UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Genetic diversity and molecular responses to drought stress in *Vigna unguiculata* L. Walp.

Doctoral Degree in Agricultural Production Chains - From Fork to Farm

Márcia Raquel Gomes de Carvalho

Supervisors: Professor Doutor Valdemar Pedrosa Carnide

Professora Doutora Maria Teresa Lino Neto

Vila Real, 2018

Universidade de Trás-os-Montes e Alto Douro

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DECLARATIONS

Esta Tese foi expressamente elaborada para cumprimentos dos requisitos necessários à candidatura ao grau de Doutor em Cadeias de Produção Agrícola - da mesa ao campo pela Universidade de Trás-os-Montes e Alto Douro tendo sido realizada sob a orientação científica do Professor Catedrático Valdemar Carnide da Universidade de Trás-os-Montes e Alto Douro e da Professora Doutora Teresa Lino Neto da Universidade do Minho.

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ABSTRACT

Climate change is considered as one of the major threats to agriculture sustainability and biodiversity. Drought is a severe environmental stress with major impacts on plant development and productivity. The use and improvement of crops with the ability to mitigate the effects of drought will be a key step for future crop sustainability. Cowpea (*Vigna unguiculata* L. Walp) is a warm-season grain legume, considered as an interesting crop, due to its high adaptability to heat and drought, as well as to its association with nitrogen fixing rhizobia. As other legumes, cowpea plays a major role in the global food security by providing an affordable dietary source of nutrients mainly proteins.

The thesis main objective is to contribute for a higher cowpea production in Europe, anticipating the upcoming climate changes. To achieve this goal, multidisciplinary approaches were undertaken involving field trials and molecular genetics, physiology and biochemistry approaches. Regarding genetic diversity, the morphological and agronomical characterization of 24 Iberian Peninsula cowpea genotypes was performed, thus emphasizing the high genetic diversity among genotypes. From this characterization, ten cowpea genotypes were selected and further used for determining the stability of morphological and agronomical traits in three different environments (two in Portugal and one in Spain), during two consecutive years. A high interaction between genotype and environment was found and Elvas (Portugal) revealed to have the most appropriated environment for the production of this set of cowpea genotypes. The recently developed Cowpea iSelect Consortium Array (Illumina, Inc.) provided an excellent opportunity for further determination of cowpea genetic diversity. This array contains 51,128 SNPs and was used in a set of 96 cowpea genotypes, 43 of which from Iberian Peninsula and 23 from 22 other worldwide countries. Cowpea genotypes were clustered in four subpopulations, mainly differentiated by their geographical origin, allowing the suggestion of a new hypothesis about cultivated cowpea dispersion routes. Most of Iberian Peninsula genotypes and those from other Southern European and Northern African countries were grouped in the same subpopulation, indicating a high genetic similarity among them. However, three Iberian Peninsula cowpea genotypes did not belong to this subpopulation, being two of them classified as ‘admixed’ and another from a different subpopulation. These genotypes could be considered as interesting sources of diversity for future cowpea breeding programs. To get new insights on cowpea drought stress responses, the selection of the best approaches

for screening cowpea genotypes with enhanced drought tolerance is fundamental. Four cowpea genotypes (two Portuguese and two international tolerant references) were submitted to three different watering regimens, during 15 days. Several physiological, biochemical and molecular approaches were tested, revealing that stomatal function parameters, free proline and anthocyanins contents were the most effective in discriminating cowpea tolerance levels. Furthermore, two drought-related genes (*VuCPRD14* and *VuHsp17.7*) were identified as the most effective for drought tolerance selection. For screening cowpea genotypes with enhanced drought tolerance, a worldwide collection of cowpea genotypes (58 genotypes) was tested for seed germination, seedling emergence and proline content under different osmotic potentials. A total of seven drought tolerant genotypes were suggested, which could represent starting material for future cowpea breeding programs.

This thesis gave a good contribution for increasing cowpea production in Europe, being the selection of more productive and drought tolerant genotypes the first step. These genotypes could be integrated into breeding programs for enhancing cowpea resilience to climate change. Furthermore, the methodologies tested and proposed in this study allow an effective and fast screening of cowpea genotypes drought tolerance.

