 2 (Te) grown for six weeks in a tunnel house in Mozambique under well-watered (closed bars) or drought conditions (open bars).

Bars represent the mean of six individual plants ± SE. Different letters at a particular growth period denote values that differed significantly at $P \leq 0.05$.

5.5 Discussion

Nitrogenase activity in cowpea nodules decreased when nodules were exposed to drought. The decline might result from depletion of carbohydrates, oxygen limitation and feedback regulation by nitrogen accumulation (Serraj *et al*., 1999). Plants of Timbawene moteado always performed best and those of Massava nhassenje worst regarding symbiotic nitrogen fixation and nodule biomass and number. These results were found with both experimental designs, greenhouse and tunnel house. Drought tolerance has recently been related to the presence of large nodules and an inherent supply of photosynthesis to the nodules, which might also be one of the reasons why Timbawene moteado plants performed best, since they had a higher nodule biomass (King and Purcell, 2001).

In this PhD study, drought also affected the protein content of nodules, with Timbawene moteado nodules less affected. A decrease in protein content might be due to either proteolysis resulting from drought treatment or less protein synthesis (Simova-Stoilova *et al*., 2006). An increase in nodule cysteine protease activity found in this study was associated with a decline in symbiotic nitrogen fixation, as well as a decrease in both nodule biomass and number. Up-regulation of cysteine proteases under drought stress has previously been reported by Jones and Mullet (1995). Furthermore, Khanna-Chopra *et al.* (1999) found that in cowpea, not watered for seven days, the amount of papain-like cysteine proteases increased, which was detectable by Western blotting using a polyclonal antiserum raised against papain. In this PhD study, more drought-tolerant Timbawene moteado plants displayed a much lower increase in nodule protease activity when compared to more drought-sensitive plants of all other landraces. A similar result was also found when protease activity was measured in leaves. Demirevska *et al*. (2008) recently also reported that drought-tolerant wheat varieties showed higher protease activity under well-watered conditions but a negligible increase in

proteolytic activity under severe drought conditions. In contrast, drought-sensitive wheat varieties increased their protease activity in drought. Nodule senescence, in particular the role of cysteine proteases causing protein degradation, has previously been investigated by several research groups with both bacteroids and nodule cells ultimately dying in response to proteolytic activity. This activity ultimately allows the recovery of nitrogenous compounds from senescing tissues (Pladys and Vance, 1993).

Chapter 6: General discussion and perspectives

6.1 General discussion

This study has found support for the set hypothesis that Mozambican cowpea landraces might exist that are well adapted to local conditions and have morphological, physiological and molecular traits allowing better performance under drought. Since only a small number cowpea accessions have been collected locally, which have not been characterized in greater detail, this study has first characterized four of these local cowpea landraces with possibly various degrees of drought tolerance. Mozambique is vulnerable to drought affecting the country's food production. More drought-tolerant crops are therefore urgently required but the exact scientific basis for drought tolerance in cowpea is, however, not very well established. This has resulted in a rather slow progress in local cowpea breeding for drought tolerance.

Two approaches are generally applied to screen any crop for drought tolerance. The first approach is determining grain yield, preferably under field conditions, which involves testing segregating material over many years at several locations. This approach has been previously applied for cowpea with rather little success (Hall and Patel, 1985; Hall *et al*., 1997). A second approach, as done in this study, is based on measuring morphological and physiological parameters under drought conditions in a greenhouse that might have an impact on final yield. A combination of both approaches likely facilitates more rapid progress in the development of drought-tolerant varieties (Fussell *et al*., 1991). Successes so far reported for Africa included the development of early maturing cowpea varieties IT84S-2246 and Bambey-21. Both were recently released and widely adopted by farmers, particularly in West Africa (Agbicodo *et al*., 2009). However, simple transfer of these varieties to other African countries is problematic due to consumer and cowpea grower preference for taste and seed appearance but also local growth conditions. This very often renders local varieties the

preferred choice for both farmers and consumers. Unfortunately, despite this demand, sufficient activities to improve local cowpea landraces have so far not been carried out in Mozambique.

