Genetic analysis of abiotic and biotic resistance in cowpea [*Vigna unguiculata* (L.) Walp.]

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Contents

This thesis is dedicated with deep gratitude to Almighty God and to our Lord Jesus Christ,
to my beloved wife Vicencia Isabelle, to my mother Melanie and my late father Alexandre

Chapter 1

General Introduction

Cowpea

Origin, Domestication and Distribution

Cowpea [*Vigna unguiculata* (L.) Walp.] ($2n = 2x = 22$) is one of the most ancient human food sources and has probably been used as a crop plant since Neolithic times (Summerfield et al. 1974). Cowpea is commonly referred to as “niébé,” “wake,” and “ewa” in much of West African countries, and “caupi” in Brazil. In the United States, other names include “southern peas,” “blackeyed peas,” “field peas,” “pinkeyes,” and “crowders.” These names reflect traditional seed and market classes that developed over time in the southern United States. The name cowpea probably originated from the fact that the plant was an important source of hay for cows in the southeastern United States and in other parts of the world (Timko et al. 2007). Cowpea most likely originates from Africa, as wild cowpeas only exist in Africa and Madagascar (Steele 1976). The centre of diversity of cultivated cowpea is found in West Africa, in an area encompassing the savannah region of Nigeria, southern Niger, parts of Burkina Faso, northern Benin, Togo, and the northwestern part of Cameroon (Ng and Marechal 1985). Carbon dating of cowpea (or wild cowpea remains from the Kimtampo rock shelter in central Ghana) has been carried out (Flight 1976) and is the oldest archaeological evidence of cowpea found in Africa. Cowpea is considered to have been domesticated in Africa from its wild ancestral form, *V. unguiculata* subsp. *dekindtiana* (Harms) Verdc. (Ng and Marechal 1985). However, the precise location of origin where cowpea was first domesticated is still under speculation. Ba et al. (2004) reported that the crop was probably domesticated by farmers in West Africa while Coulibaly et al. (2002) presented some evidence that domestication occurred in northeastern Africa, based on studies of amplified fragment length polymorphism (AFLP) analysis. Cowpea was introduced from Africa to the Indian sub-continent approximately 2000 to 3500 years ago (Allen 1983). Cowpeas had reached Europe from Asia and have been cultivated in southern Europe at least since the 8th century BC and perhaps since prehistoric times (Tosti and Negri 2002). From the West Indies, cowpea was taken to the USA in about 1700 BC (Pursglove 1968). The slave trade from West Africa resulted in the crop reaching the southern USA early in the 18th century however, many US cultivars appear more closely related to germplasm from Asia or southern Europe than West Africa (Fang et al. 2007). Presently cowpea is grown throughout the tropic and subtropic areas around the whole world.

Description and Classification

Cowpeas are generally more robust in appearance than common beans with better developed root systems and thicker stems and branches. Summerfield et al. (1974), Kay (1979) and Fox and Young (1982) described cowpea as an annual herb reaching heights of up to 80 cm with a strong taproot and many spreading lateral roots in the surface soil. Growth forms vary and include erect, trailing, climbing, or bushy, usually indeterminate growers under favorable conditions. Fruits are pods containing seeds that vary in size, shape, colour and texture (Figure 1). Pods may be held erect, crescent-shaped or coiled. They are usually yellow when ripe, but may also be brown or purple. The flowers are arranged in racemose or intermediate inflorescence at the distal ends of 5-60 cm long peduncles. Flowers are conspicuous, mostly self-pollinating, borne on short pedicels and the corollas may be white, dirty yellow, pink, pale blue or purple in colour. Flowers open in the early day and close at approximately midday.

Verdcourt (1970) and Marechal et al. (1978) classified cowpea as follows:

- ORDER: Fabales
  - FAMILY: Fabacea
    - SUBFAMILY: Faboideae
      - TRIBE: Phaseoleae
        - SUBTRIBE: Phaseolinae
          - GENUS: *Vigna*
            - SECTION: *Catiang*

Vigna has several species, but the exact number varies according to different authors. Cultivated cowpeas have been divided into five cultivar groups based mainly on pod and seed characteristics (Pursglove 1968; Pasquet 1999). Cultivar group Unguiculata is the largest and includes most medium- and large-seeded African grain and forage-type cowpeas. Cultivar group Melanophthalmus includes “blackeye pea”-type cowpea with large, somewhat elongated seeds with wrinkled seed coats and fragile pods (Pasquet 1998). Members of cultivar group Biflora (also known as “catjang”) are common in India and characterized by their relatively small smooth seeds borne in short pods that are held erect until maturity. Cultivar group Textilis is a rather rare form of cowpea with very long peduncles that were used in Africa as a source of fiber. Cultivar group Sesquipedialis (known as “yardlong bean,” “long bean,” “Asparagus bean,” or “snake bean”) is widely grown in Asia for production of its very long (40 to 100 cm) green pods that are used as “snap” beans.

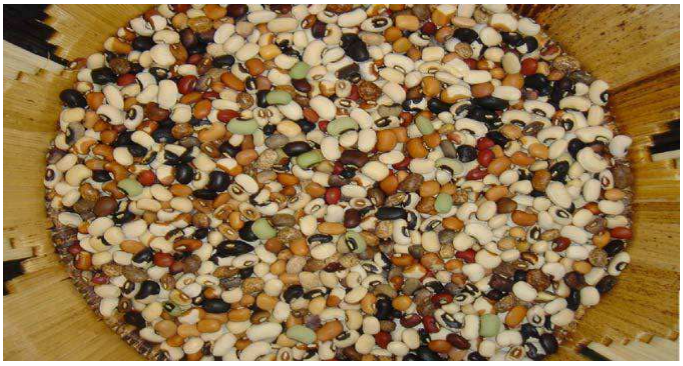

Figure 1. Diversity of seed types in cowpea. Shown in photograph is variation in seed shape, color, and texture observed in cowpea from around the world (picture courtesy of J.D. Ehlers) from Timko et al. (2007)

Importance

Members of the Phaseoleae (which cowpea belongs to) include many of the economically important warm season grain and oilseed legumes, such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), and mungbean (*Vigna radiata*) (Timko et al. 2007). Cowpea is the most economically important indigenous African legume crop and has a wide variety of uses as a nutritious component in the human diet as well as nutritious livestock feed (Langyintuo et al. 2003). It is usually the first crop harvested before the cereal crops are ready and therefore is referred to as "hungry-season crop". With more than 25% protein in dry seeds as well as in young leaves (dry weight basis), cowpea is a major source of protein, minerals and vitamins in daily diets and is equally important as nutritious fodder for livestock (Singh et al. 2003). The high protein content of cowpea grain represents a major advantage for use in infant and children’s food (Lambot 2002). The mature pods are harvested and the haulms are cut while still green and rolled into small bundles containing the leaves and vines. These bundles are stored on rooftops for uses as feed supplement in the dry season, making cowpea a key component of crop-livestock systems. Cowpea haulms fetch 50% or more of the grain price (dry weight basis). Therefore, cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world, and is a valuable and dependable commodity that produces income for farmers and traders (Singh 2002; Langyintuo et al. 2003). Additionally cowpea is a valuable component of farming systems in many areas because of its ability to restore soil fertility for succeeding cereal crops grown in rotation with it (Carsky et al. 2002;

Tarawali et al. 2002; Sanginga et al. 2003). Figure 2 summarizes the potential contributions of cowpea described (Tarawali et al. 2002).

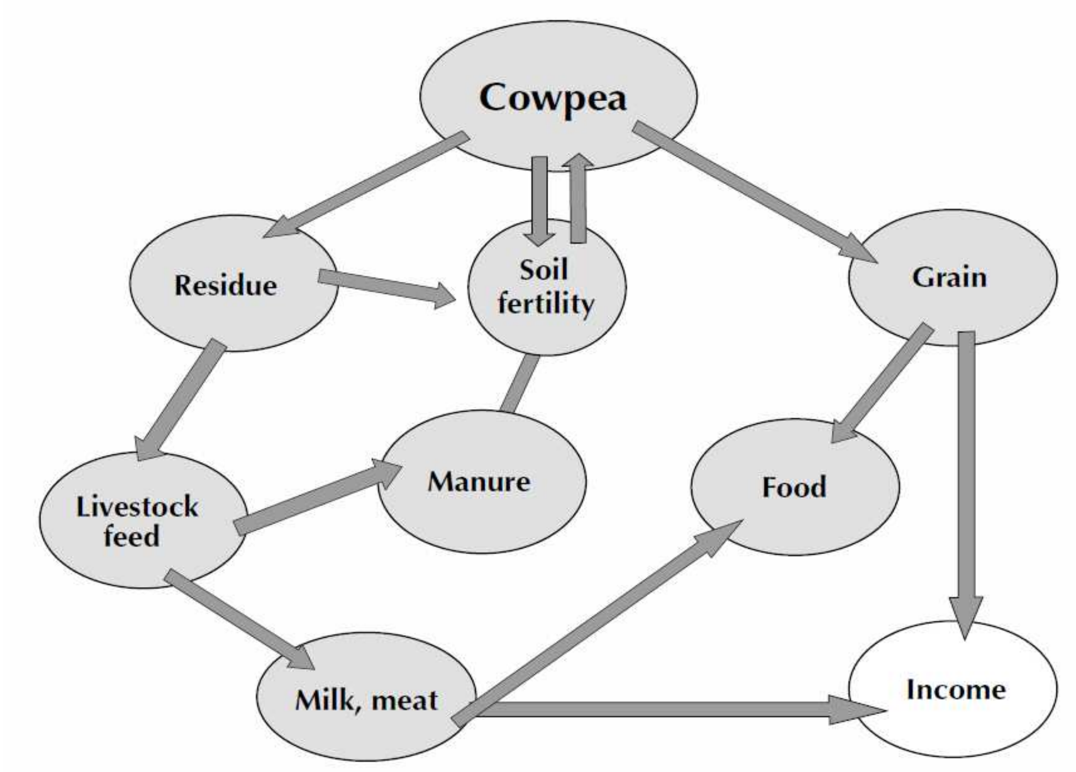

Figure 2. Schematic representation of the potential contributions of cowpea in crop-livestock systems in the dry savannas. Not all potential interactions are shown for simplicity (after Tarawali et al. 2002).

Classical and Molecular Breeding

Cowpea production is limited by numerous insect pests and diseases, parasitic weeds and environmental stresses. Grain yield and quality are primary breeding objectives of nearly all cowpea breeding programs. The accomplishments of some of these programs have been described by others (Ehlers et al. 2002; Singh et al. 2002; Hall et al. 2003). Most cowpea breeders employ backcross, pedigree, or bulk breeding methods to handle segregating populations because cowpea is a self-pollinating species and varieties are pure lines. Recently, Padi and Ehlers (2008) reported that single-seed descent (SSD) or bulk breeding methods are more efficient than pedigree breeding for developing cowpea varieties with high yield potential in semiarid West Africa. Insect damage is the number one constraint for cowpea grain production in many regions and therefore breeding insect-resistant cowpeas would have significant impact on food availability in many regions where cowpea is grown

(Timko et al. 2007). Screening methods have been developed for several major insect pests of cowpea (Ehlers and Hall 1997). Recurrent selection is being used to combine low to moderate levels of resistance to flower thrips, pod bugs, and Maruca pod borer identified in several genotypes (Singh et al. 2002). However, progress is limited by the low heritability of the traits based on the field screening methods used. Identification of molecular markers for insect resistance could facilitate transfer and pyramiding of the resistance genes via marker-assisted selection (MAS).

Target genes in a segregating population can be identified with the assistance of DNA markers so as to accelerate cultivar development (Thottappilly et al. 2000). In cowpea, molecular marker techniques such as RFLP (i.e. Fatokun et al. 1993a; 1993b; Menendez et al. 1997), RAPDs (i.e. Mignouna et al. 1998; Xavier et al. 2005), AFLPs (i.e. Coulibaly et al. 2002; Ouédraogo et al. 2002a; 2002b), microsatellites (i.e. Li et al. 2001; Wang et al. 2004) have been used. Several genetic maps of cowpea have been constructed by Fatokun et al. (1992; 1993a), Menancio-hautea et al. (1993), Menendez et al. (1997), Ubi et al. (2000) and Ouédraogo et al. (2002a). Number of biochemical and phenotypic traits have also been located on the genetic map by Ouedraogo (2002a). However, only few sequence-characterized amplified region (SCAR) markers developed from AFLP markers linked to *Rsg2-1*, [a gene that confers resistance to *Striga* Race 1 (SG1) in Burkina Faso], and to gene *Rsg4-3*, [a gene that provides resistance to *Striga* Race 3 (SG3) from Nigeria] were proven to be effective and remarkably reliable for MAS. Another two AFLP markers were discovered to be closely linked to *Rsg1-1*, a gene that also confers resistance to SG3 in Nigeria (Boukar et al. 2004). One of the AFLP markers, designated EACT/M-CAC115 and determined to be 4.8 cM from *Rsg1-1*, was converted to a SCAR marker for ease of use in breeding programs (Boukar et al. 2004). Most recently, a consensus genetic map of seven populations of cowpea recombinant inbred lines (RILs) based on single nucleotide polymorphisms (SNPs) has been reported by Muchero et al. (2009a). This consensus genetic map will offer new possibilities including comparative genomics studies that will enhance marker assisted development of improved cowpea cultivars especially for quantitative complex traits such as resistance to disease, insects and yield under drought stress conditions.

Rainfall is erractic in the semi-arid zone of Africa where most cowpea is produced. Therefore, drought stress can occur throughout the cropping season and drought represents the most important abiotic stress affecting cowpea production. Another trait of importance is bacterial blight which occurs in cowpea growing areas worldwide and can cause losses up to 92% (Kishun et al. 1989). Despite the importance of these two traits, no quantitative trait loci (QTLs) with linked markers have been identified for use in selecting for cowpea bacterial blight (CoBB) and more complex traits such as drought tolerance. It is only recently that Muchero et al. (2009b) reported QTLs for drought stress-induced premature senescence and maturity in cowpea. In Chapter 2 we review the accomplishments, constraints, and future prospects for breeding cowpea drought tolerant varieties.

Scope and outline of the thesis

In this thesis, we carried out genetic analysis of abiotic and biotic stress resistance in cowpea. Genetic analysis of drought tolerance at early (seedling stage) and terminal (adult plant stage) cropping seasons and of cowpea bacterial blight resistance to *Xanthomonas axonopodis* pv. vignicola were investigated. Recombinant inbred lines derived from a cross between Danila and TVu7778 were used in these studies.

In Chapter 3, we studied drought tolerance at the seedling stage of the RIL population derived from DanIla x Tvu7778. The lines and parents were phenotyped using the wooden box technique (Singh et al. 1999) for the following drought tolerance traits: drought induced trifoliate senescence (DTS), stay green (Stg) and survival (Sur). For QTL mapping, a genetic map with single nucleotide polymorphism (SNP) markers was constructed using data supplied by T. J. Close at University of California Riverside (UCR). Several QTLs with effects for these traits were mapped.

Traits and criterions that can be used for selection in breeding for terminal drought tolerance in cowpea are examined in Chapter 4. The RILs and parents were phenotyped in Nigeria in field trails with two contrasting water regimes during the dry season 2005-2006 (Kano) and the dry season 2006-2007 (Kano, Ibadan). Stomatal conductance (Gs), relative water content (RWC), delayed leaf senescence (DLS), days to flowering, number of pods per plant, number of seeds per pod, seed weight, grain and fodder yields were measured. Genetic variation, heritability, and relationship between the traits were evaluated.

In Chapter 5, we use the SNP genetic linkage map to carry out QTL analysis for physiological and yield parameters with an effect on terminal drought tolerance in cowpea. The extent to which physiological parameters and productivity are under common genetic control in well-watered and water limited environments is evaluated and discussed.

In Chapter 6, the genetics of resistance to cowpea bacterial blight (CoBB) was studied using two virulent strains of *Xanthomonas axonopodis* pv. vignicola. The RIL population derived from Danila and TVu7778 segregated for CoBB resistance and QTL contributing to CoBB resistance were mapped using the SNP genetic map.

In Chapter 7 we present a general discussion about results found in the different experimental chapters and point out common and specific chromosomal regions controlling drought tolerance at seedling and adult plant stages and cowpea bacterial blight resistance. The importance of these findings for cowpea breeding programs is discussed.

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Chapter 2

Breeding drought tolerant cowpea: constraints, accomplishments, and future prospects

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Abstract

This review presents an overview of accomplishments on different aspects of cowpea breeding for drought tolerance. Furthermore it provides options to enhance the genetic potential of the crop by minimizing yield loss due to drought stress. Recent efforts have focused on the genetic dissection of drought tolerance through identification of markers defining quantitative trait loci (QTL) with effects on specific traits related to drought tolerance. Others have studied the relationship of the drought response and yield components, morphological traits and physiological parameters. To our knowledge, QTLs with effects on drought tolerance have not yet been identified in cowpea. The main reason is that very few researchers are working on drought tolerance in cowpea. Some other reasons might be related to the complex nature of the drought stress response, and partly to the difficulties associated with reliable and reproducible measurements of a single trait linked to specific molecular markers to be used for marker assisted breeding. Despite the fact that extensive research has been conducted on the screening aspects for drought tolerance in cowpea only very few - like the 'wooden box' technique - have been successfully used to select parental genotypes exhibiting different mechanisms of drought tolerance. Field and pot testing of these genotypes demonstrated a close correspondence between drought tolerance at seedling and reproductive stages. Some researchers selected a variety of candidate genes and used differential screening methods to identify cDNAs from genes that may underlie different drought tolerance pathways in cowpea. Reverse genetic analysis still needs to be done to confirm the functions of these genes in cowpea. Understanding the genetics of drought tolerance and identification of DNA markers linked to QTLs, with a clear path towards localizing chromosomal regions or candidate genes involved in drought tolerance will help cowpea breeders to develop improved varieties that combine drought tolerance with other desired traits using marker assisted selection.

Key words: *Vigna unguiculata* L., drought tolerance, QTL mapping, comparative genomics, candidate genes, Marker Assisted Selection (MAS)

Introduction

Agriculture is at a crossroad due to water scarcity, climate change, population pressure and environmental degradation. Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important food legumes in the tropic and sub-tropic regions where drought is a major production constraint due to low and erratic rainfall (Singh et al. 1997). Of the world total area of about 14 million ha planted with cowpea, West Africa alone accounts for about 9 million ha (Singh et al. 2003a). With more than 25% protein in seeds as well as in young leaves (dry weight basis), cowpea is a major source of protein, minerals and vitamins in daily human diets and is equally important as nutritious fodder for livestock (Singh et al. 2003b). Among the popular crops grown in Central and West Africa, cowpea belongs to the inherently more drought tolerant ones (Singh et al. 1997; Ehlers and Hall 1997; Kuykendall et al. 2000; Martins et al. 2003). However, cowpea still suffers considerable damage due to frequent drought in the Savanna and Sahel sub-region. Early maturing varieties escape terminal drought (Singh 1987), but if exposed to intermittent moisture stress during the vegetative growth stage, they perform very poorly (Mai-Kodomi et al. 1999a). Moreover, the early maturing cowpea cultivars tend to be very sensitive to drought that occurs during the early stages of the reproductive phase (Thiaw et al. 1993). Therefore, genetic enhancement of cowpea for drought tolerance by incorporating drought tolerance into early maturity cowpea lines represents the best and most cost-effective method for insuring sustainable and improved crop yield in variable and changing climates. Unstable rainfall in the early cropping season seems to be the pattern in the sub-region. There is also a rationale for incorporating tolerance to terminal drought, which is becoming more frequent in the sub-region due to reduction in the duration of the rainy season. Unlike some other legume crops such as common bean (Blair et al. 2002; Schneider et al. 1997) and soybean (Mian et al. 1996; Mian et al. 1998; Specht et al. 2001) for which contemporary technological studies for drought tolerance are more advanced, cowpea is well studied for conventional genetics, but poorly characterized at the genomic level. Nevertheless, concerted efforts are being made worldwide to develop drought tolerant cowpea varieties (Turk and Hall 1980; Hall et al. 1997a) and good progress has been made at the International Institute of Tropical Agriculture (IITA) on breeding for enhanced drought (Okosun et al. 1998a; 1998b; Singh et al. 1999a; 1999b; Mai-Kodomi et al. 1999a; 1999b). The current state of breeding research on drought tolerance in cowpea and possibilities for genetic enhancement of drought tolerance for optimal utilization of the genetic potential of the crop are discussed in this review.

Dimension of drought on cowpea production in Central and West Africa

Cowpea is one of the most ancient crops known to man. The crop originated and domesticated from Africa (Ng and Marechal 1985) and is widely adapted and grown throughout the world. Based on information available from FAO and from scientists in several countries, cowpea researchers at IITA estimated that cowpea is now cultivated on at least 14 million hectares, with 3722 thousand metric tons worldwide in 2003 (FAO 2004). However, Africa largely predominates in production as shown in Figure 1. Central and West Africa alone account for about 9.3 million hectares. A substantial part of cowpea production in the region comes from the drier areas of northern Nigeria (about 4 million ha, with 1.7 million tons), and southern Niger Republic (about 3 million ha, with 1 million tons) (Singh et al. 1993). Millions of African farmers grow cowpea in small scale farming. Some two hundred million Africans consume cowpea, and many possibly even a majority of the farmers are women. One of the most remarkable things about cowpea is that it thrives in dry environments and this makes it the crop of choice in the semi-arid/arid zones of West and Central Africa. Additionally, cowpea used to be the first crop harvested before the cereal crops are ready and therefore is referred to as "hungry-season crop". It is the most economically important indigenous African legume crop (Langyintuo et al. 2003) and is of vital importance to the livelihood of several millions of people in West and Central Africa. Cowpea is a most versatile African crop, it feeds people, their livestock and because of its ability in nitrogen-fixation, it improves soil fertility, and consequently helps to increase the yields of cereal crops when grown in rotation and contributes to the sustainability of cropping systems. Despite all its economic and cultural importance in Sub-saharan Africa, cowpea production is subjected to a wide range of biotic and abiotic constraints.

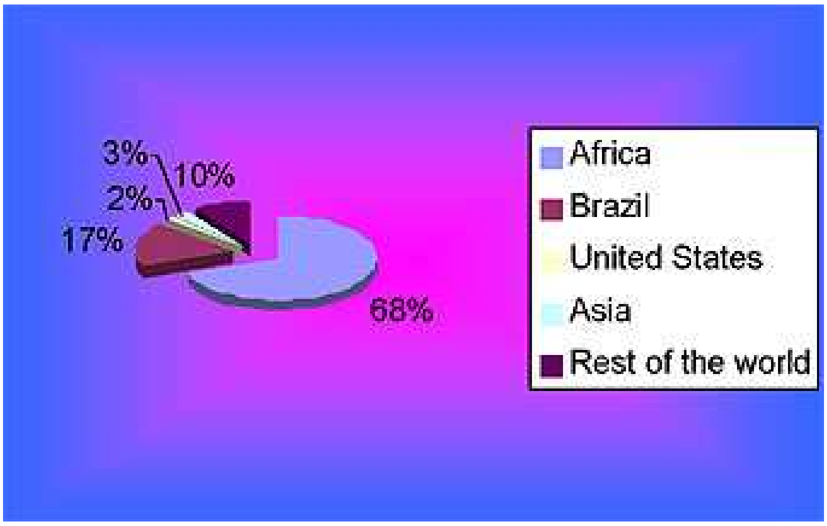

Figure 1. Cowpea production throughout the world (dry grains) (FAO, http://www.fao.org/inpho/content/compend/text/ch32/ch32.htm)

Hounam et al. (1975) and Glantz (1987) studied the effect of drought on hunger in Africa and reported that impact may range from slight personal inconvenience to endangered nationhood. Drought is the major abiotic constraint of cowpea production. Since cowpea is grown mainly in the dry savanna and Sahel areas with no irrigation facilities, irregular rainfall especially early in the season have adverse effects on the growth of the crop. The drier zones of northern Nigeria and Niger harbor the largest area of cowpea production in the world but yields are only between 100 to 500 kg/ha, despite its 5 times higher biological potential (Karsky et al. 2001). Niger is the second largest producer of cowpea after Nigeria yet it has the lowest average grain yield of 110 kg/ha (Table 1). This is probably due to the fact that the whole country is located in the Sahel where rainfall is rather low. Moreover, drought conditions weaken the plants making them more vulnerable to disease infestations and insect pests attacks. As an African crop grown in resource-poor areas, few countries have cowpea improvement programs and the continent has very low average grain yield compared to for instance the United States (Table 1). However, concerted multidisciplinary efforts including genetics, physiology and biochemistry are being developed to unravel drought mechanisms in cowpea and to develop varieties better adapted to the climate changes in Sub-saharan Africa.

Table 1. Average yield (t ha^{-1}) of cowpea production in selected countries in West and Central Africa (1990-1999) and the United States (Langyintou et al. 2003)

Countries	Average yield (t ha^{-1})	Countries	Average yield (t ha^{-1})
Nigeria	0.494	Ghana	0.663
Niger	0.110	Mauritania	0.331
Mali	0.244	Côte d'Ivoire	0.500
Burkina Faso	0.777	Chad	0.489
Togo	0.284	Cameroon	0.827
Benin	0.635	Africa	0.475
Senegal	0.341	United States	1.950

Drought tolerance mechanisms

Several factors and mechanisms operate independently or jointly to enable plants to cope with drought stress. Therefore drought tolerance is manifested as a complex trait (Krishnamurthy et al. 1996). Traditionally, drought tolerance is defined as the ability of plants to live, grow, and yield satisfactorily with limited soil water supply or under periodic water deficiencies (Ashley 1993). According to Mitra (2001), the mechanisms that plants use to cope with drought stress can be grouped into three categories viz. drought escape, drought avoidance and drought tolerance. However, crop plants use more than one mechanism at a time to cope with drought.

Drought escape is defined as the ability of a plant to complete its life cycle before serious soil and plant water deficits occur. This mechanism involves rapid phenological development (early flowering and early maturity), developmental plasticity (variation in duration of growth period depending on the extent of water deficit) and remobilization of pre-anthesis assimilates. Drought avoidance is the ability of plants to maintain relatively high tissue water potential despite a shortage of soil-moisture. Plants develop strategies for maintaining turgor by increasing root depth or developing an efficient root system to maximize water uptake, and by reducing water loss through reduced epidermal (stomatal and lenticular) conductance, reduced absorption of radiation by leaf rolling or folding and reduced evapo-transpiration surface (leaf area) (Mitra 2001). Drought tolerance is the ability of plants to withstand water-deficit with low tissue water potential. The mechanisms of drought tolerance are maintenance of turgor through osmotic adjustment (accumulation of solutes in the cell), increased cell elasticity and decreased cell size and desiccation tolerance by protoplasmic resistance.

However, all these adaptation mechanisms of the plant to cope with drought have some disadvantages with respect to yield potential. For instance, a genotype with a shortened life cycle usually yields less compared to a genotype with a normal life cycle. The mechanisms that confer drought avoidance by reducing water loss (such as stomatal closure and reduced leaf area) decrease carbon assimilation due to reduction in physical transfer of carbon dioxide molecules and increase leaf temperature thus reducing biochemical processes, which negatively affects yield. Plants try to maintain water content by accumulating various solutes that are nontoxic (such as fructans, trehalose, polyols, glycine betaine, proline and polyamines) and do not interfere with plant processes and that are, therefore, called compatible solutes (Yancey et al. 1982). However, many ions concentrated in the cytoplasm due to water loss are toxic to plants at high concentrations leading to what is termed a glassy state. In this condition whatever liquid is left in the cell has a high viscosity, increasing the chances of molecular interactions that can cause proteins to denature and membranes to fuse (Hartung et al. 1998). Consequently, crop adaptation to water stress must reflect a balance among escape, avoidance and tolerance while maintaining adequate productivity. Drought escape, avoidance, and tolerance mechanisms have been described in cowpea. However, the drought response pathways associated with these mechanisms are not yet understood, and the degree to which these adaptations operate jointly or separately to allow the crop to cope with drought still needs to be established.

Drought tolerance mechanisms in cowpea

Drought escape in cowpea

The increased incidence of drought in some cowpea growing areas has caused a shift to early maturing varieties (Mortimore et al. 1997). Early maturity of cowpea cultivars is desirable and has proven to be useful in some dry environments and years because of their ability to escape drought (Hall and Patel 1985; Singh 1987; 1994). Such early cultivars can reach maturity in as few as 60 to 70 days in many of the cowpea production zones of Africa. Earliness is important in Africa as early cultivars can provide the first food and marketable product available from the current growing season, and they can be grown in a diverse array of cropping systems. In addition to escaping drought, early maturing cultivars can escape some insect infestations (Ehlers and Hall 1997). The International Institute of

Tropical Agriculture (IITA) and the Institut Senegalais de Recherches Agricoles (ISRA) have been at the forefront in developing early maturing high yielding and pest resistant cultivars. Selection for early flowering and maturity and yield testing of breeding lines under drought conditions has been used successful in developing cowpea cultivars adapted to low rainfall areas (Hall and Patel 1985; Cisse et al. 1997). Early maturity cowpea varieties (i.e. IT84S-2246, Bambey 21) that escape terminal drought have been released and widely adopted by African farmers. However, if exposed to intermittent drought during the vegetative or reproductive stages, these varieties performed very poorly. Efforts are therefore being made to breed cowpea varieties with enhanced drought tolerance for early, mid- and terminal season drought stresses. Different RIL populations are currently under evaluation for different traits including physiological, phenological and yields for drought tolerance at seedling and flowering/ reproductive stages. These investigations aim at understanding which of the traits contribute importantly to yield under drought.

Mechanisms of drought avoidance and tolerance in cowpea

In cowpea, two types of drought tolerance have been described at the seedling stage using the wooden box technique (Mai-Kodomi et al. 1999a). At 15 days after the termination of watering, all the seedlings of the two susceptible lines TVu 7778 and TVu 8256, were completely dead. The “Type 1” drought tolerant lines like TVu 11986 and TVu 11979 stopped growth after the onset of drought stress and maintained uniformity, but displayed a declining turgidity in all tissues of the plants including the unifoliates and the emerging tiny trifoliates for over two weeks. All plant parts such as the growing tip, unifoliates and epicotyl gradually died almost at the same time. In contrast, the “Type 2” drought tolerant lines like Danila and Kanannado remained green for a longer time and continued slow growth of the trifoliates under drought stress. With continued moisture stress, the trifoliates of these varieties started wilting as well and died about 4 weeks after drought stress started. The two types of tolerance responses by cowpea seedlings to drought stress indicate that cowpea genotypes evolved different mechanisms to cope with prolonged drought encountered in the semi-arid regions of Africa where the crop is believed to have originated. Closure of stomata to reduce water loss through transpiration and cessation of growth (for Type 1 drought avoidance) and osmotic adjustment and continued slow growth (drought tolerance in Type 2) have been suggested as the possible mechanisms for drought tolerance in cowpea (Lawan 1983; Boyer 1996). Cowpea is known as dehydration avoider with strong stomatal sensitivity and reduced growth rate (Lawan 1983). This seems to be the mechanism underlying the Type 1 reaction to drought of TVu 11986 and TVu 11979. The Type 2 reaction of Danila and Kanannado appears to be a combination of three mechanisms; stomatal regulation (partial opening), osmotic control and selective mobilization with distinct visible differences in the desiccation of lower leaves compared to the upper leaves and growing tips (Mai-Kodomi et al. 1999a). It seems that the Type 2 mechanism of drought tolerance is more effective in keeping the plants alive for a longer time and ensures better chances of recovery than Type 1 when the drought spell ends. Both drought tolerant lines Danila and Kanannado are local varieties commonly grown in the Sudano- Sahelian border areas of Nigeria and Niger Republic, indicating that in these areas farmers have selected cowpea varieties with good adaptation to drought. Similarly, Muchero et al. (2008) studied 14 genotypes of cowpea at seedling stage and confirmed the existence of significant genetic variation in response to drought stress. Genotypes, IT93K-503-1 and IT98K-499-39 were consistently most tolerant whereas CB46 and Bambey 21 were most susceptible. However, the differences in phenotypic responses to seedling-stage drought among the 14 genotypes were not consistently associated to drought tolerance. As for examples, genotypes IT82E-18(232) and Sutiva 2 showed rapid loss of unifoliates but were found at opposite ends of the drought tolerance spectrum. While, genotypes CB27 and Bambey 21 preserved unifoliates but Bambey 21 was highly drought susceptible and CB27 modereately susceptible under similar stress conditions. Somehow, these clear phenotypic responses to drought stress provide an opportunity for detailed studies of specific drought responses and select genotypes to be used as parents to study the inheritance of these specific responses.

The association between crop performance and carbon isotope discrimination (Δ) has been reviewed for cowpea, common bean, and peanut (Condon and Hall 1997). Genotypic differences in the potential grain yield of cowpea have been positively associated with Δ, indicating that more productive genotypes have a higher photosynthesis rate resulting in higher internal carbon dioxide concentration in their leaves (Hall et al. 1997b; Condon and Hall 1997). Similar studies in other crops such as Pima cotton (*Gossypium barbadense*) and bread wheat (*Triticum aestivum*) have shown remarkable positive correlations between yield increases and increases in stomatal conductance (Lu et al. 1998). The authors argued that the higher Δ in more productive genotypes of cowpea, cotton (*G. barbadense* L.), and wheat (*Triticum* sp.) was probably due to their having more open stomata, which could have resulted in greater rates of photosynthesis due to diffusion effects (Condon and Hall 1997), or beneficial effects on the plant resulting from greater evaporative cooling (Lu et al. 1998). In favor to the more open stomata strategy under water stress, Cruz de Carvalho et al. (1998) compared physiological responses of cowpea and common bean genotypes and reported that the cowpea genotypes kept their stomata partially opened and had a lower decrease in their net photosynthetic rates than the common bean. Further investigations on these cowpea genotypes are needed to demonstrate whether there are significant positive effects on grain yield related to the partial opening of stomata under drought conditions.

Several other mechanisms may partially explain the extreme dehydration avoidance of cowpea. The mechanisms through which cowpea is able to resist vegetative-stage drought may be related to the limited decrease of leaf water potential even under extreme drought. The lowest leaf water potential recorded for cowpea is -18 bar (-1.8 MPa) (e.g., Turk and Hall 1980; Hall and Schulze 1980), whereas peanut has developed leaf water potentials under drought as low as -82 bar (-8.2 MPa) (Turner et al. 2000). Cowpea also changes the position of leaflets under drought (a drought avoidance mechanism). They become paraheliotropic and orientated parallel to the sun's rays when subjected to soil drought, causing them to be cooler and thus transpire less (Shackel and Hall 1979), which helps to minimize water loss and maintain water potential.

Screening approaches for drought tolerance in cowpea

Success in breeding for drought tolerance in cowpea has not been as pronounced as for many other traits (Singh et al. 1997). This is partly due to the lack of simple, cheap, and reliable screening methods to select drought tolerant plants and progenies from the segregating populations. The complexity of factors involved in drought tolerance could also have contributed to this. Nevertheless, cowpea genotypes with contrasting response to drought have been identified (Figure 2). Researchers have proposed two approaches for screening and breeding for drought tolerance in plants. The first is the empirical or performance approach that utilizes grain yield and its components as the main criteria, since yield is the integrated expression of the entire array of traits related to productivity under stress. The second is the analytical or physiological approach that identifies a specific physiological or morphological trait that will contribute significantly to growth and yield in the event of drought. Modest progress in cowpea breeding for dry environments has been achieved by selecting for yield in breeding lines over several locations and years (Turk et al. 1980; Hall and Patel 1985; Selvaraj et al. 1986; Cisse et al. 1997; Hall et al. 1997b).

However, these empirical approaches are slow, laborious, and expensive because of the need to assess the yield of a large number of lines across several locations and years, and the substantial variation from the effects of environment, and genotype– environment interactions (Blum 1985). As suggested by Blum (1983) and Fussell et al. (1991), the approach which combines selection for yield potential in favorable conditions with selection for the expression of physiological traits thought to be associated with drought tolerance under controlled, repeatable stress environments might be the most effective. This therefore requires the identification of specific traits associated with drought tolerance under adequate water management that are easy and reliable to measure (Fischer and Wood 1979).