Keywords: cowpea genotypes; morphological and agronomical traits; SNP markers; drought stress; screening methods

RESUMO

As alterações climáticas são consideradas uma das principais ameaças à sustentabilidade da agricultura e à biodiversidade global, sendo o stresse abiótico um dos seus maiores constrangimentos. A seca é um dos stresses ambientais mais severo e com um grande impacto no desenvolvimento e produtividade das plantas. A utilização e melhoramento de culturas com capacidade de mitigar os efeitos da seca assumem cada vez mais um papel relevante para o aumento da sustentabilidade das culturas. O feijão-frade (*Vigna unguiculata* L. Walp) é uma cultura de Primavera/Verão considerada muito versátil devido à sua capacidade de tolerar elevadas temperaturas e défice hídrico, tendo ainda a capacidade de fixar azoto atmosférico através da simbiose com bactérias *Rhizobium*. Como todas as leguminosas de grão, o feijão-frade possui um elevado valor nutritivo, em particular um alto teor em proteína, tornando-a assim importante na segurança alimentar global.

O principal objetivo da tese é desenvolver estudos que venham a contribuir para uma maior produção de feijão-frade na Europa tendo em consideração as futuras alterações climáticas. Para atingir este objetivo desenvolveram-se estudos integrados que envolveram ensaios de campo e abordagens de genética molecular, de fisiologia e de bioquímica.

Em relação à diversidade genética, foi realizada uma caracterização morfológica e agronómica de um conjunto de 24 genótipos de feijão-frade, da Península Ibérica, onde ficou evidenciada a elevada diversidade entre genótipos. Desta caracterização foram selecionados, com base em características morfológicas e agronómicas, os 10 genótipos mais promissores para ensaios comparativos que foram instalados, em três ambientes diferentes (dois em Portugal e um em Espanha), durante dois anos consecutivos. Este estudo revelou uma elevada interação entre genótipo e ambiente, verificando-se que Elvas (Portugal) é o ambiente mais adequado para a produção desta leguminosa. O recém-desenvolvido *Cowpea iSelect Consortium Array* (Illumina, Inc.) veio permitir uma avaliação mais precisa e pormenorizada da diversidade genética existente no feijão-frade. Esta metodologia, que contém 51.128 SNPs, foi utilizada em 96 genótipos de feijão-frade sendo 43 provenientes da Península Ibérica e os restantes de 22 países de todo mundo. Este conjunto de genótipos foram agrupado em quatro subpopulações diferenciadas principalmente pela sua origem geográfica. A maioria dos genótipos de feijão-frade da Península Ibérica foram agrupados numa única subpopulação juntamente com os de outros países do sul da Europa e do norte de África, indicando uma semelhança genética entre

eles. Contudo, dois genótipos da Península Ibérica foram classificados como "*admixed*" e um terceiro pertencente a outra subpopulação. Estes genótipos podem ser considerados interessantes fontes de diversidade para futuros programas de melhoramento de plantas. Os dados agora obtidos com os SNPs conduziram ainda a novas indicações sobre as possíveis rotas de dispersão do feijão-frade cultivado.

Para obter novos dados sobre as respostas do feijão-frade ao stresse hídrico foram analisadas diferentes metodologias para a seleção de genótipos de feijão-frade com maior tolerância à seca. Quatro genótipos de feijão-frade (dois portugueses e duas referências internacionais) foram submetidos a três regimes de rega durante 15 dias e o comportamento das plantas estudado a nível fisiológico, bioquímico e molecular. A condutância estomática, o conteúdo de prolina livre e de antocianinas foram as determinações mais eficazes na discriminação dos níveis de tolerância das plantas à seca. Para além disso, foi possível identificar os genes *VuCPRD14* e *VuHsp17.7* como sendo os que revelam maior expressão em condições de stresse hídrico. De forma a selecionar os genótipos de feijão-frade mais tolerantes à seca, uma coleção mundial de feijão-frade (58 genótipos) foi avaliada ao nível da germinação das sementes, emergência de plântulas e conteúdo de prolina livre sob diferentes potenciais osmóticos. Sete genótipos foram considerados tolerantes à seca podendo assim vir a ser incluídos em programas de melhoramento.

