Cowpea: a legume crop for a challenging environment

Running title: Cowpea for a challenging environment

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Abstract

Cowpea is a grain legume native from Africa and is a primary source of protein for millions of people in sub-Saharan Africa and other parts of the developing world. The main important characteristics of this crop include the good protein quality with a high nutritional value, the nitrogen-fixing ability, and be more drought- and heat-tolerant than most of its legume relatives. In a research perspective, the studies with cowpea are relatively scarce, despite its relevance to agriculture in the developing world and its resilience to stress. This review provides an overview on different aspects of cowpea, with special emphasis on the molecular

markers for assessing genetic diversity, as well as on the biochemical and transcriptomic data to evaluate cowpea drought stress tolerance. The integration of both datasets will be useful for cowpea improvement, since the research on drought stress tolerance is a major interest for this crop in a challenging environment.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is a member of Leguminosae family native from Africa and is currently one of the most important grain legumes growing in tropical and subtropical regions.[1–3] This legume has been used in human diet as well as forage for animal feeding. For human consumption the most important product is the dry grain that can be consumed boiled, fried (as akara), or steamed (as moi moi),[4] according to different preparations, on salads, snacks, cakes and others. Also young leaves, fresh pods and fresh seeds have been consumed in some world regions.[4,5] Green organs could be used as vegetable and are often served boiled, as well as consumed fried or fresh.[5] One of the most important characteristics of cowpea is the high nutritive content value in all plant parts.[3,4,6] The dry grain is rich in proteins (23% to 32%), essential amino acids as lysine (427 mg/g N), and tryptophan (68 mg/g N) although low in the sulphur-containing amino acids.[7,8] So, cowpea and cereals complement each other in terms of amino acids and consequently a diet combining both provides a balanced protein intake. The presence of both minerals (iron and zinc) and

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vitamins (folic acid and vitamin B) has also been reported to be important to prevent birth defects during the pregnancy stage.[3,9,10] Dry grain is also high in fibre and low in fat.[8] Taking into account these advantages, an increase of cowpea production and consumption in the European Union is highly desirable. Currently, the European Union imports from African countries almost all consumed cowpea, more specifically from Niger and Nigeria. During the 2009-2013 period, the world cowpea planting area was 5 million hectares and the production was 12 million tonnes. Africa has been responsible for 95.4% of worldwide cowpea production,[11] being the drier savannah and the Sahelian region of West and Central Africa responsible for producing 72%. Nigeria and Niger are the largest producers with 3.4 and 1.4 million tons, respectively. In contrast, Europe is only responsible for 0.4% of worldwide cowpea production and the European Union have only produced 463 thousand tonnes during the period 2009-2013.[11]

As revealed by the major producing countries, cowpea has the capacity to grow in low fertility soils, which has much to do with the ability of establishing associations with distinct microorganisms, mainly nitrogen-fixing bacteria (like rhizobia) and vesicular-arbuscular mycorrhizal fungi. Cowpea tolerance to low fertility soils[8,12,13] and to a wide range of soil pH,[14] as well as the adaptation of cowpea to high temperatures and drought,[15] makes this grain legume crop of interest for facing the predicted environmental changes (e.g., increased temperature, reduction of water availability) associated with climate change. This review provides an overview of different issues about genomic and transcriptomic studies in cowpea, with more emphasis to studies related with genetic diversity and cowpea drought stress tolerance that could be useful to integrate in cowpea breeding programs.

CLASSIFICATION AND DESCRIPTION

The cowpea cultivated form obtained from the Antilles was first described by Linnaeus as *Dolichos unguiculatus* L., being later classified by Walpers as *Vigna unguiculata* (L.) Walp..[16] This diploid species ($2n = 2x = 22$) belongs to the division Magnoliophyta, class Magnoliopsida, order Fabales, family Leguminosae, tribe Phaseoleae, genus *Vigna*. The genus *Vigna* includes more than 80 species[17] and was subdivided into six sections, namely, *Vigna*, *Comosae*, *Macrodontae*, *Reticulatae*, *Liebrechtsia*, and *Catiang*.[18] *Vigna unguiculata* (L.) Walp. includes annual cowpeas (ssp. *unguiculata*) and ten wild perennial subspecies (Table 1).[19] The subspecies *unguiculata* includes all the domesticated forms (var. *unguiculata*), as well as the wild and weedy forms [var. *spontanea* (Schweinf.) Pasquet].[19,20] The domesticated forms are subdivided into four cultivar-groups essentially based on seed and pod characters.[19,21] These cultivar-groups are *unguiculata* grown as pulse, *biflora* (catjang) used mainly as forage, *sesquipedalis* (asparagus bean) grown as a vegetable, and *textilis* cultivated for the fibres of its long floral peduncles.[19] Pasquet[16] also proposed the insertion of *melanophthalmus* (black-eyed pea) as another cultivar-group.

"Cowpea" is the *V. unguiculata* most popular worldwide name but local names such as black-eyed beans, black-eyed peas, pink-eyes or southern peas (all used in United States of America), 'frijol caupí' (Spanish speaking countries in America), 'lobia' (India), 'caupi' (Brazil), 'caupí' and 'carilla' (Spain), 'niébé' (French speaking countries of Africa) and 'feijão-frade' (Portugal) are used.