Figure 2. Field screening of cowpea lines for drought tolerance. The plants on the left are IT98K-205-8 (drought tolerant) and those on the right are, IT98K-555-1 (drought susceptible)

Morphological, biochemical and physiological traits for drought screening in cowpea

Data on changes of morphological, biochemical and physiological traits in response to drought are available for some cultivars of *Vigna unguiculata* (Turk et al. 1980; Ogbonnaya et al. 2003; Matsui and Singh 2003; Slabbert et al. 2004). These traits include water-use efficiency (WUE), water potential, relative turgidity, leaf gas exchange, relative water content (RWC), diffusion pressure deficit, chlorophyll stability index, and carbon isotope discrimination (Bates et al. 1981; Turk and Hall 1980; Morgan et al. 1991; Hall et al. 1990; 1997b; Anyia and Herzog 2004; Souza et al. 2004). While comparing physiological responses of *Phaseolus vulgaris* and *Vigna unguiculata* to drought, Cruz de Carvalho et al. (1998) demonstrated that stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}) and net assimilation rates (A, mmol CO_2 m^{-2} s^{-1}) measured during and after a water stress treatment were reliable physiological parameters to use in early screening for drought tolerance in these species. Stomatal closure in the cowpea cultivar EPACE-1 was not related to any change in relative water content (RWC) indicating that early stomatal responses to substrate water depletion are not triggered by changes in leaf water content. Therefore, RWC alone can not be used as a drought indicator for cowpea. This also suggests the possible existence of a root to leaf communication, independent of the leaf water status that informs the shoot about changes in the root zone.

Following exposure of six cowpea varieties to drought in the upper 20 cm rooting zone, Kulkarni et al. (2000) compared the rate of abscisic acid (ABA) synthesis relative to total root mass and inherent variation per unit root mass. The authors observed that the intrinsic ABA synthesizing capacity rather than the root mass is responsible for the total ABA produced in the roots of the dry soil zone. The relationship between stomatal conductance and total root ABA was assessed and found to be negative ($r = -0.90$, $n = 24$, $P = 0.05$) suggesting that the intrinsic capacity of cowpea varieties for ABA synthesis could play an important role in regulating stomatal conductance in a drying soil and provide useful selection criteria for tolerance to drought stress in cowpea. In support to these results, stomatal regulation was reported to be the common strategy used by the five different cowpea genotypes to avoid dehydration both under glasshouse and field conditions (Hamidou et al. 2007). These authors measured the physiological, biochemical and agronomic responses to water deficit at flowering stage in five cowpea genotypes, Gorom local, KVX61-1, Mouride, Bambey 21 and TN88-63, that were grown in the glasshouse and the field. The five cowpea genotypes are known to differ in their susceptibility to water stress. Water deficit significantly increased the canopy temperature and the proline content of the five genotypes while gaseous exchanges and starch content decreased significantly. Yield components of the five genotypes, with the exception of seed number per pod, were also significantly affected. Number of pods and number of seeds per plant decreased after drought treatment by 57% in the glasshouse and by 64% in the field when compared to non-stressed plants. Genotypic differences were observed for both of the yield components. Genotype TN88-63 was more productive than the other four genotypes under glasshouse conditions, while under field conditions, Mouride and Gorom local proved to be more productive than KVX61-1, which in turn performed better than Bambey 21.

As an alternative to all the above investigations which focus on some specific physiological, biochemical and agronomic traits, an integrated approach which combines cellular water relations, rooting characteristics, leaf area and biochemical and morphological changes to screen cowpea for drought tolerance has been proposed by Slabbert et al. (2004). The different screening techniques that were tested included: the antioxidative response in the form of superoxide reductase (SOD), glutathione reductase (GR), ascorbate peroxidase (AP), proline accumulation, 2,3,5- triphenyltetrazolium chloride (TTC) assays, early drought screening at the seedling stage (wooden box technique), cell membrane stability (CMS), relative water content (RWC), leaf water potential (LWP), leaf area, chlorophyll *a* and *b* and carotenoid content and chlorophyll fluorescence (JIP test). Contrary to the results of Cruz de Carvalho et al. (1998), RWC was a good parameter to discriminate genotypes under water stress in cowpea (Slabbert et al. 2004).

An important morphological trait that may contribute to drought adaptation is the delayed leaf senescence (DLS) trait (Gwathmey et al. 1992). This trait enhances plant survival after a mid-season drought damages the first flush of pods, which enables a substantial second flush of pods to be produced. Cultivars with DLS also have enhanced production of forage because their leaves remain green and attached to the plant until harvest. The DLS trait allows the crop to stay alive through midseason drought and recover when rainfall resumes. Most importantly, DLS can be easily measured by visual observation using an appropriate scale.

In summary, based on the above findings from the different studies the following methods were most suitable for screening large number of cowpea lines for drought tolerance:

a) determination of chlorophyll fluorescence,

b) stomatal conductance measurements,
c) abscisic acid (ABA) measurements,
d) measuring free proline levels
e) wooden box screening for drought tolerance at the seedling stage,
f) delayed leaf senescence (DLS)

Screening cowpea for drought tolerance at the seedling stage

Singh et al. (1999a) suggested that different cowpea plant organs (leaf, shoot and root) should be used to screen for drought tolerance. The authors argued that different tissues have different responses to abiotic stress and should therefore be studied individually. This may enable the identification of tissue-specific genetic factors underlying the drought responses and the elucidation of parts of the drought response pathways possibly making breeding for drought tolerance easier. A simple screening method using the ‘‘wooden box technique’’ (Figure 3) has been found suitable for identifying seedling drought tolerance in cowpea. This method eliminates the influences of the root system on drought tolerance, and permits nondestructive visual identification of shoot dehydration tolerance (Singh et al. 1999a). The method has proven to be efficient in screening for drought tolerance in different crop species (Singh et al. 1999b; Tomar and Kumar 2004; Slabbert et al. 2004; Ewansiha and Singh 2006). Field and pot testing of the plants of the different crop species demonstrated a close correspondence between drought tolerance in the seedling stage and reproductive stage. The wooden box screening method has been used to identify cowpea genotypes with contrasting responses to drought (Danila, IT96D-602 and TVu 11986 which exhibit seedling drought tolerance and TVu 7778 which is susceptible). The RILs developed from the cross between Danila and TVu 7778 have been evaluated for seedling survival under severe drought stress using the wooden box technique (not published). Seeds of four RILs and the two parents were planted randomly in straight rows in each wooden box. After emergence plants were thinned to one per stand. The boxes were watered daily with the same volume of water until the first trifoliate emerged and watering was completely stopped. After 4 weeks of water stress, when all the plants of susceptible parent TVu7778 appeared dead, watering was resumed. Variable number of seedlings recovered in some RILs and the tolerant parent two weeks after watering resumed (Figure 3). Similar to the wooden box technique, small plastic pots were tested to separate plant root systems and to eliminate competition among genotypes for a communal water source while still maintaining the low space requirement that is characteristic of wooden screening (Muchero et al. 2008). The pot experiments in greenhouse were used to discriminate between 14 cowpea genotypes that exhibit significant genetic variation to drought stress at seedling. These authors emphasized that stem greenness, survival and recovery dry weights in greenhouse were the useful traits to screen cowpea genotypes for their ability to withstand drought stress at the seedling stage.

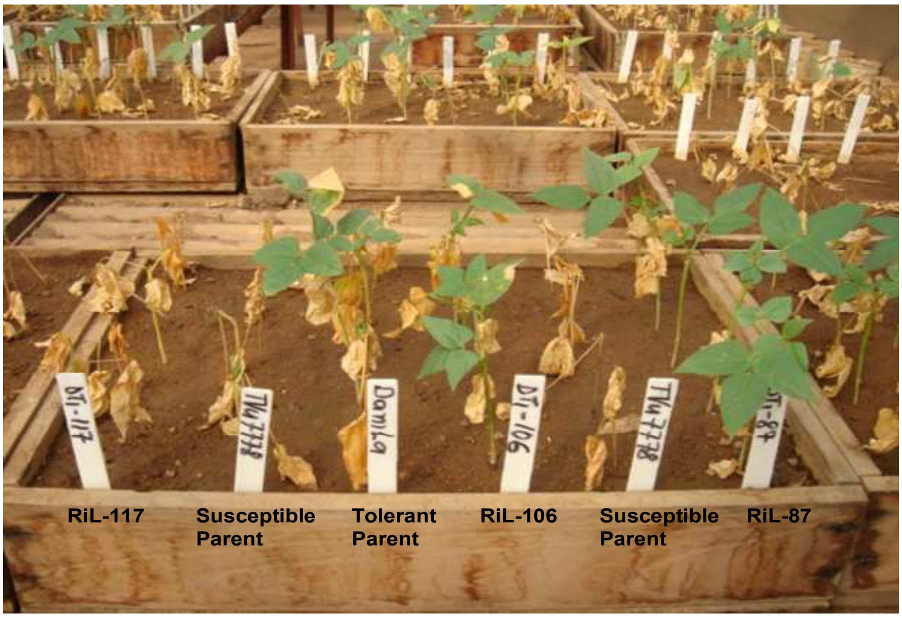

Figure 3. Cowpea seedlings survival after four weeks of drought followed by two weeks of daily re-watering. The drought tolerant parent Danila and RIL-106 had a 60% survival rate, susceptible parent TVu 7778 and RIL-117 had 0% survival, while RIL-87 had a 100% survival rate.

Root characteristics and drought in cowpea

Drought tolerance mechanisms in legume crops seem to be closely related to the root system or rooting pattern (Pandey et al. 1984; 1986; Itani et al. 1992; Silim and Saxena 1993; Matsui and Singh 2003). However, screening for root characteristics is difficult because of the underground distribution of root. The 'pin-board root-box' (Matsui and Singh 2003), herbicidal band screening (Robertson et al. 1985) and polyethylene glycol (PEG) (Badiane et al. 2004) methods were used to identify the role of cowpea root characteristics in drought tolerance. Typically, the evaluation of rooting characteristics has only been performed in a few cultivars when choosing parents for crosses or with a few promising advanced lines. With the herbicide-band screening the authors succeeded in detecting significant genotypic differences in mean numbers of days to first herbicide symptoms among five cowpea genotypes. Cowpea genotypes CB5 and Grant developed symptoms the earliest, 8006 and PI302457 developed symptoms the latest, and PI293579 was intermediate. The ranking of genotypes was consistent with estimates of relative depth of effective rooting obtained from soil moisture extraction measurements. With pin-board root-box screening two-dimensional distribution of roots can be studied. Important varietal differences were observed in cowpea architecture and some varieties have a well-spread deep root system while others have concentrated roots only on the upper soil strata. Although it has been reported that the results of this method is highly correlated with field observations (Matsui and Singh 2003), the pin-board root-box technique received much less attention compared to wooden box technique as described previously. This is probably because it is not practical to screen large number of plants. As root characteristics are important traits involved in drought avoidance, cowpea physiologists at IITA (Kano Station) and researchers from different areas are working to establish simple methods for root screening in cowpea.

Being a quantitatively inherited trait, an integrated screening approach as proposed by Slabbert et al. (2004) might be the most promising for phenotyping cowpea for drought tolerance. It is imperative that selected genotypes should always be tested in the field for confirmation of their yield performance under field drought. It would be helpful to identify traits that are associated with drought tolerance, but that are easier to measure and that have high heritability. Molecular markers closely linked to the loci with effects on these traits could be identified and later used in marker assisted selection (MAS) programs. However, any traits to be used in MAS programs for improving drought tolerance, must have a proven contribution to yield under drought conditions.

Discovery of drought tolerance genes in cowpea

The ability of cowpea to tolerate severe drought conditions and its relatively small nuclear genome size (estimated at ~620 Mb) (Arumuganathan and Earle 1991) makes it an ideal model to study the molecular mechanisms of drought tolerance in crops. Several approaches can be utilized to identify genes that underlie drought tolerance in cowpea. One of the approaches would be to identify candidate genes that are known to be relevant to drought tolerance from previous studies in cowpea and other related crops and test its functionality in cowpea. Another and often-used approach is to identify differential expression of mRNAs in drought stressed vs control plants. Contrary to the candidate genes approach, differential expression of mRNA has been used in cowpea to identify genes that are involved in the drought response. Table 2 provides an overview on genes studied in cowpea in relation to drought stress that are further discussed below.

Iuchi et al. (1996a) isolated 24 cDNA clones that corresponded to dehydration-induced genes from cowpea variety IT84S-2246-4 by a differential screening method. Variety IT84S-2246-4 possesses higher drought tolerance and produces higher seed yield compared to other cultivars in semi-arid areas (Singh 1993). The cDNA clones represented ten different genes collectively named CPRD (cowpea clones responsive to dehydration) (Table 2). Nine of the CPRD genes were induced by drought, while one gene (CPRD29) was not. However, the timing of induction varied among the nine CPRD genes. Five of the cDNAs (*CPRD8*, *CPRD14, CPRD22, CPRD12* and *CPRD46)* were further characterized by Iuchi et al. (1996a; 1996b). Two additional novel drought-inducible genes were reported from the same cowpea variety (IT84S-2246-4) by Iuchi et al. (2000). One of these genes, *VuNCED1*, encodes a 9-cisepoxycarotenoid dioxygenase that catalyzes the key step in ABA biosynthesis (Schwartz et al. 1997; Tan et al. 1997; Iuchi et al. 2000). Drought-stressed cowpea plants accumulated ABA to a level that was 160 times higher than that in unstressed plants. Both the accumulation of ABA and expression of *VuNCED1* were strongly induced by drought stress in eight day old cowpea plants, whereas drought stress did not trigger the expression of the *VuABA1*gene that encodes zeaxanthin epoxidase (Iuchi et al. 2000). Based on genomic Southern-blot analysis, the *VuNCED1* gene is part of a small gene family. The importance of this gene in drought stress response and tolerance of cowpea is however still to be proven (Iuchi et al. 2000).

The regulation of protein degradation through the use of protease-specific inhibitors is a common mechanism in metabolic processes and adaptive processes, including adaptation to drought stress in cowpea (Fernanders 1993; Diop et al. 2004). To elucidate the role of the cowpea leaf protease inhibitor cystatin in response to abiotic stresses, *V. unguiculata* cultivars with contrasting response to water stress were subjected to controlled drought stress, desiccation and exogenous ABA. Expression of the cowpea cystatin gene was studied at the mRNA and protein level, using Northern blot and Western blot analysis

(Diop et al. 2004). To elucidate the role of the cowpea leaf protease inhibitor cystatin in response to abiotic stresses, *V. unguiculata* cultivars with contrasting response to water stress were subjected to controlled drought stress, desiccation and exogenous ABA. Expression of the cowpea cystatin gene was studied at the mRNA and protein level, using Northern blot and Western blot analysis (Diop et al. 2004). It was demonstrated that two cystatin transcripts were present in the leaves of stressed plants, which translated into two polypeptides. The polypeptide with the lowest molecular weight, which was also the weakest, corresponded in size to the deduced polypeptide of the *Vu*C1 cDNA (the two-domain cystatin *VU*C1). Identity of the band with the highest molecular weight could not be determined. In cowpea seeds, multiple minor cystatin-like polypeptides were identified in addition to the major cystatin-like polypeptides of 25 kDa (Flores et al. 2001). The authors concluded that this multiplicity of forms was related to multiple biological roles, as was also the case in rice (Kondo et al. 1990).

In cowpea it has been shown that severe drought led to a massive degradation of membrane lipids (Monteiro de Paula et al. 1993). Phospholipase D (*Vu*PLD1) the main enzyme responsible for the drought-induced degradation of membrane phopolipids was isolated and characterized from two cowpea cultivars (El-Maaroof et al. 1999). The expression and enzymatic activity of *Vu*PLD1 gene were highly stimulated by drought stress in the susceptible cultivar (1183) and remained almost unchanged in the tolerant cultivar (EPACE-1). It seems that the drought-tolerant plants have the capacity to regulate the expression of enzymes responsible for the degradation of membrane lipids, which could be related to its previously shown capacity to maintain a remarkable stability of its membrane structure and functioning (Monteiro de Paula et al. 1993). From the leaves of the same cultivars, Matos et al. (2001) isolated a putative patatin-like (*Vu*PAT1) gene encodes for galactolipid acyl hydrolase. The hydrolysis of galactolipids the main components of chloroplast membrane is stimulated by drought stress. The susceptible cultivar (1183) showed a rapid increase of *Vu*PAT1 expression at mild drought stress while the tolerant (EPACE-1) was able to maintain lower levels of transcripts (Matos et al. 2001). This might be an indication of premature cell death and subsequently tissue death under water stress condition.

Two cDNAs encoding putative phosphatidate phosphatases (PAPs) designated *Vu*PAP-α and *Vu*PAP-β were cloned from cowpea leaves (Marcel et al. 2000). PAP is thought to play a role in the enzymatic cascade leading to membrane lipid degradation under environmental stresses or senescence (Todd et al. 1992; Sahsah et al. 1998). Unlike *Vu*PAP-β, *Vu*PAP-α has an N-terminal transit peptide and is targeted *in vitro* to the chloroplasts. The effect of water deficit on gene expression of *Vu*PAP-α and *Vu*PAP-β was studied in leaves of cowpea plants subjected to progressive drought by withholding water or in cut leaves subjected to rapid air-desiccation. Gene expression of *Vu*PAP-α remained very low during the drought treatments, but was strongly stimulated on rehydration. *Vu*PAP-β expression did not vary in plants submitted to water stress by withholding irrigation, but increased rapidly in air-desiccated leaves (Marcel et al. 2000).

Water deficit (drought and desiccation) is known to induce the production of reactive oxygen species (ROS). Among these, H_2O_2 is produced mainly in the chloroplasts and mitochondria of stressed cells and is the source of major cell damage (Foyer et al. 1994; Dat et al. 2000). Among the detoxification systems two enzymes, glutathione reductase (GR) and ascorbate peroxidase (APX), play key roles. To study the variation in cytosolic and dual-targeted GR gene expression in the leaves, cowpea plants 'EPACE-1' (drought tolerant) and 1183 (drought sensitive) were subjected to progressive drought, rapid desiccation and application of exogenous abscisic acid (ABA) (Contour-Ansel et al. 2006). Two new cDNAs encoding a putative dual-targeted (dtGR) and a cytosolic GR (cGR) were cloned and sequenced from leaves of *V. unguiculata*. Drought stress induced an up-regulation of the expression of the cGR gene directly related to the intensity of stress in both cultivars. The regulation of the expression of dtGR upon drought stress was different in a drought resistant cultivar (EPACE-1) compared with susceptible one (1183). In EPACE-1, the progression of the drought treatment down-regulated dtGR expression, whereas in the susceptible cultivar it highly stimulated dtGR expression, at least until moderate water stress was reached. In summary, these results demonstrate a noticeable activation in both cultivars of the antioxidant metabolism under progressive water stress, which in the susceptible cultivar 1183 involves both GR genes.

Gazendam and Oelofse (2007) used suppression subtractive hybridization (SSH) on a drought tolerant (IT96D-602) and a susceptible (TVu7778) line to obtain differentially expressed transcripts. Preliminary sequencing revealed that four out of five randomly selected cDNA clones from this procedure coded for known genes found in a variety of plant species. Two are known to be stress-related genes glutathione-S-transferase (GST) and pathogenesis related protein-1 (PR-1). Analysis of additional clones may result in identification of more interesting differentially expressed genes with known protein functionality related to drought tolerance.

Table 2. Overview of different genes identified as being involved in drought tolerance in cowpea

Gene designation	Accession number	Gene function	Authors
*Vu*NCED1	(AB030293)	9-cisepoxycarotenoid dioxygenase catalyzes the key step involved in ABA biosynthesis	Iuchi et al. (2000)
CPRD86	(AB030294)	9-cisepoxycarotenoid dioxygenase catalyzes the key step involved in ABA biosynthesis	Iuchi et al. (2000)
*Vu*ABA1	(AB030295)	zeaxanthin epoxidase, an enzyme involved in early step of ABA biosynthesis	Iuchi et al. (2000)
CPRD12	(D88121)	Cowpea response to dehydration stress	Iuchi et al. (1996b)
CPRD46	(D88122)	Water stress-inducible gene for neoxanthin cleavage enzyme involved in ABA biosynthesis	Iuchi et al. (1996b)
CPRD8	(D83970)	Cowpea response to dehydration stress	Iuchi et al. (1996a)
CPRD14	(D83971)	Cowpea response to dehydration stress	Iuchi et al. (1996a)
CPRD22	(D83972)	Cowpea response to dehydration stress	Iuchi et al. (1996a)
dtGR	(DQ267474)	Dual-targeted glutathione reductase key enzyme involved in detoxication of (AOS)	Contour-Ansel et al. (2006)
cGR	(DQ267475)	Cytosolic glutathione reductase key enzyme involved in detoxication of (AOS)	Contour-Ansel et al. (2006)
*Vu*cAPX	(U61379)	Cytosolic ascorbate peroxidase key enzyme involved in detoxication of (AOS)	D'Arcy-Lameta et al. (2006)
*Vu*pAPX	(AY466858)	Peroxisomal ascorbate peroxidase key enzyme involved in detoxication of (AOS)	D'Arcy-Lameta et al. (2006)
*Vu*tAPX	(AY484492)	Thylakoidal ascorbate peroxidase key enzyme involved in detoxication of (AOS)	D'Arcy-Lameta et al. (2006)
*Vu*sAPX	(AY484493)	Stromatic ascorbate peroxidase key enzyme involved in detoxication of (AOS)	D'Arcy-Lameta et al. (2006)
*Vu*PLD1	(U92656)	Putative phospholipase D a major lipid-degrading enzyme in plant	El-Maarouf *et al.* (1999)
*Vu*PAP-α	(AF165891)	PAP important for enzymic cascade leading to membrane lipid degradation under environmental stresses or senescence	Marcel et al. (2000)
*Vu*PAP-β	(AF171230)	PAP important for enzymic cascade leading to membrane lipid degradation under environmental stresses or senescence	Marcel et al. (2000)
*Vu*C1	(AF278573)	Protein inhibitors of cystein proteinases belonging to the papain family.	(Diop et al. (2004)
*Vu*PAT1	(AF193067)	Galactolipid acyl hydrolase involes in membrane degradation induced by drought stress	Matos et al. (2001)

D'Arcy-Lameta et al. (2006) studied ascorbate peroxidases (APX) gene expression in response to progressive drought, rapid desiccation and application of exogenous abscisic acid in the leaves of the same cowpea varieties. Four new *V. unguiculata* cDNAs (Table 2) encoding putative cytosolic (*Vu*cAPX), peroxisomal (*Vu*pAPX), chloroplastic (stromatic *Vu*sAPX) and thylakoidal (*Vu*tAPX) ascorbate peroxidases were isolated and characterized. The four cowpea APX deduced proteins were aligned and compared with a pea cytosolic APX (Mittler and Zilinskas 1991). Amino acid residues essential for enzymatic activity were conserved in the cowpea sequences *Vu*cAPX (Y62077) and pea (Jespersen et al. 1997; Shigeoka et al. 2002). Important increases in steady-state transcript levels of *Vu*cAPX and *Vu*pAPX were observed after 2 h of ABA treatment and after 30 min of desiccation in 1183, while in EPACE-1 air-desiccated leaves, no significant changes were observed in steady-state levels of *Vu*cAPX and *Vu*pAPX transcripts in response to rapid water loss and exogenous ABA treatment. Stimulation of the stromal isoform of 1183 occurred much later, at severe water deficits. Chloroplastic APX gene expression was strongly stimulated already at low levels of water stress in EPACE-1. Although in the less-tolerant cowpea cultivar 1183 the stimulation of chloroplastic APX genes occurred later than for EPACE-1 (D'Arcy-Lameta et al. 2006), the plant was still able to early activate the expression of genes coding for cytosolic isoforms. This shows that cowpea is a drought-tolerant species compared to other cultivated plants, and even the more sensitive cultivars have some level of resistance to water deficits.

Muchero et al. (2008) investigated the correlation of restriction fragment length polymorphisms markers derived from 12 known drought responsive cDNA in cowpea with seedling drought tolerance phenotypes. Such approach offers an opportunity to identify potential targets that would help to assign a specific contribution of cDNAs in conferring tolerance or susceptibility to drought stress. Putative fragments generated from CPRD12, CPRD46, galactolipid acyl hydrolas, phospholipase D, and 9-cis-epoxycarotenoid dioxygenase (Table 2) showed promising correlations with drought related phenotypes. Such information would guide for further genetic studies and help plant breeders to select potential parents for generating mapping populations. Although drought tolerance is a highly quantitative trait, it has been demonstrated that the expression of a single gene can confer drought tolerance in plants. It was shown that over-expression of the AP2/ERF factors CBF1, DREB1A and CBF4 resulted in drought/salt/cold tolerance in Arabidopsis

(Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Haake et al. 2002). AP2 transcription factor SHINE was shown to confer drought tolerance in Arabidopsis (Aharoni et al. 2004) using a different mechanism than that of the DREB/CBF genes. WXP1 is another AP2 domain containing transcription factor gene that increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*) (Zhang 2005). Further analysis of cowpea transgenic plants in which those above mentioned genes will be over-expressed or suppressed by anti-sense RNA should give more information on their functions under water stressed conditions in cowpea.

An important step elucidating the molecular mechanisms underlying the genetically complex abiotic stress responses such as drought is the rapid discovery of genes by the large-scale sequencing of randomly selected cDNA clones or expressed sequence tags (ESTs). There are now 183000 EST as a result of the University of California Riverside (UCR) project, and the earlier IITA-Generation Challenge Program (GCP) project, from 13 genotypes. Recently, sequencing and analysis of the gene-rich, hypomethylated portion of the cowpea genome has been initiated (Timko et al. 2008). Over 250,000 gene-space sequence reads (GSRs) with an average length of 610 bp were generated. Sixty-two (62) out of 64 well characterized plant transcription factor (TF) gene families are represented in the cowpea GSRs. The generated GSRs sequences may provide a source for functional markers in genes linked to drought tolerance traits in cowpea which could be used for marker-assisted selection.

Breeding options to enhance drought tolerance in cowpea

Attempts to improve drought tolerance of crops through conventional breeding programs have met with limited success because drought tolerance is physiologically and genetically a complex trait. The use of molecular markers to identify and locate different genes and genomic regions possessing factors which influence drought tolerance in cowpea will help to gain insight into the complex trait of drought tolerance. In addition these markers can be used to select for multiple traits and combine genes underlying these traits in cultivars with improved drought tolerance. These properties and prospects have initiated an increased interest in the application of Marker-Assisted Selection (MAS) for improving drought tolerance in many crops including cowpea. For better understanding of different biochemical and physiological pathways involved in drought tolerance in cowpea, three main approaches using molecular marker tools can be used.

The first approach assumes no prior knowledge about genes and is based on the so-called quantitative trait loci (QTL) method. On the most recent genetic map of cowpea (Ouedraogo et al. 2002), consisting of 11 linkage groups (LGs) spanning a total of 2670 cM, with an average distance of approximately 6 cM between markers, no genes/QTLs related to drought tolerance were mapped. However, different RIL populations are being currently screened at IITA for mapping and identification of QTLs with effects on drought tolerance across populations. The development of a set of ESTs from drought-stressed and non-stressed drought-sensitive and tolerant cowpea lines will be helpful in genotyping. The ESTs are utilized to develop other molecular markers such as simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs) and COS markers. The COS markers would facilitate cross-legume studies and allow better integration of cowpea into legume functional genomics. Currently cowpea genomics is receiving increased attention, which has resulted in projects that are producing large sets of ESTs and other genome sequences which has recently applied an Illumina Goldengate SNP array with 1536 SNPs (UCR) to several RIL populations and diverse array of genotypes. This is an opportunity for the cowpea community to use a common set of markers in a wide collection of crosses and germplasm for construction of a densely populated consensus genetic map and for connecting genetics and QTLs/genes in cowpea. All the efforts in improving genetic maps and increasing available sequence data are only useful for QTL analysis if drought tolerance parameters can be measured as heritable traits. For cowpea these include the traits mentioned earlier like stomatal conductance, chlorophyll fluorescence, abscisic acid (ABA) levels, free proline levels, wooden box screening for drought tolerance at the seedling stage, and delayed leaf senescence (DLS).

The second approach is to make an ‘educated guess’ from published data, i.e. select candidate genes (CG) that are known to be functionally relevant for drought tolerance and test in cowpea plants whether these genes can be linked to drought tolerance. Candidate genes refer either to cloned genes presumed to affect a given trait (‘functional CGs’) or to genes suggested by their close proximity on linkage maps to loci controlling the trait (‘positional CGs’) (Pflieger 2001). The final validation of a CG will be provided through physiological analyses, and genetic transformation. The most detailed studies relating candidate genes to drought QTLs have looked at genes that determine ABA levels, at genes involved in dehydrin production, at invertase activity and transcription factors (Pflieger 2001). However, there has also been interest in mapping a wide range of regulatory and structural candidate genes to determine QTLs with effects on drought tolerance and this approach has been particularly effective in the case of rice (Nguyen et al. 2004). As mentioned in Table 2, genes involved in ABA biosynthesis, ascorbate peroxidase, glutathione reductase and transferase, and putative phosphatidate phosphatases have been cloned from cowpea under water stress conditions. However, clear evidence that these genes affect drought tolerance for instance through transgenic analyses has not been reported so far. Other CGs can be inferred from studies in related crops and model crops. Cowpea orthologues of these genes that have been characterized in other species and crops as being involved in drought tolerance will be increasingly easy to discover, as the number of cowpea EST sequences as well as genespace sequences is increasing rapidly. An interesting group of GCs are transcription factors that are involved in the drought response including Myb genes, WRKY genes, AP2 and ERF genes.

The third approach is comparative genomics. Earlier studies indicated that members of Papilionoideae subfamily to which cowpea belongs exhibit extensive genome conservation, based on comparative genome analysis between mungbean and cowpea (Menancio-Hautea et al. 1993), between pea and lentil and orthologous seed weight genes in cowpea and mungbean (Fatokun et al. 1992). Recent advances in comparative mapping among the legumes has clarified the genetic relationship of model and crop legumes and enabled linking of the genomes of the tropical and temperate legumes that represent the major clades of the legume family (Choi et al. 2004a; Choi et al. 2004b). Drought tolerance is a highly appropriate target for comparative plant genomics because this information-rich approach has the potential to unveil the key genetic contributors to the complex physiological processes involved (Bennetzen 2000). With the already extensive and rapidly increasing publicly available genomic data for cowpea, comparative genomics of cowpea with other legumes such as common bean (Blair et al. 2002; Schneider et al. 1997) and soybean (Mian et al. 1996; Mian et al. 1998; Specht et al. 2001) could be applied. This will allow aligning of drought QTLs between legume species including cowpea and determine the most important regions for saturated mapping. Moreover, the micro and macrosyntenic relationships detected between cowpea and other cultivated and model legumes (Timko et al. 2008) would simplify the identification of informative markers for marker-assisted trait selection and map-based gene isolation necessary for cowpea improvement.

Conclusion

A multidisciplinary approach including breeding, physiology and biotechnology is required for efficient germplasm improvement for drought tolerance in cowpea. Concerted efforts are being made worldwide to develop drought tolerant cowpea varieties. At IITA RIL mapping populations are being used to identify markers associated with QTLs with effects on different traits with particular emphasis given to the genetic dissection of both yield component and physiological drought adaptive traits.

Important drought related cDNAs and genes have been isolated from cowpea. The advances that are currently being made in cowpea genomics will unlock even more candidate genes. The next step will be to select promising candidate genes and functionally characterize these genes. For candidate genes with well-known functions functional markers can be used for MAS. The molecular analysis of drought responses in plants has reached a stage where research can now build upon a large collection of well characterized genes. The use of novel approaches combining genetic, physiological, biochemical, and molecular techniques should provide exciting results in the development of drought tolerant cowpea varieties in the near future.

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Chapter 3

QTL mapping for seedling drought tolerance traits in cowpea [*Vigna unguiculata* (L.) Walp.] using a SNP-based genetic map.

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Abstract

A cowpea population of recombinant inbred lines (RILs) derived from a cross between two parental genotypes with contrasting reactions to drought stress, Danila (tolerant) and TVu7778 (susceptible) was evaluated for drought tolerance at seedling stage. The plants were screened for stem greenness (Stg), drought-induced trifoliate senescence (DTS), and plant survival (Sur) at seedling stage. Stem greenness was an excellent predictor of seedling survival to drought ($r^2 = 0.91$) and stem greenness was inversely related to drought-induced trifoliate senescence ($r^2 = -0.714$). In order to identify cowpea genes/QTLs that contribute to drought tolerance and survival, a genetic linkage map of SNP markers was constructed. Out of 1536 SNPs mined from EST sequences from several sources and analyzed on an Illumina GoldenGate genotyping array, 302 SNPs were polymorphic between the parents and segregated within the RILs with minor allele frequency ≥ 0.3. The constructed linkage map has 282 loci covering a map distance of 633 cM distributed over 11 linkage groups (LG). The sizes of LGs and the number of markers assigned to the different LG varied between 31.6 cM for LG1 (21 loci) and 111.62 cM for LG3 (58 loci). Two QTLs were identified for each of the three traits DTS, Stg and Sur on LG3 and LG7. QTLs were discovered at identical regions for Stg and Sur on LG7 and on LG3 suggesting that similar genes may explain variation in stem greenness and survival. These chromosomal regions warrant further studies for map-based cloning of genes that maintain plant greenness in cowpea and that might be also useful in other crops.

Keywords: Single nucleotide polymorpisms (SNP), genetic map, QTL, Drought tolerance, Seedling

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important crop grown extensively as a food and fodder in West Africa, lower elevation areas of eastern and southern Africa, north-eastern Brazil, part of Middle East, India, and south-eastern and the south-western regions of North America (Ehlers et al. 1997). Cowpea production is limited by numerous insect pests and diseases, parasitic weeds and environmental stresses. Significant long-term genetic improvement efforts of cowpea have been described (Ehlers et al. 2002; Singh et al. 2002; Hall et al. 2003). However, progress in this area is hampered by many factors including the low heritability of the traits based on the field screening methods.

Although cowpea has considerable adaptation to high temperatures and drought when compared to other crop species (Hall et al. 2002; Hall 2004) it still suffers important yield reduction due to erractic rainfall. Efforts to develop cowpea varieties with enhanced drought tolerance have focused on mid- and terminal-season drought stress because of the negative effects on yield (Hall 2004; Dadson et al. 2005). However, due to the increased frequency of drought stress over the last 30 years (Hall et al. 2003) and the irregular rainfall pattern especially at the beginning of the cropping season, tolerance to drought at seedling stage has become more important. Therefore, there is a rational for incorporating drought tolerance genes/QTLs into elite cowpea lines so that survival of drought stress at seedling stages is improved. For QTL analysis a genetic map with sufficient markers density is required. Several genetic maps of cowpea have been published using different types of molecular markers including RLFP, RAPD, AFLP, SSR, SNP (Fatokun et al. 1992; 1993a; Menancio-hautea et al. 1993; Menendez et al. 1997; Ubi et al. 2000; Ouédraogo et al. 2002a; 2002b; Omo-Ikerodah et al. 2008; Muchero et al. 2009a; 2009b). Only a few useful SCAR markers converted from AFLP markers for some Striga races, Race 1 (SG1) in Burkina Fasso and Race 3 (SG3) in Nigeria (Ouédraogo et al. 2002a; Boukar et al. 2004), have been used for marker-assisted selection (MAS).

Single nucleotide polymorphisms (SNPs) are markers of choice as they are the most abundant type of genetic polymorphism in most if not all genomes (Slate et al. 2009). As a consequence, SNPs can be found in a gene of interest. SNPs are sites in the genome where individuals differ in DNA sequence by a single base pair. In recent years, SNPs markers have gained much interest in the scientific and breeding community. EST sequence libraries provide an important source for genetic variation in expressed genes, including SNPs. For cowpea, 183000 EST from 13 genotypes are available as a result of a project at the University of California Riverside (UCR, Dept. of Botany and Plant Sciences), and the earlier IITA-Generation Challenge Program (GCP) project. These ESTs were mined for more than 10000 SNPs, and 1536 SNPs selected and collected on a Illumina GoldenGate Genotyping SNP array by UCR, were used to genotype 7 cowpea RIL mapping populations (Muchero et al. 2009a).

We phenotyped recombinant inbred lines (RILs) derived from two parents with contrasting drought tolerance properties (Danila and TVu7778) for drought tolerance at seedling stage. Three important traits were evaluated including drought-induced trifoliate senescence (DTS), maintenance of stem greenness (Stg) under severe water stress and plant survival (Sur) after severe water stress followed by two weeks re-watering. The cowpea SNP array was used to map quantitative traits loci (QTLs) for the three traits and the importance of these QTLs for improving cowpea varieties with tolerance drought at seedling are discussed.