Chilule (2010) recently assessed the effect of drought on Mozambican cowpea landraces in a field study. Landraces could be grouped into four categories: high yielding-drought tolerant genotypes, high yielding-drought susceptible genotypes, low yielding-drought tolerant genotypes and low yielding-drought susceptible genotypes with Tete-2 identified as drought-tolerant. Although drought affected the performance of all landraces, variability was found among the investigated landraces when 13 above-ground and 8 below-ground characteristics were measured (Table 6.1). Further, application of the SSR technology, which can also be carried out in Mozambique due to the existence of molecular biology lab, indicated that genetic diversity exists between the four cowpea landraces. This study therefore clearly confirmed that the Mozambican cowpea germplasm deposited in the seed-bank is diverse and contains traits to be useful for any national breeding program.

Among the landraces, Massava nhassenje was the most drought-sensitive landrace. This landrace had the lowest biomass accumulation, lowest protein content and also highest increase in proteolysis under drought conditions. In contrast, landrace Timbawene moteado performed best of all landraces under drought with highest leaf biomass and lowest increase in proteolytic activity. These characteristics were directly related to higher leaf protein content as well as higher nodule number and nodule biomass and also SNF. Better nodule performance enables Timbawene moteado to fix more nitrogen, supply more N compound via xylem to the shoot which finally allows better growth. Such significant relationships between SNF and leaf parameters (photosynthetic CO_2 assimilation rates, stomatal conductance values

Table 6.1. Tolerance level of above- and below-ground plant characteristics in cowpea landraces

Above-ground traits

Landrace	Leaf DB*	Shoot DB*	Total-DB*	Leaf area*	Photosynthesis	Stomatal conductance	IWUE	Transpiration	Leaf water potential	Chlorophyll*	Leaf protein*	Proline*	Leaf protease
Massava nhassenje	-	-	-	-	-	-	+/-	-	+/-	-	-	-	-
Namarua	+	+/-	+/-	+	+/-	-	+/-	-	-	+/-	-	+/-	+/-
Tete-2	+/-	+/-	+/-	+/-	+/-	-	-	-	+/-	+/-	-	+/-	+/-
Timbawene moteado	+	+	+	+	+	+	-	+	+	+	+	+	+

Below-ground traits

Landrace	Root DB	Root/shoot ratio*	Soil water potential	Nodule FB*	Nodule number*	Nodule protein*	SNF	Nodule protease
Massava nhassenje	-	-	-	-	-	-	-	-
Namarua	-	+/-	-	+/-	-	-	-	+/-
Tete-2	+/-	+/-	+/-	+/-	-	+/-	+/-	+/-
Timbawene moteado	+/-	+	+	+	+	+	+	+

DB = Dry biomass; FB =Fresh biomass; *measurable under Mozambican conditions; (+) = tolerant; (+/-) + intermediate; (-) = sensitive

and intracellular CO_2 concentration) have been recently also reported for soybean (Fenta *et al*., 2012). SNF activity is, however, rapidly inhibited in dry soil affecting the life-span of nodules and causing premature nodule senescence limiting the nitrogen supply for plants (Fenta *et al.*, 2012). A relationship between drought tolerance and the presence of larger nodules has also been previously reported by King and Purcell (2001).

Tete-2 was recently identified as a relatively high yielding landrace under drought conditions in a field assessment study (Chiulele, 2010). However, Timbawene moteado was not included in this field evaluation. In the greenhouse and tunnel house study, Tete-2 was surprisingly rather moderately drought-tolerant when compared to Timbawene moteado (Tabe 6.1). Timbawene, originating from an area characterized by low and unpredictable precipitation, would be well-suited for growth under drought conditions. This also indicates the importance of characterizing landraces in Mozambique to prevent loss of favored traits. Timbawene moteado, despite that the darker seed color of the landrace is less preferred by Mozambican farmers, has potential as a source for breeding programs and should also be tested in the future in the field particularly in the central, semi-arid very warm areas of Mozambique prone to drought where Tete-2 is performing well. In comparison, Massava nhassenje is poor-yielding in the southern part of Mozambique where periods of drought are experienced. This study confirmed such poor performance under drought (Table 6.1). This landrace might therefore be better suited for areas under irrigation and not for rain-fed areas. Landrace Namarua is an early flowering and seed maturing landrace with characteristics of a drought escaper very likely suitable for arid and semi-arid areas with a short rainfall growing season. Namarua maintains a high photosynthetic rate during a relatively long period of water deficiency where the soil moisture content is sufficient at beginning of the season but quickly reduces due to drought.