Cowpea is described as an herbaceous warm-season annual plant with a great variability in morphology. This crop is autogamous but around 5% outcrossing was reported in the cultivated varieties probably due to insect activities.[22,17] Its growth habit could be prostate (trailing), semi-prostate, semi-erect, erect or climbing, depending mostly on genotype but also on photoperiod and growth conditions, being the pattern of growth determinate or indeterminate.[8,23] This crop is well adapted to a wide range of soil types from sands to heavy,

including low fertility soils.[24] Plants grow in an extensive range of temperatures, being 28 ºC the optimal temperature. Early flowering cowpea can produce a crop of dry grain in only 60 days, while longer season cowpeas may require more than 150 days to produce mature pods, depending on photoperiod.[8]

According to the International Institute of Tropical Agriculture (IITA) and Bioversity International (ex-International Board for Plant Genetic Resources, IBPGR), the leaves can be classified into four categories: sub-globose, sub-hastate, globose and hastate/lanceolate.[23] Flowers emerge in alternate pairs on racemes at the distal ends of long peduncles, with usually two flowers per inflorescence. Flowers have a short life cycle, opening in the early day and closing at approximately midday, after which they usually wilt and collapse.[25] Corollas can be purple, mauve-pink, yellow or white.[23] Each peduncle commonly develops two or three pods and pods differ in size, shape, colour and texture.[8] They are cylindrical, but could be straight, slightly curved, curved or coiled and when they ripe the colour can vary from yellow to brown or dark purple.[23] The sub-species/cultivar-group *Sesquipedalis* (more common in Asia) have very long green pods (40 to 100 cm) that are often used as green beans (or snap beans),[8] while the other groups have standard pods (10 to 25 cm). Seeds differ in size and colour, ranging from white, cream, green, buff, red, brown or black and can be kidney, ovoid, crowder, globose or rhomboid and are characteristic by the presence of an eye, due to the different pigmentations encircling the hilum.[23]

Environmental conditions, including photoperiod and growing conditions (temperature, rainfall, etc.), can also affect the plant height and morphology.[8,24] Cowpea root system is dense and well-developed[26] and has a beneficial effect on the structure and tilth of the topsoil layer. Most root growth occurs within the topsoil layer, but in drought conditions a long taproot can grow for reaching the deeper moisture in the soil profile.[27] These

characteristics furnish cowpea plants with a high resistance to drought in comparison with other legumes.

ORIGIN, DOMESTICATION AND DISTRIBUTION

Africa was suggested as the centre of origin of cowpea.[28] This belief was not contested, since wild cowpea plants have been found in tropical Africa and Madagascar,[1] where it was presumably domesticated since Neolithic age.[29] Pasquet[30] suggested that the most likely progenitor of domesticated cowpea is *V. unguiculata* ssp. *unguiculata* var. *spontanea*. For determining the precise domestication site and the cowpea diversity centres, several studies have been performed in the last decades, although a conclusive result has been difficult to reach. Several hypotheses have been proposed for cowpea domestication, such as Ethiopia,[1,31,32] West Africa,[33–37] Eastern and Southern Africa.[38] Coulibaly *et al.*[19] using amplified fragment length polymorphisms (AFLPs) and morphologic data concluded that the wild species was originated from Eastern Africa. In this case, domestication should have occurred in Northeastern Africa and the domesticated plant was then probably dispersed to Western Africa. According to Ng and Padulosi,[39] West Africa seems to be the centre of diversity of cultivated forms. A 'diffuse' domestication in the African savanna after the dispersal of cereals was also hypothesized.[1,40] This last hypothesis was presented by Harlan,[41] who considered that the cowpea was domesticated in the African Non-Center. Whatever the place of domestication, cowpea is an ancient legume that was domesticated by African gatherers, cultivators and farmers from its wild forms in Africa dating back to Neolithic times.[2] During the Neolithic period, the cowpea was first introduced into India, which was then considered a secondary centre of cowpea genetic diversity.[42] The spread of cowpea in Asia occurred at the end of Neolithic period (third millennium BC), where the subspecies

asparagus bean or yardlong (*V. unguiculata* ssp. *sesquipedalis*) is still cultivated for long immature pods,[43] and in America between the sixteenth and seventeenth centuries (AD).[44] Although some reports suggest that cowpea has been cultivated in Europe at least since the eighteenth century BC and possibly since prehistoric times,[19,45] others suggest that it was only introduced in Europe around 300 BC, where it still remains as a minor crop in the southern part.[17] From Europe, more specifically from Portugal and Spain, this legume was exported in the seventeenth century to the New World.[17,46] Other important result was obtained by Fang *et al.*[46] that provided evidences for the common origin of cowpea germplasm from Asia and North America different from the West Africa. However, these authors have mostly used breeding lines and consequently the introgression of extra regional germplasm could have occurred. Huynh *et al.*[47] analysing a worldwide collection of cowpea landraces and African ancestral wild cowpeas by using more than 1200 single nucleotide polymorphism (SNP) markers verified that accessions from Asia and Europe were more related to those from western Africa, while accessions from Americas appeared more closely related to those from Eastern Africa.

EVALUATION OF GENETIC DIVERSITY

Cowpea has been referred as a worldwide crop with more prevalence in tropical areas, displaying a high phenotypic/morphological variability.[8] Genetic diversity assessment is then useful for the preservation and utilization of germplasm resources, as well as for the improvement of varieties/cultivars.[3] Genetic diversity can be evaluated using morphological traits, biochemical and molecular markers. Each of these markers has different applications in several areas, such as plant breeding, phylogenetic studies, gene mapping, genetic

engineering, micropropagation and genetic resources characterization, and can be used individually or combined.