Materials and Methods

Plant material

Cowpea genotypes Danila and TVu7778 were crossed and the F_2 generation was advanced by repeated selfing and keeping one single seed per plant to generate the next generation up to F_{10} giving rise to the 120 recombinant inbred lines (RILs). The two parents were selected based on their contrasting responses to drought tolerance at seedling stage. Danila (tolerant parent) is a local variety commonly grown in the Sudano-Sahelian border areas of Nigeria and Niger Republic (Mai-Kodomi et al. 1999), while TVu7778 (susceptible parent) is a germplasm line maintained at IITA. Seeds of the RILs were multiplied in pots placed in the screen house and harvested seeds of each RIL were kept and later used for further field and greenhouse trials.

Genotyping

Growth of plants and DNA isolation were conducted at the University of California, Riverside (UCR) as described in Muchero et al. (2009a). DNA was isolated using the Qiagen Plant DNeasy DNA isolation kit. Parental genotypes and RILs from the mapping population were genotyped for 1536 SNPs using the Illumina GoldenGate assay. Among these, 1248 SNP were selected from ESTs derived from 11 cowpea genotypes representing important lines involved in cowpea breeding in Africa. Most of the remaining 288 SNPs were selected from UCR cowpea genotypes breeding lines (Muchero et al. 2009a). All marker processing steps were carried out at the University of California, Riverside. Processing steps included the exclusion of SNPs that had poor technical performance in the GoldenGate assay as well as SNPs exhibiting segregation distortion, defined as having a minor allele frequency (MAF) less than 0.30. In addition, monomorphic SNP, and RILs with excessive heterozygosity, non-parental alleles, and "no-calls" (valid call could not be made) which suggested cross-contaminated DNA samples, recent out-crossing, or poor quality DNA were excluded from further analysis. The final genotype calls were provided by Timothy Close at UCR in an Excel spreadsheet for further analysis.

RIL phenotyping at seedling stage using the wooden box technique

The experiment was carried out during dry season 2007-2008 in the greenhouse at IITA Ibadan (7^{o}30'N, 3^{o}54'E and 243 m altitude) which is in the Guinean zone in South-West Nigeria. Wooden boxes of 130 cm length, 65 cm width and 15 cm depth made of 2.5 cm thick planks were kept on benches as described by Singh et al. (1999a). The boxes were lined with paper sheets and filled with the same volume of 1:1 mixture of top soil and sand. The boxes were filled up 12 cm and leaving approximately 3 cm space on the top for watering. Each box contained 6 straight rows of which 4 rows were planted with 4 different RILs and 2 rows planted with the 2 parents arranged randomly. Five equidistant holes were made per row and 2 seeds were sown in each hole. After germination, plants were thinned to one plant per hill. Five plants from the parental lines were planted. The boxes were watered daily with the same volume of water until 18 days after planting where the first trifoliate emerged and watering was completely stopped. The number of vigorous plants was recorded at the beginning of the water stress treatment. Thereafter, drought-induced trifoliates senescence was visually scored based on 0 to 5 scale where 0 meant the trifoliate stayed completely green and 5 completely wilted. The number of days for trifoliates to be completely wilted was recorded daily until watering resumed. Stem greenness was scored based on 0 to 5 scale, with 0 being a completely dried stem and 5 being a stem that stayed completely green until the end of the experiment. After 4 weeks of water stress, when all the plants of TVu7778 the susceptible parent were apparently dead, watering was resumed.

Plants were re-watered every alternative day for 2 weeks. Survival was recorded as 1 when the plant completely recovered and 0 when the plant had not recovered at the end of experiment. The percentage of plants that recovered after re-watering was calculated and used for statistical analysis.

Statistical analysis

Statistical analyses were carried out with GenStat 11th Edition. In total 160 plants of each of the parental lines were randomly planted in 32 boxes. This data set was used to evaluate the box and row effects and to provide estimates of environmental variability and the heritability of traits measured. The general linear model procedure of ANOVA was used for parental data and one-way ANOVA was used to evaluate the variation among the RILs. Pearson's correlation analyses were performed between the traits measured. Heritability was calculated according to the formula:

$$h^2_m= \sigma^2_g / \sigma^2_g + \sigma^2_e$$

Where h^2_m is heritability based on mean entry, genetic variance σ^2_g = $(MS_g\text{-}MS_e)/r$, variance due to errors σ^2_e = MS_e/r, r = number of replications. MS_g is considered as total phenotypic variation and MSe is an estimate of non-genetic variation.

Map construction

We used JoinMap 4.0 (Van Ooijen 2006) to construct the genetic linkage map of the DanIla x TVu7778 RIL population. The Kosambi mapping function (Kosambi 1944) was used to convert recombination frequencies to Centimorgans. To assign markers to linkage groups, a step-wise reduction of LOD score above 3 with maximum recombination of 0.45 was used. Highly skewed markers were omitted and only markers that showed highest congruency were used to construct the map.

QTL analysis

The software program MapQTL 5.0 (Van Ooijen 2004) was used for QTL analysis. Entry means for visual scoring of drought-induced trifoliate senescence, stem greenness and seedling survival after 4 weeks of water stress and re-watering for 2 weeks were analysed separately. The analysis started first with non-parametric Kruskal-Wallis test to identify markers that showed significant (stringent > 0.005) association with phenotypic traits. The next step was an Interval Mapping (IM) to get better positioning of putative QTLs. Markers located in the vicinity of QTL were selected as initial set of cofactors. The Multiple-QTL model mapping (MQM) method was used to locate precisely QTL using the automatic cofactor selection. A permutation test was applied to each data set (1000 permutations) to decide the LOD (Logarithm of Odds) thresholds ($p = 0.05$). A LOD value of genome wide (GW) was used as threshold to declare QTL for traits measured. The chromosomal location with the maximum LOD score was considered to be the most likely position of a QTL. Graphics were produced by MapChart software (Voorrips 2002).

Results

SNP analysis

Several high-throughput technologies have been developed to genotype SNPs efficiently including the Illumina GoldenGate platform. A total of 117 RILs and their parents were genotyped with 1536 SNPs using the Illumina GoldenGate assay. Different criteria were used to exclude bad SNPs. GenTrain scores measure the reliability of SNP detection based on the distribution of genotypic classes (http://www.illumina.com) and SNPs to be considered had a minimum GenTrain score of 0.25. Subsequently SNPs that were not polymorphic in the parents or in the RILs were excluded from analysis (more than 50% of the SNPs). Of the remaining ca. 400 SNPs, only markers with minor alleles frequency (MAF) higher than 0.30 were considered. SNPs loci where both parents were monomorphic (AA) and RILs showed (BB) or (AB) and SNPs with monomorphic (BB) parent scores and RILs showed (AA) or (AB) were excluded. For a RIL population, there are two possible genotypes for a diploid individual: AA and BB. Four RILs (DT1-27, DT1-28, DT1-66 and DT1-115) showed a high number of heterozygosities for many SNPs. These lines were considered as off-types and were excluded for further analysis. After processing, 302 SNP (~20%) were left and used for mapping.

Genetic linkage map

The 302 SNP markers were used for construction of a genetic linkage map using JoinMap 4 software program. The order of markers on each linkage group (LG) was determined at LOD ≥ 3.0. Twenty SNPs were not linked to any LG. These markers were used as unmapped loci for QTL analysis. The constructed linkage map consists of 282 SNP loci covering a map distance of 633 cM (Kosambi mapping function) in 11 linkage groups in agreement with the expected 11 haploid chromosome number of cowpea (Table 1). The linkage groups were designated LG1 to LG11. The number of markers assigned to LG and the map distances of LGs varied considerably (Table 1). Linkage group (LG 3) had the largest distance (111.66 cM) with 58 SNP loci and the smallest (31.6 cM) with 21 SNP loci was LG1. The average distance between markers was about 2 cM, markers density also varied between LGs. Marker orders were mainly consistent with the consensus SNP map developed from seven cowpea RILs populations including the RIL population studied here (Muchero et al. 2009a).

Table 1. Features of the genetic linkage map of 282 SNP markers segregating among RILs developed from the cross of Danila X TVu7778.

LG	No Markers*	Length cM	Distance between Markers
1	21	31.6	1.5
2	26	66.3	2.6
3	58	111.6	2
4	26	60.4	2.3
5	28	52.8	2
6	17	40	2.3
7	21	44.1	2
8	12	54.3	4.5
9	27	78.6	3
10	27	59.6	2.2
11	19	33.7	1.7
Total	**282**	**633**	**2.2**

* Markers with minor allele frequency (MAF) ranging from 0.381 to 0.495 indicating normal allelic distribution (1:1)

Trait variation and correlations

Visual scoring data as described in Material and Methods for drought-induced trifoliate senescence (DTS), stem greenness (Stg) and survival (Sur) were used for statistical analyses. The two parents showed contrasting responses for all drought tolerance traits measured at the seedling stage under greenhouse conditions. Danila (tolerant parent) displayed a strong ability to maintain leaf and stem greenness longer and showed better survival to the severe seedling drought conditions compared to TVu7778 (susceptible parent) (Figure 1). ANOVA analysis revealed highly significant (P=0.001) difference between the two parents for all traits. Box and row effects were not significant indicating that performance of plants is independent of boxes and row position within a box (Table 2). Trifoliates of the most susceptible RIL lines were completely wilted within 2 weeks after water stress treatment. All the traits showed high heritability, the highest being for Stg (h^2 = 0.96) (Table 2). The traits measured segregated among RILs under water stress imposed at seedling stage and highly significant (0.001) differences were observed for trifoliate senescence, stem greenness and plant survival. Our results indicate that the traits were quantitatively inherited with transgressive segregations towards both directions. For distribution of the traits, mean entry of visual data scores for DTS, Stg at the end of the drought period were used while percentage of surviving plants per RIL was used for Sur. Histogram distribution and mean performance of parents for all traits are shown in Figure 1. Drought-induced trifoliate senescence, stem greenness and survival showed high correlations ranging from 0.541 to 0.911. Seedling survival after severe water stress showed the highest correlation (0.911) with stem greenness and correlated negatively (-0.662) with drought-induced trifoliate senescence. Stem greenness was highly correlated with increased drought-induced trifoliate senescence as shown by correlation (-0.714) between the two traits suggesting a common physiological mechanism may be involved. Danila and the most tolerant lines maintained their stem greenness as well as showing reduced trifoliate senescence.

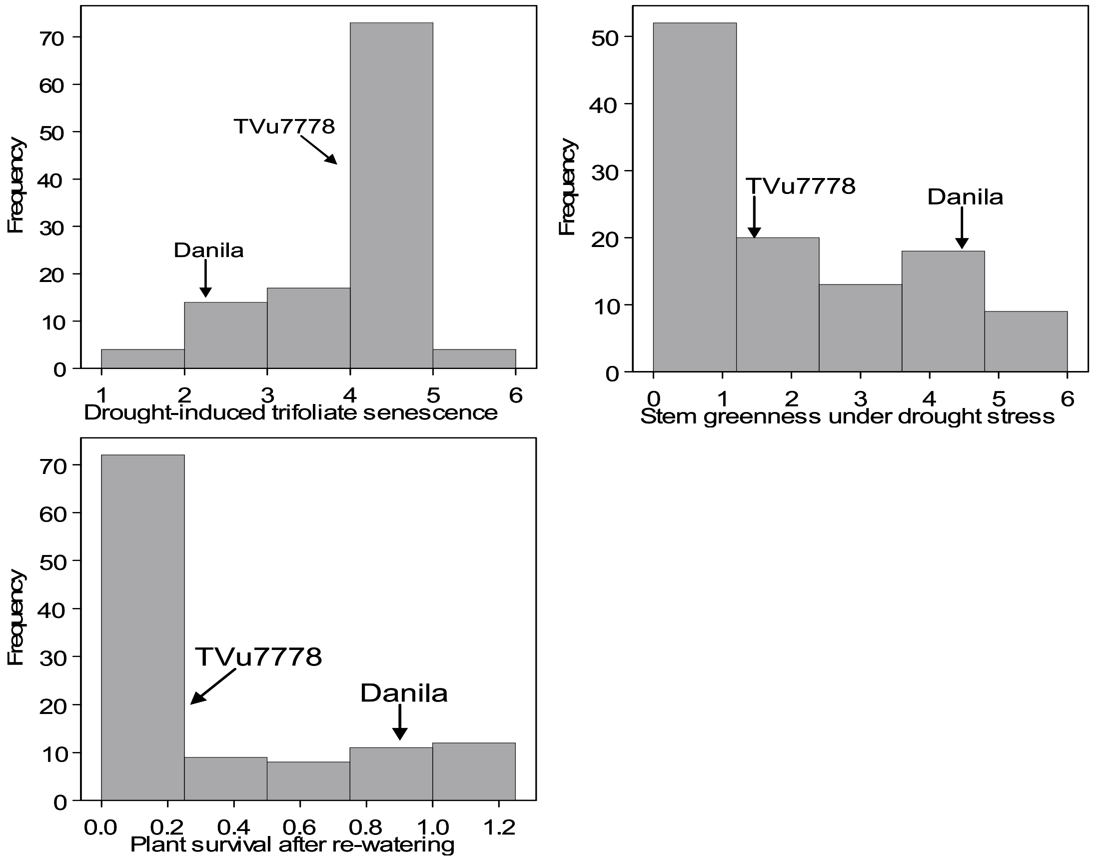

Figure 1. Frequency distribution of drought tolerance traits measured at seedling stage under greenhouse conditions.

Arrows indicate the average values of Danila (tolerant parent) and TVu7778 (susceptible parent). DTS: drought-induced trifoliate senescence Stg: stem greenness, Sur: Plant survival after 2 weeks of re-watering, evaluations were performed by visual observations in five classes for DTS and Stg (1 to 5 according to sensitivity of plant) and two classes for Sur (0: non-survival plant and 1: survival plant).

Table 2. Mean square, level of significance and heritability of the traits measured using the data set of the two parents.

	DTS			Stg			Sur		
Source of Variation	m.s.	Fpr.	H^2	m.s.	Fpr.	h^2	m.s.	Fpr.	h^2
RILs	4.86	0.001	0.90	13.9	0.001	0.96	0.7	0.001	0.88
RILs x Box	1.19	0.120		3.88	0.401		0.37	0.07	
RILs x Box x Row	1.07	0.274		3.27	0.756		0.21	0.14	
Error	0.44			0.47			0.08		

DTS: drought-induced trifoliate senescence Stg: stem greenness, Sur: Plant survival after 2 weeks of re-watering, evaluations were performed by visual observations in five classes for DTS and Stg (1 to 5 according to sensitivity of plant) and two classes for Sur (0: non-survival plant and 1: survival plant).

QTL analysis

The phenotypic data for Sur, DTS and Sur and the genetic map described above were used for QTL analysis. QTLs were identified by IM and MQM analyses and were declared significant based on the LOD threshold of a genome wide (GW) permutation test. In total six QTLs were identified for drought-induced trifoliate senescence (DTS), stem greenness (Stg) and survival (Sur) measured under drought stress at seedling stage, on LG 3 and LG 7 (Table 3). QTLs for DTS, Stg and Sur identified on LG7 overlapped in the chromosomal region from 13.37 to 37.68 cM. On LG3 QTLs for Stg and Sur also overlapped, but the QTL for DTS (on LG3) was found at the extreme end of the linkage group (105.45 to 110.41 cM). The strongest QTLs were found on LG7 with high LOD scores (8.36) and highest proportion of phenotypic variation explained for DTS (16.2%), Stg (20.2%) and Sur (25.2%). Of the QTLs on LG3, the highest variation was explained by the QTL for Stg (15.2%) and around 10% for the DTS and Sur QTLs.

Table 3. Biometrical parameters of QTLs identified for seedling drought tolerance traits as revealed by MQM analysis in cowpea recombinant inbred lines derived from a cross between Danila (drought tolerant) and TVu7778 (drought susceptible)

Trait	LG	Position (cM)	Flanking Markers	Permutation Test (GW)	MQM LOD	MQM Exp%
DTS	3	105.45 - 110.41	1_1206 - 1_0183	2.90	3.68	8.9
DTS	7	13.37 - 37.68	1_0864 - 1_0168	2.90	6.23	16.2
Stg	3	31.66 - 50.56	1_1292 - 1_0352	3.00	7.26	15.2
Stg	7	17.68 - 37.68	1_0270 - 1_0168	3.00	8.36	20.2
Sur	3	48.94 - 53.56	1_0984 - 1_0400	3.00	5.27	9.5
Sur	7	16.96 - 37.68	1_0270 - 1_0168	3.00	6.65	25.2

LG: linkage group, GW: genome wide LOD values for permutation test, MQM: Multiple-QTL model mapping, DTS: drought-induced trifoliate senescence, Stg: stem greenness, Sur: Plant survival after two weeks of every two days re-watering

Discussion

Over the last 30 years the frequency of drought is increasing (Hall et al. 2003) and due to irregular rainfall pattern especially at the beginning of the cropping season in the semi-arid zones in West Africa where a large percentage of the cowpea crop is produced, tolerance to drought at seedling stage is now receiving more attention. We screened a RIL population at seedling stage using the wooden box technique for drought tolerance as described by Singh et al. (1999a) and identified QTLs on LG3 and LG7 for seedling survival, delayed trifoliate senescence and stem greenness.

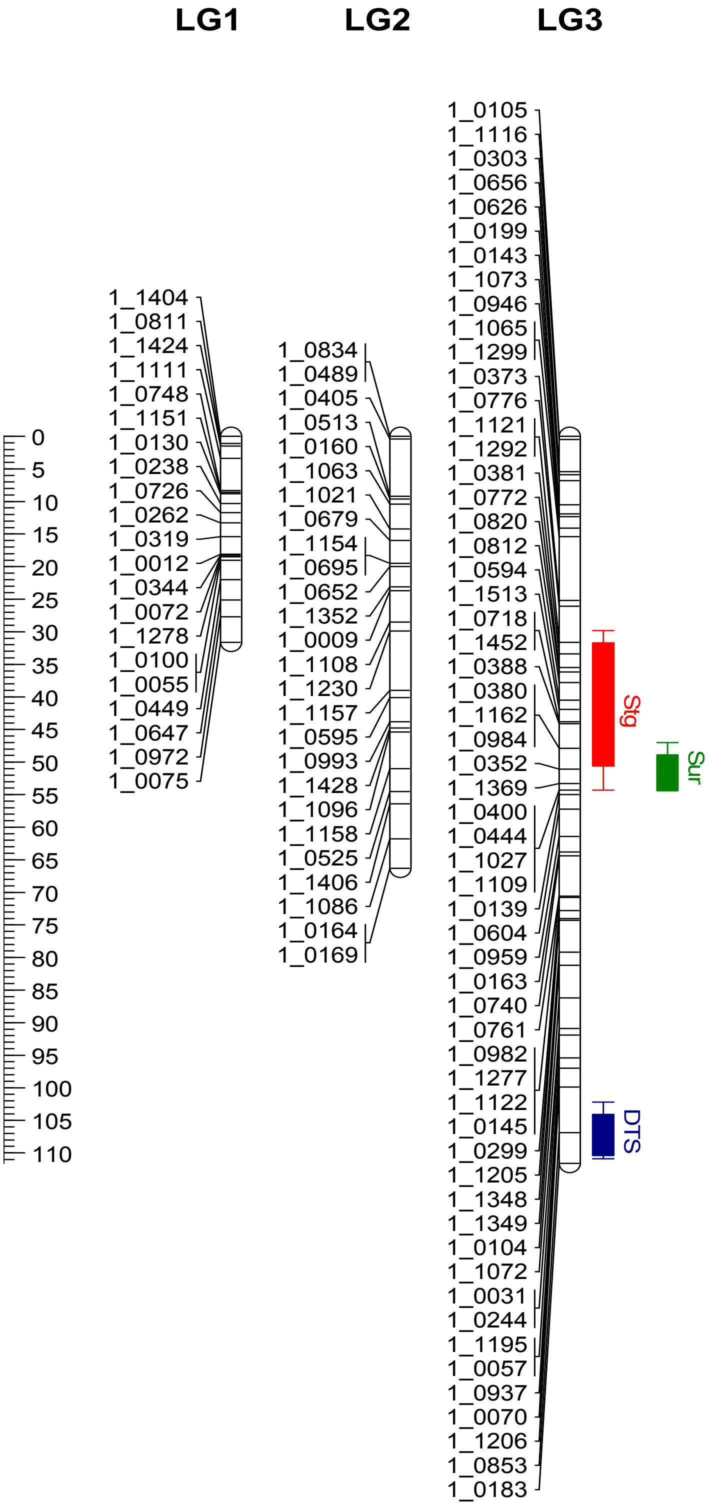

Figure 2. Single nucleotide polymorphisms (SNPs) genetic map of cowpea developed from recombinant inbred lines (RILs) derived from cross between Danila and TVu7778 showing 11 LGs named from LG1 to LG11.

Positions of QTLs for drought-induced trifoliate senescence (DTS), stem greenness (Stg) and plant ability to survive four weeks water stress and two weeks daily re-watering (Sur) are depicted as colored boxes.

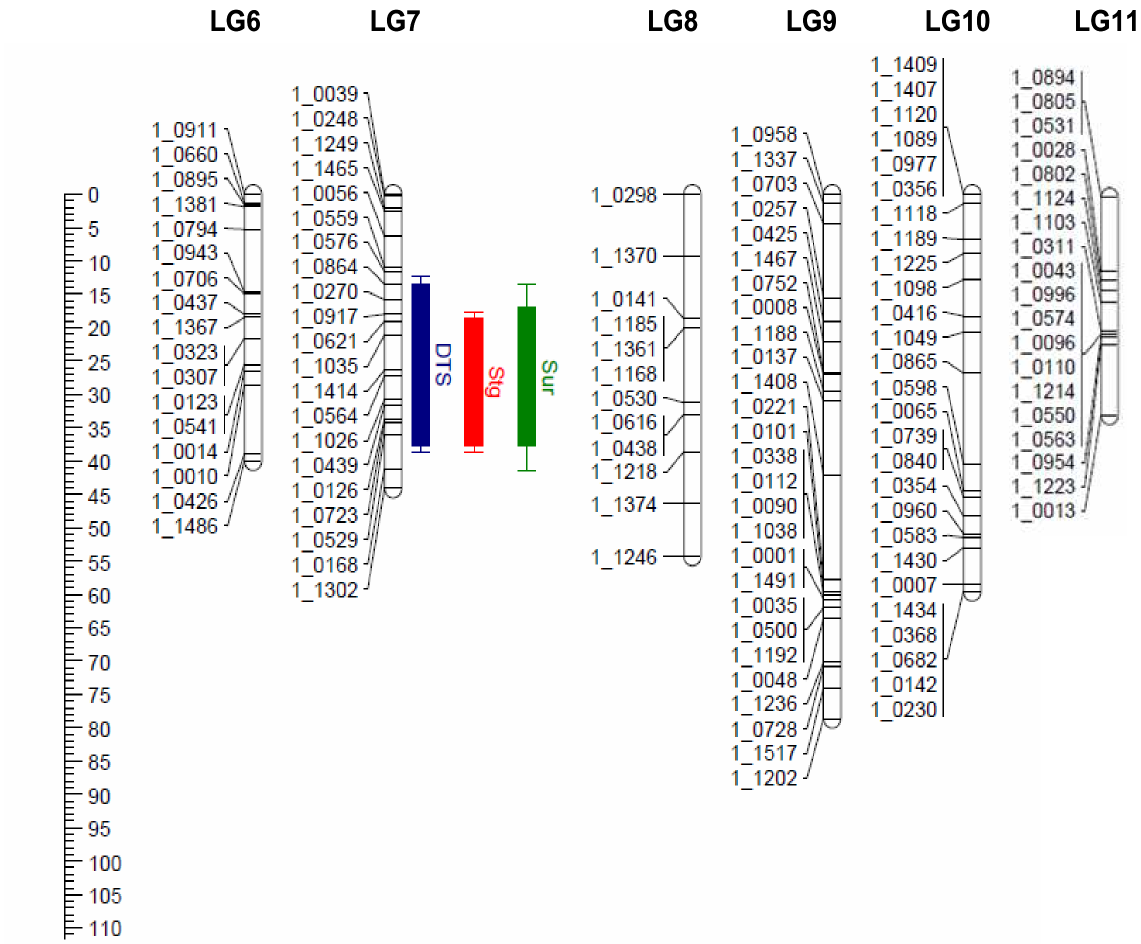

Figure 2. Continued

Use of SNP marker array and genetic map

The genetic map used for QTL analysis was constructed using a 1536 Golden Gate SNP genotyping array (Muchero et al. 2009a). Until recently only limited use was made of molecular marker techniques to enhance cowpea breeding. Restriction fragment length polymorphisms (RFLPs) have produced only a limited number of markers that could not facilitate QTL studies (Fatokun et al. 1992; 1993a; 1993b; Menancio-Hautea et al. 1993; Myers et al. 1996; Menendez et al. 1997). Random amplified polymorphism DNAs (RAPDs) were used by several researchers (Menendez et al. 1997; Mignouna et al. 1998; Fall et al. 2003; Sylla Ba et al. 2004; Badiane et al. 2004; Diouf and Hilu 2005; Xavier et al. 2005). However, RAPDs are not very reproducible between laboratories, and therefore their use for breeding is limited. Simple sequences repeats (SSRs) markers are being used in cowpea breeding, but the number of markers is still limited (Li et al. 2001; Wang et al.

2004; Diouf and Hilu 2005). Amplified fragments length polymorphisms (AFLPs) were found to be the most informative and were used successfully in many studies (Fatokun et al. 1997; Ouédraogo et al. 2001; 2002a; 2002b; Coulibaly et al. 2002; Tosti and Negri 2002; Boukar et al. 2004; Omo-Ikerodah et al. 2008; Muchero et al. 2009b). Currently, SCAR markers converted from AFLP markers are being used for implementation of marker assisted breeding for only some Striga resistance genes SG1 in Burkina Fasso (Ouedraogo et al. 2002a) and SG3 in Nigeria (Boukar et al. 2004). The availability of a 1536 SNP array opens up new possibilities for cowpea genomics and breeding, in particular for quantitative analysis of more complex traits such as grain yield for the drought prone environments of Africa. Single nucleotide polymorphisms (SNPs) offer important advantages over the above-described marker systems used for cowpea breeding so far. SNPs are the most abundant type of genetic polymorphism in most, if not all genomes (coding and non-coding regions) (Slate et al. 2009). SNPs have greater utility than the other marker types for their higher genotyping efficiency, data quality, genome-wide coverage, analytical simplicity and cost effectiveness (Morin et al. 2004). For more details on the advantages (and disadvantages) of SNPs relative to other types of molecular marker-systems see Morin et al. (2004). The 1536 SNP array of cowpea was developed using EST information of 11 different cowpea genotypes, and has been used for genotyping 7 cowpea mapping populations and the construction of a consensus genetic map. This enables the integration of many breeding research results, comparing QTL loci between different genetic backgrounds, which can enhance breeding for improved varieties in cowpea.

Out of the 1536 SNP used for genotyping the Danila and TVu7778 RIL population 302 SNPs (~20%) were polymorphic between the parents and segregating in the RIL population. More than 50% of the SNPs screened were monomorphic. This may be the result of limited allelic variation between the parents. However, it should be taken into account that the 1536 SNPs screened were mainly selected from 11 different cowpea genotypes and from synteny regions of soybean and Medicago. DanIla and Tvu7778 were not included. Many of the SNP available in the cowpea genotypes used for EST sequencing may not be present in the mapping population under study, depending on the genetic relatedness of the parents of the mapping population with the EST-donating genotypes. This is exemplified by results from a similar effort in our laboratory with a 384 SNP genotyping GoldenGate array based on EST sequence variation in 3 potato cultivars that was used for mapping in two mapping populations, yielding about 45% monomorphic SNP (Kumari personal communication). Six more RIL populations were screened with the same set of 1536 SNPs and most of the populations had only slightly less monomorphic loci than the RIL population used in this study (Muchero et al. 2009a). In general cowpea as a self-pollinating plant has limited diversity in its gene pool as was reported by several researchers. Genetic background within the germplasm used for plant breeding is very narrow and represents only a small part of genetic variation of the entire species (Tanskley and McCouch 1997).

In our RIL population, which is the 10th generation of selfing, we can expect most of the loci used for mapping to be homozygous. However, four RILs (DT1-27, DT1-28, DT1-66 and DT1-115) showed a high level of heterozygosity. This suggests that these genotypes were not genuine RILs, and possibly were resulting from recent intercrossing or outcrossing. Alternatively, there might have been a mix-up of DNA samples. In support of the former explanation, several of the genotypes that displayed a high level of heterozygosity also behaved as off-types in the terminal drought field trials described in Chapter 4.

A total of 282 SNPs with minor allele frequency between 0.381 and 0.495 were successfully mapped. The size of LGs and the number of loci per LG varied significantly, the largest being LG 3 (111.6 cM) with 58 loci and the smallest being LG1 (31.6 cM) with 21 loci. Variations in LGs is in agreement with Barone and Saccardo (1990) who studied the karyotypes of cowpea and reported that cowpea has one long chromosome and nine chromosomes of intermediate sizes. Size variation in LGs was also observed for wild type of cowpea *Vigna vexillata* (Ogundiwin et al. 1999; 2005). Compared to previous cowpea genetic linkage maps, similar distances 643 cM, (Muchero et al. 2009b), 669.8 cM (Ubi et al. 2000), 684 cM (Menancio-Hautea et al. 1993) were observed, but also larger distances, 2670 cM (Ouédraogo et al. 2002a), 972 cM (Menéndez et al. 1997) and 1620 cM (Omo-Ikerodah et al. 2008) were reported. The differences in map distance could be a result of the use of different software (JoinMap or Mapmaker) or other factors such as differences in plant material and/or marker-systems and marker scoring. The genetic map for DanIla x Tvu7778 presented here spans 633 cM and has similar distance (680 cM) with the integrated genetic map of in total 928 SNPs loci developed from 7 RILs population including the RILs population studied herein (Muchero et al. 2009a). Linkage groups in our map aligned with the consensus map and marker order agreement between the two maps suggesting that our map is accurate. Still a few marker order conflicts were observed compared with the consensus map which may indicate that chromosomal rearrangement and/or translocations events differentiate the different cowpea genotypes.

QTLs for drought tolerance at seedling stage

Using a shallow wooden box for drought stress tolerance assessment at the seedling stage served to eliminate the effect of the cowpea root system in up-taking water from deeper soil, allowing one to screen the role of leaves and stem for cowpea seedling survival under drought stress. All the three traits examined in this study (DTS, Stg, Sur) varied significantly between parents and between RILs. In our results, maintenance of stem greenness appeared to be the best indicator of cowpea plant seedling survival as shown by high correlation (0.911) between the two traits. Our results are in agreement with those of Muchero et al. (2008) who studied 14 cowpea genotypes using small plastic pots to eliminate competition among genotypes for communal water source while testing leaves and stems characteristics for cowpea seedling survival. The authors found that some genotypes preserved stem greenness much more than others, and stem greenness was a reliable predictor of survival ($r \geq 0.6011$). The higher correlation between the Stg and Sur in our study may be due to the scoring methods. We scored survival in two classes: 0 (plant did not survive) and 1 (plant did survive) while Muchero et al. (2008) scored three categories of plant survival 1 (when recovery occurred from apical meristem), 0.5 (when recovery occurred from the basal meristem) and 0 (when plant did not recover). Using the wooden box screening and different scoring Mai-Kodomi et al. (1999) described two mechanisms of drought tolerance (Type 1 and Type 2) in cowpea including Danila and TVu7778. Danila was described as showing Type 2 mechanism while TVu7778 was one the most susceptible to drought stress. In a Type 1 response, plants ceased all growth and conserved moisture in all plant tissues, thereby allowing subsequent recovery of the entire shoot following rehydration. In contrast, a Type 2 response involved plants mobilizing moisture from lower leaves to sustain growth of new trifoliates, with rapid senescence of unifoliates at the onset of drought conditions. Inheritance studies suggested single-gene control for both Type 1 and 2 tolerance mechanisms (Mai-Kodomi et al. 1999). We used the same wooden box technique as described by Singh et al. (1999a) and used by Mai-

Kodomi et al. (1999), but our findings did not confirm the single-gene control described by these authors. This might be due the intensity of water stress and to differences in scoring methods. We imposed water stress on plants for about 30 days while water stress was applied for only fifteen days with Singh et al. (1999a). Within 2 weeks of water stress plant may be classified in two categories while with prolonged water stress the plants show even more variations in their response to drought stress. Muchero et al. (2008) imposed water stress for 30 days and in line with our results showed the quantitative nature of drought tolerance traits at the seedling stage.

QTL analysis revealed two QTLs for each of the traits, on LG3 and LG7. QTLs for the DTS, Stg and Sur fall in the same regions on LG7 while on LG3 QTLs were found in different regions. This agrees with the high correlation found between these two traits. The QTL identified on LG7 between 13.37 cM and 37.68 cM controls maintenance of stem and leaf greenness as well as survival ability of the cowpea plant under drought condition. These results confirm those of Muchero et al. (2009b) who used AFLP genetic map of a RILs population derived from cross IT93K503-1 x CB46 and reported consistent and co-localizing QTLs for stem greenness and plant survival under greenhouse. These QTLs were also highly reproducible in field conditions (Muchero et al. 2009b). The RIL population from IT93K503-1 x CB46 is one of the populations used for the consensus map. Comparison of QTL results from Danila x TVu7778 and IT93K503-1 x CB46 RILs populations revealed that QTLs for DTS, Stg and Sur we reported here perfectly co-localize in the same regions of LG7 with those identified for seedling drought-induced senescence traits (Muchero et al. 2009b). Obviously the LG7 QTL is important for seedling survival under drought in different genetic backgrounds, in different greenhouse experiments and in field conditions. This also exemplifies the potential of the SNP consensus map, which allowed confirmation of our QTLs with other populations in multiple environments.

In previous studies it was shown that cowpea genotypes exhibiting seedling drought tolerance were more tolerant to terminal drought under field conditions than genotypes exhibiting seedling sensitivity to drought (Singh et al. 1999a; 1999b; Muchero et al. 2008; 2009b). The importance of maintenance of plant greenness designated as delayed leaf senescence (DLS) for adaptation to water-limiting conditions was reported in cowpea by Gwathmey et al. (1992). A similar trait coined “stay-green” has been reported in sorghum (*Sorghum bicolor* L. Moench) with post-flowering drought tolerance mediated by the “stay-green" trait (Subudhi et al. 2000). Stay-green is a drought tolerance mechanism exhibited in some sorghum genotypes subjected to post-flowering drought stress. The trait allows tolerant genotypes to maintain green leaf area during the grain-filling stage, thereby allowing more productivity (Crasta et al. 1999). However, it is not clear yet whether the stay-green trait in sorghum, which is a post-flowering phenomenon and the cowpea, delayed drought-induced leaf and stem senescence traits observed at the seedling stage are regulated by similar mechanisms.

The consistency of the QTL expression for maintenance of plant greenness across populations suggests that the genomic regions harboring the identified QTL carry genes that are of major importance in determining cowpea response to drought. Moreover, the SNP marker loci in LG7 where QTL for delayed leaf senescence and stem greenness coincided across populations fall within a syntenic region between cowpea, soybean and Medicago (Muchero et al. 2009a). Further studies are needed to identify the genes that these SNP Marker loci represent or whether they are close to genes of interest. These markers loci represent potential candidates for marker-assisted selection (MAS) for seedling stage drought tolerance in cowpea that also might be important for adult plant drought tolerance.

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Chapter 4

Genetic variation, heritability and relationships among drought tolerance traits in cowpea [*Vigna unguiculata* (L.) Walp.]