Esta tese pretendeu oferecer uma contribuição para o aumento da produção de feijão-frade na Europa, para o qual a seleção de genótipos mais produtivos e tolerantes à seca foi o primeiro passo. Os genótipos selecionados podem vir a ser integrados em programas de melhoramento de forma a aumentar a resiliência do feijão-frade às alterações climáticas. Para além disso, disponibilizaram-se metodologias que permitem de uma forma expedita identificar os genótipos com maior tolerância à seca.

Palavras-chave: genótipos de feijão-frade; parâmetros morfológicos e agronómicos; marcador SNP; stresse hídrico; seleção de metodologias

LIST OF PUBLICATIONS

In the scope of the work carried out during this PhD thesis was possible to perform a total of four published and two submitted papers in international peer-review journals, one published paper in national peer-review journal and seven oral communications and twelve poster communications in international and national meetings. The list of these publications is presented below.

Publications in international journals

Carvalho M., Castro I., Moutinho-Pereira J., Correia C., Egea-Cortines M., Matos M., Rosa E., Carnide V., Lino-Neto T. Screening cowpea responses to drought stress *Submitted to Plant Science* (PSL_2018_785). If = JIF (2017) = 3.712; SJR (2017) Agronomy and Crop Science/Plant Science = Q1.

Carvalho M., Matos M., Castro I., Monteiro E., Rosa E., Lino-Neto T., Carnide V. (2018). Screening of world-wide cowpea collection to drought tolerant at a germination stage. Scientia Horticulturae 247: 107-115 (doi: 10.1016/j.scienta.2018.11.082). JIF (2017) = 1.760; SJR (2017) Horticulture = Q1.

Carvalho M., Muñoz-Amatriaín M., Castro I., Lino-Neto T., Matos M., Egea-Cortines M., Rosa E., Close T.J., Carnide V. 2017. Genetic diversity and structure of Iberian Peninsula cowpeas as compared to worldwide cowpea accessions using high density SNP markers. BMC Genomics, 18: 891 (doi: 10.1186/s12864-017-4295-0). JIF (2017) = 3.730; SJR (2017) Genetics = Q1.

Martos-Fuentes M., Fernández J.A., Ochoa J., **Carvalho M**., Carnide V., Rosa E., Pereira G., Barcelos C., Bebeli P., Egea-Gilbert C. 2017. Genotype by environment interactions in cowpea (*Vigna unguiculata* L. Walp.) grown in the Iberian Peninsula. Crop & Pasture Science, 68:924-931 (doi: 10.1071/CP17071). JIF (2017) = 1.354; SJR (2017) Agronomy and Crop Science = Q1.

Carvalho M., Bebeli P.J., Pereira G., Castro I., Egea-Gilabert C., Matos M., Lazaridi E., Duarte I., Lino-Neto T., Ntatsi G., Rodrigues M., Savvas D., Rosa E., Carnide V. 2017. European cowpea landraces for a more sustainable agriculture system a novel foods. Journal of the

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Publications in national journals

Carvalho M., Castro I., Matos M., Lino-Neto T., Silva V., Rosa E., Carnide V. 2016. Caracterização agro-morfológica de acessos de feijão-frade (*Vigna unguiculata*): bases para o melhoramento. Sociedade de Ciências Agrárias de Portugal, 39, 4, 38-49. doi: 10.19084/RCA16091

Other publications

Domínguez-Perles R., Carnide V., Marques G., de Castro I., Matos M., **Carvalho M**., Rosa E. 2015. Relevance, constraints and perspectives of cowpea crops in the Mediterranean Basin. Legume Perspectives. 10:40-42

Carvalho M., Castro I., Matos M., Carnide V. 2014. Feijão-frade: uma cultura promissora para Portugal com grande biodiversidade. Voz do campo 178: VI-VII.