Several studies have been referring the characterization of cowpea by morphological and quantitative traits.[16,48–52] This characterization is followed by using a set of descriptors: i) parameters related with plant morphology, such as growth habit, leaf type, flower colour, seed shape and colour, and ii) parameters related with plant production, namely the number of pods and seeds per plant and seed weight. Morphological characterization does not require any complex equipment or experiments, being simple and inexpensive to score. These are the reasons responsible for the constant use of morphological traits as a first step for evaluating genetic relationships. The main disadvantage is that the observed characteristics do not exclusively reflect the genotype, but reflects the interaction between genotype and environment.[53]

The first biochemical marker to be used for genetic diversity analysis was the isozyme markers in the 60's.[54] These enzymes differ in amino acid sequence and are encoded by different genetic *loci* (isozymes) or by different alleles at the same *locus* (allozymes), yet catalyse the same reaction.[55] Until the end of 80's, isozymes were the main marker used to analyse the genetic variability and taxonomy in plants, helping to define the phylogenetic relationships and population genetics. Over the years, several studies were developed in cowpea that made use of this biochemical marker. Panella and Gepts[56] and Vaillancourt *et al.*[57] characterized wild and cultivated accessions of cowpea by using 10 and 26 isoenzyme *loci*, respectively, and concluded that the genetic diversity in the evaluated collections was low. Besides isozyme markers, seed storage protein profiling is another method used to reveal genetic variation between cowpea cultivars.[58–62] Often, in these studies, the obtained results were not very conclusive by the lack of domesticated cowpea and progenitor representative samples.

In comparison with morphological and biochemical markers, DNA molecular markers have a set of characteristics that turn them ideal to several studies such as their highly polymorphic nature and frequent occurrence in the genome, allowing a direct comparison of genetic material in an environmental independent way.[54,63] DNA-based molecular markers have been extensively used in cowpea genetic diversity research, variety identification, phylogenetic analysis, gene mapping, and resource classifications (Table 2). The first study using amplified fragment length polymorphism (AFLP) markers in cowpea was performed by Coulibaly et al.,[19] in which the genetic relationship among a total of 117 cowpea accessions [including 47 domesticated cowpeas (ssp. *unguiculata*) and 52 wild and weed annuals (ssp. *unguiculata* var. *spontanea*)] was pretended. This study showed that the wild cowpeas were more diverse than domesticated ones, also suggesting an Eastern African origin for the wild taxon. This result was corroborated by Ba et al.[2] using random amplified polymorphic DNA (RAPD) markers, and by Ogunkanmi et al.[64] with single sequence repeat (SSR) or microsatellites markers. The variation within and among cowpea populations from different agro-ecological regions and germplasm accessions has been also evaluated using AFLP[46] and RAPD markers.[65–68] In addition, RAPD markers were used to eliminate the putative duplicates of Senegal cowpea accessions in a germplasm bank and identify elite varieties.[69] Currently, SSR is the most frequently used molecular marker in cowpea genetic diversity analyses, namely in cowpea landraces accessions from China, Africa and other Asian countries,[70] Korea,[71] Ghana,[72] Southwestern Nigeria,[73] and Senegal,[74] where a high genetic diversity was observed. To evaluate the genetic diversity of asparagus bean (*V. unguiculata* ssp. *sesquipedalis*) cultivars from different Chinese geographical origins, SSR markers derived from *V. unguiculata* ssp. *unguiculata* sequences were used, attesting the transferability of SSR markers between these two subspecies.[75] In all these studies, SSR markers also showed sufficient genetic variance that could be useful for improvement

strategies in cowpea. Single nucleotide polymorphism (SNP) markers have gained an increasing importance, due to their bi-allelic nature, higher frequency in the genome than SSRs and other markers, and to their easily automated genotyping.[76] In a study for the characterization of 113 cowpea accessions, comprising 108 from Ghana and five from abroad, 458 SNPs (out of 477) revealed high polymorphism.[77] The results suggested an unexpected high level of heterozygosity. The chip-based SNP detection technology is being widely used in plant genetic applications.[78–80] In cowpea, Illumina chip-based SNP detection platforms (GoldenGate and more recently iSelect) have been developed and can be very useful for molecular characterization,[77,81] genetic diversity analysis[47,82] and genetic mapping.[43,83–85] Researchers at the University of California, Riverside, in partnership with institutions from several African countries have designed a 60,000-assay iSelect BeadArray for cowpea that successfully assayed 51,128 SNPs.[86]

The combined use of different molecular markers could better assist the evaluation of genetic diversity. Diouf & Hilu[87] used a combination of RAPD and SSR markers for assessing genetic variability of local cowpea varieties and breeding lines from Senegal and identified 12 polymorphisms due to the broad genome coverage used. The combinations of AFLP and SAMPL (selectively amplified microsatellite polymorphic locus) markers,[88] as well as AFLP and SSR markers,[89] were used to determine the genetic variation within and among closely related *V. unguiculata* accessions, while the combined use of RAPD and ISSR markers allowed the evaluation of genetic variations of seven *Vigna* species.[90] A combination of molecular and classical markers has been considered essential to turn the results of genetic diversity more reasonable for genetic cowpea breeding and evaluation of germplasm resources.[3] The combined use of molecular markers (SSR and ISSR) and classical markers (morphological traits) was described to estimate the genetic diversity and relatedness of 23 asparagus bean (*V. unguiculata* ssp. *sesquipedalis*) accessions and 7 accessions of a hybrid

between cowpea (*V. unguiculata* ssp. *unguiculata*) and dwarf asparagus bean in Thailand.[91] Morphological characters were diverse among most accessions, but their exclusive use did not allowed to distinguish between accessions. Indeed, ISSR markers showed higher efficiency for estimating the levels of genetic diversity and relationships among the two subspecies than SSR markers.[91] The combined use of morphological traits, RAPD and ISSR markers was also employed for discriminating landraces of cowpea scattered from all Algeria regions[92] and evaluate the genetic variability and relationships between two cowpea cultivars and nine elite genotypes.[93] Both studies showed that ISSR markers were better linked to morphological variation than RAPD markers.