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Abstract

Traits that enhance drought stress tolerance are not easily assayable in large populations or do not show enough genetic variation and heritability to serve as selection criterions in breeding programs. Two cowpea genotypes with contrasting response to drought stress were used to develop a set of 120 recombinant inbred lines (RILs). The objectives of this study were to, (i) evaluate the performances of the RILs (ii) estimate genetic variation and heritability of traits and (iii) examine relationships among traits and their effects on grain yield under water stressed and nonstressed conditions. The RILs were sown in two plots of randomized complete blocks design with three replications in the field at one location (Kano, Year 1) and two locations (Ibadan and Kano, Year 2) in Nigeria. Plants in one plot were watered to maturity while plants in the second plot were moisture stressed from four weeks after sowing. Stomatal conductance (Gs), relative water content (RWC), delayed leaf senescence (DLS), days to flowering, number of pods per plant, number of seeds per pod, seed weight, grain and biomass yields were recorded on each RIL. Moisture stress significantly affected RILs performances with number of pods per plant as the yield component most adversely affected by water stress. Except for RWC, genetic variations and heritabilities were quite important under both water regimes but were higher in well-watered conditions for all the traits. However, Gs showed greater genetic variation and heritability under drought conditions in one location (Kano, Year 2). Correlation and path analyses revealed that grain yield components (mainly number of pods per plant) and plant biomass had the largest direct effects on grain yield under moisture stress and irrigation. Gs and DLS appeared to favor grain yield indirectly through pod development and fodder yield respectively specially under drier conditions of Kano. Number of pods per plant is the most stable genetic component and a key selection criterion to determine grain yield under optimum and drought conditions. Fodder yields appear to be genetically and/or functionally linked to number of pods per plant as they were positively correlated in all cases.

Keywords: *Vigna unguiculata* L., terminal drought tolerance, DLS, delayed leaf senesecence, Gs, stomatal conductance, number of pod/plant.

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is economically the most important indigenous African legume crop (Langiyntuo et al. 2003) which is of vital importance to the livelihood of several hundred millions of people in West and Central Africa. This sub-region is where the most important cowpea production takes place, especially in drought prone areas of Northern Nigeria and Southern Niger. The ability of cowpea to tolerate drought makes it the crop of choice in these areas where annual rainfall is between 250 and 500 mm. Nevertheless, drought-related yield losses are important in cowpea. Where available, early maturing cowpea genotypes may complete their life cycle within a short period of time to escape terminal-season drought (Ehlers and Hall 1997), although they tend to perform poorly when exposed to mid-season drought (Thiaw et al. 1993). Therefore it is rational to breed cowpea varieties with enhanced drought tolerance to early-, mid- and terminal season drought stresses. Tolerance to mid- and terminal-season drought stress has received considerable attention, due to their negative effects of these stresses on yield (Dadson et al. 2005).

Breeding approaches to develop varieties with better yields in drought-prone environments have increasingly neglected empirical yield testing over several locations and years in favor of selection for physiological traits that confer drought tolerance (Blum 1988; 2005). In cereals, progress has been made by using physiological traits as selection criteria to develop better yielding varieties for water-limited areas (Richards 2004). In cowpea, morphological, biochemical and physiological traits affecting responses to drought have been identified (Turk et al. 1980; Kulkarni et al. 2000; Ogbonnaya et al. 2003; Matsui and Singh 2003; Slabbert et al. 2004; Anyia and Herzog 2004a; Souza et al. 2004; Hamidou et al. 2007). The bottleneck is that in many cases these traits did not show enough genotypic variation to allow specific breeding efforts and genetic studies. Measuring these traits in large populations can be difficult and time consuming to allow further genetic studies. However, previous studies on the parents of RILs studied herein (unpublished) revealed that traits such as stomatal conductance (Gs), relative water content (RWC), flowering time and delayed leaf senescence (DLS) are easy to measure, show genetic variation and thus were suitable criteria for screening large numbers of cowpea lines in the field for drought tolerance.

Plant stomata play a central role in plant responses to environmental conditions. In many plant species stomatal closure due to water stress reduces CO_2 fixation and in turn contributes to yield reduction (Costa Franca et al. 2000; Charves et al. 2003; Grassi and Magnani 2005; Gallé et al. 2007). Cowpea is considered a drought-avoiding plant with stomata that are extremely sensitive to water stress (Shackel and Hall 1979; Hamidou et al. 2007). Because of its negative effect on yield, complete stomata closure is not useful in breeding for drought tolerance (Mitra 2001). An alternative strategy is partial opening of stomata which has shown to be beneficial for plant yield performances under drought. Remarkable positive correlations were found between yield performance and carbon isotope discrimination (Δ) in cowpea (Hall et al. 1997; Condon and Hall 1997). Increased Δ in more productive genotypes of cowpea was probably due to more open stomata, which could have resulted in greater rates of photosynthesis. A linear correlation was found between stomatal density of cowpea and Δ across phosphorus, water and CO_2 environments examined (Sekiya and Yano 2008). Cruz de Carvalho et al. (1998) compared cowpea and common bean cultivars and found that cowpea genotypes kept their stomata partially opened and had a lower decrease in their net photosynthetic rates than the common bean genotypes.

The ability of cowpea plants to stay green or to delay leaf senescence (DLS) under drought is an important trait (Gwathmey et al. 1992) that can be assessed easily by visual observations. This trait allows the plant to stay alive through mid-season drought and enable recovery when rainfall resumes. The combination of DLS with early flowering which allow plants to produce a second flush of pods offers potential to circumvent adverse effects due to both mid- and terminal-season drought conditions (Gwathmey et al. 1992). Muchero et al. (2009) mapped QTLs mediating drought-induced senescence using a cowpea RIL population. Markers associated with QTL for delaying drought stress-induced senescence under field at seedling and post-flowering stages will enhance breebing programs for drought tolerance in cowpea.

In this study, a set of cowpea RIL population was evaluated under irrigation and drought conditions in the first year (Kano) and the second year (Kano and Ibadan). Stomatal conductance, relative water content (RWC), days to flowering and DLS were measured as well as grain and fodder yields on each RIL. The ojectives were to assess the effects of water stress on performance of the RILs, determine genetic variation and heritability of traits measured and using path coefficient analysis to identify traits that contribute directly or indirectly, to greater grain yield under both water stressed and well-watered conditions. Path coefficient analysis has been used by researchers to assess the importance of yield components, and to establish direct or indirect relationships between physiological and productivity traits in different crops (Yao et al. 2002; Condon et al. 2004; Rebetske et al. 2002; Hui et al. 2008; Ehsani-Moghaddam and DeEll 2009). This study represents a first step towards elucidating genetic factors underlying tolerance to drought. The potential of the traits examined and the relationships between the traits for breeding of cowpea varieties with increased tolerance to terminal drought are discussed.

Materials and methods

Plant material

Two cowpea genotypes, Danila and TVu7778 were crossed and the F_2 generation was advanced by repeated selfing and keeping one single seed per plant to generate the next generation for up to F_{10} giving rise to the 120 recombinant inbred lines (RILs). Danila (drought tolerant parent) is a local variety commonly grown in the Sudano-Sahelian border areas of Nigeria and Niger Republic (Mai-Kodomi et al. 1999), while TVu7778 (susceptible parent) is a germplasm line maintained at IITA. Previous physiological and yield performance studies carried out at IITA-Kano station revealed that Danila and TVu7778 showed contrasting responses to drought in the traits investigated herein (unpublished). Seeds of the RILs were multiplied in pots placed in the screenhouse and harvested seeds of each RIL were kept and later used for further field and greenhouse trials.

Field trials and Experimental design

The RILs and the parental lines were grown at two different locations in Nigeria: Kano and Ibadan IITA experimental fields. Kano (12°03’N, 8°32’E and 476m altitude) is located in Sahelian zone in Northern Nigeria while Ibadan (7°30’N, 3°54’E and 243 m altitude) is in the forest-savanna transition zone in South-West Nigeria. The experiments were carried out during the dry seasons in 2005-2006 (Kano) and 2006-2007 (Kano and Ibadan) when rainfall had ceased. This allowed imposition of drought stress on the plants. The experiments started in October in Kano while in Ibadan due to wetter soil moisture it started in December. The experiment was a randomized block design with three replications and two irrigation treatments. Irrigation and non-irrigation plots separated by a distance of 10 m were used for the study. Plants in one plot were irrigated from planting to maturity (well-watered treatment) while those in the second plot were irrigated for four weeks after sowing and watering was therefater stopped (water stress treatment). Each RIL was planted in rows spaced 75 cm apart in three replications per plot. Two seeds were sown per hole at 40 cm spacing within rows. There were ten hills per RIL per replication. Plant protection measures during experiments consisted of weeding by hand and applying insecticides several times. The mean maximum/minimum temperatures during the period of the study were 36.4/19.1°C in Kano and 32.0/22.4°C in Ibadan, while the mean relative humidity was 28% in Kano and 73% in Ibadan. Each RIL was planted in rows spaced 75 cm apart. Spacing within rows was 40 cm.

Soil samples were taken randomly (10 different points) in dry and wet plots at 0-20 cm and 20-40 cm from both locations for chemical and physical soil characterizations (Table 1). Soil temperature was measured continuously during experiments at 20 cm and 40 cm soil depths. Soil moisture was measured three times during the experiment: at the beginning of the stress treatment, 3 and 5 weeks after water withholding.

Three weeks after termination of watering, stomatal conductance (Gs) was measured on two young fully expanded leaves per row using a Steady State Diffusion Porometer (SC1, Decagon Devices). In both locations, Gs measurements were only done on sunny days from around 10 a.m. to noon when the temperatures were about 28°C ±2. The leaves used for Gs measurements were detached and weighed to get fresh weight (FW), after that they were put in small plastic bags containing water and kept on ice for 4 hours. The turgid weights (TW) were measured as well as dry weight (DW) after drying the leaves in the oven for 48 hours at 60°C. Relative water content (RWC) was determined using the formula: RWC = (FW-DW)/(TW-DW)*100 (Kramer 1980). Stomatal conductance and RWC were measured for the second time at five weeks after termination of watering, when differences among RILs were becoming obvious. Plants of the RILs were rated for drought tolerance using a 1 to 5 scale as described by Mai-Kodomi et al. (1999): 1 (normal green turgid leaves), 2 (green with slight wilting), 3 (yellowish grey with moderate wilting), 4 (yellow and light brown leaves with severe wilting), 5 (completely dried). At maturity, five plants were harvested per row and the following yield parameters were measured: number and weight of pods, total seed weight and 100 seed-weight, fresh and dry fodder weight.

Statistical procedures

Yield and its components were calculated on a plant basis and the resuling data were used for statistical analysis. Dry fodder weight was considered fodder yield (FY). Grain yield (GY) was calculated based on three independent variables using the following formula:

GY = Seed weight * Number of Seeds/pod * Number of Pods/plant

Using the average yield of each RIL under irrigation (X_i) and average yield of each line under drought (X_d), relative reduction (RR) was calculated according to the formula:

$$RR = (X_i - X_d)/X_i*100$$

Statistical analyses were carried out with GenStat 11th Edition. The data for each trait were subjected to analysis of variance using the general linear model for randomized block design with two treatments, three replications and two years and two locations. In addition, data sets of dry and wet treatments in both locations were considered as six random environments and data were analyzed separately using one-way ANOVA. For traits that showed a significant F test following ANOVA, regression and correlation analyses were performed between the traits measured in each particular environment. Data from the parents were excluded from the data set and heritability was calculated according to the formula:

$$h^2_m = \sigma^2_g / \sigma^2_g + \sigma^2_e$$

where genetic variance $\sigma^2_g = (MS_g - MS_e)/r$, variance due to errors $\sigma^2_e = MS_e/r$, r = number of replications. MS_g is considered as total phenotypic variation and MSe is an estimate of non-genetic variation. To investigate the inter-relationships among the variables and their direct and indirect contributions to yield performance under well-watered and water-stressed conditions, entry means of variables that showed high genetic variation were subjected to correlation and path analyses (Dewey and Lu 1959; Lal et al. 1997).

Table 1. Some chemical and physical characterisations of soil at the beginning of the experiments.

Site	Soil depth	Zn	Cu	Fe	Mn	N	Sand	Silt	Clay
		ppm				%			
Kano	0-20cm	6.82	0.44	50.35	33.45	0.02	83	7	10
	20-40cm	6.15	0.37	48.10	26.78	0.02	80	9	11
Ibadan	0-20cm	10.24	2.71	130.73	275.27	0.09	71	15	14
	20-40cm	8.92	4.17	148.13	347.2	0.09	69	11	20

Results

Genetic variation of traits observed under well-watered and water-stressed conditions

There were highly significant differences between the RILs and interactions between RILs and treatments, locations and treatment x locations for all the traits except for RWC and days to flowering. The mean sums of squares (MS) and experimental errors (MSe) of analysis variance for randomized block design with three replicates, two treatments and two locations are presented in Table 2. The ANOVA indicate the presence of highly significant G x E interactions and also show that the RILs performed differently under irrigation and drought conditions in both locations. The interactions of RILs with treatment, location and treatment x location were not significant for RWC. Flowering time showed significant variation for interactions between the RILs and location but RILs x treatment and treatment x location interactions were not significant.

When data were analyzed separately as treatment per location, using one-way analysis of variance, all response variables with exception of RWC showed significant variations under irrigation as well as drought conditions in both locations. However, the variations tended to be greater under well-watered conditions for all traits, except Gs which conversely showed greater variation under drought. Not much variation was observed in stomata behaviour among the RILs when plants were well-watered. However, water stress induced more genetic variations between RILs for Gs, and plants showed different reactions to the water stress by closing their stomata partially or completely.

Table 2. Mean sum of squares (MS) and experimental errors (MSe) for the traits evaluated derived from ANOVA general linear model.

Source of Variation	Gs	RWC	DLS	Flowering	Pod/plant	Seed/pod	Seed weight	GY	FY	TY
RIL	4232**	29.51*	1**	31.81**	52.77**	9.6**	0.18**	115.81**	123.19**	412.19**
Site x RIL	4057**	24.91	0.88**	28.97**	27.64**	5.14**	0.12**	37.9**	40.98**	108.96**
RIL x Treat	3208**	19.04	0.68*	16.13	14.31**	6.02**	0.06**	26.18**	33.45**	66.69**
Site x RIL x Treat	3143**	21.36	0.77**	16.34	14.7**	7.11**	0.06**	18.33**	22.01**	46.07**
Errors	1755	23.71	0.46	16.05	6.66	3.46	0.03	10.24	10.65	22.89

*, ** significant ar 5% and 0.1% levels of probability respectively

Gs: stomatal conductance (mmol $s^{-2}m^{-1}$), RWC: relative water content, DLS: delayed leaf senescence, GY, grain yield (g/plant), FY, fodder yield (g/plant), TY, total yield (g/plant)

Heritability of the traits

Variation among plants observed in field experiments is due to the combined action of genetic and environmental factors. Heritability is a measure for the proportion of variance observed among plants that is due to genetic differences. Trait-specific heritabilities obtained from the studies under irrigation and water stress over the two years are presented in Table 3. Heritability varied over treatment, location and year. Of all the traits, RWC showed the lowest heritability ($h^2_m \leq 0.10$), followed by seed weight under drought in Kano ($h^2_m = 0.16$), while for the other traits heritabilities varied between 0.20 and 0.88. The highest heritability ($h^2_m = 0.88$) was observed for total yield under well-watered conditions in Kano. With exception of that for stomatal conductance in Kano, all heritabilities obtained from the trials in Kano and Ibadan were greater under irrigation than under drought. Heritabilities did not differ very much between locations under well-watered conditions, while under water stress, the estimates for h^2_m of Gs and seed weight were strikingly higher in Kano (0.85 and 0.73) than in Ibadan (0.24 and 0.16, respectively). These results are a consequence of higher experimental errors for the traits in the Ibadan trial.

Effects of water stress on RIL performances

Adequate water supply is critical to plant survival, growth and crop yield. An overview of the performance of the RIL population and its parents under irrigation and drought is presented in Table 3. Water stress caused a significant reduction in stomatal conductance (Gs), while no reduction was observed for relative water content (RWC) in both locations. The reduction of Gs was greater at five weeks compared to three weeks after cessation of watering, indicating that cowpea plants close their stomata with progressive Soil drying. Percentage of reduction of Gs was lower in P_1 (Danila, the tolerant parent) compared with P_2 (TVu7778, the susceptible parent) indicating that Danila kept its stomata partially opened compared to TVu7778. Flowering time varied in the RIL population but was not correlated with drought tolerance in either location. The response to drought by delayed leaf senescence was described with five different classes (1-5). Plant greenness was higher under irrigation compared to those in water stressed plots in both locations. However, under water stress onset of senescence occurred early among some RILs while others kept their leaves green for a longer period than others (Figure 1). Under well-watered conditions yield performances of the two parents at both locations were similar while reductions due to water stress were much higher with TVu7778 compared to those for the tolerant Danila. For yield performances of the RILs, the reductions due to water stress were considerable for number of pods/plant, grain yield (GY), fodder yield (FY) and total yield (TY) in over the two years in both locations. There was a moderate reduction in seed weight and a negligable reduction observed in number of seeds/pod due to water stress. Of the three grain yield components, number of pods/plant was most drastically reduced by 63.6 percent in Kano and 59.4 percent in Ibadan. A moderate reduction was observed for seed weight and a negligable reduction for number of seeds/pod. Of the three grain yield components, only number of pods/plant reduced drastically by 36.4% in Kano and 40.6% in Ibadan. These results suggested that under water stress, cowpea plants reduced yields is mainly attributed to a decrease in the number of pods while the number of seeds per pod and size of seed was maintained at the same level as in well-watered conditions.

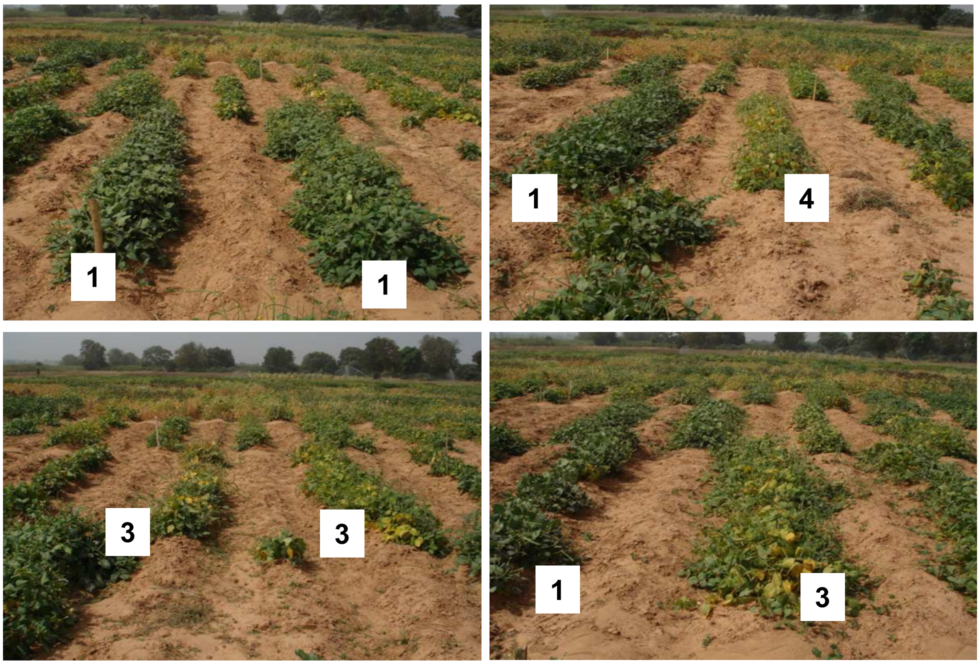

Figure 1. Shows variations in the ability of RILs to delay leaf senescence (DLS).
1 (normal green turgid leaves), 2 (green with slight wilting), 3 (yellowish grey with moderate wilting), 4 (yellow leaves with severe wilting).

Table 3. Mean performances of parents and RILs, genetic and non-genetic variances and heritability of measured traits in two contrasting water regimes in Kano and Ibadan

				Performance							
	Factor			Parent		RILs			Variance		
Trait	Treat	Site	Year	P_1	P_2	Mean	min.	max.	σ^2_g	σ^2_e	h^2_m
Gs	W	K	Y2	200.3	238.4	175.4	50.8	410.8	336.0	850.3	0.28
		I	Y2	218.6	230.9	246.0	136.0	404.0	313.7	520.7	0.37
	D	K	Y2	158.7	97.2	95.2	17.7	227.6	1542.1	271.0	0.85
		I	Y2	154.8	75.7	121.1	10.9	297.5	225.0	700.7	0.24
RWC	W	K	Y2	87.5	84.7	89.5	52.4	98.5	0.4	7.4	0.05
		I	Y2	83.9	86.5	86.3	60.6	99.5	1.0	8.1	0.10
	D	K	Y2	91.0	91.9	89.4	74.2	99.1	0.1	5.8	0.02
		I	Y2	80.9	87.3	86.0	61.8	99.1	0.5	9.2	0.04
Flow	W	K	Y2	49.3	51.3	49.5	38.0	62.0	3.8	2.4	0.62
		I	Y2	47.0	43.7	43.4	36.0	76.0	1.9	4.5	0.30
	D	K	Y2	50.6	46.7	48.3	33.0	60.0	3.3	7.0	0.32
		I	Y2	41.0	39.7	40.6	34.0	57.0	1.8	6.6	0.21
DLS	W	K	Y2	1.3	1.5	2.1	1.0	4.0	0.2	0.1	0.75
		I	Y2	1.0	1.7	1.9	1.0	3.0	0.1	0.4	0.45
	D	K	Y2	2.3	4.7	3.3	1.0	5.0	0.1	0.1	0.60
		I	Y2	2.7	3.3	4.1	1.0	5.0	0.1	0.1	0.36
Pod/P	W	K	Y2	25.6	23.7	17.1	4.2	40.0	11.2	3.5	0.76
		K	Y1	27.8	26.7	24.6	9.4	56.3	14.3	8.5	0.62
		I	Y2	14.7	13.3	13.7	8.0	27.5	3.8	0.8	0.83
	D	K	Y2	23.9	16.9	10.9	0.0	26.0	6.7	6.3	0.51
		K	Y1	22.7	22.1	14.6	0.0	29.8	10.4	6.7	0.63
		I	Y2	10.2	7.5	8.1	0.0	18.5	4.6	1.2	0.79
S/P	W	K	Y2	7.7	11.1	6.9	3.0	18.7	0.9	0.7	0.58
		K	Y1	7.8	7.7	6.8	3.5	13.4	1.4	0.8	0.63
		I	Y2	8.2	8.0	7.6	3.5	14.5	1.6	0.7	0.68
	D	K	Y2	7.9	6.4	6.4	0.0	15.3	1.0	1.9	0.33
		K	Y1	10.8	8.9	6.7	0.0	13.0	1.0	1.7	0.37
		I	Y2	8.7	6.2	7.4	0.0	13.7	0.8	2.0	0.29
SW	W	K	Y2	15.0	12.4	12.1	8.0	20.0	0.3	0.1	0.85
		K	Y1	15.8	14.5	13.6	8.2	23.6	0.4	0.1	0.80
		I	Y2	14.6	13.7	12.0	8.0	17.2	0.1	0.1	0.66
	D	K	Y2	13.1	11.2	10.0	0.0	16.0	0.4	0.1	0.78
		K	Y1	15.2	9.8	11.4	0.0	19.4	0.3	0.2	0.60
		I	Y2	14.2	6.9	10.0	0.0	17.0	0.0	0.2	0.16
GY	W	K	Y2	29.6	32.5	14.7	4.0	74.3	30.7	5.9	0.84
		K	Y1	30.6	30.4	17.3	6.2	80.4	34.5	10.3	0.77
		I	Y2	17.4	14.6	12.1	4.4	45.1	9.4	2.9	0.76
	D	K	Y2	23.9	14.9	8.1	0.0	30.2	8.8	4.4	0.66
		I	Y1	25.1	8.7	15.1	0.0	38.5	28.4	10.1	0.74
		I	Y2	12.1	6.1	6.5	0.0	20.6	3.3	1.8	0.64

Table 3. continued

				Performance							
	Factor			Parent		RILs			Variance		
Trait	Treat	Site	Year	P_1	P_2	Mean	min.	max.	σ^2_g	σ^2_e	h^2_m
FY	W	K	Y2	28.2	32.4	20.8	10.5	61.0	16.6	4.1	0.80
		K	Y1	30.2	33.5	20.3	12.7	64.2	30.3	15.2	0.66
		I	Y2	28.6	31.3	27.2	10.6	54.2	15.5	4.8	0.76
	D	K	Y2	20.8	11.6	10.3	2.1	40.6	11.4	6.6	0.64
		K	Y1	23.1	22.4	20.1	5.6	39.8	25.7	13.6	0.65
		I	Y2	15.5	13.7	12.8	2.8	45.1	10.7	3.8	0.73
TY	W	K	Y2	54.2	63.9	35.5	19.3	129.5	84.8	11.3	0.88
		K	Y2	57.7	55.1	39.2	15.7	143.5	80.2	15.6	0.84
		I	Y1	46.0	45.9	39.3	19.6	86.4	36.9	7.7	0.83
	D	K	Y2	44.7	26.5	18.4	6.0	70.7	31.6	16.2	0.66
		K	Y1	47.4	32.5	28.3	8.9	111.3	87.8	21.6	0.80
		I	Y2	27.6	19.8	19.3	6.8	64.9	19.4	5.9	0.76

Gs; stomatal conductance (mmol s-2m-1), RWC; relative water content (%), Flow days to flowering, DLS; delayed leaf senescence, Pod/P; number of pod per plant, S/P; number of seed per pod, SW; seed weight (g), GY; grain yield (g/plant), FY; fodder yield (g/plant), TY; total yield (g/plant) σ^2_g: genetic variance, σ^2_e: variance that is not explained by genetic effects, P_1: Danila (torerant parent), P_2: TVu7778 (susceptible parent), K: Kano, I: Ibadan, Y1: first year dry season 2005-2006, Y2: second year dry season 2006-2007, W: well-watered, D: water-stressed, min. and max.: minimun and maximum values measured, h^2_m: heritability based on entry mean.

Correlations and path analyses

A correlation coefficient is a measure of the relationship between two variables while a path coefficient is a standardized partial-regression coefficient and a measure for the direct influence of one dependent variate on the variance for the response variate. A path analysis allows the separation of correlation coefficients into components of direct and indirect effects. RWC was excluded from the path coefficient analysis as Pearson's simple correlation test revealed no correlation between RWC and all the other traits. Eight variables including stomatal conductance, delayed leaf senescence, flowering time, number of pod per plant, number of seed per pod, seed weight, grain and fodder yields were included in the path analyses. The direct and indirect effect of 7 variables on grain yield performances under drought and irrigated conditions were calculated for each location. Correlation coefficients of each performance variable under water stress and irrigation with GY_D and GY_I in both locations are presented in Table 4. Low to high correlations were found between the 7 variables and grain yields. Correlations were generally higher between grain yield and its components and fodder yields, moderate to negligible between grain yield and Gs, days to flower and DLS. However, because of the inter-relationships among variables, different variables contribute positively or negatively to the observed coefficients.

Table 4. Correlation coefficients of 7 variable performances under drought and irrigation on GY_D and GY_I in Kano and Ibadan.

		GY_D		GY_I	
Variable	Location	Drought	Irrigation	Drought	Irrigation
Pod/P	K	0.845	0.594	0.650	0.767
	I	0.683	0.217	0.272	0.597
SW	K	0.437	0.266	0.209	0.238
	I	0.233	0.072	0.117	0.217
S/P	K	0.561	0.148	0.266	0.521
	I	0.459	0.133	0.069	0.667
Gs	K	0.223	0.073	0.179	0.143
	I	-0.085	0.007	-0.012	0.010
Flowering	K	0.034	0.123	0.065	0.236
	I	0.024	0.073	0.171	-0.011
DLS	K	-0.147	-0.284	-0.314	-0.379
	I	-0.002	-0.086	0.023	0.131
FY	K	0.543	0.600	0.463	0.674
	I	0.330	0.412	0.311	0.374

GY_D: grain yield under drought, GY_I: grain yield under irrigation, Pod/P: number of pod per plant, SW: seed weight, S/P: number of seed per pod, Gs: stomatal conductance, Flowering: days to flowering DLS: delayed leaf senescence, FY: fodder yield (g/plant), K: Kano, I: Ibadan.

For instance, the correlation coefficient of number of pods/plant under drought with GY_D in Kano is r = 0.845 (Table 4). The following example illustrates the path analysis partitioning (Table 5). Partitioning of the correlation coefficient of pod number/plant with GY_D into its components clearly shows that the indirect contributions of the other variables, with main contributors of fodder yield (0.107) and seeds per pod (0.100) only partly contribute to the high correlation (0.845) between pod number/plant and grain yield under drought (GY_D). Examination of correlation components of path analysis as shown in Figure 2 revealed that the largest direct contribution to grain yield under drought (GY_D) was that of number of pods/plant, followed by fodder yield. Plant fodder yield performance in both water regimes showed important direct effects on grain yield under drought. This is an indication that bigger plants maintain better grain production under drought than small plants (Fig 2A). Conversely, plant size played a negligible direct effect on grain yield under irrigation (GY_I)

and the most important direct contributors to GY_I are number of pods/plant, seed size and number of seeds/pod (Fig 1 B). These patterns as shown in path diagram for Kano (Fig. 2) were different in Ibadan (Fig 3). In all cases (Fig. 2, 3), number of pods/plant is the only trait that showed strong direct influences on grain yield production in both water regimes and locations. Additionally, different variables contribute indirectly to pod development as they showed positive inter-relationships with number of pods per plant with plant biomass being the main contributor (Figs. 2 and 3). The correlation coefficient of Gs with GY_D under drought in Kano is r = 0.223 which is partitioned mainly into its relationship with pod number/plant (r = 0.232) (Fig 2A). Delayed leaf senescence (DLS) had negligible direct effects on GY_D and GY_I, but its indirect effects via days to flowering and fodder yield were high thereby counterbalancing the very low direct effects on GY_D and GY_I in Kano. However in Ibadan, the Gs and DLS seem to not be informative as both indirect and direct effects were negligible. To some extent, flowering time did show direct effects on GY_I (Figs. 2B, 3B) but no direct or indirect influence on GY_D in both locations.

Table 5. Shows the separation of the the total correlation coefficient between pod number/plant and GY_D into components of direct and indirect effects

Direct and indirect effects of variables on GY_D	$[r_{ij}]$ x $[P_{i\text{-}Y}]$	$[r_{i\text{-}Y}]$
Indirect effect via seed weight	0.431 x 0.086	0.037
Indirect effect via number of seed/pod	0.501 x 0.200	0.100
Indirect effect via stomatal conductance	0.232 x 0.055	0.013
Indirect effect via flowering time	0.001 x 0.034	0.000
Indirect effect via delayed leaf senescence	0.045 x 0.023	0.001
Indirect effect via fodder yield	0.438 x 0.246	0.107
Direct effect of number of pods/plant with GY_D		0.587
Total (correlation between number of pod/plant and GY_D)		0.845

$[r_{ij}]$ = simple correlations among variables and number of pods per plant, $[P_{i\text{-}Y}]$ = represent the path coefficients, $[r_{i\text{-}Y}]$ = simple correlations between number of pod per plant and grain yield under dry condition (GY_D)

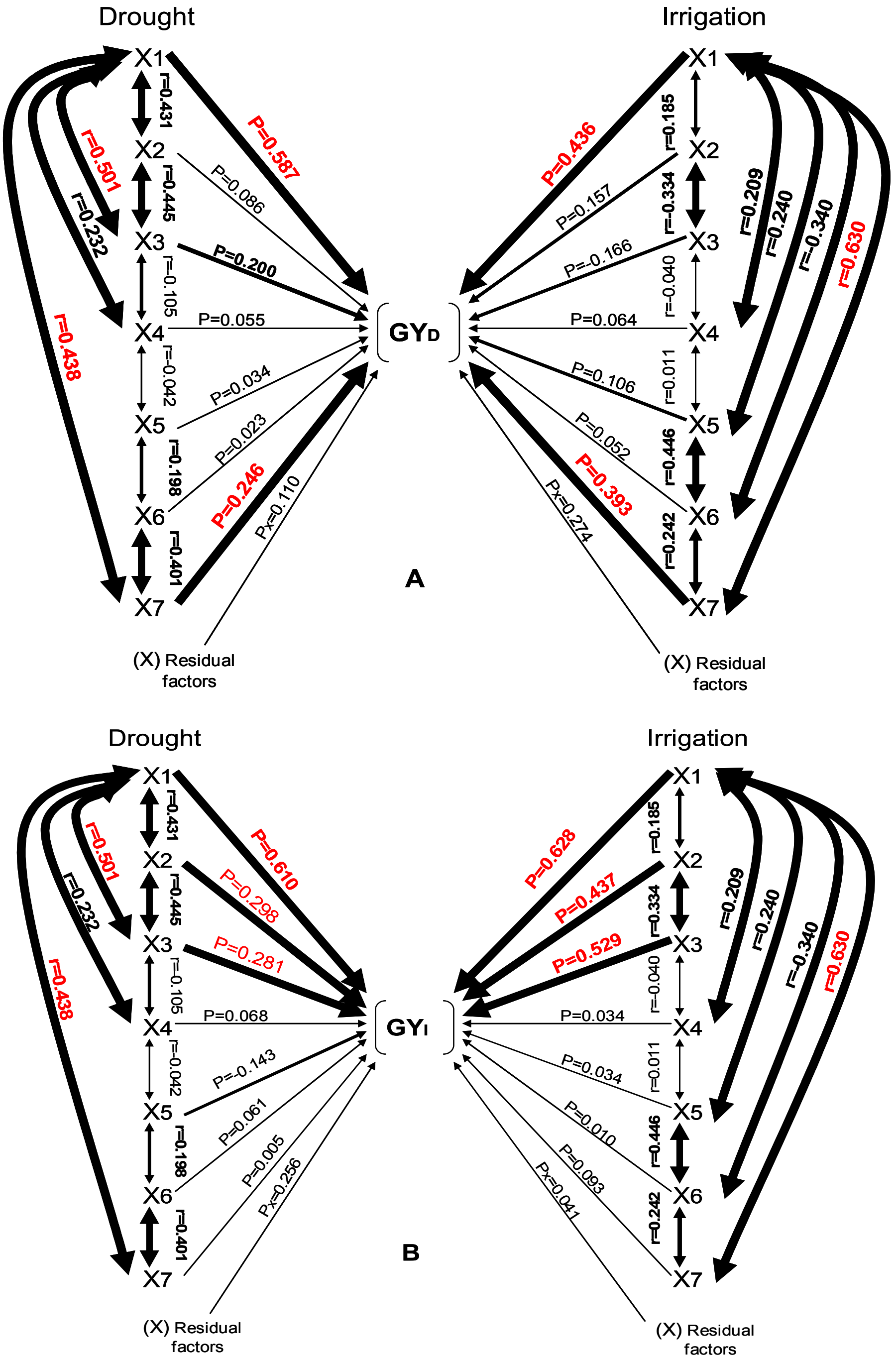

Figure 2. Diagram showing correlations and path coefficients 7 factors influencing grain yield production under drought (A) and under irrigation (B) in Kano. Double-arrowed lines indicate mutual association as measured by correlation coefficients (r) and the single-arrowed lines represent direct influence as measured by path coefficients (P). Bold lines indicate stronger effect. Negligible correlation and path coefficients are omitted.

GY_D: grain yield under drought, GY_I: grain yield under irrigation, X1: number of pod per plant, X2: seed weight (g), X3: number of seed per pod, X4: stomatal conductance (mmol $m^{-2}s^{-1}$), X5: days to flowering, X6: delay of leaf senescence (DLS), X7: fodder yield (g/plant), (X): consists of all residual factors that influenced GY_D and GY_I.