Oral communications in international meetings

Carvalho M., Matos M., Egea-Cortines M., Lino-Neto T., Castro I., Rosa E., Carnide V. 2017. Effects of drought stress in cowpea: a gene expression analysis during seed development. In International Conference Advances in grain legume breeding, cultivation and uses for a more competitive value-chain, Novi Sad, Serbia, 27-28 September 2017.

Carvalho M., Castro I., Matos M., Lino-Neto T., Close T.J., Muñoz-Amatriaín M., Carnide V. 2016. Characterizing the genetic diversity of cowpea accessions using a high-density SNP array. In 2nd International Legume Society Conference, Setúbal (Tróia), Portugal, 11-14 October 2016.

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Carvalho M., Pereira G., Castro I., Duarte I., Matos M., Rosa E., Carnide V. 2017. Portuguese cowpea (*Vigna unguiculata* L. Walp.) landraces – adaptation to different envrionments. In 1º Congresso Luso-Brasileiro de Horticultura, Lisbon, Portugal, 1-4 November 2017.

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Carnide V., Lazaridi E., Fernández J.A., **Carvalho M**., Pereira G., Castro I., Ntatsi G., Martos-Fuentes M., Barcelos C., Matos M., Savvas D., Duarte I., Egea-Gilabert, Bebeli P.J., Rosa E. 2017. Cowpea genetic resources: their use in plant breeding and for new uses. In International Conference Advances in grain legume breeding, cultivation and uses for a more competitive value-chain, Novi Sad, Serbia, 27-28 September 2017.

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Poster communications in national meetings

Carvalho M., Lino-Neto T., Castro I., Rosa E., Matos M., Carnide V. 2018. Antioxidant enzymes changes in responses to drought stress in cowpea genotypes. In X Jornadas Nacionais de Genética e Biotecnologia, Vila Real, Portugal, 7-9 March 2018 (P1).

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LIST OF ABBREVIATIONS

A	Net CO_2 assimilation rate
ABA	Abscisic acid
AD	Anno Dommi
AFLP	Amplified fragment length polymorphism
A/g_s	Intrinsic water use efficiency
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AUA	Agricultural University of Athens
BAC	Bacterial artificial chromosome
BC	Before Christ
bp	Base pairs
CAT	Catalase
cDNA	Complementary DNA
cM	Centimorgans
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
E	Environment
ETR	Photosynthetic electron transport rate
FAOSTAT	Food and Agriculture Organization of the United Nations Statistics
FC	Field capacity
F_v/F_m	Maximum quantum efficiency of photosystem II
G	Genotype
GHG	Greenhouse gas
GPX	Glutathione peroxidase
GR	Germination rate
g_s	Stomatal conductance
GST	Glutathione S-transferase
GWAS	Genome-wide association studies
H^2	Heritability
He	Expected heterozygosity

H_2O_2	Hydrogen peroxide
INIAV	National Institute for Agrarian and Veterinarian Research
ISSR	Inter simples sequence repeat
LG	Linkage group
MAF	Minor allele frequency
MAS	Marker assisted selection
MCMC	Monte Carlo Markov Chain
MDA	malondialdehyde
ND	No data
NGS	Next Generation Sequencing
NJ	Neighbor-joining
NPK	Nitrogen-phosphorus-potassium
PAP	Phosphatide phosphatases
PCA	Principal component analysis
PEG	Polyethylene glycol
PGR	Plant Genetic Resources
PIC	Polymorphism information content
POX	Guaiacol peroxidase
QC	Quality control
qP	Photochemical quenching
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative trait locus
R	Precipitation
R&D	Research and Development
RAPD	Random amplified polymorphism DNA
REML	Restricted maximum likelihood
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred lines
RL	Root length
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain reaction
SAMPL	Selectively amplified microsatellite polymorphism locus