Genetic mapping and marker-assisted selection

Currently, the construction of the cowpea genetic map is mainly based on the use of efficient molecular markers, such as SSR and SNP, which show sufficient genetic variability.[43,83,84,94–97] A consensus genetic linkage map using EST-derived SNPs led to the integration of 928 markers into a cowpea genetic map spanning 680 cM with 11 linkage groups (0.73 cM of average marker distance).[98] These authors reported a significant macrosynteny with *Glycine max* and *Medicago truncatula* genomes, and some microsynteny with *Arabidopsis thaliana* genome. The first genetic map of asparagus bean based on SNP and SSR markers was reported by Xu *et al.*.[43] This map consisted of 375 *loci* mapped on 11 linkage groups, with 191 *loci* detected by SNP markers and 184 *loci* by SSR markers. The development of a high-density genetic map offers a powerful tool for analysing the inheritance of target genes, monitoring specific genes or genomic regions transmitted from parents to progeny.[3] Using the recently developed Illumina iSelect genotyping assay for cowpea, Muñoz-Amatriain *et al.*[85] genotyped five biparental RIL populations and developed a consensus genetic map containing

over 37,000 SNPs mapped to ~3,200 bins in ~800 cM. These results are being used to genetically anchor an initial whole-genome shotgun (WGS) assembly of the cowpea accession IT97K-499-35. To this assembly, sequences from about 4,000 minimal tiling path BACs are being incorporated with the aim to increase the number of anchored scaffolds and help resolve the order within recombination bins.

The biotechnology based on such genetic maps and use of DNA markers brings a great hope in cowpea breeding, as specific molecular markers could be used to select target traits with marker assisted selection (MAS).[17] The association of 18 SNPs with seed size in cowpea varieties from Ghana suggested that these molecular markers could be useful for marker assisted breeding of larger seeded cowpea plants.[99] Performing a RFLP analysis of 29 polymorphic markers, among 14 drought-tolerant genotypes, it was possible to find a correlation between seven RFLP markers and different drought-related cowpea phenotypes.[100] The additional use of other high-density DNA markers in the genome could speed even more the selection process in breeding programs. For breeding to resistance to the parasitic weed *Striga gesnerioides*, SSR[101] and AFLP[102,103] markers have been used. Similarly, SNPs have been used to identify markers associated to cowpea resistance to foliar thrip.[104] The asparagus bean rust disease, caused by the fungus *Uromyces vignae*, was also associated to a specific AFLP marker that can now be effectively used for MAS.[105] Sequencing and analysis of the gene-rich hypomethylated portion of the cowpea genome was performed by Timko *et al*.[106] More than 250,000 gene-space sequences reads (GSRs) were generated, thus providing a source of functional markers for detailed comparative studies of cowpea with other plant species and positional cloning of key genes of agronomic interest.

TOLERANCE TO DROUGHT STRESS

Drought is one the most severe environmental stresses with major impact on plant development and productivity thus causing serious agricultural yield losses.[107,108] Drought tolerance is a complex trait defined as the ability of plants to live, grow, and reasonably produce with limited soil water supply or under periodic water deficiencies.[109] Mitra[110] grouped the plant mechanisms used to cope with drought stress into three groups: drought escape, drought avoidance and drought tolerance. Crop plants could use more than a single mechanism to cope with drought stress. One of the most important food legumes in tropical and sub-tropical regions, where drought is a major constraint for production due to low and erratic rainfall, is cowpea. Indeed, some authors pointed cowpea as one of the most tolerant crops to drought, due to its capacity of growing in areas with no irrigation facilities and irregular rainfall.[24,111–113] This tolerance has been attributed to the three drought tolerance mechanisms,[112] although several drought avoidance mechanisms were extensively described, including deep rooting, strong stomatal sensitivity, reduced growth rate, leaf area reduction, delayed leaf senescence, hastened or delayed reproductive cycle, osmotic adjustment and sensitive moisture remobilization to the upper leaves and growing tips.[109,113] Since cowpea has the ability to tolerate severe drought conditions and displays a relatively small nuclear genome size (estimated at ~620 Mb), this legume has been considered as an ideal model to study the molecular mechanisms of drought tolerance in crops.[112]

Morphological, biochemical and physiological traits for drought

Changes of morphological, biochemical and physiological traits in response to drought stress for several *V. unguiculata* cultivars have been reported.[114–117] The root system or rooting pattern are closely related to drought-tolerance mechanisms in legume crops.[118,119] To evaluate and screen cowpea drought-tolerance, several parameters of the root system have been used,

such as root length density, rooting depth and root dry matter.[119] To examine cowpea drought tolerance ability, water potential, relative turgidity, diffusion pressure deficit, chlorophyll stability index measurements, or carbon isotope discrimination are typically evaluated.[109,120] However, most of these methods have the disadvantage of being slow, laborious, expensive, and influenced by environmental conditions.[109,112] Slabbert *et al.*[114] tested and proposed other methods to screen cowpea for drought tolerance, such as proline accumulation, 2,3,5-triphenyltetrazolium chloride (TTC) assays, cell membrane stability (CMS), relative water content (RWC), leaf water potential (LWP), leaf area, chlorophyll *a* and *b* contents, chlorophyll fluorescence, carotenoids content, evaluation of anti-oxidative responses through enzyme activities determination [superoxide reductase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX)], as well as the early drought screening at the seedling stage (wooden box technique). Altogether, these methods pretend to evaluate the most typical changes that occur in plants after a drought imposition.