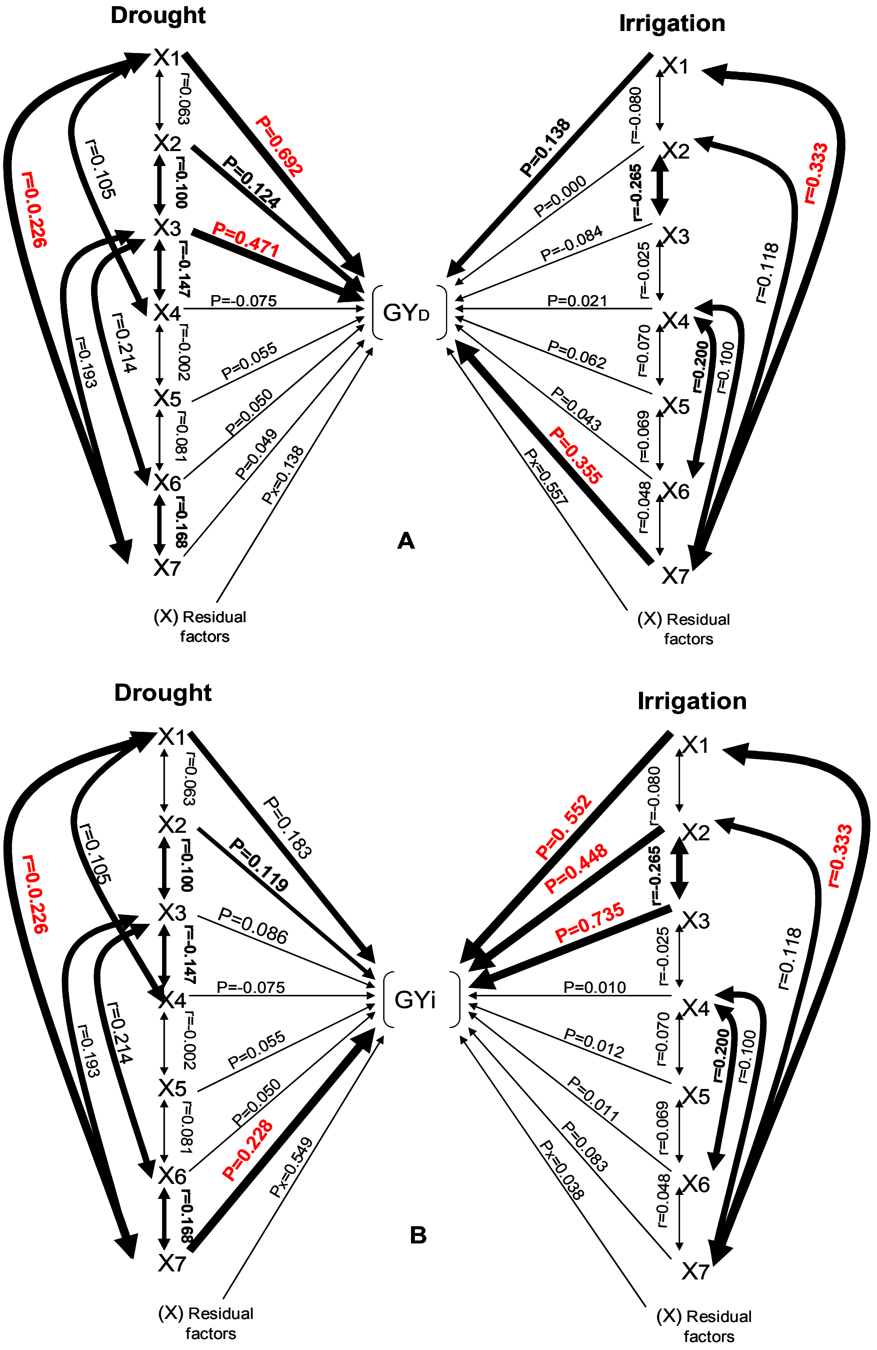

Figure 3. Diagram showing correlations and path coefficients 7 factors influencing grain yield production under drought (A) and under irrigation (B) in Ibadan. Double-arrowed lines indicate mutual association as measured by correlation coefficients (r) and the single-arrowed lines represent direct influence as measured by path coefficients (P). Bold lines indicate stronger effect. Negligible correlation and path coefficients are omitted.

GY_D: grain yield under drought, GY_I: grain yield under irrigation, X1: number of pod per plant, X2: seed weight (g), X3: number of seed per pod, X4: stomatal conductance (mmol $m^{-2}s^{-1}$), X5: days to flowering, X6: delay of leaf senescence (DLS), X7: fodder yield (g/plant), (X): consists of all residual factors that influenced GY_D and GY_I.

Discussion

Genetic variation, heritability and correlation of the traits

Traits with high genetic variation, heritability and strong correlations with grain yield under drought are desirable for making progress in plant breeding for drought tolerance. The results of our studies showed that genetic variation and heritability estimates for the measured traits, except for RWC, are important under both water regimes but higher in well-watered conditions. Under water stressed conditions in Kano genetic variation and heritability estimates were highest for stomatal conductance (Gs). The performances of the RILs did not seem to follow similar trends across locations and whether plants were stressed or not. We have therefore used path analysis to identify selection criteria for increasing grain yield in cowpea under drought and well watered conditions. While genetic correlation analysis simply measures the relationships between two traits, and is unable to elucidate the related mechanisms among them path analysis can dissect the correlation coefficient into direct and indirect effects and quantify the relative contributions of each component to the overall correlation (Yao et al. 2002; Condon et al. 2004; Rebetske et al. 2002; Hui et al. 2008; Ehsani-Moghaddam and DeEll 2009).

In Kano (with drier weather and poorer soil), number of pods per plant and fodder yield had the largest direct contributions to grain yield under drought (GY_D) and therefore would be good traits for selection under drought. The bigger the plant size, the greater the pod number per plant under drought. Conversely under well watrered conditions plant size is not an indicator for grain yield in Kano. In Ibadan (cooler weather and soil with greater clay content), each of the grain yield components and fodder yields could be used for selection under both irrigation and drought. In both locations number of pods per plant is the only trait that showed strong direct influence on grain yield production in both water regimes. Number of pods per plant therefore is genetically the most stable component and possibly a key selection criterion for increasing grain yield under optimum and drought conditions. Number of pods per plant and fodder yields appeared to be genetically or functionally linked as they were positively correlated in all cases.

Other traits such as Gs and DLS positively affect the number of pods per plant and indirectly grain yield, especially under the drier conditions of Kano. RamirezVallejo and Kelly (1998) already suggested that number of pods per plant is a quantitative trait in which multiple genes are involved. Gs and DLS in Kano could be good physiological indicators for drought tolerance in Kano but not in Ibadan. Mitra (2001) indicated that stomatal closure it is not useful in breeding for drought tolerance because of its negative effect on yield. Nevertheless, several studies indicate that keeping stomata at least partially open is a trait that can be a useful target for breeding for drought tolerance (Cruz de Carvalho et al. 1998). Some cowpea genotypes do not close their stomata completely in water deficit conditions. This was the case with Danila (tolerant parent) of which stomatal conductance in water deficient was higher under drought when compared to TVu7778 (susceptible parent) (Table 3), indicating that the tolerant parent kept its stomata partially opened under drought. Partial opening of stomatal aperture with cowpea genotypes was also reported by Cruz de Carvalho et al. (1998). The little direct effects of Gs and DLS on grain yield under drought (GY_D) and irrigation (GY_I) as revealed by path coefficients (Fig 2, 3) indicate that these traits have only partial contributions to GY_D and GY_I. Evaluation of more morphological, physiological and biochemical traits is needed that may add increments to yield and yield components under drought and well-watered conditions.

Breeding for phenological traits such as early flowering has been very successful and a number of improved cowpea varieties (i.e. IT84S-2246, Bambey 21) have been released which can reach maturity at 60 days after planting. Lines such as these can escape the effects of terminal drought and still produce appreciable grain yield. Hence positive association between reduction of days to flowering and grain yield is desirable. This was not the case in this RIL population. Although significant genetic variation was observed for flowering time both under irrigation and drought, correlation and path coefficients indicated no strong relationship between earliness and grain yield under drought condition. The time of year that the studies were carried out coincided with when day lengths were short. Cowpea responds to day length as typical of quantitative short day plants (Craufurd et al. 1997). Long days delay flowering but do not prevent flowering in cowpea (Lush and Evans 1980). Compared to TVu7778, Danila takes longer to flower under long day length than the former. Being more sensitive to day length Danila would have taken a longer time to flower had the trials been carried out during the normal cropping season which however, is same time as when rainfall is highest.

Responses of Gs and RWC to water stress

Cowpea is considered a drought-avoiding plant with stomata that are extremely sensitive to water stress (Shackel and Hall 1979; Hamidou et al. 2007). In the present study water stress caused appreciable reductions in Gs in Kano (45.7%) and Ibadan (50.7%) but with no effect on RWC. However, closure of stomata due to water stress was not responsible for the maintenance of leaf water status under drought as no correlation was found between Gs and RWC. Stomatal closure as response to water deficit in cowpea has been reported by several researchers (Turk et al. 1980; Bates and Hall 1981; Osonubi 1985; Hall et al. 1997; Cruz de Carvalho et al. 1998; Anyia and Herzog 2004a; Souza et al. 2004; Hamidou et al. 2007). However the findings reported by these authors did not show consistent correlation between Gs and RWC. As in the present study, Bates and Hall (1981), and Cruz de Carvalho et al. (1998) did not find any correlation between GS and RWC. It could be suggested that there exists communication between root and leaf such that the shoot responds to changes in the plants root zone, independent of the leaf water status. Number of studies have reported the importance of root systems or rooting pattern in drought tolerance in legume crops, including cowpea, (Pandey et al. 1984; Robertson et al. 1985; Itani et al. 1992; Silim and Saxena 1993; Matsui and Singh 2003; Badiane et al. 2004) which may favour the hypothesis of root to shoot communication. The independence of RWC from stomatal conductance in our population may also indicate that other processes than stomatal conductance are responsible for maintaining water status, like for instance osmotic adjustment. This may be one of the mechanisms that underly the inherent drought tolerance of cowpea. However, Anyia and Herzog (2004b) using ten cowpea varieities and have found a positive correlation (0.71) between Gs and RWC. The authors argued that reduction of water loss through stomatal closure maintained leaf water status under drought. The divergent findings about the role of stomatal regulation in maintaining water status is most propably due to the fact that different cowpea genotypes evolve different strategies to survive dry conditions.

Effect of water deficit on yield, yield components, leaf senescence and flowering time

Grain, fodder and total yields were significantly reduced by up to 50 percent when the RILs were subjected to water stress four weeks after sowing as compared to when there was ample water supply. This means that water stress imposed at early flowering and pod formation stages caused considerable damage to plant functions and thus total biomass yield. High reductions in cowpea yield when exposed to water stress at flowering have been reported by Anyia and Herzog (2004a; 2004b) and Hamidou et al. (2007). It is well established that total plant biomass production depends on the amount of water used for growth (Anyia and Herzog 2004b). Water stress resulted in a great reduction in the number of pods per plant up to 32.8 % (Year 1 in Kano) and 36.4% and 40.6% for Year 2 in Kano and Ibadan respectively, moderate reduction in mean seed weight (up to 16.6%) and negligeable reduction for number of seeds per pod. These results imply that in response to drought stress cowpea plants reduce the number of pods produced per plant while it maintained size of seeds and number of seeds per pod. Our results confirm the findings of Hamidou et al. (2007) who reported significant depressive water deficit effect on yield components except number of seeds per pod in cowpea. They however reported higher reduction in pod number per plant up to 57 percent and 64 percent in cowpea when drought was imposed under glasshouse and field conditions respectively. Grain yield reduction due to drought stress that occurs at flowering is mostly attributed to decrease of pod development rather than reduction in size of seeds and number of seeds per pod in several legume crops such as soybean, (Liu et al. 2004), dry bean (Acosta-Gallegos and Shitaba 1989; Acosta-Gallegos and Adams 1991) and common bean (RamirezVallejo and Kelly 1998; Aminian et al. 2007; Ghassemi-Golezani and Mardfar 2008).

Conclusions

Although drought stress at flowering and pod developmental stages adversely reduced grain and fodder yield in the DanIla x TVu7778 cowpea population, some drought tolerant lines delayed their leaf senescence (DLS) with higher stomatal conductance (Gs) and better yields. However, we observed that these drought tolerant lines in one location might perform poorly in other location and over the years for the same traits as shown by GxE interactions (Table 2). Therefore, selection should focus on different traits in both locations under water stress and non-stress conditions. DLS and Gs seem to be good physiological traits for selection in Kano especially under drought stress but not in Ibadan. Additionally under drought in Kano plant size is important for pod formation and subsequently for grain yield. Under irrigation however plant size did not have a direct effect on grain yield and grain yield components seem to be more important for selection. While in Ibadan (a more humid weather and richer soil) yield components are the best indicators for grain yield and plant size seems to be unimportant for selecting grain yield under both water regimes. Further investigations will focus on identifying QTLs with effect on grain yield under drought condition in the DanIla x TVu7778 population.

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Chapter 5

QTL analysis for terminal drought tolerance traits in cowpea [*Vigna unguiculata* (L.) Walp.]

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To be submitted

Abstract

In cowpea, drought stress that occurs towards the end of the cropping season (so-called terminal drought) has gained attention because of the negative effects on yield. Potential terminal drought tolerance traits have been identified in cowpea but molecular genetic analysis of these traits is lacking. The objectives of this study were (i) to map QTLs for physiological and yield parameters with an effect on terminal drought tolerance in cowpea and (ii) to evaluate the extent to which physiological parameters and productivity were under common genetic control in well-watered and water-limited environments. To these ends, we carried out QTL analyses for stomatal conductance (Gs), delayed leaf senescence (DLS), days to flowering, number of pods per plant, number of seeds per pod, seed weight, grain and fodder yields. The traits were measured in three field experiments, each with two contrasting water regimes using recombinant inbred lines (RILs) developed from a cross between Danila and TVu7778. A total of 42 QTLs were detected using a single nucleotide polymorphisms (SNPs) genetic map. QTLs were located on 9 linkage groups, and 14 QTLs (localized on LG4, LG6 and LG10) were specific to yield parameters. QTLs for Gs, DLS and flowering time co-localized with yield parameters on LG2, LG3, LG5, LG7, LG8 and LG9. QTL analysis confirmed the quantitative nature of all traits investigated. QTL-treatment and QTL-location interactions as well as association between QTLs of different traits sharing a common genomic region were observed. The strongest QTLs were discovered for delayed leaf senescence and flowering time, with phenotypic variation explaining up to 46% of the variation in one of the environments. QTLs for number of pods per plant and grain yield were spread out on different linkage groups with less phenotypic variation explained (about 10%). QTLs specific to treatment suggested that partly different sets of genetic loci account for plant performance and productivity under well-watered conditions and water stress conditions.

Keywords: QTL mapping, Terminal drought tolerance, Delay leaf senesncence (DLS), Productivity

Introduction

Terminal drought that occurs at the end of the cropping season constrains cowpea [*Vigna unguiculata* (L.) Walp.] production especially in Africa's arid Sahel where most cowpea seed production takes place. Agriculture in these areas is mainly rainfall dependent. Due to its negative effects on yield, mid- and terminal-season drought stress have received considerable attention (See Chapter 2 (Agbicodo et al. 2009) for a review). Drought tolerance is manifested as a highly complex trait (Krishnamurthy et al. 1996). Several studies have identified important morphological, biochemical, physiological and productivity traits related to drought tolerance in cowpea. Breeding efforts to improve crop adaptation to water-limited conditions through direct selection have been hindered by the complex genetic basis of plant productivity and drought responses (Blum 1988; Ceccarelli and Grando 1996; Mittler 2005). QTL mapping is an effective approach for studying such genetically complex traits. It offers opportunities to dissect quantitative traits into their single genetic determinants, quantitative trait loci (QTLs), thus enabling transfer of specific genomic regions between different genetic backgrounds through marker assisted selection (MAS) (Tuberosa and Salvi 2006). This approach has only been limitedly applied for cowpea. To the best of our knowledge only Muchero et al. (2009a) reported QTLs which were shown to be associated with drought stress-induced premature senescence and maturity in cowpea. These authors suggested the possibility of pyramiding early maturity with delayed drought-induced senescence to manage both early and terminal season drought stress in cowpea.

In order to circumvent the complexity of drought tolerance, the strategy of dissecting drought tolerance into several physiological components that determine drought tolerance is employed. In many crop species QTLs were identified for physiological traits that are likely to be associated with stress tolerance such as osmotic adjustment (Lilley et al. 1996; Robin et al. 2003), stomatal conductance (Price et al. 1997; Ulloa et al. 2000), carbon isotope discrimination (Price et al. 2002; Rebetzke et al. 2008; Takai et al. 2006) chlorophyll and chlorophyll fluorescence parameters (Guo et al. 2008). However, there are fewer studies in which QTLs for productivity and different physiological variations were mapped in the same population. Using genetic mapping to dissect the inheritance of a number of complex traits in the same population is a powerful means to distinguish common heredity from casual associations between such traits (Paterson et al. 1988). In principle, such an approach can provide a direct test for the role of specific physiological traits in determining genetic potential for plant productivity under abiotic stresses, such as those imposed by arid conditions (Saranga et al. 2004). Some of such studies found that productivity was related to relative water content (RWC) in barley (Teulat et al. 1998), and to $\delta^{13}C$ in cotton (Saranga et al. 2004), but not in soybean (Mansur et al. 1993; Specht et al. 2001). Saranga et al. (2004) found that QTL likelihood intervals for high seed cotton yield and low leaf osmotic potential corresponded to three genomic regions, implicating osmotic adjustment as a major component of improved cotton productivity under arid conditions.

In this study, we performed QTL analysis for stomatal conductance (Gs), delayed leaf senescence (DLS), days to flowering, number of pods per plant, number of seeds per pod, seed weight, grain and fodder yield using a single nucleotide polymorphisms (SNPs) genetic map. The traits were measured in three field experiments, each with two contrasting water regimes, using recombinant inbred lines (RILs) developed from a cross between Danila and TVu7778. The objectives of this study were (i) to map QTL for physiological and yield parameters with effect on terminal drought tolerance in cowpea and (ii) to evaluate the extent to which physiological parameters and productivity were under common genetic control in well-watered and water-limited environments.

Materials and Methods

Plant material

Two cowpea genotypes, Danila and TVu7778 were crossed to generate cowpea recombinant inbred lines (RILs). Danila (tolerant parent) is a local variety commonly grown in the Sudano-Sahelian border areas of Nigeria and Niger Republic (Mai-Kodomi et al. 1999), while TVu7778 (susceptible parent) is an IITA advanced line. The RILs were obtained through the single seed descent method by repeated selfing and keeping one single seed per plant to generate the next generation for up to 10 generations starting from a random set of F_2 plants. Seeds of RILs were multiplied in a pot experiment in the greenhouse. Harvested seeds of each RIL were kept and served as source for further field and greenhouse trials. Previous physiological and yield performance studies carried out at Kano IITA station revealed that Danila and TVu7778 showed contrasting responses to drought for the traits investigated herein (unpublished results).

Field trials and Experimental design

The 117 RILs and the parental lines were grown at two different locations in Nigeria: Kano and Ibadan IITA experimental fields. Kano (12°03'N, 8°32'E and 476m altitude) is located in the Sahelian zone in Northern Nigeria while Ibadan (7°30'N, 3°54'E and 243 m altitude) is in the Guinean zone in South-West Nigeria. The experiments were carried out during the dry seasons in 2005-2006 and 2006-2007 without rainfall allowing imposition of drought stress on plants. In the first year (2005-2006) a trial was only conducted in Kano. The experiments started in October in Kano while in Ibadan due to wetter soil moisture it started in December. Each RIL was planted in rows spaced 75 cm apart. Spacing within rows was 40 cm twenty seeds per RIL and 2 seeds per hole were planted. Experimental units were one-row plots. The experiments had a randomized block design with three replications and two treatments. At each location plants were irrigated twice a week for a period of 4 weeks prior to starting of the stress treatment. Subsequently drought was imposed to three blocks by withholding irrigation water while irrigation continued until harvesting in the non-stressed blocks. The distance between wet and dry blocks was 10 m. Plant protection measures during experiments consisted of weeding by hand and applying insecticides several times. The mean maximum/minimum temperatures were 36.4/19.1°C in Kano and 32.0/22.4°C in Ibadan, while the mean relative humidity was 28% in Kano and 73% in Ibadan. Soil samples were taken randomly (10 different points) in dry and wet blocks at 0-20 cm and 20-40 cm from both locations for chemical and physical soil characterizations (Chapter 4). Soil temperature was measured continuously during experiments at 20 cm and 40 cm soil depths using a temperature sensor. Soil moisture was measured three times during the experiment: at the beginning of the stress treatment, at 3 and 5 weeks after water withholding.

Three weeks after termination of watering, stomatal conductance (Gs) was measured on two young, but fully expanded, leaves per row using a Steady State Diffusion Porometer (SC1, Decagon Devices). In both locations, Gs measurements were only done on sunny days from around 10 a.m. to noon when the temperatures were about 28°C ±2. The leaves used for Gs measurements were detached and weighed to get fresh weight (FW), after that they were put in small plastic bags containing water and kept on ice for 4 hours. The turgid weights (TW) were measured as well as dry weight (DW) after drying the leaves in an oven for 48 hours at 60°C. Five weeks after termination of watering, when differences among lines were more pronounced, stomatal conductance and RWC were measured for the second time. RILs were rated for drought tolerance using a 1 to 5 scale as described by Mai-Kodomi et al. (1999): 1 (normal green turgid leaves), 2 (green with slight wilting), 3 (yellowish grey with moderate wilting), 4 (yellow and light brown leaves with severe wilting), 5 (completely dried). At maturity, five plants were harvested per row and the following yield parameters were measured: number and weight of pods, total seed weight and 100 seed-weight, fresh and dry fodder weight.

DNA sources

Growth of plants and DNA isolation was conducted at the University of California, Riverside as described in Muchero et al. (2009b). DNA was isolated using the Qiagen Plant DNeasy kit. Parental genotypes and RILs from the mapping population as described above were genotyped at the University of California, Los Angeles with an Illumina GoldenGate SNP genotyping array containing 1536 SNPs, as described in Chapter 3.

Data processing

All DNA data processing steps beginning with raw data were carried out at the University of California, Riverside as described in Muchero et al. (2009b). Processing steps included the exclusion of SNPs that had poor technical performance in the GoldenGate assay as well as SNPs exhibiting segregation distortion, defined as having a minor allele frequency (MAF) less than 0.30. In addition, RILs with excessive heterozygosity, non-parental alleles, and “no-calls” which suggested cross-contaminated DNA samples, recent intercrossing or out-crossing, or poor quality DNA were excluded from further analysis (for more details see Chapter 3). The final genotype calls were collected in an Excel spreadsheet.

QTL Mapping

We used the SNP genetic map of the RIL population derived from DanIla x TVu7778 (described in Chapter 3) to perform molecular marker based genetic analyses of 8 traits measured under three experiments each with 2 water regimes. We used the software program MapQTL 5.0 (Van Ooijen 2004) for QTL analysis. Entry means of traits under each water regime, each year and location, and relative reduction values (as defined above), were used for QTL analyses. The analysis started first with a non-parametric Kruskal-

Wallis test to identify markers that showed significant (stringency > 0.005) association with phenotypic traits. The next step was an Interval Mapping (IM) to get better positioning of putative QTLs. Markers located in the vicinity of the QTL were selected as initial set of cofactors. Multiple-QTL Model mapping (MQM) was used to even more precisely locate QTL using the automatic cofactor selection. A permutation test was applied to each data set (1000 permutations) to decide the Logarithm of Odds (LOD) thresholds ($p = 0.05$). Genome wide (GW) LOD values were used to declare QTL for traits measured. The chromosomal location with the maximum LOD score was considered to be the most likely position of a QTL. Graphics were produced by MapChart software (Voorrips 2002).

Results

Phenotypic characterization of the population

Cowpea recombinant inbred lines (RILs) of 117 individuals were evaluated under water stress and well-watered conditions over two years in two locations in Nigeria. For all traits genetic variation, heritability and relationships among traits are presented in Chapter 4. Phenotypic distributions of RILs for each trait in each water regime for both locations for the second year (dry season 2006-2007) are presented in Figure 1. All traits investigated showed continuous distribution typical of quantitatively inherited characters controlled by multiple genes. However data for the first year (dry season 2005-2006) were subjected to log transformation to normalize distributions. With exception of number of seeds per pod (Fig.1e, Fig.1m) and seed weight (Fig.1f, Fig.1n) the parents of the RILs performed differently in both locations especially under dry conditions, whereas under well-watered conditions, parents showed similar performances for all traits over the two years in both locations. Some RILs showed extreme performances when compared to the tolerant or susceptible parent indicating transgressive segregation. For all traits, relative reduction due to water stress was less with Danila (drought tolerant parent) compared to TVu7778 (drought susceptible parent). Large variation was observed among RILs for their relative reduction for all traits.

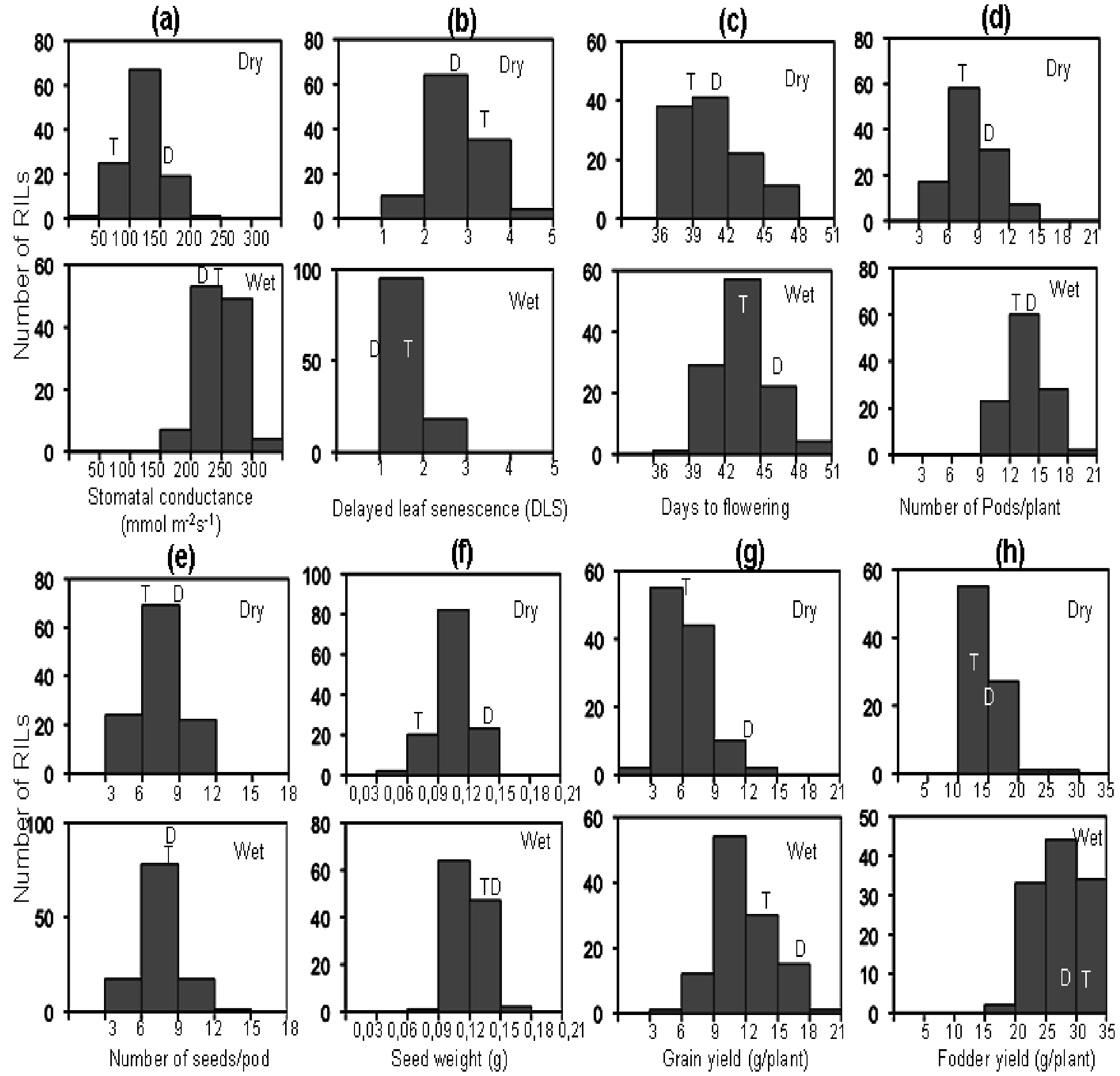

Figure 1 Phenotypic distribution of physiological and yield component traits measured under water stress (Dry) and well-watered conditions (Wet) in Kano Nigeria.
Performances of parents are shown by D: Danila (drought tolerant parent) and T: TVu7778 (drought susceptible parent). Each panel shows the dry and wet condition for the different traits as indicated below the panel a to p.

QTL controlling physiological traits and productivity

A SNP genetic map constituting of 282 loci as described in Chapter 3 was used for QTL analysis using MapQTL5. Detailed biometrical parameters for each QTL detected in each year and location, under each water regime and in relative reduction values [(Wet-Dry)/Wet*100], are provided in Table 1. Relative reduction values were used to identify QTL due to water treatment effects. In total 42 QTLs were detected including four for stomatal conductance (Gs), six for delayed leaf senescence (DLS), five for flowering time

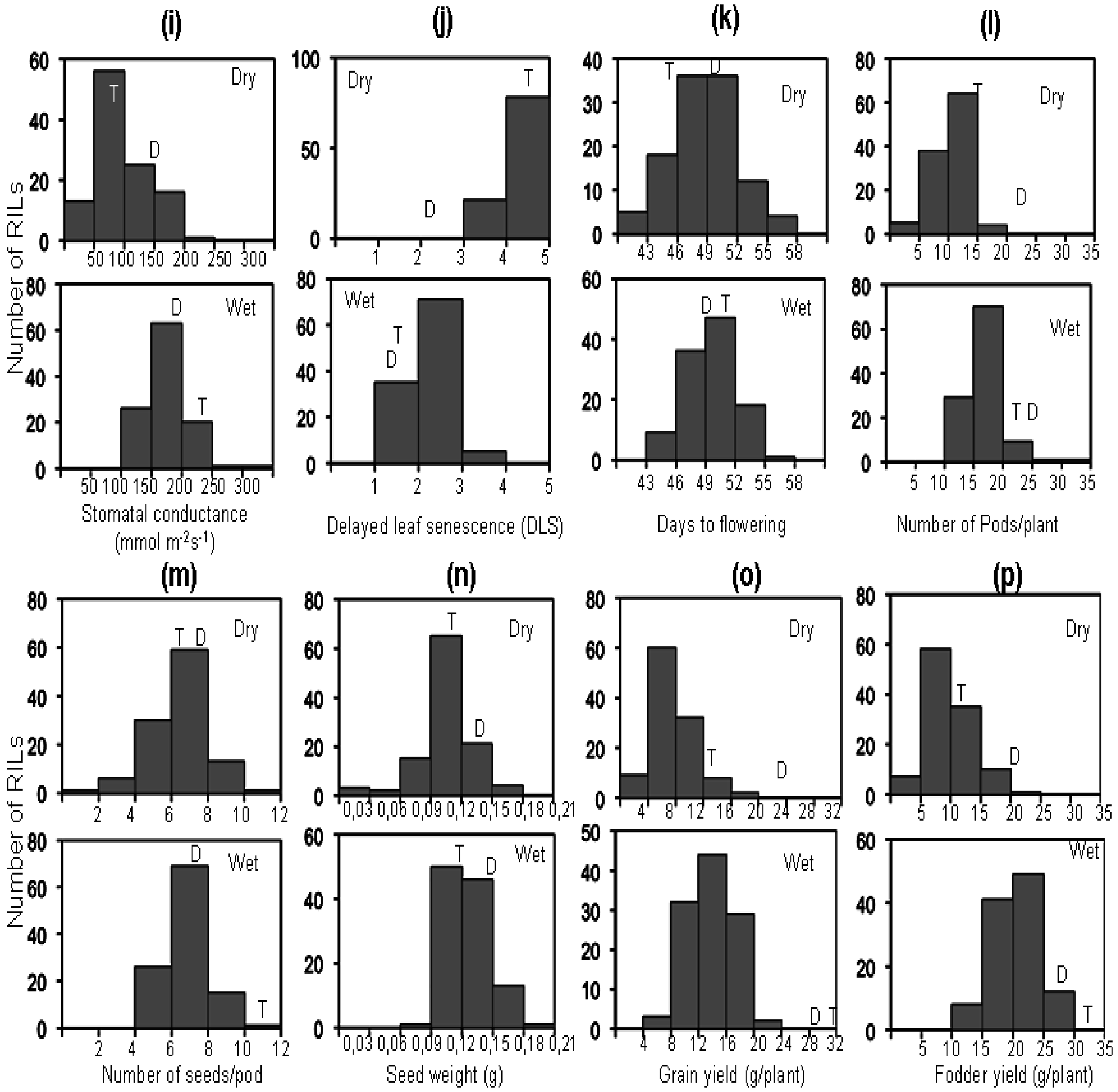

Figure 1 (Continued): Phenotypic distribution of physiological and yield component traits measured under water stress (Dry) and well-watered conditions (Wet) at the second location Ibadan.

and sixteen for grain yield components (pod number/plant, seed number/pod, seed weight), six for grain yield and five for fodder yield. With exception of days to flowering, no QTLs for relative reduction due to water stress were found. Graphical representation of common genomic regions were QTLs overlapped or are closely adjacent are presented in Figure 2.

Stomatal Conductance

A total of four QTLs were identified for stomatal conductance designated as Gs-1 (LG2), Gs-2 (LG7), Gs-3 (LG7) and Gs-4 (LG8). Of these, 2 QTLs showed significant effects only in Kano: LG2 (for drought) and LG8 (for both water regimes). The other 2 QTLs overlapped on LG7 and were identified in both locations under water stress regime. The phenotypic variation explained by these QTLs ranged from 2.1 to 18.5%, the highest being for Gs-4 on LG8 with highest LOD score of 3.12. No significant QTL was detected for the relative reduction of stomatal conductance in both locations.

Delayed leaf senescence (DLS)

Six QTLs were detected for delayed leaf senescence (DLS). Three of the QTLs identified for DLS were located on LG3 of which two overlapped (DLS-1, DLS-2). In both locations, two of the QTLs (DLS-2, DLS-3) found on LG3 showed significant LOD values above threshold (3.60) for water stress treatment only and one QTL (DLS-1) was significant under irrigated conditions at both locations and when data were pooled. Another QTL (DLS-4) found on LG5 was specific to Kano and was significant for dry and irrigated trials. The two QTLs detected on LG7 overlapped and showed the highest LOD value (10.75) and phenotypic variation explained (46.3%) of all the QTLs in this study. In Kano, DLS-5 on LG7 was significant for dry and wet conditions. DLS-6 was significant in Kano for dry data only. However, in Ibadan DLS-5 was significant for dry conditions only and QTL (DLS-6) was significant in both water regimes. No QTL were identified for relative reduction (RR) due to water stress.

Flowering time

Five QTLs were detected for days to flowering on 4 different linkage groups (LG5, LG7, LG8, LG9). The two QTLs Flow-1 and Flow-2 found on LG8 were significant for water stress treatment in Kano for the first year. In the second year these two QTL on LG8 were consistently found in both dry and irrigated conditions for both locations, and for relative reduction form irrigated to drought conditions. Day to Flowering is the only trait for which QTLs for relative reduction due to water stress were found. Phenotypic variation explained ranged from 2.2 to 27.8 the highest being for Flow-1. One QTL (Flow-5) on LG7 was detected only for water stress treatment over the two years in Kano explaining about 10.2%

of phenotypic variation. Two QTLs (Flow-3 and Flow-4) were specific for Kano, Flow-3 (LG5) for water stress and Flow-4 (LG9) for well-watered conditions. Flow-4 showed the lowest phenotypic variation explaining ranged from 3.9 to 8.6%.

Number of pods/plant

A total of seven QTLs were identified for number of pods per plant in three environments. QTLs Pod-1 (LG3) and Pod-3 (LG4) were found consistently under water stress treatment over years and locations; they were significant for well-watered conditions in Year 2 (Kano) as well as Ibadan in Year 2. QTL Pod-2 (LG4) was detected for well-watered conditions in Year 2 (in both Kano and Ibadan). QTLs Pod-1, Pod-2, Pod-3 were treatment-independent in Kano (Year 2). Pod-4, Pod-5, Pod-6, Pod-7 were environment dependent. Pod-4 (LG5) and Pod-5 (LG8) were only detected for water stress treatments in Ibadan (Pod-4) or Kano (Pod-5) while Pod-6 (LG9) and Pod-7 were identified for water stress data and well-watered in Year 1 and Year 2 in Kano, respectively. The highest variation explained ranged from 9.1 to 13.9% for Pod-6 and the lowest for Pod-4 which explained 2.5 to 8.5 % variation.

Number of seeds/pod

Four QTLs were identified for number of seeds per pod. Seed/P-2 (LG3) was identified for both water regimes in Year 2 (Kano) and for well-watered in Year 2 (Ibadan), another one Seed/P-3 (LG4) for well-watered in Year 2 (Kano) and well-watered in Year 2 (Ibadan). The two remaining QTLs were only found in specific environments. Seed/P-1 (LG2) was significant for water stress treatment in Year 1 (Kano) and Seed/P-4 (LG4) for both water regimes in Year 2 (Ibadan). Seed/P-4 showed the highest phenotypic variation (up to 17.3%) and Seed/P-3 explained less than 10% phenotypic variation.