SD	Standard deviation
SE	Standard error
SL	Shoot length
SOD	Superoxide dismutase
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
$\mathbf{T_m}$	Melting temperature
$\mathbf{T_{max}}$	Maximum air temperature
$\mathbf{T_{min}}$	Minimum air temperature
UPCT	Technical University of Cartagena
UTAD	University of Trás-os-Montes and Alto Douro
VI	Vigor index
Y	Year
% G	Germination percentage
$\boldsymbol{\Phi_{PSII}}$	Quantum effective efficiency of PSII
$\mathbf{\sigma^2_g}$ **or** $\mathbf{V_g^2}$	Genotype
$\mathbf{\sigma^2_{ge}}$ **or** $\mathbf{V_{ge}^2}$	Genotype x environment interaction

CHAPTER 1

General Introduction

Food is the most basic requirement for sustaining all living organisms on Earth, being the source of energy used for their growth and development. Population growth is drastically increasing and consequently the demand for food also increases (Edgerton 2009). Vadez *et al*. (2013) referred that food production needs to increase about 50% for facing the additional three billion people expected by 2050. The increase of meat consumption (animal protein) is not sustainable, mainly due to the associated greenhouse gas (GHG) emissions and global warming (Rojas-Downing *et al*. 2017). An approach to supply an increasing demand of food can be the production and consumption of agricultural crops with high levels of proteins (like grain legumes) and choose those crops more adapted to specific agro-environments (Edgerton 2009).

Human activities are the main cause of predicted climate change. Upcoming climate alterations include global average temperature increases, and alteration of rainfall patterns that increase the risks of both heavy rains and extreme droughts (Thornton *et al*. 2014). Projections for the Mediterranean area, namely for the Southern Europe, reveal an increase of temperature and decrease of rainfall (Kröner *et al*. 2017). Besides a global biodiversity loss, these events will induce a greater requirement for water to agricultural crops that ultimately reduce their yield (Kang *et al*. 2009). Therefore, the global climate change scenario is one of the most important concerns to agricultural development all over the world, but with a major impact in developing countries (Vadez *et al*. 2012).

Legumes, belonging to Fabaceae (Lewis and Schrire 2003) or Leguminoseae (Lewis *et al*. 2005) family, represent the second most economically important family of crop plants, following grass family (Poaceae) (Smýkal *et al*. 2015). Their agronomic role is important, as legumes can be divided into weeds of cereals agriculture and grain legumes (or pulses), thus providing important sources of food, fodder, oil and fiber products (Smýkal *et al*. 2015). In terms of biodiversity, Fabaceaee family is the third largest family of flowering plants and comprises a total of 770 genera and 19,500 species. Traditionally, and based on morphological characters, three subfamilies were identified: Caesalpinioideae, Mimosoideae and Papilionoideae (Lewis *et al*. 2005; Smýkal *et al*. 2015). However, more recently and based on plastid *matK* gene sequencing, Fabaceae family was divided into six subfamilies: Caesalpinioideae, Mimosoideae, Cercidoideae, Detarioideae, Duparquetioideae, Dialiodeae and Papilionoideae, (LPWG 2017). Papilionoideae subfamily contains most of the major cultivated food and feed legumes (Lewis *et al*. 2005; Smýkal *et al*. 2015), being grain

legumes the most important. This subfamily can be divided into cold-season legumes, such as faba bean (*Vicia faba* L.), lentil (*Lens culinaris* L.), lupin bean (*Lupinus albus* L.) and pea (*Pisum sativum* L.), and warm-season legumes, as common bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.), chickpea (*Cicer arietinum* L.) and cowpea (*Vigna unguiculata* L.).

Grain legumes, considered as *poor man's meat*, have an important place in human nutrition, especially in the dietary pattern of low-income people from developing countries (Tharanathan and Mahadevamma 2003). Grains are mainly appreciated for their high content on protein and source of slow release carbohydrates (Tharanathan and Mahadevamma 2003; Zhu 2005). Grain legumes also contain several beneficial substances to health, such as folate, lignans, saponins, antioxidants, dietary fibre and resistant starch, offering potential protection against some cancers, diabetes and obesity (Mousavi-Derazmahalleh *et al*. 2018). In addition, one of the most important attributes of grain legumes is their unique capacity for bacterial symbiotic nitrogen fixation, playing an important role in natural and agricultural ecosystems (Zhu 2005; Stagnari *et al*. 2017). For example, soybean is estimated to fix up to 300 kg N ha^{-1} (Hungria *et al*. 2015), lentil approximately 8-14 kg N ha^{-1} (Zafar *et al*. 2003), and cowpea 200 kg N ha^{-1}(Kyei-Boahen *et al*. 2017). Therefore, legume cropping will lead to a reduction of key greenhouse emissions and a slight fossil energy, which are usually needed for other food and forage production (Jensen *et al*. 2012). These attributes are useful in sustainable farming through crop rotations that allow increasing soil fertility and are a valuable strategy to mitigate climate change.