As the complex regulatory processes of drought adaptation involves the control of water flux and cellular osmotic adjustments via the biosynthesis of osmoprotectants,[108] such compounds determination has often been used for screening tolerant cowpea genotypes. The osmoprotectants are classified into three major groups: amino acids (e.g. proline), polyol/sugars (e.g. trehalose, fructans, mannitol), and quaternary amines (e.g. glycine betaine).[121–123] However, these compounds are not accumulating in all plant species in sufficient amounts to avoid adverse effects of drought stress.[122,124] Studies in drought stress cowpea and osmoprotectants are still scarce. But, the application of chitosan in drought stress cowpea plants has been described to allow the maintenance of osmotic balance.[125]

Physiological changes related with photosynthesis and stomatal conductance have also been frequently used in drought evaluation studies. Indeed, one of the processes largely affected by water deficit is photosynthesis, due to a decline of stomatal conductance that

limits the carbon assimilation, as well as due to biochemical and photochemical adjustments.[126,127] The dynamics of photosynthesis (A), stomatal conductance (gs) and intrinsic water-use efficiency (WUE=A/gs) were evaluated in 14 cowpea genotypes over a period of drought and post-stress.[128] Under water stress conditions, a decrease in photosynthesis and stomatal conductance accompanied with an increase in the intrinsic water-use efficiency was detected in all genotypes, although differences between genotypes were found.[128] When cowpea genotypes, differing in drought resistance, were subjected to three distinct water stress conditions (unstressed, moderate and severe stressed), an increase on root biomass and a reduction on chlorophyll content were detected with water stress imposition.[115]

One of the main regulators of plant drought tolerance is the abscisic acid (ABA) that, not only regulates many essential processes of plant development, including the inhibition of germination and control of stomatal closure, but also several adaptive responses to a variety of environmental stresses.[129,130] Kulkarni *et al.*[131] studying the response of six cowpea cultivars to drought stress suggested that the intrinsic capacity for ABA synthesis could play an important role in regulating stomatal conductance. ABA accumulation is higher in drought-stressed plants than in unstressed plants.[112] In cowpea, some studies have been developed to understand the role of ABA in the drought tolerance.[132,133]

As membranes are the key targets of degradative processes induced by drought, membrane integrity parameters have also been used for assessing drought stress severity. A decrease in membrane lipid content was reported under water stress,[134] which seems to be correlated to the inhibition of lipid biosynthesis and stimulation of lipolytic and peroxidative activities.[135,136] The degradation of membrane lipids and the enzymatic antioxidant activity seem to be a useful method to evaluate the level of plant drought stress. However, data are still scarce in cowpea.[111,114,136–138]

Agbidoco *et al.*[112] suggested that the most suitable parameters for screening a large number of cowpea lines for drought tolerance are the measurements of chlorophyll fluorescence, stomatal conductance, ABA and free proline levels. Besides these parameters, the wooden box screening for drought tolerance at the seedling stage and delayed leaf senescence (DLS) could be interesting to evaluate and determine drought tolerance. Physiological, biochemical and agronomic responses to water deficit at flowering stage of cowpea detected an increase of canopy temperature and proline content, as well as a decrease of gaseous exchanges and starch content that eventually affect the yield components with exception of seed number per pod.[139]

The knowledge transfer between plant species and cultivars should be taken with care, since differences in drought tolerance were detected when evaluating distinct plant species or cultivars. For example, a comparison of physiological responses to drought between *Vigna unguiculata* and *Phaseolus vulgaris* demonstrated that both species significantly differ in the responses evaluated by leaf gas exchange parameters.[140]

Drought tolerance genes

Transcriptomic studies have been developed to identify genes, pathways and processes important in controlling plant response to multiple abiotic or biotic stresses, thus providing candidate targets for stress tolerance improvement.[141] Many cowpea drought-related genes have been deduced from previously recognized candidate genes for drought tolerance in other related species, and were then confirmed by their differential expression in drought-stressed versus non-stressed cowpea plants. On the other hand, studies on the differential expression of cowpea genes in experimental plants subjected to different levels of water privation have led to the identification of cowpea genes involved in drought responses.[112]

Many cowpea genes are now recognized as being involved in drought responses (Table 3). Using a differential screening method, Iuchi et al.[142] isolated 24 cDNA clones that corresponded to dehydration-induced genes from a cowpea variety (IT84S-2246-4) displaying a high drought tolerance. These cDNA clones represented ten different genes, nine of which were specifically induced by dehydration stress. Five of these drought-associated genes were further characterized (*CPRD8*, *CPRD14*, *CPRD22*, *CPRD12* and *CPRD46*),(142,143) being followed by the description of two additional drought-inducible genes all from the same cowpea variety (*VuNCED1* and *VuABA1*).(132) *VuNCED1* encodes a 9-cis-epoxycarotenoid dioxygenase that catalyses a key step in ABA biosynthesis, while *VuABA1* encodes a zeaxanthin epoxidase[132] involved in another important key step of ABA biosynthesis. Indeed, zeaxanthin epoxidase has been reported as required for resistance to osmotic and drought stress, ABA-dependent stomatal closure and regulate the expression of stress-responsive genes.[144]