The study also clearly showed that the four landraces reacted biochemically different to drought exposure. Protein and chlorophyll degradation was less affected in Timbawene moteado, the more drought-tolerant landrace, than in Massava nhassenje, the drought sensitive cowpea landrace. Landraces further showed differences in protease activity. The drought-tolerant landrace Timbawene moteado had only a slight increase in proteolytic activity under drought. In contrast, the drought-sensitive landrace Massava nhassenje had higher protease activity and possibly degraded proteins much faster leading to premature senescence. Although having the longest maturation time, Timbawene moteado plants also had much more vigorous growth, very likely overcoming a drought period more easily with fast recovery and re-growth, and accumulated more biomass when compared to Massava nhassenje and Namarua plants. Further, the proline content was significantly increased in Timbawene under drought stress compared to the other three landraces. Proline content, associated with tolerance to drought stress, might therefore also be used as a simple marker for selecting drought-tolerant cowpea landraces. However, Lawlor (1979) reported that proline accumulation is only an effective indicator during the initial stages of stress development.

Only a limited number of growth parameters could be, however, determined in Mozambique. This included biomass, protein, chlorophyll, and proline accumulation. Unfortunately, a more detailed physiological study could not be carried out in Mozambique due to the current lack of more sophisticated equipment. For example, correct measurement of photosynthesis, stomatal conductance or analysis of a greater number of metabolites to allow objective comparison of data (Lawlor, 2009) was not possible. According to Lawlor (2009), assessing the effects of water deficit on photosynthetic metabolism particular attention should be paid to the conditions during growth and application of water deficit, such strict control of growth conditions was not achievable in Mozambique. However, data obtained at the University of

Pretoria, by growing plants in a temperature-controlled greenhouse and using more sophisticated equipment, for example for photosynthesis measurement, were comparable to the results obtained in Mozambique where only a sheltered field plot and very basic equipment and methods were used for plant characterization. Both locations correctly identified plants with tolerance and sensitivity to drought.

6.2 Conclusions

Characterization of landraces is crucial for effective conservation and exploitation of genetic resources in any crop improvement program. Screening and selection of different cowpea landraces for drought tolerance are important for the development of new drought-tolerant cowpea cultivar(s). In particular, certain above- and below-ground characteristics measured in this study, which can also be easily measured in Mozambique with locally existing infrastructure (Table 6.1), are valuable selection criteria in germplasm screening for drought tolerance. To my knowledge, this study was also the first more detailed study on the phenotypical and physiological characterization of Mozambican cowpea landraces for drought tolerance under both greenhouse and tunnel house conditions. Knowledge generated by this study is therefore a useful addition to information for cowpea already kept by the IIAM Mozambican gene-bank and performance data for cowpea landraces obtained in this study therefore extend currently available landrace data in the Mozambican national gene bank.

Tools developed in this study might further be applied in the future to screen a much greater number of cowpea accessions. In particular above-ground biomass (leaf dry biomass) determination and below-ground nodule biomass and number, were simple methods for cowpea germplasm screening. Shenkut and Brick (2003) already demonstrated that shoot biomass accumulation highly correlates with seed yield. Measuring above-ground biomass

would, therefore, also provide direct information on plant productivity. This study has also confirmed the existence of a close relationship between capacity for N acquisition in nodules and the above-ground performance of cowpea under drought. Further, determination of nodule number is a simple technique and the technique can also be directly applied in the field. In a recent soybean field study, nodule number was found to be positively and significantly correlated with seed yield under drought (Fenta, unpublished result).

6.3 Recommendations

Since the gene-bank in Mozambique holds collections that have been only characterized so far by morphological markers, understanding the morphological, physiological and biochemical responses of cowpea landraces to typical local conditions as well as the identification of the mechanisms responsible for plant adaptation/tolerance to stress should be among the future actions in Mozambique. In particular, cowpea germplasm screening to identify superior more stress-tolerant accessions in the Mozambican gene-bank collection should be carried out. This study has provided a first technical basis allowing the screening a much greater number of landraces for drought tolerance. Such screening should ultimately also be linked with yield measurement under field conditions. Since the tunnel house experiment gave comparable results to the greenhouse experiment, a less expensive tunnel house set-up for rapid screening of the gene bank's existing cowpea germplasm might be first established. Applying methods used in this study for screening might also be initially sufficient without requiring more sophisticated infrastructure for a basic evaluation of plant growth, in the longer term there is a need to improve the current facilities e.g. green-house with controlled conditions and adequate temperature control and equipment to perform also more sophisticated physiological studies. Finally, future work in Mozambique might also focus on the application of the SSR technology by attempting to develop a SSR marker linked to drought tolerance.

Chapter 7: References

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