Grain legumes represents 27% of world crop production, providing 33% of the dietary protein consumed by humans (Smýkal *et al*. 2015). However, some studies have revealed that grain legumes production in Europe declined comparatively to other regions, as in Canada and Australia (Schilizzi and Kingwell 1999; Zentner *et al*. 2002; Preissel *et al*. 2015). Indeed, Europe is facing a deficit of about 70% of high-protein materials, which are mainly supplied (in 87%) by the importation of soybean and soymeal (Watson *et al*. 2017). Although a negative trade scenario shows that grain legumes are underrepresented in European agriculture (Table 1), in the last years the harvested area of grain legumes has been increasing in Europe (Table 2). The yield of most grain legumes depends on the adaptation of available cultivars to a broad range of environmental conditions and susceptibility to pests and diseases (Rubiales *et al*. 2015).

Table 1 - Most important grain legumes imports and exports for Europe (adapted from FAOSTAT 2018). Values correspond to 1,000$US. ND – no available data.

Crops		2012	2013	2014	2015	2016
Dry bean	Import	$73x10^4$	$81x10^4$	$95x10^4$	$73x10^4$	$64x10^4$
	Export	$18x10^4$	$20x10^4$	$22x10^4$	$16x10^4$	$18x10^4$
Green bean	Import	$63x10^4$	$73x10^4$	$79x10^4$	$70x10^4$	$72x10^4$
	Export	$23x10^4$	$25x10^4$	$25x10^4$	$22x10^4$	$24x10^4$
Pea	Import	$21x10^4$	$24x10^4$	$26x10^4$	$22x10^4$	$22x10^4$
	Export	$11x10^4$	$12x10^4$	$11x10^4$	$10x10^4$	$11x10^4$
Fava bean	Import	$5.1x10^4$	$5.4x10^4$	$5.2x10^4$	$5.7x10^4$	$7.2x10^4$
	Export	$19x10^4$	$18x10^4$	$14x10^4$	$16x10^4$	$17x10^4$
Chickpea	Import	$20x10^4$	$21x10^4$	$16x10^4$	$15x10^4$	$20x10^4$
	Export	$13x10^4$	$9.3x10^4$	$14x10^4$	$19x10^4$	$22x10^4$
Cowpea	Import	ND	ND	ND	ND	ND
	Export	ND	ND	ND	ND	ND
Lentil	Import	$9.9x10^4$	$9.8x10^4$	$21x10^4$	$95x10^4$	$95x10^4$
	Export	$18x10^4$	$0.88x10^4$	$2.0x10^4$	$18x10^4$	$4.4x10^4$
Soybean	Import	$893x10^4$	$956x10^4$	$948x10^4$	$765x10^4$	$775x10^4$
	Export	$229x10^4$	$191x10^4$	$174x10^4$	$180x10^4$	$148x10^4$

Table 2 - Harvested area (in 10^3 ha) and yield (in 10^3 kg/ha) of different grain legumes in Europe (adapted from FAOSTAT 2018).