According to the degradation of membrane lipids that occur under drought stress conditions,[134] several other cowpea drought-related genes are recognized to be involved on lipid metabolism. El-Maarouf et al.[135] isolated and characterized the cowpea *VuPLD1* gene that encodes a phospholipase D, which is the main enzyme responsible for the drought-induced degradation of membrane phospholipids. In a drought stress susceptible cultivar, phospholipase D activity and *VuPLD1* expression were highly stimulated by drought stress, while remained unchanged in a tolerant cultivar.[135] From the leaves of the same cultivars, Matos et al.[136] isolated a *VuPAT1* (putative patatin-like) gene that encodes for galactolipid acyl hydrolase. A rapid increase of *VuPAT1* expression was also observed in the susceptible cultivar under drought conditions, while the tolerant exhibited lower levels of transcripts. These results suggest that drought stress in cowpea stimulates the hydrolysis of galactolipids, the main components of chloroplast membrane. *VuPAP-α* and *VuPAP-ß* are two cDNAs

encoding putative phosphatidate phosphatases (PAPs) that were cloned from cowpea leaves by Marcel *et al.*.[145] PAP plays a role in the enzymatic cascade that leads to membrane lipid degradation under environmental stresses or senescence.[137] Marcel *et al.*[145] revealed that gene expression of *VuPAP-α* remained very low during drought treatments, being strongly stimulated after rehydration. On the other hand, *VuPAP-ß* expression did not vary in plants submitted to water stress by withholding irrigation, but increased rapidly in air desiccated leaves.

Metabolic and adaptive processes, in which the adaptation to drought stress is included, comprise the regulation of protein degradation through the use of protease-specific inhibitors[146] and cellular protection against oxidative damage through the regulation of anti-oxidant enzymes and free radical scavengers.[147] The expression of cowpea cystatin (cowpea leaf protease inhibitor; *VuCI*) gene, evaluated at mRNA (Northern analysis) and protein (Western analysis) levels, suggested that two cystatin transcripts producing two distinct polypeptides would lead to a multiplicity of forms related to multiple biological roles.[146]

A noticeable activation of cowpea antioxidant metabolism has been detected under progressive water stress by studying drought-related genes. The cloning and sequencing of two new cDNAs encoding a putative dual-targeted (*dtGR*) and a cytosolic (*cGR*) glutathione reductase (GR) from cowpea leaves was performed by Contour-Ansel *et al.*.[138] The expression of both genes in cowpea leaves of drought-sensitive and drought-tolerant plants subjected to different drought stress conditions revealed that up-regulation of *cGR* expression is directly related to the intensity of stress in both cultivars, but *dtGR* expression was different in susceptible and resistant cultivars. The results revealed the participation of GR in drought responses of both cowpea cultivars, which in susceptible cultivar involves both GR genes.[138] The expression of other antioxidant enzyme genes (ascorbate peroxidases; APX) was also studied in cowpea response to progressive drought, rapid desiccation and application of

exogenous ABA. Four new cowpea cDNAs encoding putative cytosolic (*VucAPX*), peroxisomal (*VupAPX*), chloroplastic (stromatic *VusAPX*) and thylakoidal (*VutAPX*) ascorbate peroxidases were isolated and characterized.[111] When the expression levels of *VucAPX* and *VupAPX* were followed in drought-tolerant and sensitive cultivars, an increase in steady-state transcripts levels was observed in response to rapid water loss and exogenous ABA treatment in drought-sensitive cultivar, while no significant changes in drought-tolerant cultivar were registered. Also, the *VusAPX* gene expression was strongly stimulated at low levels of water stress in drought-tolerant cultivar. The higher expression of all these genes in tolerant cultivars, comparatively to sensitive ones, suggested once more that cowpea is a drought-tolerant species compared to other crops, indicating that even the more sensitive cultivars have some level of resistance to water deficits.[111] Two other well-recognized stress-related genes, *GST* (glutathione-S-transferase) and *PR-1* (pathogenesis-related-protein-1), were identified in cowpea by suppression subtractive hybridization (SSH) using drought-tolerant and susceptible lines.[148] Silva *et al.*[149] followed the effect of drought and heat stresses on cowpea nodules by evaluating the differential gene expression, using a cDNA-AFLP approach, and identified 14 differentially expressed nodule stress responsive genes. These genes are involved in different metabolic processes, five (*VuNSR4*, *VuNSR10*, *VuNSR44*, *VuNSR47* and *VuNSR49*) of which were related with the nodule protection under abiotic stress conditions as revealed by their expression levels.[149]

MicroRNA drought regulation

MicroRNAs (miRNAs) regulate gene expression at posttranscriptional level through the recognition of target RNAs by nearly perfect base complementary. Several functional analyses have demonstrated that miRNAs are involved in a variety of plant developmental

processes and play important roles in plant resistance to abiotic and biotic stresses.[150,151] From two cowpea genotypes, one drought-tolerant and another drought-sensitive, 157 miRNAs were identified, 44 of which were drought-associated, being 30 upregulated and 14 downregulated in drought conditions. Cowpea miRNAs from leaves and roots of plants subjected to drought treatment were also identified and validated by real-time-quantitative PCR.[152] The results demonstrated that the same miRNAs in different tissues respond differently to drought stress. Both studies suggest that miRNAs could play an important role in cowpea response to drought stress by regulating the expression levels of drought-related genes.