Seed weight

One of the QTLs for seed weight, Seedwt-4 (LG10) was significant for water stress in Year 1 in Kano and for well-watered datasets in both locations in Year 2. QTL Seedwt-2 (LG6) was detected under both water regimes in Year 1 (Kano) only. Three QTLs (Seedwt-1 (LG4), Seedwt-3 (LG6) and Seedwt-5 (LG10)) were significant for water stress treatment over the 2 years in Kano. One of these QTL (Seedwt-3) was detected for well-watered in

Year 1 (Kano) and one (Seedwt-1) for well-watered in Year 2 (Kano). All the 5 QTLs were significant for water stress treatment in Year 1 (Kano). The highest phenotypic variation explained was for Seedwt-3 ranging from 6 to 22.8% and the lowest being for Seedwt-2 with variation about 10.5%.

Fodder yield

Five QTLs were identified for fodder yield with one QTL detected in all the three environments. Of these QTLs, FY-5 (LG4) and FY-3 (LG10) were specifically detected under both water treatments in Year 1 (Kano) and Year 2 (Ibadan) respectively while FY-2 (LG6) was only detected under both water treatments and in Year 2 (Kano). FY-1 (LG5) was significant for Year 2 under both water regimes in Kano and only under water stress in Ibadan. FY-4 (LG6) was significant under water stress only in Year 1 in Kano and both water regimes in Year 2 (Ibadan). Phenotypic variation was higher ranging from 7.2 to 17.2% for FY-5 and the lowest variation being about 6 to 9% for FY-1 and FY-2.

Grain yield

Five QTLs for grain yield were identified using dry and wet datasets in three year-location environments. However, no QTL was found in all the three environments. QTLs GY-6 (LG6) and GY-1 (LG8) were detected only for water stress for Year 2 in Ibadan and Kano respectively. QTLs GY-2 (LG5) and GY-4 (LG7) were treatment-specific, and detected with well-watered and water stress data sets respectively in Year 1 and Year 2 in Kano. For Year 2, GY-3 (LG9) was detected for both water treatments in Kano and well-watered in Ibadan and GY-5 (LG3) for both water regimes in Kano and water stress in Ibadan. Phenotypic variation explained was about 10% for all QTLs detected for grain yield.

Discussion

Although several morphological and physiological traits varying in response to drought tolerance have been identified in cowpea, genetic mapping of these traits in cowpea has hardly been done. Only Muchero et al. (2009a) reported QTLs for drought stress-induced premature senescence and maturity in cowpea. In the current study, eight physiological and yield parameters, e.g. stomatal conductance (Gs), delayed leaf senescence (DLS), days to flowering (Flow), number of pod/plant (Pod), number of seed/pod (Seed/P), seed weight

(Seedwt), grain and fodder yield (GY and FY) were measured in three field trials each with two contrasting water regimes. QTL analysis using a SNP genetic map produced a total of 42 QTLs for the traits. QTL-environment interactions and association between QTLs of different traits sharing a common genomic region were observed.

QTL-environment interactions

Environmental sensitivity of QTLs was observed, with QTLs effective under a specific water regime, year or location. Out of the four QTLs discovered for stomatal conductance, three (Gs-1, Gs-3, Gs-4) were discovered under water stress conditions in Kano and one (Gs-2) was specifically detected in Ibadan. Among these only one (Gs-3) was confirmed under water-limited conditions in both locations in Year 2. This QTL-water treatment and location effect agreed with estimations of heritability (Chapter 4) which was higher ($h^2 = 0.85$) under water stress in Kano and low ($h^2 < 0.4$) under well-watered in Kano and under both water regimes in Ibadan. This is most likely due to experimental errors during measurements related to unstable weather conditions in Ibadan at the time of measurement of stomatal conductance. To a lesser extend flowering time and delayed leaf senescence (DLS) also showed QTL-water treatment and location effects. The slight QTL-water treatment and location effects for flowering time may be the result of photoperiod and phenological sensitivity of cowpea.

Cowpea responds to day length typical of quantitative short day plants (Craufurd et al. 1997). Long days delay flowering but do not prevent flowering in cowpea (Lush and Evans 1980). Compared to TVu7778, Danila generally takes longer to flower under long day length. However, because the studies were carried out in the dry seasons which coincided with short day length Danila and TVu7778 flowered and matured at about the same time, so the effect of photoperiod sensitivity will be small, if any. If the trials would have been carried out in the normal cropping season, which corresponds to longer day length and highest rainfall, parents and RILs might have shown more variation for flowering time. Although flowering time is the only trait where 2 QTLs (Flow-1 and Flow-2) were found for relative reduction due to water stress both in Kano and Ibadan, variation did not correlate with grain yield. This may be an indication that reduction of flowering time due to water stress in order to escape drought has a negligible effect on yield production.

Table 1. Biometrical parameters of QTLs identified for 8 terminal drought tolerance traits under contrasting water regimes in three different year-location environments using recombinant inbred lines (RILs) derived from Danila and TVu7778.

				Kruskal-Wallis	Year 1 (Kano)			Year 2 (Kano)			Year 2 (Ibadan)			% Variation
QTL	LG	Position (cM)	Flanking Markers	Significant level	Dry	Wet	RR	Dry	Wet	RR	Dry	Wet	RR	Explained
Stomatal Conductance Gs; permutation threshold (GW)* 2.10														
Gs-1	2	40.03 - 50.98	1_0595 - 1_1158	0.01 -0.001	na	na	na	**2.91**	1.34	0.28	1.12	1.03	0.62	2.1 - 10.7
Gs-2	7	2.06 - 10.95	1_1249 - 1_0559	0.01 -0.001	na	na	na	1.97	1.05	0.45	**2.29**	0.94	0.24	3.4 - 9.7
Gs-3	7	2.06 - 20.68	1_1249 - 1_1414	0.01 -0.001	na	na	na	**2.63**	1.14	1.32	**2.15**	0.66	0.87	3.8 - 12.8
Gs-4	8	23.13 - 31.32	1_1168 - 1_0530	0.01 -0.001	na	na	na	**3.12**	**2.35**	0.92	1.82	2.05	1.05	5.5 - 18.5
Dealyed leaf senescence (DLS); permutation threshold 3.60														
DLS-1	3	7.73 -32.66	1_0853 - 1_1349	0.01 - 0.001	na	na	na	**4.20**	**4.31**	2.34	**3.78**	**4.09**	2.08	9.3 - 17.8
DLS-2	3	19.33 - 28.96	1_1195 - 1_0104	0.01 - 0.001	na	na	na	**3.97**	2.87	1.98	**4.33**	3.11	2.19	3.9 - 16.2
DLS-3	3	54.25 - 70.41	1_1027 - 1_0594	0.05 - 0.001	na	na	na	**3.91**	2.71	2.03	**3.75**	2.06	2.02	6.1 -19.4
DLS-4	5	5.81 - 38.03	1_0309 - 1_0037	0.05 - 0.001	na	na	na	**4.87**	**3.67**	1.98	2.76	2.32	1.82	7 - 15.2
DLS-5	7	17.68 - 37.68	1_1414 - 1_0056	0.001 - 0.0001	na	na	na	**10.75**	**4.89**	3.10	**5.21**	2.59	2.26	10.3 - 46.3
DLS-6	7	20.68 - 41.89	1_1414 - 1_1249	0.001 - 0.0001	na	na	na	**3.91**	2.82	2.81	**7.86**	**3.95**	3.00	8.7 - 32.7
Days to flowering; permutation threshold 3.20														
Flow-1	8	0 - 15.19	1_0298 - 1_0141	0.01 - 0.001	**4.07**	2.90	1.77	**7.19**	**5.42**	2.32	**3.39**	2.01	**3.26**	3.8 - 27.8
Flow-2	8	0 - 9.19	1_0298 - 1_1370	0.01 - 0.0001	**3.89**	3.10	2.46	**5.29**	**3.42**	**3.64**	**4.61**	**3.43**	**3.29**	5.6 - 19.6
Flow-3	5	16.34 - 31.70	1_0924 - 1_0800	0.001 - 0.0001	1.78	1.07	0.37	**3.62**	3.10	2.09	1.97	0.59	0.21	7.9 - 16.2
Flow-4	9	23.18 - 39.99	1_1467 - 1_1408	0.01 - 0.001	2.65	2.03	1.02	2.05	**3.54**	0.93	1.04	0.22	0.09	3.9 - 8.6
Flow-5	7	7.37 - 14.51	1_0056 - 1_0864	0.01 - 0.0001	**3.36**	1.39	0.96	**3.43**	0.98	1.01	0.97	0.23	0.09	2.2 - 10.2
Number of pod/plant; permutation threshold 2.10														
Pod-1	3	73.83 - 78.30	1_0299 - 1_1349	0.01 - 0.0001	**2.17**	0.97	0.76	**3.06**	**2.32**	0.47	**2.33**	1.98	0.83	5.3 - 10.8
Pod-2	4	11.46 - 15.842	1_0275 - 1_0856	0.01 - 0.001	1.88	0.43	1.02	**2.36**	**3.24**	1.04	1.99	**2.88**	1.02	0.6 - 12.6
Pod-3	4	9.88 - 18.22	1_0304 - 1_1013	0.05 - 0.001	**2.10**	0.89	0.32	**3.24**	**3.23**	1.21	**2.88**	2.00	1.22	5.2 -12.7
Pod-4	5	25.58 - 32.70	1_0362 - 1_0800	0.05 - 0.001	1.22	0.88	0.46	0.27	0.43	0.01	**2.21**	0.37	0.08	2.5 - 8.5
Pod-5	8	18.68- 22.12	1_0141 - 1_0530	0.01 - 0.001	0.25	0.76	0.03	**2.27**	0.91	0.23	0.27	0.08	0.02	8.5 - 10.1
Pod-6	9	57.72 - 69.64	1_0221 - 1_1236	0.05 - 0.001	0.09	0.06	0.37	2.00	**3.50**	1.98	0.87	0.24	0.05	9.1 - 13.9
Pod-7	10	19.28 - 31.81	1_0416 - 1_0598	0.05 - 0.001	**3.20**	0.97	0.54	1.20	0.49	0.19	0.76	0.06	0.01	12.1 - 13.7

Table 1. Continued

QTL	LG	Position (cM)	Flanking Markers	Kruskal-Wallis Significant level	Year 1 (Kano) Dry	Year 1 (Kano) Wet	Year 1 (Kano) RR	Year 2 (Kano) Dry	Year 2 (Kano) Wet	Year 2 (Kano) RR	Year 2 (Ibadan) Dry	Year 2 (Ibadan) Wet	Year 2 (Ibadan) RR	% Variation Explained
Number of seed/pod; permutation threshold 2.00														
Seed/P-1	2	23.05 - 29.95	1_1352 - 1_1230	0.05 - 0.001	**2.29**	1.98	0.69	1.64	1.97	0.41	0.90	0.76	0.25	5.8 - 9.2
Seed/P-2	3	13.328 - 18.42	1_1073 - 1_0373	0.05 - 0.001	0.01	0.02	0.01	**2.26**	**2.20**	0.43	1.03	**2.40**	0.02	4.2 - 10
Seed/P-3	4	11.46 - 17.22	1_0275 - 1_0398	0.05 - 0.001	0.81	0.82	0.01	1.56	**2.25**	0.87	1.22	**2.73**	0.51	5.3 - 9.4
Seed/P-4	4	25.03 - 33.41	1_0106 - 1_0774	0.05 - 0.001	0.02	0.01	0.01	0.87	2.30	0.20	**2.10**	**4.66**	1.92	4.7 - 17.3
Seed weight; permutation threshold 2.40														
Seedwt-1	4	7.88 - 21.27	1_0304 - 1_0106	0.001 - 0.001	**2.59**	2.30	0.90	**3.62**	**2.49**	0.65	2.10	1.04	0.72	5.7 - 13.8
Seedwt-2	6	2.93 - 8.32	1_1381 - 1_0943	0.01 - 0.001	**5.97**	**2.58**	0.45	1.93	1.22	0.32	2.06	2.01	0.67	8.8 - 10.9
Seedwt-3	6	0 - 12.32	1_0911 - 1_0943	0.001 - 0.001	**5.97**	**2.58**	1.93	**2.46**	0.99	0.39	2.29	2.21	1.09	6 - 22.8
Seedwt-4	10	47.37 - 55.99	1_0840 - 1_0007	0.001 - 0.001	**2.41**	0.90	0.08	2.19	**4.29**	1.93	2.03	**2.89**	1.03	12.1 - 19.3
Seedwt-5	10	6.77 - 19.28	1_1189 - 1_1049	0.01 - 0.001	**3.41**	0.53	0.12	**2.93**	0.90	1.32	1.22	0.87	0.09	7.4 -15.1
Grain yield; permutation threshold 2.00														
GY-1	8	17.19 - 27.13	1_1370 - 1_0530	0.01 - 0.001	0.21	0.09	0.00	**2.71**	0.91	0.04	1.24	1.01	0.38	8.5 - 13.8
GY-2	5	32.72 - 43.25	1_0800 1_0819	0.01 - 0.001	0.91	**2.01**	0.82	1.24	**2.77**	0.03	0.78	0.54	0.19	4.6 - 10.1
GY-3	9	10.5 - 30.49	1_0703 - 1_0137	0.01 - 0.001	0.02	0.98	0.32	**2.36**	**2.92**	1.03	1.87	**2.44**	0.21	8.5 - 12.2
GY-4	7	2.06 - 13.52	1_0248 - 1_0864	0.01 - 0.001	**2.63**	1.25	0.52	**2.76**	1.98	1.00	1.98	1.32	0.08	4.2 - 8.3
GY-5	3	0 - 14.09	1_0105 - 1_1065	0.001 - 0.001	1.98	0.91	0.09	**2.10**	**3.11**	1.08	**2.13**	1.92	0.05	6.7 - 12.9
GY-6	6	16.95 - 23.59	1_0706 - 1_0123	0.001 - 0.001	1.34	1.21	0.03	0.91	0.42	0.02	**2.67**	0.76	0.01	4.7 - 9.8
Fodder yield; permutation threshold 2.00														
FY-1	5	23.54 - 29.67	1_1359 - 1_0510	0.05 - 0.001	1.23	1.98	0.91	**3.10**	**2.77**	1.22	**2.12**	0.03	0.01	6.7 - 9.1
FY-2	6	19.61 - 32.06	1_1381 - 1_0943	0.01 - 0.001	0.65	0.90	0.01	**2.03**	**2.97**	0.90	0.01	0.01	0.07	6.3 - 9.6
FY-3	10	31.42 - 53.05	1_0865 - 1_0354	0.05 - 0.001	0.03	0.01	0.01	1.82	1.43	0.76	**4.53**	**2.41**	0.09	6.7 -12.50
FY-4	6	8 - 31.06	1_0323 - 1_0943	0.05 - 0.001	**2.01**	0.04	0.06	0.98	0.62	0.03	**2.07**	**3.96**	0.21	7.4 - 13
FY-5	4	35.75 - 52.68	1_1221 - 1_1147	0.01 - 0.001	**2.95**	**2.15**	1.02	1.06	0.92	0.01	0.18	1.03	0.10	7.2 - 17.2

(*) genome wide values from Permutation test, MQM LOD values > permutation threshold is written in bold. Dry, Wet, All and RR indicated water stress, well-watered, relative reduction due water stress calculated as (Wet-Dry)/Wet*100. Stomatal conductance (Gs) and delayed leaf senescence (DLS) were measured only in year 2 and hence some statistical parameters were not applicable (na). Flanking markers are markers adjacent to the QTLs regions.

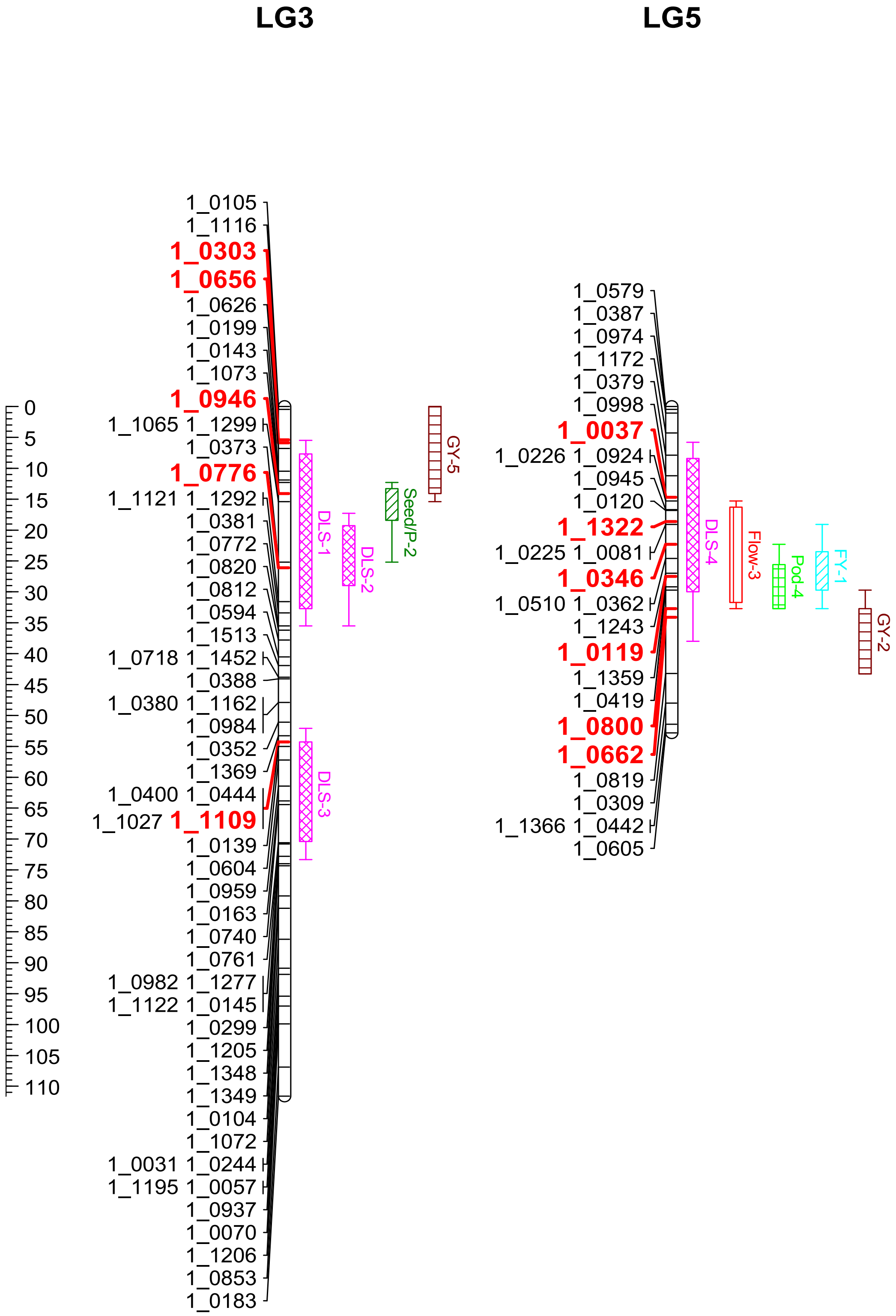

Figure 2. QTLs for physiological, yield and yield components overlapping or closely adjacent on LG 3, LG5, LG7 and LG8.

SNP loci in bold and red represent markers at QTLs peak positions.

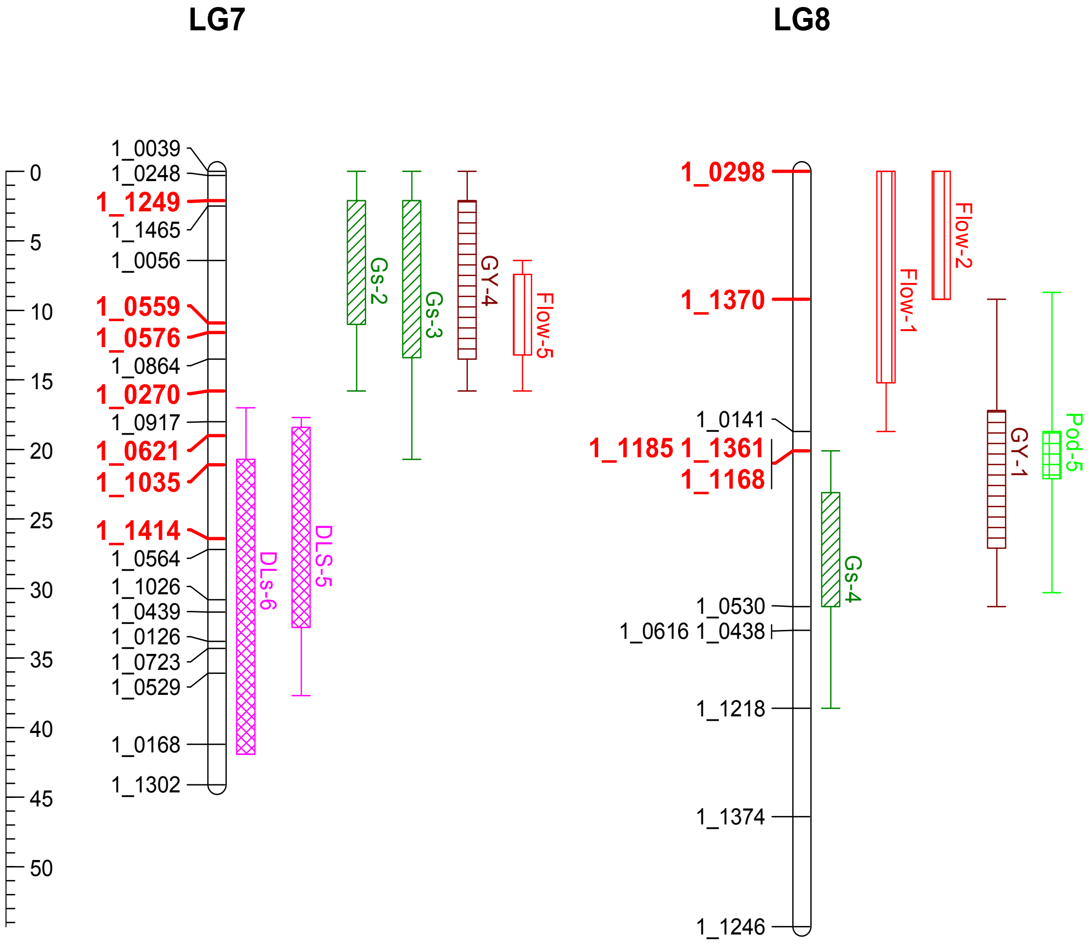

Figure 2 Continued

QTLs for delayed leaf senescence DLS-1 (found under both water regimes in Kano and Ibadan Year 2), DLS-2 (found under water stress in both locations Year 2), number of seed per pod Seed/P-2 and grain yield GY-5 (found under both water regimes in Kano and under well-watered in Ibadan Year 2) overlapped on LG3. On LG5, QTLs for delayed leaf senescence DLS-4 (discovered under both water regimes in Kano), flowering time Flow-3 (detected under water stress in Kano Year 2), number of pod per plant Pod-4 (only found in Ibadan in water stress condition) and fodder yield FY-1 (detected under both water regimes in Kano in Year 2 and water stress in Ibadan) overlapped. QTLs for stomatal conductance Gs-2 (detected under water stress in Year 2 Ibadan), Gs-3 (detected under water stress both in Kano and Ibadan Year 2), grain yield GY-4 and Flow-5 (detected under water stress in Kano both in Year 1 and 2) and flowering time overlapped on LG7. On LG8 Stomatal conductance Gs-4 (identified in both water regimes in Kano Year 2), grain yield GY-1 (identified under stress in Kano Year 2) and number of pod per plant Pod-5 (detected under water stress in Kano Year 2) overlapped.

Out of 6 QTLs identified for DLS only one was location specific (identified in Kano Year 2); all the others were present at least in one of the water regimes at both locations indicating that these QTLs are less influenced by environment. Still, the presence of only one QTL under both water regimes in Kano but not in Ibadan might be due to higher relative humidity and richer soil in Ibadan compared to Kano. The drier weather induced extra QTL in Kano but not in Ibadan. This is in agreement with the path analysis in Chapter 4 where DLS was concluded to be more suitable for indirect selection criterions for grain yield in Kano. Similarly, in cowpea only few QTL-environment interactions were reported by Muchero et al. (2009a) who measured drought-induced senescence over three years at University of California-Riverside Coachella Valey Agricultural Research Station (CVARS). They reported ten QTLs for drought-induced senescence of which some were consistent over the three years and some QTLs were found at least in two experiments and only one QTL was detected in a single experiment.

Among all traits assessed, yield parameters (especially number of pods per plant and grain yield) were most affected by the environment with QTLs specific for water regime, location and year (Table 1). To some extent these findings supported the phenotypic data analysis where higher GxE interactions for those traits were found indicating that plants performed differently under different water treatments, locations and over the years. Out of the 42 QTLs discovered, 14 QTLs were specific to yield parameters on LG 4, LG6 and LG10. This might indicate that genetic loci defining these QTLs are specific for pod and seed formation, and seed weight which is the reflection of seed size contributing to grain yield production. Among the grain yield components, seed weight showed the highest LOD values and variation explained in Year 1 (Kano). For number of pods per plant and grain yield, smaller effect QTLs were spread out on different linkage groups. Our path analysis confirms the highly quantitative nature of these traits since several traits inter-related with grain yield and number of pods/plant (Chapter 4). The highly complex nature of grain yield and number of pods/plant under drought and the G x E interaction are widely reported in several crop species including cowpea (Turk and Hall 1980; Hall and Patel 1985; Selvaraj et al. 1986; Cisse et al. 1997; Hall et al. 1997a). Our QTL analysis is in line with the results of Ramirez and Kelly (1998) who suggested that number of pods per plant is a quantitative trait in which multiple genes are involved. Similar results were reported for soybean

(Mansur et 1993; Specht et al. 2001), and for cotton (Saranga et al. 2001; 2004) where specific QTLs for physiological and productivity traits were discovered under limited and well-watered conditions. The fact that we found different QTLs for dry and watered conditions in the two locations over the years suggests that different sets of genes account for plant performance and productivity under well-watered and water stress conditions. Such QTL–environment interactions for yield parameters are reported (Saranga et al. 2001; 2004; Levi et al. 2009). The phenotypic data were in three experiments each with two contrasting water treatments in two environments, and several factors (like climate/weather, soil, temperature) may account for the QTL-environment effects. Soil characteristics between locations were quite different, with Ibadan having a richer soil with more clay compared to Kano, indicating higher soil water retention capacity in Ibadan. Although we have tried to minimize the differences in soil moisture by starting experiments in Ibadan two months later compared to Kano, we cannot rule out the effects on plant performance due to chemical and physical soil characteristics.

Association between QTLs of different traits

Correlations between QTLs, drought-related physiological traits and yield can aid breeding strategies. Via marker-assisted selection (MAS) desirable traits can be combined using molecular markers specific for the QTLs that have a positive effect on yield. The more QTLs are present in a relatively small genomic region, the easier these traits can be combined in a single introgression. Our results demonstrate that on LG7 two QTLs for stomatal conductance (Gs-2, Gs-3), two for delayed leaf senescence (DLS-5, DLS-6) and one for grain yield (GY-4) overlapped or were closely adjacent. On LG8 one QTL for stomatal conductance (Gs-4), two for flowering time (Flow-1, Flow-2) and two for yield parameters (Pod-5 and GY-1) overlapped. Three QTLs for delayed leaf senescence (DLS-1, DLS-2, DLS-3) overlapped and were closely adjacent to QTLs for grain yield parameters (Pod-1, Seed/P-2, GY-5) on LG3. Remarkably single QTLs for delayed leaf senescence, flowering time, number of pod per plant, grain and fodder yields (DLS-4, Flow-3, Pod-4, GY-2 and FY-1) fell within the same chromosomal region (5.81 to 38.03 cM) on LG5. These results showed that a significant part of the variation in physiological and productivity parameters is explained by a few genomic regions, and hint at the importance of DLS, Gs and flowering time for grain and fodder yields production. Our results are in line with Gwathmey et al. (1992) who suggested that combination of DLS with early flowering may allow cowpea plants to produce a second flush of pods and this will reduce yield loss due to both mid- and terminal-season drought conditions. The relevance of DLS was also recently reported by Muchero et al. (2009a) who mapped QTLs for drought stress-induced premature senescence in cowpea and suggested drought-induced senescence is a reliable indicator of seedling drought tolerance that can be utilized in both greenhouse and field screening in cowpea. Several sorghum studies reported QTLs for stay-green trait (Crasta et al. 1999; Borrell et al. 2000; Subudhi et al. 2000; Tao et al. 2000; Sanchez et al. 2002). In most cases, visual scoring of premature leaf senescence has been a reliable predictor of green leaf area at maturity in sorghum subjected to post-flowering drought stress (Crasta et al. 1999; Borrell et al. 2000; Subudhi et al. 2000; Tao et al. 2000).

Common genomic regions to different traits may suggest that the same set of genetic loci contribute to the effects of stomatal conductance, delayed leaf senescence, flowering time and yield parameters. The chromosomal segments of LG5, LG7, LG8 where QTLs for physiological traits and productivity from different enviroments co-localized represent hot spot regions for drought-tolerance traits. In cowpea, grain yield reduction under drought is mainly attributed to the decrease in pod number per plant while number of seed per pod and seed weight reflecting seed size were hardly affected (Chapter 4). According to Momen et al. (1979) in soybean, decrease in number of pods per plant under stress is due to an increased rate of flower abortion which is a result of a limitation of photosynthetic activities. Genotypic differences in the grain yield potential of cowpea have been positively associated with carbon isotope discrimination (Δ) (Condon and Hall 1997). The authors argued that the higher Δ in more productive genotypes of cowpea was probably due to more open stomata, facilitating a higher photosynthesis rate. Photosynthesis is an essential process to maintain crop growth and many studies indicated that the stay-green trait is associated with improved yield and transpiration efficiency under water stress conditions in cereal crop species such as sorghum, maize and wheat (Benbella and Paulsen 1998; Baenziger et al. 1999; Borrell et al. 2000, Haussmann et al. 2002; Verma et al. 2004). Our results demonstrate that gene loci that regulate the plant’s ability to delay leaf senescence and maintain higher stomatal conductance and photosynthetic activities and promote pod formation colocalize in similar regions of the genome. Stomatal conductance and DLS showed indirect effects on grain yield through pod development and fodder yield (plant size) respectively (Chapter 4). Pod number and plant size showed positive correlations in both water regimes in both locations and our results confirmed that these two traits are genetically linked. Breeding for such a QTL hot spot region may help to improve several aspects of the drought response of the cowpea plant and yield under dry conditions.

Implications for improving cowpea productivity under drought condition

In many crop species, QTL mapping for drought related physiological as well as plant productivity traits in the same population is receiving more attention (Mansur et al. 1993; Teulat et al. 1998; Specht et al. 2001; Sangara et al. 2001; 2004; Levi et al. 2009). We showed here and in Chapter 4 that this approach allows assessment of the importance of physiological traits in determining plant productivity under drought stress. These findings represent an important step towards a better understanding of the genetics that underlies grain yield productivity in cowpea under drought and the genetics of related traits. Marker loci defining the QTLs, especially those in hot spots, may even represent candidate genes for further molecular studies, and these present valuable tools for introgression of traits to produce a cowpea cultivar with tolerance to drought via MAS. Information from the Medicago genome sequence can also be used for further identification of putative candidate genes. The cowpea SNP markers originate from EST sequences, which can be located in the Medicago genome and identify the Medicago syntenic region of the cowpea QTL. The genes present in this region can be identified, and based on (functional) annotation information, putative candidate genes may be selected. Alternatively, new markers can be developed within the QTL region to try and delimit the QTL interval even more, zooming in on a subset of putative candidate genes. In cowpea, genetic variation of morphological, biochemical and physiological traits in response to drought have been reported (Turk et al. 1980; Hall et al. 1990; 1997b; Ogbonnaya et al. 2003; Matsui and Singh 2003; Slabbert et al. 2004; Anyia and Herzog 2004; Souza et al. 2004). Therefore there is a rationale to carry out genetic mapping studies for more physiological traits on a wider set of cowpea germplasm, which will increase the chance to locate new genes and alleles with effect on cowpea productivity under drought stress that can be used in breeding. In this respect, mapping QTL alleles associated with carbon isotope discrimination (Δ) may be particularly interesting. Loci that contribute to Δ are expected to be involved in stomatal conductance/photosynthetic capacity relationships; this may minimize flower abortion (Momen et al. 1979) which is an important problem contributing to grain yield reduction in water limited conditions such as those imposed by arid conditions in Africa's Sahel region.

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Chapter 6

Identification of markers associated with bacterial blight resistance loci in cowpea [*Vigna unguiculata* (L.) Walp.]

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Abstract

Cowpea bacterial blight (CoBB), caused by *Xanthomonas axonopodis* pv. vignicola (*Xav*), is a worldwide major disease of cowpea (*Vigna unguiculata* L. Walp). Among different strategies to control the disease including cultural practises, intercropping, application of chemicals, and sowing pathogen-free seeds, planting of cowpea genotypes with resistance to the pathogen would be the most attractive option to the resource poor cowpea farmers in SSA. Breeding resistance cultivar would be facilitated by marker assisted selection (MAS). In order to identify loci with effects on resistance to this pathogen and map QTLs controlling resistance to CoBB, eleven cowpea genotypes were screened for resistance to bacterial blight using 2 virulent *Xav*18 and *Xav*19 strains. Two cowpea genotypes Danila and Tvu7778 were identified to contrast in their responses to foliar disease expression following infection with bacterial blight. A set of recombinant inbred lines (RILs) comprising 113 individuals derived from Danila (resistant parent) and Tvu7778 (susceptible parent) were infected with CoBB using leaf inoculation method. The experiments were conducted under greenhouse conditions (2007 and 2008) and disease severity was visually assessed using a scale where 0 = no disease and 4 = maximum susceptibility with leaf drop. A single nucleotide polymorphism (SNP) genetic map with 282 SNP markers contructed from the same RIL population was used to perform QTL analysis. Using Kruskall-Wallis and Multiple-QTL model of MapQTL 5, three QTLs, CoBB-1, CoBB-2 and CoBB-3 were identified on linkage group LG3, LG5 and LG9 respectively. Two of the QTLs CoBB-1, CoBB-2 were consistently confirmed in the two experiments accounting for up to 22.1 and to 17.4% respectively for the first and second experiments. Whereas CoBB-3 was only discovered for the first experiment (2007) with less phenotypic variation explained of about 10%. Three of the SNP loci (1_0946, 1_0604, 1_0225) in the QTLs regions were highly similar to putative extracellular matrix proteins with defense functions important for restricting movement of bacteria from cell to cell.

Keywords: Cowpea, Bacterial blight, Source of resistance, SNP, QTL mapping

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp., Fabaceae ($2n = 2x = 22$)] is an essential leguminous crop in less-developed countries of the tropics and subtropics, especially in sub-Saharan Africa, Asia and Latin America (Singh et al. 1997). Besides fungal and viral diseases, bacterial blight and pustules caused by *Xanthomonas axonopodis* pv. *vignicola* (*Xav*) (Vauterin et al. 1995), formerly *X. campestris* pv. *vignicola* (Burkholder 1944) is the most important disease of cowpea. CoBB is prevalent in all major cowpea growing areas of the world (Gitaitis 1983; Emechebe and Florini 1997), causing severe grain yield loss of more than 64% in some areas of West Africa (Sikirou 1999). When highly susceptible cultivars are sown the crop may even be completely destroyed (Emechebe and Shoyinka 1985). The symptoms of CoBB appear as tiny, water-soaked, translucent spots, which are more clearly visible from the abaxial surface of the leaves (Williams 1975). The spots enlarge, coalesce and develop to big necrotic spots, usually with a yellow halo, leading to premature leaf drop. The pathogen also invades the stem causing cracking with brown stripes. Pod infection appears as dark green water-soacked areas, from where the pathogen enters the seeds and causes discolouration and shrivelling (Sikirou 1999). CoBB is seed-borne (Sikirou 1999) and the pathogen can be spread by wind-driven rain and insects (Zandjanakou-Tachin et al. 2007), but also crop debris and weeds can play a role as inoculum source (Sikirou and Wydra 2004). Among different strategies to control the disease including cultural practises (Emechebe and Florini 1997), intercropping (Sikirou 1999; Sikirou and Wydra 2008), application of chemicals (Rao and Hiremath 1985; Kotchoni et al. 2007), and sowing pathogen-free seeds (Emechebe and Soyinka 1985; Soni and Thind 1991) cultivation of resistant cowpea genotypes appears to be a promising strategy with potential to control CoBB (Emechebe and Shoyinka 1985; Khatri-Chhetri 1999; Sikirou 1999). Thus there is a rationale to develop high yielding cowpea varieties combining important agronomic traits with resistance to bacterial blight. Marker assisted selection (MAS) would help to achieve this goal.