CONCLUSION

Global climate changes have an enormous impact on plant diversity patterns with significant current negative effects. In Europe, Mediterranean countries are the ones where is expected a higher impact of climate changes will be expected, including an increase in drought, high temperatures, and water scarcity. Drought is a critical constraint for agricultural production yield, which is currently expanding worldwide and affecting an increased number of countries. New strategies are thus required to overcome this major challenge in agricultural production systems, such as the development of new farming systems and use of undervalued crop varieties. Due to its natural tolerance to water scarcity conditions and high temperatures, cowpea could be considered as a valued crop for increasingly drought scenarios. Besides drought tolerance, cowpea also presents high levels of protein and the capacity to establishing symbiotic associations with distinct microorganisms (rhizobia and mycorrhizal fungi, mainly) that turns it as an environmentally friendly crop. This legume could also be a useful plant model for understanding the mechanisms involved in drought tolerance. The existence of

several cowpea varieties and cultivars, displaying different tolerance levels to drought conditions, provides an excellent germplasm resource for identifying new candidate genes involved in responses to drought stress tolerance and also for being used in future breeding programmes. DNA molecular markers have shown to be a good tool for germplasm evaluation and selection of the most interesting drought stress/tolerant genotypes. As the marker assisted selection (MAS) can facilitate the selection of elite germplasm and accelerate plant breeding programs, the identification of the precise position of drought-related known genes and of new candidate genes should be done. The integration of data from phenotype, biochemical and molecular characterization will help to understand the resilience and resistance of cowpea under drought and provide sufficient cowpea knowledge for the development of drought-tolerant varieties. For these reasons, cowpea can also be an important plant model for the development of other crop varieties more drought tolerant.

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TABLES

Table 1 – Taxonomic classification of cowpea.

Species	Subspecies	Variety	Cultivar group
Vigna unguiculata	*unguiculata*	*spontanea*	
		unguiculata	*unguiculata* *biflora* *sesquipedalis* *textilis* *melanophthalmus*
	baoulensis *burundiensis* *letozeyi* *aduensis* *pawekiae* *dekindtiana* *stenophylla* *tenuis* *alba* *pubescens*		

Table 2 – DNA-based molecular markers that have been used for specific cowpea studies.

Molecular Marker	Sub-species	Objective	References
AFLP	*V. unguiculata* ssp. *unguiculata*	Markers linked to cowpea	Ouédraogo *et al.,*

	Species	Focus	Reference
		parasitism resistance	2001[153]
	V. unguiculata ssp. *unguiculata*	Phenetic organization and	Coulibaly *et al*.,
	V. unguiculata ssp. *spontanea*	genetic diversity	2002[19]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Fang *et al*., 2007[46]
	V. unguiculata ssp. *unguiculata*	Markers linked to cowpea golden mosaic virus	Rodrigues *et al*., 2012[154]
RFLP	*V. unguiculata* ssp. *unguiculata* *Vigna radiata*	Markers linked to orthologous seed weight genes	Fatokun *et al.,* 1992[155]
	V. unguiculata ssp. *unguiculata*	Markers linked to aphid resistance gene	Myers *et al.,* 1996[156]
	V. unguiculata ssp. *unguiculata*	Diversity of indigenous bradyrhizobia	Krasova-Wade *et al.,* 2003[157]
	V. unguiculata ssp. *unguiculata*	Markers linked to genotypic and phenotypic responses to seedling-stage drought	Muchero *et al.,* 2008[100]
RAPD	*V. unguiculata* ssp. *unguiculata*	Genetic diversity	Fall *et al.,* 2003[69]
	V. unguiculata ssp. *unguiculata*	Genetic relatedness and gene flow	Nkongolo, 2003[158]
	V. unguiculata ssp. *unguiculata* *V. unguiculata* ssp. *spontanea*	Genetic diversity	Ba *et al.,* 2004[2]
	Vigna unguiculata ssp. *unguiculata*	Genetic diversity	Zannou *et al.,* 2008[65]
	V. unguiculata ssp. *unguiculata* *Phaseolus vulgaris*	Genetic diversity and markers linked to cowpea resistance to pests weevil pests	Abdel-Sabour *et al.,* 2010[159]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Malviya *et al.,* 2012[66]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Prasanthi *et al.,* 2012[67]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Patil *et al.,* 2013[68]
SSR	*V. unguiculata* ssp. *unguiculata* *V. unguiculata* ssp. *dekindtiana* var. *pubescens*	Genetic diversity and relationships	Li *et al*., 2001[105]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Xu *et al*., 2007[70]
	V. unguiculata ssp. *dekindtiana* *V. unguiculata* ssp. *ovata* *V. unguiculata* ssp. *kgalagadensis* *V. unguiculata* ssp. *rhomboidea* *V. unguiculata* ssp. *Pubescens* *V. unguiculata* ssp. *mensensis*	Genetic diversity	Ogunkanmi *et al*., 2008[64]