Development of resistant crop varieties requires reliable methods of screening for the traits of interest. In cowpea, reliable assays have been established for screening for resistance to bacterial blight. The assays are based on leaf spray-infiltration with bacterial suspensions on the abaxial surface without injuring the leaves and inoculation of the stem by inserting a sharp tooth-pick, contaminated with bacterial suspension (Sikirou 1999;

Sikirou and Wydra 2004). The identification and characterization of *Xanthomonas axonopodis* pv vignicola which causes bacterial blight in cowpea crop has been carried out in West Africa. A number of sources of resistance among cowpea genotypes to the several strains of the bacteria causing this disease (CoBB) has been detected (Bua et al. 1998; Sikirou 1999; Khatri-Chhetri 1999; Okechukwu and Ekpo 2004). However, very little is known about the inheritance of resistance to this disease in cowpea. Prakash and Shivashankar (1984) studied the inheritance of resistance to CoBB in the field by crossing a resistant parent '779' with four susceptible cultivars and reported that susceptibility was dominant over resistance and segregating patterns did not fit into simple genetic ratios. The resistance appeared to be inherited quantitatively and segregation was affected by the genetic background of parents and modifying factors.

In the present study we focused on foliar bacterial blight expression of cowpea. The objectives of this study were to i) determine the genetics of resistance to CoBB, ii) identify molecular markers with strong associations to foliar CoBB resistance and iii) map chromosomal regions (QTLs) involved in the resistance. To this end we used a single nucleotide polymorphism (SNP) based genetic linkage map derived from a set of recombinant inbred lines (RILs) which resulted from a cross between Danila (resistant parent) and TVu7778 (susceptible parent). We report here the identification of regions of the genome with QTLs for resistance to CoBB by artificial leaf inoculation in cowpea.

Materials and Methods

Sites

Experiments were conducted in the pathology laboratory and greenhouses of the International Institute of Tropical Agriculture (IITA) in Ibadan (7°30'N, 3°54'E and 243 m altitude) located in the forest-savanna transition zone of South-West Nigeria.

Inoculum preparation

Highly virulent *Xanthomonas axonopodis* pv vignicola strains *Xav*18 and *Xav*19 were grown on nutrient agar (NA) medium for 48 hours at 28ºC. After harvesting bacterial colonies with sterile distilled water, the concentration was adjusted to an optical density (OD) = 0.06 corresponding to 10^8 colony forming units/ml (CFU/ml) with a spectrophotometer. A few drops of Tween 20 were added both to the bacterial suspension and to the sterile distilled water to be used for control plants just before inoculation.

Plant materials and inoculation

Cowpea genotypes IT81D-1228-14 (reported to be resistant to CoBB; Singh et al. 1997; Amusa and Okechukwu 1998; Okechukwu and Ekpo 2004), IT90K-76 and IT84S-2246-4 (known to be susceptible to CoBB; Sikirou 1999; Okechukwu and Ekpo 2004) were used to determine bacterial concentration and the optimal growth stage most effective for inoculations under the greenhouse conditions in Ibadan IITA station. Two inoculation methods were employed, one on the leaf and the second on the stem. A total of eleven genotypes including three parental lines of existing RIL populations (Danila, TVu11986 and TVu7778) were screened using the two highly virulent strains of *Xanthomonas axonopodis* pv vignicola (*Xav*). Plastic pots (8 cm diameter) were filled with sterilized topsoil and genotypes were planted in four sets of which each set consisted of 22 pots with two pots per genotypes. Four seeds were sown per pot and were watered with tap water. The seedlings were thinned to two plants per pot. Three weeks after planting, three sets of 22 pots each were arranged in randomized block design with three replications. Plants were inoculated with bacterial suspension at a concentration of 2 x 10^6 (CFU/ml). Twenty four hours before inoculation, plants were placed under high humidity conditions to allow stomata opening. Plants were covered with plastic bags and kept under humidity for 48 h after inoculation to enhance the establishment of infection. The inoculation was done by spraying the abaxial surface of the first two trifoliates until water-soaked spots apeared using a hand-operated atomiser as described (Sikirou 1999; Okechukwu and Ekpo 2004). In the fourth set, two plants per genotype were used for stem inoculation by inserting a sharp tooth-pick, contaminated with 48 hours old bacterial suspension and two plants per genotype were inoculated with sterile distilled water as control.

After the initial analysis was carried out to determine which genotypes were susceptible or resistant to CoBB, a set of recombinant inbred lines (RILs) comprising 113 individuals derived from the cross between Danila (CoBB resistant line) and TVu7778 (CoBB susceptible line) was evaluated for reaction to cowpea bacterial blight infection using the most virulent strain (*Xav*18). This experiment used a completely randomized design with two replications. Four plants per line and two trifoliates per plant were inoculated with bacterial suspension as described above. The experiments were repeated twice under controlled conditions (October 2007 and March 2008).

Evaluation of disease reactions

Disease severity was visually scored for 27 days after inoculation (dai) on the infested leaves using a severity scale of 0 (no symptom), 1 (leaf spots symptoms, i.e. translucent and water-soacked spots), 2 (leaf blight: 10-50% leaf area infected), 3 (severe blight symptoms: > 50% leaf area infected), 4 (inoculated trifoliate is shed). Seven evaluations were performed, 5, 7, 9, 12, 16, 21 and 27 days after inoculation. Foliar disease severity data of genotypes and RILs were subjected to analysis of variance and Duncan Multiple Rank Test (test level 5%) was used to determine significant differences between genotypes. The area under disease progress curve (AUDPC) was calculated for each RIL from the disease reaction scores 5, 7, 9, 12, 16, 21 and 27 dai by using the formula AUDPC = $\sum_i[(D_i + D_{i-1}) \times (t_i - t_{i-1})]/2$, where D_i = disease score at time t_i using the 0 to 4 scores and t_i = time measured in days after inoculation (Shaner and Finney 1977).

QTL Mapping

The genetic linkage map of cowpea described in Chapter 3 of this thesis was used to identify markers associated with QTLs that have effects on resistance to cowpea bacterial blight using the computer program MapQTL 5.0 (Van Ooijen 2004). Entry means for disease scores for each of the 7 evaluation days separatelty and overall means of all evaluations days and AUDPC means for each experiment were used for QTL analyses. The analysis started first with non-parametric Kruskall-Wallis test to identify markers that showed significant (stringent > 0.005) association with phenotypic traits. The next step was Interval Mapping (IM) to identify putative QTLs. Markers located in the vicinity of the QTL were selected as initial set of cofactors. The Multiple-QTL model Mapping (MQM) method was used to more precisely locate QTL using automatic cofactor selection. A permutation test was applied to each data set (1000 permutations) to determine the LOD (Logarithm of odds) thresholds. A LOD value of 3.0 was used as genome wide (GW) threshold for QTL significance at 95% confident interval. The chromosomal location with the highest GW LOD score was considered to be the most likely position of a QTL. Graphics were produced by MapChart software (Voorrips 2002).

Results

Screening cowpea genotypes for novel sources of resistance to CoBB

Cowpea bacterial blight (CoBB) symptoms start with small water soaked spots on leaves which enlarge to irregular brown necrotic lesions surrounded by yellow haloes. The pathogen also invades cowpea stem causing canker symptoms on susceptible plants. A cowpea cultivar with resistance to both foliar and stem disease expressions is therefore desirable. We inoculated leaf and stem with the pathogen, but no stem canker symptoms were observed in all the eleven genotypes tested. Two types of foliar symptoms were observed on the cowpea plants: blight translucent spots that enlarge leading to premature leaf drop on the susceptiple genotypes and brown leaf spots with limited lesion areas on the resistant genotypes (Figure 1). The eleven cowpea genotypes screened in this study differed significantly ($P < 0.01$) in severity of their foliar disease symptoms following inoculation with both *Xav*18 and *Xav*19 strains. The overall mean of visual scores for disease severity and disease reactions are shown in Table 1. After the first leaf translucent spots' appearance, lesion areas of the inoculated leaves continued to increase with yellow surroundings in the susceptible genotypes (IT84S-2246-4, TVu7778, IT90K-76, IT98K-205-8, IT98K-216-44, TVu14676). In both experiments, only the known susceptible control IT84S-2246-4 showed systemic expression of leaf spots on non-infested leaves when inoculated with *Xav*18 and the infected leaves dropped early. When inoculated with *Xav*19 the cowpea genotypes TVu11986, IT81D-994 and IT98K-205-8 were classified as moderately susceptible (MS) with disease severity ranging between 2.5 and 3 while the same genotypes were classified as susceptible with severity greater than 3 when inoculated with *Xav*18. In the MS genotypes blight spots enlarged up to 75% of leaf area infected but no leaves were shed. The resistant genotypes Danila and Aloka local developed brown leaf spots with limited lesion area and severity score ranged between 0.33 and 1. Genotype IT81D-1228-14 (1) showed disease scores ≤ 0.5 for both *Xav*18 and *Xav*19. IT81D-1228-14 (1) therefore was the most resistant genotype, followed by Aloka local and Danila in that order. The CoBB strain *Xav*18 was more aggressive with a shorter latency period compared to *Xav*19. Since the genotype Danila was among the most resistant lines and due to availability of a set of RILs developed from a cross involving Danila and another cowpea line TVu7778 it was decided to dissect the genetics of the resistance.

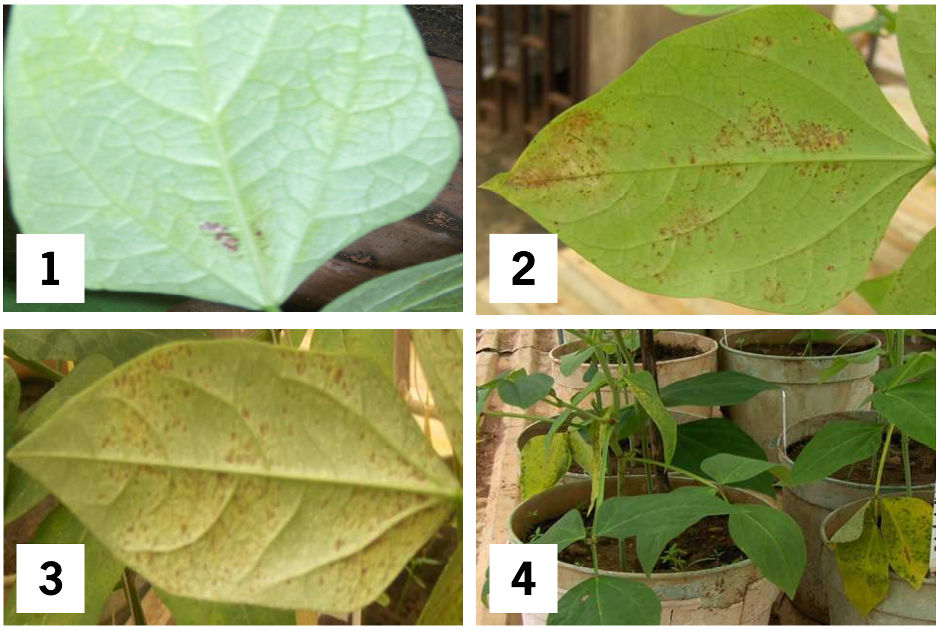

Figure 1 Visual scoring of cowpea plant response when infested with 2 x 10^6 CFU/ml CoBB virulent strain *Xav*18.

0: no symptom comparable with control plant when inoculated with sterile distilled water, 1: brown spots with limited lesion area observed for reristant genotypes, 2: blight leaf symptoms with < 50% of leaf area infected 3: blight leaf symptoms with >75% of leaf area infected, 4: infected trifoliate dropped as observed for susceptible genotypes.

CoBB resistance segregation

Disease severity was assessed by visual inspection of lesion areas on infected leaves and scored in five classes (0 to 4). The area under disease progress curve (AUDPC) was calculated using severity score data according to the formula explained in methodology part. The correlations between the disease rating and AUDPC were 0.96 and 0.94 for the first (2007) and second (2008) experiments respectively indicating good agreement between the two methods. Disease scores were used to study segregation patterns in the set of RILs. Considerable differences were observed among the plants making up the set of RILs derived from the cross between Danila and TVu7778 for the first leaf spots

Table 1. Disease severity and reaction of foliar symptoms to CoBB strains (*Xav*18 *Xav*19) in eleven cowpea genotypes

	Experiment 1 (March2007)				Experiment 2 (Aug. 2007)			
	*Xav*18		*Xav*19		*Xav*18		*Xav*19	
Genotype	S	DR	S	DR	S	DR	S	DR
IT81D-1228-14(1)*	0.25a	R	0.20a	R	0.5a	R	0.42a	R
Aloka	0.33a	R	0.33a	R	0.75a	R	0.75a	R
Danila	0.5a	R	0.42a	R	1a	R	1a	R
TVu11986	3b	S	2.5b	MS	3b	S	2.75b	MS
IT81D-994	3b	S	2.75b	MS	3b	S	2.75b	MS
IT98K-205-8	3b	S	2.75b	MS	3b	S	2.85c	MS
IT90K-76	3.25bc	S	3bc	S	3.5b	S	3.5c	S
IT98K-216-44	3.5cd	S	3.5cd	S	3.8cd	S	3.75c	S
TVu4676	3.75de	S	3.75d	S	4d	S	4d	S
TVu7778	3.83de	S	3.75d	S	4d	S	4d	S
IT84S-2246-4	4e	S	3.85d	S	4d	S	4d	S

* IT81D-1228-14 (1) is the most resistant genotype selected from the original IT81D-1228-14 received from IITA genebank over 3 generations based on single plant selection with best resistance to both *Xav*18 and *Xav*19.

Mean values in the same column followed by the same letter(s) are not significant different ($P \leq 0.05$) using Duncan Multiple Rank Test. S: severity, DR: disease reaction; R: resistant (severity score ≤ 1), MS: moderately susceptible (1< severity score < 3); S: susceptible (severity ≥ 3).

appearance on the abaxial surface and days to first leaf drop. Analysis of variance of score data collected from the RILs for their foliar disease expressions to *Xav*18 is presented in Table 2. The latency period was longer during the second experiment where disease assessment started seven dai compared to five dai in the first experiment when the first leaf spot symptoms appeared on the most susceptible lines including TVu7778. Based on mean square (MS_g) considered as total phenotypic variation and experimental errors (MSe) as an estimate of non-genetic variation of ANOVA (Table 2), the estimated heritability for disease reactions to CoBB were 0.93 and 0.92 in the first and second experiments respectively. Disease reactions in both experiments were highly correlated (0.87). The frequency distribution of the RIL population based on foliar disease severity scores showed a bimodal pattern in both experiments (Figure 2) with some transgressive lines towards resistance and susceptibility. Each line was classififed based on disease rating of 0 (no symptom) to 1 (symptom with limited lesion area ≤ 10%) as resistant (R), a line with disease rating between 2 and 3 (10% < lesion area < 75%) as moderately susceptible (MS) and disease rating greater than 3 (lesion area > 75%) to 4 (leaf drop) as susceptible (S).

Based on this classification, in both experiments there was a larger number of lines with intermediate and susceptible reactions and the distribution of intermediates was skewed toward susceptibility. The fact that RILs were not classified into two discrete classes (R and S) indicate the presence of modifiers with minor effects influencing the expression of resistance.

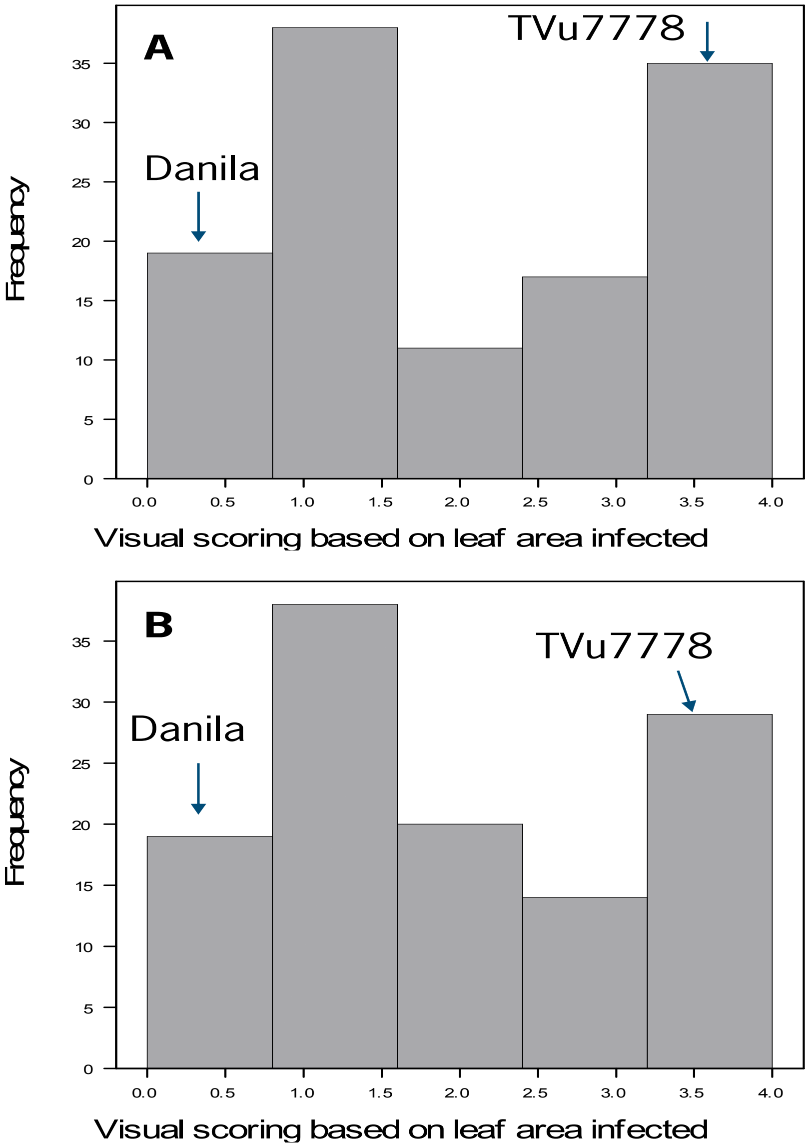

Figure 2. Frequency distribution of foliar disease severity of bacterial blight *Xav*18 strain in cowpea RILs derived from cross between Danila and TVu7778. Fig 3A: first experiment (October 2007), Fig 3B: second experiment (March 2008).Arrows indicate the disease scoring values for Danila (tolerant parent) and TVu7778 (susceptible parent)

Table 2. ANOVA for foliar disease severity of 113 cowpea recombinant inbred lines screened for bacterial blight resistance using *Xav*18 strain.

	Experiment (October 2007)			Experiment (March 2008)		
Source of variation	m.s.	v.r.	F pr.	m.s.	v.r.	F pr.
RIL	4.5625	14.1	<.001	4.3293	15.74	<.001
Block	0.1238	0.38	0.537	0.3196	1.16	0.282
RIL x Block	1.0538	3.26	<.001	0.3374	1.23	0.095
Residual	0.3235			0.2751		

m.s. mean square, v.r.: variation ratio (is the mean square divided by the residual mean square), Fpr. F probability, (v.r. and Fpr.) allow to access whether the disease reactions in the RIL population are larger enough not to have been caused by random variability.

QTL mapping

A genetic linkage map of cowpea generated from the set of RIls and consisting of 282 single nucleotide polymorphism (SNP) markers with total size of 633 cM (as described in Chapter 3) was used to perform QTL analysis using entry means of disease rating for each evaluation day separately and overall means disease rating and AUDPC values per experiment. Results of a (non-parametric) Kruskall-Wallis Test revealed 11 SNP loci significantly associated (at a significance $0.01 < P < 0.0001$) with phenotypic data expressed as AUDPC and disease rating. These marker loci associated with phenotypic data were of minor allele frequency (MAF) ranging from 0.381 to 0.496 indicating a normal (1:1) segregation pattern. The 11 SNP loci defined five QTLs on LG3, LG5 and LG9 as assessed with interval mapping, and confirmed with Multiple QTL mapping. Another five SNP loci including two unmapped loci 1_1186 and 1_0884 (showing high segregation distortions) were also significantly associated with CoBB in the Kruskal Wallis test. Biometrical characteristics of QTLs are presented in Table 3. Of the five QTLs, three QTLs named CoBB-1, CoBB-2, and CoBB-3 were detected in the first experiment. In the second experiment two QTLs CoBB-1, and CoBB-2 on LG3 and LG5 respectively were confirmed in the same chromosomal regions. The two QTLs CoBB-1 and CoBB-2 were consistently discovered for the data sets of the last three evaluations days (16, 21 and 27 dai) and also confirmed for overall means of disease rating and AUDPC. The QTL CoBB-3 on LG9 was discovered when disease ratings (16, 21 and 27 dai) were used in the first experiment only. No QTL was found for disease rating and AUDPC five and seven dai in either experiment. The two chromosomal regions on LG 3 (99.9 - 111.6 cM) and LG5 (4.3 - 16.8 cM) where QTLs CoBB-1 and CoBB-2 were consistently identified for the first and second experiments represent two major regions for CoBB resistance. The QTL on LG3 explained up to 22.1 % and up to 17.4% of the phenotypic variance associated with response of *Xav*18 in the first and second experiments respectively. The most significant QTL (CoBB-2) was located in LG5 and marker 1_0037 showed the highest LOD (3.36) and variation explained (22.1%). The QTL CoBB-3 detected only for the first experiment showed the lowest phenotypic variation explained of about 10%.

Table 3. Biometrical parameters of QTLs identified showing linkage groups, position of QTLs, LOD scores, phenotypic variation explained and the most significant SNP loci associated with lesion areas on leaves expressed as AUDPC and disease scores for 113 RILs population after leaf inoculation with highly virulent strain of *Xanthomonas axonopodis* pv. vignicola (*Xav*18).

QTL	LG	Position(cM)	Marker	K-W significant	Relevant data set	MQM	
						LOD	% Explained
CoBB-1	3	95.7 - 111.6	1_0853	0.001	Exp1	2.98	5.6-15.8
CoBB-1	3	99.9 - 111.6	1_0183	0.001	Exp2	2.69	4.7-13.9
CoBB-2	5	4.3 - 16.8	1_0037	0.0001	Exp1	3.36	9.2-22.1
CoBB-2	5	4.3 - 18.6	1_0037	0.0001	Exp2	3.10	6.7-17.4
CoBB-3	9	71.2 - 78.6	1_1202	0.001	Exp1	2.28	4.3-9.72

SNP markers are those of higest LOD scores and variation explained within the respective QTL regions, relevant data set indicates the experiment for which QTL parameters are shown, 0.001, 0.0001 significant level as revealed by Kruskall-Wallis Test. QTLs on LG3 and LG5 were discovered in identical chromosome regions for the first experiment (Exp1) and second experiment (Exp2) and were therefore named CoBB-1 and CoBB-2. K-W: Kruskal-wallis test.

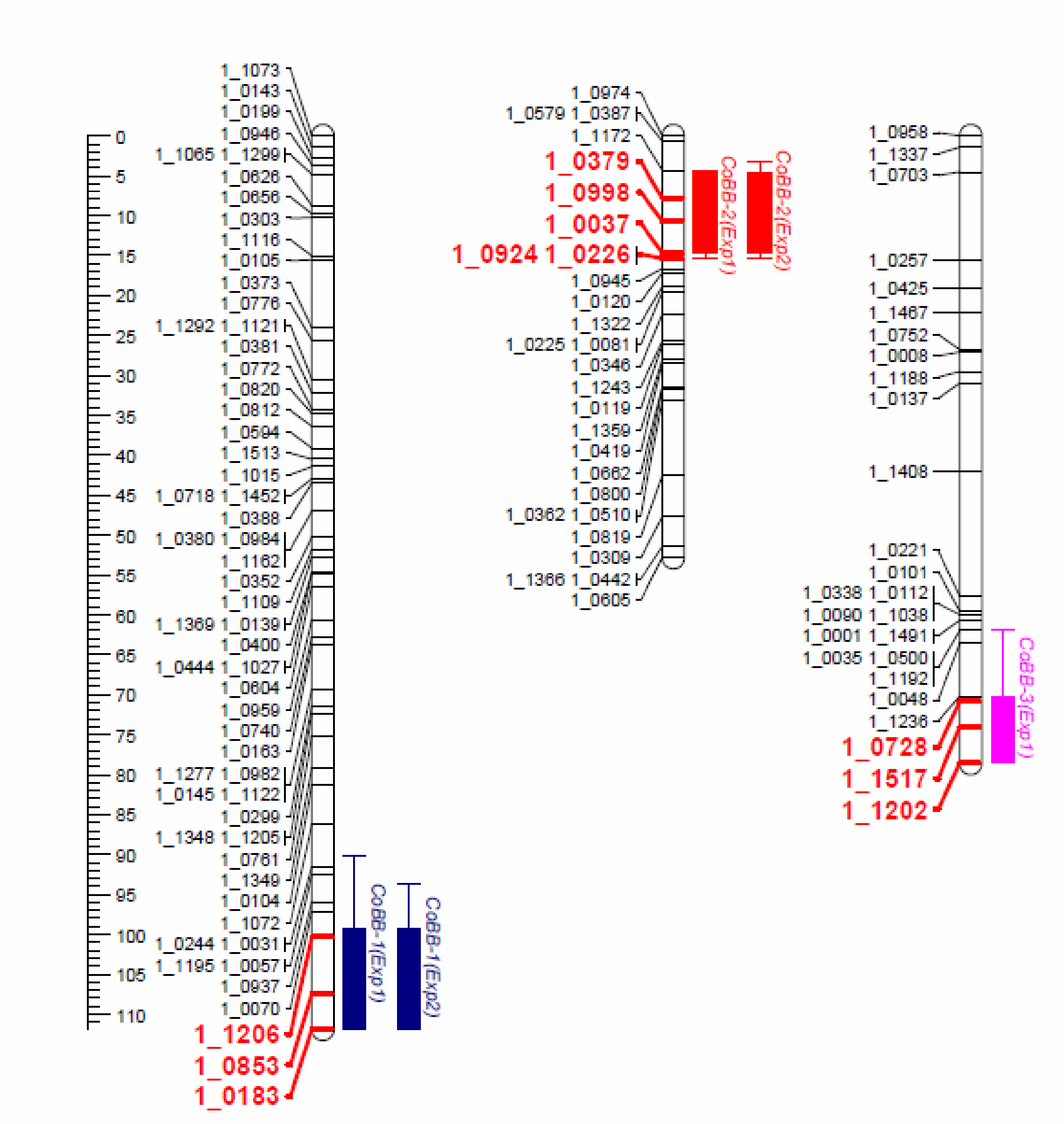

Figure 3. Localisation of QTLs for CoBB resistance to single virulent strain of *Xanthomonas axonopodis* pv. vignicola on RILs population derived from Danila and TVu7778 on LG3, LG5 and LG9.

QTLs [CoBB-1(Exp1), CoBB-2(Exp1), CoBB-3(Exp1)] and [CoBB-1(Exp2), CoBB-2(Exp2)] represent QTLs identified at LOD >2 for first (carried out October 2007) and second (carried out march 2008) experiments respectively. SNP loci defining the QTLs are shown in bold with red color.

Discussion

Bacterial blight caused by *Xanthomonas axonopodis* pv. vignicola (*Xav*) is one of the major diseases of cowpea giving rise to yield loss in all cowpea growing areas. The disease could be particularly devastating in drought prone areas of SSA. The development of cowpea lines with resistance to this disease would be most attractive to farmers as a means of ameliorating the adverse effects of the disease in cowpea fields. Cultural methods such as intercropping cowpea with maize or cassava could also help to minimize yield losses due to the disease (Sikirou and Wydra 2008). Although genetic inheritance of CoBB is still poorly understood, previous research efforts on quick detection, identification and characterization of *Xa*v have been carried out in the framework of the project 'Integrated control of CoBB' at IITA (1993-1999). By using two strains (*Xav18* and *Xav19*) of CoBB a new source of resistance (cv Danila) to the disease has been found and markers associated with QTLs that have effects on resistance to the disease CoBB have been identified. These markers have also been placed on the cowpea genetic linkgae map based on SNP markers.

Bacterial blight symptoms were observed on leaves inoculated with 2 x 10^6 CFU/ml bacterial suspension in the susceptible lines. However, symptoms were observed on non-infested leaves of the susceptible line IT84S-2246-4 conforming with the systemic nature of the disease. Stem inoculation by inserting a sharp tooth-pick contaminated with bacterial suspension as suggested by Sikirou (1999) and Sikirou and Wydra (2004) using two CoBB strains did not induce canker symptoms on stems in both susceptible and resistant cowpea lines tested. It appears that most of the genotypes investigated here were indeed resistant to stem canker expression. The absence of stem canker expression even in susceptible genotypes that showed high expression of leaf symptoms may indicate that different genes could be responsible for CoBB expressions in leaf and stem. In an earlier study Nebane (1980) found cowpea varieties with leaves that were resistant to blight development while the stems showed canker expression. The author suggested that phytoalexins which confer resistance to the disease may be produced more in the leaves than in the stems of such varieties. Okechukwu and Ekpo (2004) reported that stem canker expression on cowpea is dependent on genotype and although it can not be ruled out that stem inoculation was not carried out properly in the experiments reported here. Further studies are needed to ascertain this observation.

With a RIL population, a 1:1 ratio for resistant and susceptible is expected in case of Mendelian single gene segregation. However, this was not the case with the RILs used in this study in which plants were inoculated with *Xav(18)*. The frequency distribution of disease rating displayed a bimodal pattern in both experiments with intermediate classes indicating that at least 2 complementary genes confer resistance to CoBB in this population, with putative modifying factors. For the resistance to CoBB in this population we identified two QTLs named CoBB-1 and CoBB-2 located on LG3 and LG5 which were consistent over the two experiments both for disease rating and AUDPC. Possibly a few minor QTLs, one of which may be CoBB-3 detected on LG9 in the first experiment, may be modifying factors influencing disease resistance, which may explain the partial resistance of some of the RILs. The 5 SNP loci detected to be associated to CoBB by Kruskal-Wallis test, did not define significant QTLs. Although, the Kruskal-Wallis test is not a poweful statistical tool as a non-parametric with no assumptions for the probability distribution of these loci, the highly significant (0.0001) association of these loci with disease expression supports the existence of modifying factors.

Our greenhouse inoculation procedure provided a rapid and reliable method for discriminating between CoBB resistant and susceptibe cowpea genotypes. Based on the results obtained it was possible to identify molecular markers that define QTL regions with effects on resistance to this bacterial disease. Two QTLs with effects on resistance to CoBB were detected, in agreement with Jorge et al. (2001) who found two consistent QTLs for cassava bacterial blight when screening was performed under greenhouse conditions. Varietal resistance to CoBB has been reported (Sikirou 1999) suggesting the existence of pathogenic variation in isolates of *Xav*. Therefore, screening of the RIL population with more strains of *Xav* might result in identification of additional QTLs or might confirm the absence of resistant genes to other isolates in this population. Field investigations are also required to confirm the two major QTLs and SNP markers before they can be implemented in marker-assisted selection (MAS) programs for improving resistance to CoBB. SNP marker loci associated to CoBB and QTLs identified here confirmed the quantitative nature of resistance to CoBB and agreed with the results of Prakash and Shivashankar (1984) who reported quantitative inheritance of resistance to bacterial blight in cowpea and segregation was affected by the genetic background of parents with modifying factors.

Mechanisms for bacterial blight resistance in cowpea plants are not well understood. Our results showed that only limited lesion areas developed on resistant lines while lesion areas enlarged leading to premature leaf drop in the most susceptible lines. This finding agrees with what is known about cowpea's defense response mechanism to *Xav*, represented by a brown-red discoloration without complete collapse of the tissue (Gitaitis 1983). Plants employ a variety of defense mechanisms in response to pathogens, including the use of mechanical barriers, defense proteins and defensive enzymes (Pereira et al. 2003). Interestingly, some of the SNP loci that were found to be associated to CoBB resistance in this study were present in genes with putative functions related to pathogen resistance. Three of SNP markers linked to CoBB resistance were homologous to extracellular dermal glycoprotein (1_0946), pectinacetylesterase family protein (1_0604) and ribosomal protein fibronectin (1_0225). The localization of these potential proteins in the dermal tissues might suggest that these proteins may have defense functions important for restricting movement of bacteria from cell to cell. Plant peroxidases can be directly involved in defense mechanisms acting as catalysts for the polymerization of phenolic compounds to form lignin and suberin in the cell wall, which can act as barriers to block the spread of the pathogen in the plant (Fritig et al. 1987). Flood et al. (1995) also suggested that peroxidases might play an important role bacterial blight of cassava. A cationic peroxidase gene, MEPX1, was isolated from cassava and DNA sequence of MEPX1 showed high homology with other plant peroxidase genes and contained a large intron typical of peroxidase genes (Pereira et al. 2003). The amino acid sequence had 75 % homology with two *Arabidopsis thaliana* peroxidases. In cowpea, Kotchoni et al. (2007) detected a significant increase in H_2O_2-producing peroxidase (NADH-peroxidase) activity when cowpea plants were treated with H_2O_2 at seedling and vegetative growth stages. Although the mechanism of resistance is not elucidated for cowpea, the authors argued that treatment with H_2O_2 induces the synthesis of pathogenesis-related proteins, which help the plants to resist the pathogen attack. In cassava, Kpemoua et al. (1996) found that the production of phenolic compounds in the phloem and xylem of bacterial blight resistant cassava cultivars was significantly higher than in susceptible ones. There was also a higher accumulation of lignin and a greater formation of callose and tyloses in resistant cultivars which potentially obstruct the passage of the bacteria from cell to cell (Kpemoua et al. 1996).

High heritability was found for the disease reaction to *Xav* in this population: $h^2 = 0.93$ and 0.92 for the first and second experiment respectively. However, this estimate obtained under greenhouse condition might not reflect the reality observed under field conditions. Sikirou (1999) indicated that the resistance to CoBB is severely influenced by environmental factors. Okechukwu and Ekpo (2004) reported a number of CoBB resistant varieties identified under field trials that were susceptible under greenhouse conditions. In common bean, Miklas et al. (1996) demonstrated that different QTLs for resistance to bacterial blight were identified under greenhouse and field conditions. Selection of cowpea varieties with more widespread resistance after inoculation with different pathotypes is recommended to breeders (Wydra and Singh 1998). Additional screening in greenhouses and in the field with different pathotypes is needed to detect other QTLs and molecular markers associated to CoBB resistance in order to breed for broad CoBB resistance. In order to confirm QTLs across populations, cowpea genotype IT81D-1228-14(1) identified as most resistant to CoBB among the genotypes studied herein (Table 1) was crossed reciprocally with the most suscepticles genotypes IT84S-2246-4 and IT90K-76. These populations represent potential mapping populations for confirmation of QTLs associated to CoBB resistance. As the combination of resistance to CoBB in different plant organs (leaf and stem) into elite cultivars is recommended to breeders (Wydra and Singh 1998), there is also a need to identify sources of resistance to stem canker expression in cowpea germplasm. The high-throughput SNP marker system as utilized in this study offers a good chance to identify candidate markers useful for pyramiding different CoBB resistance QTLs into cowpea varieties, and to produce elite cowpea varieties with broad resistance to bacterial blight by marker assisted selection.

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Chapter 7

General Discussion

Introduction

Cowpea production is limited by numerous biotic and abiotic stresses including drought stress and cowpea bacterial blight (CoBB) caused by *Xanthomonas axonopodis* pv. vignicola. Although cowpea is well studied for classical genetics, attempts to improve the crop for resistance to these stresses through conventional breeding programs have met with limited success. Marker-assisted (MAS) has been initiated for cowpea, but useful markers for implementation of MAS are limited. Therefore, identification of more markers or QTLs tightly linked with genes of important traits is needed for cowpea. Two cowpea genotypes with contrasting responses to two important traits, drought tolerance and CoBB resistance were used to develop a recombinant inbred line (RIL) mapping population. Using single nucleotide polymorphism (SNP), a genetic linkage map was constructed on which SNP loci and QTLs for seedling and terminal drought tolerance traits, and CoBB resistance traits were mapped. In this chapter, we present a general discussion of the results and the importance of these findings for developing cowpea cultivars with tolerance to seedling and terminal drought and CoBB resistance.