	V. unguiculata ssp. *grandiflora*		
	V. unguiculata ssp. *congolensis*		
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Lee *et al*., 2009 [71]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Asare *et al.,* 2010[72]
	V. vexillata	Genetic diversity and SSR transferability between *Vigna* species	Gupta and Gopalakrishna, 2010[160]
	V. umbellate		
	V. glabrescens		
	V. aconitifolia		
	V. trilobata		
	V. angularis		
	V. radiata		
	V. radiata		
	V. radiate var. *setulosa*		
	V. radiate var. *sublobata*		
	V. mungo		
	V. mungo var. *silvestres*		
	V. unguiculata ssp. *unguiculata*	Genetic diversity of cowpea cultivars resistant to *Striga gesnerioides*	Sawadogo *et al.,* 2010[101]
	V. unguiculata ssp. *sesquipedalis*	Genetic diversity and SSR transferability between sub-species	Xu *et al.,* 2010[75]
	V. unguiculata ssp. *unguiculata*		
	V. unguiculata ssp. *unguiculata*	Genetic distance and diversity	Adewale *et al*., 2011[48]
	V. unguiculata ssp. *unguiculata*	Genetic map and identification of QTLs	Andargie *et al*., 2011[96]
	V. unguiculata ssp. *unguiculata*	Markers linked to Yellow Mosaic Virus Resistance genes	Gioi *et al.,* 2012[161]
	V unguiculata ssp. *unguiculata*	SSR transferability to other *Vigna* species	Bansal *et al*., 2012[162]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Badiane *et al*., 2012[74]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Adetiloye *et al.,* 2013[73]
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Ali *et al*. 2015[163]
SNP	*V. unguiculata* ssp. *unguiculata*	Consensus genetic linkage maps	Muchero *et al*., 2009[83]
	V. unguiculata ssp. *unguiculata*	Linkage mapping and synteny to other legumes	Lucas *et al.,* 2011[84]
	Glycine max		
	V. unguiculata ssp. *unguiculata*	Markers linked to resistance to foliar thrips	Lucas *et al.,* 2012[104]
	V. unguiculata ssp. *unguiculata*	Gene pool structure	Huynh *et al*., 2013[47]

V. unguiculata ssp. *dekindtiana*	Phylogenetic relantioships	
V. unguiculata ssp. *unguiculata*	Markers linked to seed size	Egbadzor *et al*., 2013[99]
V. unguiculata ssp. *unguiculata*	Genetic diversity	Egbadzor *et al*., 2014[52]
V. unguiculata ssp. *unguiculata*	Genetic mapping and synteny of aphid resistance	Huynh *et al*., 2015[164]
V. unguiculata ssp. *unguiculata*	Genetic diversity and population structure	Xiong *et al.* 2016[82]
V. unguiculata ssp. *unguiculata*	Consensus genetic map	Muñoz-Amatriaín *et al.* 2016[85]
V. unguiculata ssp. *spontanea*		
V. unguiculata ssp. *sesquipedalis*	Pod length QTLs	Xu *et al.* 2016[165]

Table 3 – Genes identified as being involved in drought tolerance in cowpea.

Gene designation	Code number	Gene function	Author
CPRD8	D83970	Response to dehydration stress	Iuchi *et al*. 1996 (142)
CPRD14	D83971	Response to dehydration stress	Iuchi *et al*. 1996(142)
CPRD22	D83972	Response to dehydration stress	Iuchi *et al*. 1996(142)
CPRD12	D88121	Response to dehydration stress	Iuchi et *al*. 1996(143)
CPRD46	D88122	Neoxanthin cleavage enzyme involved in ABA biosynthesis	Iuchi *et al*. 1996(143)
VuNCED1	AB030293	9-Cis-epoxycarotenoid dioxygenase involved in a key step of ABA biosynthesis	Iuchi *et al*. 2000(132)
VuABA1	AB030295	Zeaxanthin epoxidase involved in early step of ABA biosynthesis	Iuchi *et al*. 2000(132)
VuPLD1	U92656	Putative phospholipase D, a major lipid-degrading enzyme in plant	El-Maarouf *et al*.1999(135)
VuPAP-α	AF165891	Putative phosphatidate phosphatase, important for the enzymatic cascade leading to membrane lipid degradation under environmental stresses or senescence	Marcel *et al*. 2000(145)
VuPAP-ß	AF171230	Putative phosphatidate phosphatase, important for the enzymatic cascade leading to membrane lipid degradation under environmental stresses or senescence	Marcel *et al*. 2000 (145)
VuPAT1	AF193067	Galactolipid acyl hydrolase involved in membrane degradation induced by drought stress	Matos *et al*.2001(136)
VuC1	AF278573	Protein inhibitor of cysteine proteinase belonging to the papain family	Diop et al. 2004(146)
dtGR	DQ267474	Dual-targeted glutathione reductase, a key enzyme involved in detoxification of AOS	Contour-Ansel *et al.* 2006(138)
cGR	DQ267475	Cytosolic glutathione reductase, a key enzyme involved in detoxification of AOS	Contour-Ansel *et al.* 2006(138)
VucAPX	U61379	Cytosolic ascorbate peroxidase, a key enzyme involved in detoxification of AOS	D'Arcy-Lameta *et al.* 2006(111)
VupAPX	AY466858	Peroxisomal ascorbate peroxidase, a key enzyme involved in detoxification of AOS	D'Arcy-Lameta *et al.* 2006(111)
VusAPX	AY484493	Stromatic ascorbate peroxidase, a key enzyme involved in detoxification of AOS	D'Arcy-Lameta *et al.* 2006(111)
VutAPX	AY484492	Thylakoidal ascorbate peroxidase, a key	D'Arcy-Lameta *et al.*

		enzyme involved in detoxification of AOS	2006(111)
GST		Glutathione-S-transferase, a well-recognized stress-related gene	Gazendam and Oelofse 2007(148)
PR-1		Pathogenesis-related-protein-1, a well-recognized stress-related gene	Gazendam and Oelofse 2007(148)
VuNSR4	ABA55727.1	Digalactosildiacilglicerol sintase 1	Silva *et al*. 2012(149)
VuNSR10	AAC49405.1	Kinase protein calcium dependent	Silva *et al*. 2012(149)
VuNSR44	BAA13541.1	CPRD12 protein	Silva *et al*. 2012(149)
	BAA12161.1	CPRD12 protein	
VuNSR47	BAA12160.1	CPRD8 protein ("old yellow" enzyme)	Silva *et al*. 2012(149)
VuNSR49	BAB11932.1	CPRD65 protein	Silva *et al*. 2012(149)