QTLs for delayed leaf senescence at seedling and post-flowering stages co-localize

Drought stress can occur at anytime throughout the cropping season with negative effects on yield. Therefore, it is rational to incorporate drought tolerance genes/QTLs into elite cowpea lines which will survive drought stress at early stage as well as later in the cropping season. We phenotyped 113 RILs for their ability to maintain plant greenness under water stress conditions at seedling and post-flowering stages. The parents and RILs were screened for drought-induced trifoliate senescence (DTS), stem greenness (Stg), and plant survival (Sur) at seedling stage under greenhouse conditions (Chapter 3) and for delayed leaf senescence (DLS) at post-flowering stage under field conditions (Chapter 5). Out of 6 QTLs discovered under field conditions for delayed leaf senescence, DLS-5 (identified under both water regimes in Kano and under water stress in Ibadan), DLS-6 (identified under both water regimes in Ibadan and under water stress in Kano) and QTLs for DTS, Stg and Sur overlapped on LG7 (Figure 1). We show in Chapter 4 that drought-induced senescence is one of the factors that reduces plant biomass in water limited conditions which consequently affect grain yield. Our results indicate that DLS is the most reliable indicator to evaluate plant survival at seedling stage. Our yield performances confirmed the results of Muchero et al. (2009a) who mapped QTLs for drought stress-induced premature senescence in cowpea and suggested drought-induced senescence is a reliable indicator of seedling drought tolerance that can be utilized in both greenhouse and field screening in cowpea. Comparison of QTL results from our population (Danila x TVu7778) and the (IT93K503-1 x CB46) RILs population (Muchero et al. 2009a) revealed that QTLs for DTS, Stg, Sur, DLS-5 and DLS-6 we reported in our population perfectly co-localize in the same regions of LG7 with those identified for seedling drought-induced senescence traits using the consensus map (Muchero et al. 2009a). The chromosomal region (13.37 cM to 37.68 cM) on LG7 where QTLs for drought-induced plant senescence at different plant stages across environments and populations were found indicates the presence of potential loci controlling senescence in this genomic region. Moreover, this genomic region on LG7 represents a syntenic region between cowpea, soybean and Medicago (Muchero et al. 2009b). Different pathways regulating drought tolerant traits in cowpea have been reviewed (Chapter 2). Putative candidate pathways may include the jasmonic acid and lipid signaling pathways. The isolation of transcripts homologous to lipoxygenase (Iuchi et al. 1996a) and 12-oxophytodienoic acid reductase (Iuchi et al. 1996b) from cowpea leaves subjected to dehydration stress suggests a potential role for the jasmonic acid pathway in cowpea's response to drought. The jasmonic acid pathway mediating premature leaf senescence in *Arabidopsis* under abiotic stress has been reported (He et al. 2002). In addition, the ethylene pathway has been reported in numerous plant species to be involved in stress-induced leaf senescence (John et al. 1995; Young et al. 2004; Buchanan-Wollaston et al. 2005). Medicago and soybean sequence information will be used to identify the putative functions of the loci defining the QTLs which might be valuable tools for introgression of DLS to produce a cowpea cultivar with tolerance to seedling and terminal drought via MAS.

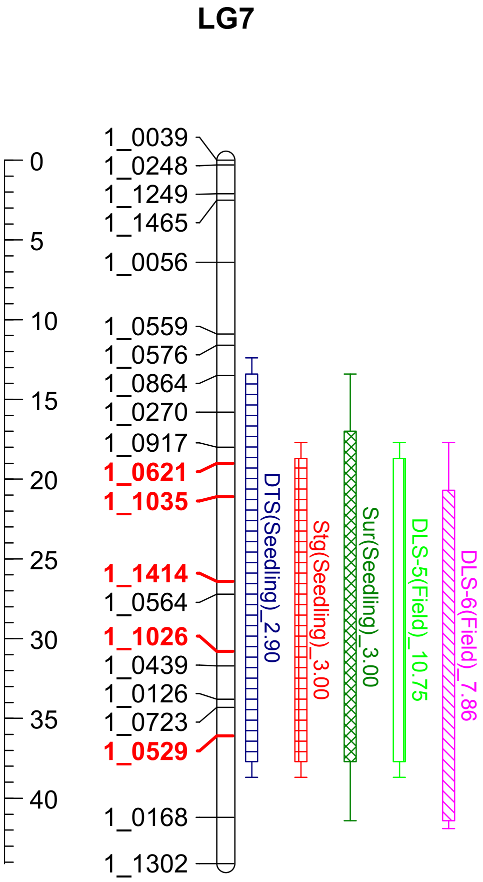

Figure 1. Co-localization of QTLs for Stg, DTS, Sur (under greenhouse: Chapter 3) and DLS (under field trials: Chapter 5) on LG7.

SNP loci in bold and red represent markers at QTLs peak positions. DTS: drought-induced trifoliate senescence (0 to 5 scale where 0 meant the trifoliate stayed completely green and 5 completely wilted), Stg: stem greenness (0 to 5 scale, with 0 being a completely dried stem and 5 being a stem that stayed completely green until the end of the experiment). Sur: survival (recorded as 1 when the plant completely recovered and 0 when the plant had not recovered at the end of experiment), DLS: delayed leaf senescence [1 (normal green turgid leaves), 2 (green with slight wilting), 3 (yellowish grey with moderate wilting), 4 (yellow and light brown leaves with severe wilting), 5 (completely dried)]. DLS-5 (identified under both water regimes in Kano and under water stress in Ibadan), DLS-6 (identified under both water regimes in Ibadan and under water stress in Kano).

Common genetic inheritance between physiological and productivity traits

In total four genomic regions on LG3, LG5, LG7 and LG8 were discovered where QTLs for DLS, Gs, flowering time, yield and yield parameters co-localized (Chapter 5). QTLs for delayed leaf senescence DLS-1 (found under both water regimes in Kano and Ibadan Year 2), DLS-2 (found under water stress in both locations Year 2), number of seeds per pod Seed/P-2 and grain yield GY-5 (found under both water regimes in Kano and under well-watered in Ibadan Year 2) overlapped on LG3. On LG5, QTLs DLS-4 (discovered under both water regimes in Kano), flowering time Flow-3 (detected under water stress in Kano

Year 2), number of pods per plant Pod-4 (only found in Ibadan under water stress condition) and fodder yield FY-1 (detected under both water regimes in Kano in Year 2 and water stress in Ibadan) overlapped. QTLs for stomatal conductance Gs-2 (detected under water stress in Year 2 Ibadan), Gs-3 (detected under water stress both in Kano and Ibadan Year 2), grain yield GY-4 and Flow-5 (detected under water stress in Kano both in Year 1 and 2) and flowering time overlapped on LG7. On LG8 Gs-4 (identified in both water regimes in Kano Year 2), grain yield GY-1 (identified under stress in Kano Year 2) and number of pods per plant Pod-5 (detected under water stress in Kano Year 2) overlapped. QTL mapping approaches give better insight to test the importance of DLS, Gs and flowering time and imply that these traits are major components for improving cowpea productivity under both water regimes. Drought and environmental stress factors can induce the onset of senescence (Noodén et al. 1997; Buchanan-Wollaston 1997) and drought-induced senescence is one of the most important factors that prematurely damages plant biomass in water limited conditions. Leaf senescence is a highly regulated, ordered series of events involving cessation of photosynthesis, disintegration of chloroplasts, breakdown of leaf proteins, loss of chlorophyll and removal of amino acids (Buchanan-Wollaston 1997). Therefore, any defense mechanism that postpones the onset of senescence and maintains leaf function will be beneficial for plant productivity. This indicates that gene loci that regulate the plant's ability to delay leaf senescence would maintain higher stomatal conductance and photosynthetic activities and promote pod formation with positive effect on grain yield. Introgression of these common genomic regions associated with DLS, and productivity via MAS is advantageous assuming that QTLs in these regions will co-segregate over generation. Such an approach was used successfully in which QTLs for yield and a single physiological trait were targeted for breeding 'stay green' sorghum (Tuinstra et al. 1998), earliness in maize (Bouchez et al. 2002) and osmotic adjustment in cotton (Saranga et al. 2004).

Overlap between QTLs for CoBB resistance and DLS

The RILs were screened for cowpea bacterial blight (CoBB) resistance using a leaf inoculation method. Two major QTLs (CoBB-1 and CoBB-2) on LG3 and LG5 respectively, reproducible over the two experiments and one QTL (CoBB-3) on LG9 discovered only for Exp1 were mapped (Chapter 6). CoBB-1 and seedling drought-induced trifoliate senescence (DTS), CoBB-2 and DLS-4 (under both water regimes in Kano) overlapped on LG3 and LG5 respectively. These results may indicate that common genes mediate CoBB resistance and DLS confirming the studies that suggested coordination of plant responses to pathogens and abiotic stresses including the expression of overlapping sets of genes in responses to pathogen and abiotic stresses (Cheong et al. 2002; Fujita et al. 2006; AbuQamar et al. 2006). A cationic peroxidase gene, MEPX1, was isolated from cassava and the DNA sequence of MEPX1 showed high homology with other plant peroxidase genes and contained a large intron typical of peroxidase genes (Pereira et al. 2003). Although the mechanism of resistance to CoBB is not elucidated, Kotchoni et al. (2007) detected a significant increase in H_2O_2-producing peroxidase (NADH-peroxidase) activity when cowpea plants were treated with H_2O_2 at seedling and vegetative growth stages. Several plant hormones including ethylene (ET), salicylate (SA), jamonate (JA) and abscisic acid (ABA) act synergistically or antagonistically to regulate plant responses to pathogens and abiotic stress factors (AbouQamar et al. 2009). Although the pathways involved in senescence and CoBB resistance are not known, the fact that QTLs for CoBB resistance and delayed drought-induced resistance co-localized may suggest a synergistic action of the QTLs where the ability of the plant to delay leaf senescence might lead to an enhanced resistance to CoBB. This corroborated the result of Govrin and Levine (2000) who suggested that cell death promotes plant susceptibility to some necrotic fungi. SNP loci defining these QTLs represent potential candidate markers for incorporating DLS genes that confer CoBB resistance.

QTL mapping and path analysis

We have studied the genetic variation, heritability and inter-relationship among terminal drought tolerance traits including stomatal conductance (Gs), relative water content (RWC), delayed leaf senescence (DLS), days to flowering, number of pods per plant, number of seeds per pod, seed weight, grain and biomass yields using the RILs developed from a cross between Danila and TVu7778 (Chapter 4). Genetic variation and heritability were quite high for all traits except for RWC which was excluded for further analysis. Genotype, treatment, location and year interaction effects were observed for all traits indicating the influence of environmental parameters (i.e. soil nutrient and moisture, temperature, relative humidity) on plant performance. Path analysis was used to study the inter-relationship among traits which revealed the relevance of different traits to be used for grain yield selection in different locations under water stress and well-watered conditions. However, the application of QTL analysis provided opportunities to identify chromosomal regions controlling the physiological and productivity traits. QTL analyses of the traits corroborated the G x E interactions of phenotypic data analysis indicating that productivity of cowpea in well-watered versus water-limited conditions are partly accounted for by different QTLs (Chapter 5). An important issue that has been discussed in the past in connection to breeding strategies for dry environments was whether breeding for stress environments should rely on selection under favourable conditions and subsequent yield testing in stress environments or on direct selection under stress conditions (Ceccarelli and Grando 1996). The first strategy assumes that varieties that give good yield in favourable conditions will also yield relatively well in unfavourable conditions, while conversely the second indicates that direct selection of varieties in stress environments will result in genotypes that most likely perform good under favourable conditions. Genomic approaches may offer a better option in the sense that these different QTLs found in different water regimes can be combined in the same cowpea genotype via MAS.

Conclusions and implications for improving drought tolerance in cowpea and CoBB resistance

Increasing grain yield potential and stability is the ultimate goal of breeding programs. Yield is known as a low-heritability complex trait. Breeding for yield under stress conditions is even more complex due to the difficulty to define and apply a precise set of environmental conditions relevant to the range of naturally occurring scenarios (Levi et al. 2009). Genetic mapping allows the dissection of complex traits and our findings provide evidence for QTL mediating seedling and terminal drought tolerance and CoBB resistance in cowpea and represent a starting point for the identification of genetic factors determining resistance to these traits. Despite the high number of 42 QTLs discovered in total, 4 main regions contain several QTLs for physiological and productivity traits. QTL analysis helps to unravel specific and common chromosomal regions controlling stomatal conductance (Gs), delayed drought-induced leaf senescence (DLS), flowering time, number of pods per plant, number of seeds per pod, seed weight, grain and biomass yields. QTL and phenotypic data analyses showed that with the ability of cowpea plant to delay leaf senescence (DLS) under drought it should be possible to pyramid CoBB resistance with seedling and terminal drought tolerance. The specific and common genomic regions where QTLs for DLS, Gs, flowering time, productivity, and CoBB resistance were found, provide two complementary options. In the first option, different QTLs that are positive in favourable and unfavourable water regimes for particular traits can be combined into a single cowpea cultivar. The second option is to investigate the markers detected in the common genomic regions where QTLs for different traits co-localized for marker assisted breeding cowpea variety with eventually better adaptation to drought and resistance to CoBB. Similar approaches were attempted in cotton by Levi et al. (2009) who combined different QTLs for yield and major drought related physiological traits in the same near isogenic lines (NILs), which permitted testing of MAS efficiency for yield versus underlying physiological traits. However, our results represent a first step for such approaches and further molecular and bioinformatic studies are needed to identify useful markers to be used in MAS. The fact that we used a SNP genetic map which has been integrated in a consensus map of seven RIL populations offers the advantage to identify common markers for delayed drought-induced leaf senescence on LG7 (13.37cM to 37.68cM) across Danila x TVu7778 and IT93K503-1 x CB46 RILs populations (Muchero et al. 2009a). The fact that this region represents a syntenic region between cowpea, soybean and Medicago (Muchero et al. 2009b) will enhance identification of functional markers for MAS of seedling and terminal drought tolerance and CoBB resistance.

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Summary

Cowpea [*Vigna unguiculata* (L.) Walp.] is a most versatile African crop, it feeds people, their livestock and because of its ability in nitrogen-fixation, it improves soil fertility, and consequently helps to increase the yields of cereal crops when grown in rotation and thus contributes to the sustainability of cropping systems. Despite its economic and cultural importance in Sub-saharan Africa, cowpea production is subjected to a wide range of biotic and abiotic constraints. In this thesis we carried out genetic analyses of seedling and terminal drought tolerance and cowpea bacterial blight (CoBB) resistance. Two cowpea genotypes with contrasting reactions to drought stress, Danila (tolerant) and TVu7778 (susceptible) were used to develop recombinant inbred lines (RILs) mapping population. The RILs and parents were phenotyped for seedling and terminal drought tolerance. At seedling stage they were evaluated for drought-induced trifoliate senescence (DTS), stem greenness (Stg), and plant survival (Sur) under greenhouse conditions in Ibadan. RILs and parents were evaluated for stomatal conductance (Gs), delayed leaf senescence (DLS), days to flowering, number of pods per plant, number of seeds per pod, seed weight, grain and biomass yields under three field trials each with two contrasting water regimes in Kano and Ibadan, Nigeria. The parental lines showed also different responses to CoBB, Danila being resistant and TVu7778 susceptible and RILs were evaluated in two experiments under greenhouse conditions for CoBB resistance using a leaf inoculation method. From a total of 1536 SNPs mined from EST sequences derived from several sources and analyzed on an Illumina GoldenGate genotyping array, only 302 SNPs were polymorphic between the parents and segregated within the RILs with minor allele frequency ≥ 0.3. The constructed linkage map has 282 loci covering a map distance of 633 cM distributed over 11 linkage groups (LG). The sizes of LGs and the number of markers assigned to the different LG varied between 111.62 cM for LG3 (58 loci) and 31.58 cM for LG1 (21 loci).

Stem greenness after a drought was an excellent predictor of seedling survival to drought ($r^2 = 0.91$) and stem greenness was inversely related to drought-induced trifoliate senescence ($r^2 = -0.714$). Using the SNPs genetic linkage map, two QTLs were identified for each of the three traits DTS, Stg and Sur on LG3 and LG7. QTLs for Stg and Sur on LG7 were discovered at identical regions (13.37 cM to 37.68 cM) moreover, on LG3 QTLs for Stg and Sur overlapped, indicating that common genes may explain variation in stem greenness and survival. For all traits measured under field trials, a total of 42 QTLs were

detected, 4 for Gs, 6 for DLS, 5 for flowering time and 16 for grain yield components (pod number/plant, seed number/pod, seed weight), 6 for grain yield and 5 for fodder yield. QTLs were located on 9 linkage groups, and 14 QTLs (localized on LG4, LG6 and LG10) were specific to yield parameters. QTL analyses of the traits corroborated the G x E interactions of phenotypic data analysis indicating that productivity of cowpea in well-watered versus water-limited conditions partly are accounted for by different QTLs. However, association between QTLs of different traits sharing a common genomic region was observed on LG3, LG5, LG7 and LG8 where QTLs for Gs, DLS, and flowering time co-localized with QTLs for yield parameters. Three QTLs were detected for CoBB resistance, with two major ones (named CoBB-1 and CoBB-2 confirmed over the two experiments) on LG3 and LG5 and one minor QTL (CoBB-3 only for experiment 1) on LG9. These results confirm the bimodal pattern of frequency distribution indicating that at least two complementary genes conferred resistance to CoBB with modifying factors. The ability of plants to delay leaf senescence shared common QTLs with CoBB resistance, CoBB-1 and seedling drought-induced trifoliate senescence (DTS), CoBB-2 and DLS-4 (under both water regimes in Kano) overlapped on LG3 and LG5 respectively. These results suggest that common genes might mediate CoBB resistance and DLS confirming several studies where overlapping sets of genes in response to pathogen and abiotic stress were reported. Two out of six QTLs detected for delayed leaf senescence in the field, DLS-5 (identified under both water regimes in Kano and under water stress in Ibadan), DLS-6 (identified under both water regimes in Ibadan and under water stress in Kano) co-localized with QTLs under greenhouse for seedling drought tolerance traits DTS, Stg and Sur on LG7. This indicates the presence of potential loci controlling senescence in this genomic region. Moreover, this genomic region is identified as a syntenic region between cowpea, soybean and Medicago by the cowpea research team in the University of California Riverside (UCR) where they also found QTLs for seedling drought-induced senescence traits in the same region of LG7 using another RIL population, IT93K503-1 x CB46.

Our findings provide evidence for QTLs mediating seedling and terminal drought tolerance and CoBB resistance in cowpea. QTL and phenotypic analysis revealed that it should be possible to pyramid CoBB resistance with seedling and terminal drought tolerance. The fact that the genetic map of Danila x TVu7778 is integrated in a consensus map of cowpea will permit comparative genomic studies which will enhance the discovery of functional markers for MAS of seedling and terminal drought tolerance and CoBB resistance.

Samenvatting

Cowpea [*Vigna unguiculata* (L.) Walp.] is een uiterst veelzijdig Afrikaans gewas, dat dient als voedsel voor mensen maar ook voor hun vee. Daarnaast wordt de vruchtbaarheid van de grond waarop cowpea groeit verbeterd doordat cowpea uitstekend stikstof bindt. Hierdoor heeft verbouwen van cowpea een positief effect op de opbrengst van granen die in rotatie met cowpea worden gegroeid en draagt het bij aan de duurzaamheid van deze gewassystemen.

Ondanks het economische belang van cowpea voor de sub-Sahara regio, wordt de productie van cowpea beperkt door een breed scala aan biotische an abiotische factoren. In dit proefschrift is een genetische analyse uitgevoerd van tolerantie voor droogte van cowpea in het stadium van zaailingen en aan het eind van de groeicyclus (terminale droogte). Ook de genetische achtergrond van resistentie tegen Cowpea Bacterial Blight (CoBB) is onderzocht.

Twee cowpea genotypen met contrasterende reacties op droogtestress, te weten Danila (tolerant) en TVu7778 (gevoelig) zijn gebruikt om een populatie te maken van recombinante terugkruisingslijnen (RILs). De RILs en de ouders zijn gefenotypeerd voor droogtetolerantie als zaailingen en in de laatste fase van de levenscyclus. In het zaailingen stadium zijn de RILs geëvalueerd met betrekking tot droogte-geinduceerde trifoliaat veroudering (Trifoliate Leaf Senescence, DTS), groen blijven van de stengel (stem greenness, Stg) en overleven van de plant (Survival, Sur) in de kas in Ibadan, Nigeria. Terminale droogte tolerantie in de RILs en ouders is bestudeerd door analyse van stomataire weerstand (Gs), DLS, dagen tot bloei, aantal peulen per plant, aantal zaden per peul, zaadgewicht, zaad- en biomassa opbrengst bepaald in drie veldproeven met in elke proef contrasterende water regimes (droog en geïrrigeerd), op twee locaties in Nigeria (Kano en Ibadan).

De RILs en de ouders zijn gegenotypeerd met Single Nucleotide Polymorphism (SNP) merkers. De SNPs zijn ontdekt in een collectie van EST sequenties van verschillende cowpea genotypen and 1536 SNPs zijn geanalyseerd met behulp van een Illumina GoldenGate genotyping array. Slechts 302 SNPs konden worden gescoord als polymorf tussen de ouders en segregerend in de RIL populatie met een frequentie van het minst voorkomende allel van $\geq 0{,}3$. De genetische kaart gemaakt met deze merkers is 633cM

groot, met 11 koppelingsgroepen (Linkage Groups, LG). The lengtes van de LGs varieerden van 111,62 cM (LG3, 58 SNPs) tot 31,58 cM (LG1, 21 SNPs).

Het groen blijven van de Stengel (St) was een uitstekende voorspeller van de overlevingskans van zaailingen (r^2 = 0,91) en St was omgekeerd evenredig met droogtegeïnduceerde verouderingsverschijnselen in het trifoliaat (DTS) (r^2 = -0,714). Voor DTS, Stg en SUR zijn met behulp van de SNP genetische kaart elk twee QTLs gevonden op LG3 en LG7. De QTLs voor Stg en Sur zijn gelokaliseerd op hetzelfde deel van LG7 (13,37-37,68 cM). Ook de QTLs voor Stg en Sur op LG3 overlappen, wat erop zou kunnen wijzen dat de groene kleur van de Stengel en overlevingskans worden bepaald door dezelfde genen. Er zijn in totaal 42 QTLs gevonden voor de eigenschappen die bepaald zijn in de veldproeven: 4 voor Gs, 6 voor DLS, 5 voor tijd tot bloei en 16 voor eigenschappen die te maken hebben met zaadopbrengst (aantal peulen/plant, aantal zaden/peul, zaadgewicht), 6 voor zaadopbrengst en 5 voor voederopbrengst (bladeren en stengels). De QTLs zijn gelegen op 9 koppelingsgroepen, en 14 QTLs (gelegen op LG4, LG6 en LG10) waren specifiek voor opbrengstparameters. De resultaten van de QTL analyses van de verschillende eigenschappen laten zien dat de productiviteit van cowpea onder goed geïrrigeerde en onder droge omstandigheden lang niet altijd worden bepaald door dezelfde QTLs. De gevonden G x E interacties van de gemeten eigenschappen wezen ook al in die richting. Desalniettemin zijn ook een aantal QTLs voor verschillende eigenschappen gevonden die wel op hetzelfde gebied in het genoom zijn gekarteerd: QTLs voor Gs, DLS en tijd tot bloei worden op dezelfde lokatie gekarteerd als QTLs voor opbrengst parameters.

The ouderlijnen van de RILs reageerden ook verschillend op infectie met CoBB, waarbij Danila resistent en TVu7778 gevoelig was. De RILs zijn getoetst op CoBB resistentie in twee afzonderlijk experimenten in de kas gebruikmakend van een blad-inoculatie protocol. Er werden drie QTLs gevonden voor CoBB resistentie, waarvan 2 QTLs met een groot effect (CoBB-1 en CoBB-2, bevestigd in twee experimenten) op LG3 en LG5, en een QTL met een kleiner effect op LG9 (CoBB-3, in één van de twee experimenten gedetecteerd). Deze resultaten komen overeen met de tweetoppige frequentieverdeling voor resistentie in de RIL populatie, wat erop wijst dat minstens twee complementaire genen verantwoordelijk zijn voor resistentie in de populatie, mogelijk met modulerende factoren. QTLs voor uitstel van bladveroudering (senescence, DLS) werden op dezelfde lokatie gekarteerd als QTLs voor CoBB resistentie; CoBB-1 werd gevonden op dezelfde lokatie als DTS (droogte-geïnduceerde veroudering van het trifoliaat) op LG3, CoBB-2 en DLS-4 (onder droge en geïrrigeerde omstandigheden in Kano) werden beide op dezelfde lokatie op LG5 gekarteerd. Deze resultaten suggereren dat CoBB resistentie en DLS mogelijk worden aangestuurd door dezelfde genen, wat past in het beeld geschetst door andere studies dat overlappende sets van genen worden gestimuleerd in reactie op abiotische stress en ziektedruk. Twee van de zes gevonden QTLs voor DLS, DLS-5 (aangetoond onder droge en geïrrigeerde omstandigheden in Kano en droogtestress in Ibadan) en DLS-6 (aangetoond onder droge en geïrrigeerde omstandigheden in Ibadan, en onder droge omstandigheden in Kano) werden op dezelfde lokatie op LG7 gekarteerd als QTLs voor DTS, Stg en Sur tijdens het zaailingenstadium. Mogelijk zijn loci die bladveroudering reguleren in verschillende ontwikkelingsstadia van de cowpea plant gelegen in dit gebied op LG7. Bovendien is dit gebied van het genoom aangewezen door het cowpea research team van de University of California Riverside (UCR) als een interessant overeenkomstig gebied in de genomen van cowpea, soya en Medicago waarin tevens QTLs zijn geïdentificeerd voor door droogte geïnduceerde verouderingseigenschappen in een andere cowpea RIL populatie IT93K503-1 x BC46.

Dit proefschrift heeft QTLs opgeleverd die van belang zijn voor tolerantie tegen droogte in het zaailingen stadium en terminale droogte, en voor CoBB resistentie in cowpea. QTL- en fenotypische analyses laten zien dat er mogelijkheden bestaan om droogtetolerantie in een vroeg en een laat ontwikkelingstadium tegelijk met CoBB resistentie in te brengen in Cowpea. De integratie van de genetische kaart van Danila x TVu7778 met de consensus genetische kaart van cowpea maakt het bovendien mogelijk om vergelijkende genomische studies uit te voeren met andere kruisingen en andere soorten, wat de ontdekking en ontwikkeling van functionele merkers voor merker-gestuurde selectie voor droogtetolerantie in verschillende ontwikkelingstadia en CoBB resistentie verder zal stimuleren.

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About the author

Eugene M. Agbicodo was born on July 13, 1971 in Paouignan, Benin. He grew up in central part of the country where he did his high school at Lycee Houffon Abomey (Benin). During his academic studies at Polytechnic College of University of Abomey-Calavi (EPAC/UAC), Eugene did several practical trainings at the International Institute of Tropical Agriculture (IITA) Cotonou-Station on integrated pest management (IPM) and got his BSc in Environmental Protection and Management in 1999. He worked as research assistant at IITA from 1999-2001 on integrated control of bacterial diseases of cassava and cowpea. He was a German Academic Exchange Service (DAAD) scholarship holder from 2001 to 2003 and did his MSc major in Genetics and Plant Breeding at Applied Genetics Institute of Leibniz University of Hanover in Germany with the distinction (Magma Cum Laude). His MSc thesis was on molecular and classical genetic analyses on cytoplasmic male sterility in garlic and leek. Between 2004 and 2005, Eugene worked on characterization of genetic diversity of yam and cowpea in the convergence of science (COS) collaborative project of WUR and Faculty Agronomic Science, University of Abomey-Calavi (FSA/UAC).

In 2005, Eugene was appointed by the IITA-Lukas Brader Scholarship fund, a program that provides graduate training for outstanding students from sub-Saharan Africa to conduct research towards a PhD program in agriculture and related sciences at Wageningen University and Research Centre (WUR). He started his PhD in January 2006 at Laboratory of Plant Breeding on the topic entitled "Genetic analysis of abiotic and biotic resistance in cowpea [*Vigna unguiculata* (L.) Walp.]" during his PhD, he did extensive multi-traits environments field works in Nigeria.

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List of publications

Articles submitted and published

Agbicodo EM, Fatokun CA, Muranaka S, Dolstra O, Visser RGF, Linden van der CG (2009) Genetic variation, heritability and relationships among terminal drought tolerance traits in cowpea. (Crop Science in review)

Agbicodo EM, Fatokun CA, Wydra K, Visser RGF, Linden van der GC (2009) Identification and mapping of quantitative resistance for bacterial blight resistance in cowpea [*Vigna unguiculata* (L.) Walp.]. (Euphytica in review)

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Zannou, A, **Agbicodo, EM**, Zoundjihékpon, J, Struik, PC, Ahanchédé A, Kossou DK, and Sanni A (2009) Genetic variability in yam cultivars from the Guinea-Sudan of Benin assessed by amplified polymorphic DNA. African Journal of Biotechnology 8:026-036

Zannou A, Kossou DK, Ahanchédé A, Zoundjhekpon J, **Agbicodo EM**, Struik PC, Sanni A (2008) Genetic variability of cultivated cowpea in Benin assessed by random amplified polymorphic DNA, African Journal of Biotechnology 7:4407-4414

Engelke T, **Agbicodo EM,** and Tatlioglu T (2004) Mitochondrial genome variation in *Allium ampeloprasum* and its wild relatives. Euphytica 137:181-191

Articles in preparation

Agbicodo EM, Fatokun CA, Muranaka S, Diop N, Muchero W, Ehlers JD, Close T, Visser RGF, Linden van der GC, QTL analysis for seedling and terminal drought tolerance traits in cowpea (*Vigna unguiculata* L. Walp.) (in preparation.)

Articles in Refereed International Conference and Proceedings

Agbicodo EM, Fatokun CA, Muranaka S, Visser RGF, Linden van der CG Genetic analysis and relationship of drought tolerance traits in Cowpea. International Conference on Plant Abiotic Stress Tolerance Vienna, Austria Feb. 8 -11 2009

Agbicodo EM, Fatokun CA, Muranaka S, Linden van der CG and Visser RGF Phenotyping cowpea recombinant inbred lines (RILs) for drought tolerance. Gordon Conference on Salt & Water Stress in Plants Big Sky Resort (USA) Sept. 7-12, 2008

Agbicodo EM (2008) Coping with water scarcity in developing countries: What role for Agricultural biotechnologies. FAO 14th Conference March 5 -April 1, 2007 Abstract 45 http://www.fao.org/biotech/logs/c14/Summury.htm

Wydra K, **Agbicodo EM**, Ahohuendo B, Banito A, Cooper RMC, Dixon A, Jorge V, Kemp B, Kpémoua K, Rudolph K, Verdier V, Witt F, Zandjanakou M and Zinsou V (2003) Integrated control of cassava bacterial blight by (1) combined cultural control measures and (2) host plant resistance adapted to agro-ecological conditions, and (3) improved pathogen detection. In: Akoroda, M. (ed.) Proceedings Eighth Triennial Symposium International Society Tropical Root Crops - Africa Branch, ISTRC-AB, November 2001, Ibadan, Nigeria. pp. 506-515

Zinsou V, Wydra K, **Agbicodo EM**, Ahohuendo B, and Rudolph K (2001) Studies on resistance of cassava genotypes to cassava bacterial blight. In: Plant Pathogenic bacteria, 10th Inter. Conf., Charlottetown, Canada. S. DeBoer (ed). Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 301-305

Zinsou V, Wydra K, **Agbicodo EM**, Ahohuendo B and Rudolph K (2000). Population dynamics of *Xanthomonas campestris* pv. *manihotis* in cassava genotypes with different resistance to bacterial blight. Deutsche Pflanzenschutztagung, München. Mitteilungen Biologische Bundesanstalt 376, 661

Academic Thesis

Agbicodo EM (2009) Genetic analysis of drought tolerance traits in cowpea (*Vigna unguiculata* L.). PhD thesis University of Wageningen 168 p. (ISBN 978-90-8585-477-7)

Agbicodo EM (2003) Molecular and Classical Genetics investigations on male sterility in garlic (Allium sativum L.) and leek (Allium ampeloprasum L.), MSc., Thesis, University of Hanover Germany, 65 p.

Agbicodo EM (1999). Studies of mechanisms of resistance and host-pathogen interactions of cassava bacterial blight. BSc. thesis EPAC/UAC 60 p.

Education Certificate

The Graduate School EXPERIMENTAL PLANT SCIENCES

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Eugene M. Agbicodo
Date: 27 October 2009
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Genetic analysis of drought tolerance and bacterial blight resistance in cowpea	May 29, 2006
► **Writing project proposal**	
Genetic analysis of drought tolerance and bacterial blight resistance in cowpea	Jan 10-Mar 17, 2006
► **Writing a review or book chapter**	
Breeding drought tolerance cowpea: constraints, accomplishments and future prospects, *Euphytica 167*	Jan 2009
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day 2009, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 22, 2009
EPS Theme 3 Symposium 'Metabolism and Adaptation', Amsterdam University	Feb 18, 2009
► **NWO Lunteren days and other National Platforms**	
Lunteren Meeting Experimental Plant Sciences	Apr 03-04, 2006
Lunteren Meeting Experimental Plant Sciences	Apr 06-07, 2009
► **Seminars (series), workshops and symposia**	
Seminars (series) PRI and laboratory of Plant Breeding (estimated 10x)	2006-2009
Seminars (series) IITA (estimated 10x)	2006-2008
WUR Biometrics and Generation Challenge Program (GCP) LD workshop	Jun 19, 2008
► **Seminar plus**	
► **International symposia and congresses**	
Reconsidering Intellectual Property Policies in Public Research "Sharing the benefits of biotechnology with developing countries	Apr 11, 2008
FAO 14th Conference: Coping with water scarcity in developing countries: What role for Agricultural Biotechnologies	Mar 05 -Apr 01, 2007
► **Presentations**	
Poster: WUR Plant Breeding Research Day, WICC, Wageningen	Jun 17, 2008
Poster: International Gordon Research Conference: salt & drought tolerance in plant, Big Sky, Montana, USA	Sep 07-12, 2008
Poster: International conference on Plant Abiotic Stress tolerance, Vienna	Feb 08-11, 2009
Poster: EPS Theme 3 Symposium 'Metabolism and Adaptation', Amsterdam University	Feb 18, 2009
Poster: Lunteren Meeting	Apr 06-07, 2009
► **IAB interview**	Dec 05, 2008
Subtotal Scientific Exposure	*9.7 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
SAS/Statistical analysis course for Graduate Research Fellows (IITA Ibadan, Nigeria)	Nov 02 -Dec 08, 2006
12th Annual Summer Institute in Statistical Genetics University of Washington Seattle, USA	Jun11-26, 2007
Summer School: 2nd Annual Wisconsin Entrepreneurship Bootcamp, Madison, USA	Jul 20-25, 2008
PhD workshop: Natural variation in plants, Botanical Centre, Wageningen WUR	Aug 26-29, 2008
► **Journal club**	
► **Individual research training**	
Practical training of LI-COR AFLP and Quantar scoring techniques	Mar 20 -Apr 14, 2006
Visit to Prof. Timko Lab University of Virginia, Charlotteville USA to Learn the Cowpea Genomics Initiative	Jun 27-30, 2007
Visit to University of California Riverside, USA for cowpea SNP data processing	May 03-10, 2009
Subtotal In-Depth Studies	*11.7 credits**

4) Personal development	*date*
► **Skill training courses**	
PhD competence Assessment and Individual Interview	Apr 11 & 25, 2006
Working with Endnote	Mar 09 & 15, 2006
Proposal writing at IITA	Sep 06, 2007
Career orientation	May 16-Jun 13, 2008
► **Organisation of PhD students day, course or conference**	
Organisation IARSAF/IITA 13th Annual Symposium 2008: Sustainable Agriculture in Sub-Saharan Africa: A critical look into the constraint and prospects	Feb 13, 2008
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.9 credits**
TOTAL NUMBER OF CREDIT POINTS*	**38.8**

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Back page: cowpea plant taken from IITA web site

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