Crops		2012	2013	2014	2015	2016
Dry bean	area	272.7	275.2	321.6	421.9	430.0
	yield	1.93	2.04	2.38	2.48	2.47
Green bean	area	110.0	109.5	105.9	105.9	107.2
	yield	6.76	7.35	7.56	7.61	7.58
Pea	area	200.1	190.1	204.4	213.4	215.6
	yield	5.27	5.50	5.61	5.45	5.33
Fava bean	area	237.3	242.6	256.5	314.2	317.9
	yield	3.19	2.89	3.16	2.73	2.73
Chickpea	area	125.1	136.9	176.1	164.4	421.0
	yield	0.95	1.07	1.01	1.07	0.93
Cowpea	area	8.0	7.4	7.9	7.8	8.9
	yield	2.98	3.39	3.26	3.27	3.18
Lentil	area	106.1	76.9	79.5	89.1	121.2
	yield	0.76	0.88	0.88	0.86	1.00
Soybean	area	3 446.3	3 225.0	4 497.5	5362.7	5038.1
	yield	1.57	1.84	1.95	1.77	2.09

In conclusion, grain legumes cultivation in Europe provides several environmental benefits to agricultural landscape, allowing an increase of nutrient use efficiency of plant,

while balancing European deficit in plant protein production (reviewed by Preissel *et al*. 2015). To counteract some specific constraints in relation to production and consumption of grain legumes, particularly in Europe, several actions should be taken: (1) exploration of genetic resources through breeding programs for improving production yield and increasing food legume competitiveness, while adapting crops for mitigating climate change effects; (2) improvement of crop management by using microbial inoculation, irrigation and pest and disease control; (3) increase consumers awareness for grain legume quality and expand pulse market by creating novel products with increased value.

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1.1. Cowpea: State of art

Cowpea: a legume crop for a challenging environment

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*Márcia Carvalho contribution: all review and manuscript writing.

1.1.1. 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 a good protein quality with a high nutritional value, its nitrogen-fixing ability, and an ability to be more drought- and heat-tolerant than most of its legume relatives. In a research perspective, studies of cowpea are relatively scarce, despite its relevance to agriculture in the developing world and its resilience to stress. The present review provides an overview of different aspects of cowpea, with a special emphasis on the molecular markers for assessing genetic diversity, as well as on biochemical and transcriptomic data with respect to evaluating cowpea drought stress tolerance. The integration of both datasets will be useful for the improvement of cowpea because research on drought stress tolerance is of major interest for this crop in a challenging environment.

1.1.2. 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 (Steele 1976; Ba *et al*. 2004; Tan *et al*. 2012). This legume has been used in the human diet, as well as in 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) (Boukar *et al*. 2015), according to different preparations, in salads, snacks and cakes, amongst others. Also, young leaves, fresh pods and fresh seeds have been consumed in some world regions (Singh *et al*. 2003; Boukar *et al*. 2015). Green organs could be used as a vegetable and are often served boiled, as well as being consumed fried or fresh (Singh et al. 2003). One of the most important characteristics of cowpea is the high nutritive content value of all plant parts (Tan *et al*. 2012; Sebetha *et al*. 2014; Boukar *et al*. 2015). The dry grain is rich in proteins (23–32%), as well as essential amino acids such as lysine (427 mg g^{-1} N) and tryptophan (68 mg g^{-1} N), although it is low in sulphur-containing amino acids (Singh *et al*. 2002; Timko *et al*. 2007). Accordingly, 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 vitamins (folic acid and vitamin B) has also

been reported to be important in preventing birth defects during pregnancy (Nielsen *et al*. 1993; Diouf 2011; Tan *et al*. 2012). Dry grain is also high in fibre and low in fat (Timko *et al*. 2007). Taking into account these advantages, an increase in cowpea production and consumption in the European Union is highly desirable. Currently, the European Union imports almost all of the cowpea consumed from African countries, more specifically from Niger and Nigeria. During the period 2009–2013, the world cowpea sowing area was 5 million hectares and the worldwide production was 12 million tonnes. Africa has been responsible for 95.4% of worldwide cowpea production (FAOSTAT 2016), with the drier savannah and the Sahelian region of West and Central Africa being responsible for producing 72% of the total. Nigeria and Niger are the largest producers, with 3.4 and 1.4 million tonnes, respectively. By contrast, Europe is only responsible for 0.4% of worldwide cowpea production and the European Union has only produced 463 thousand tonnes during the period 2009–2013 (FAOSTAT 2016).

As revealed by the major producing countries, cowpea has the capacity to grow in low fertility soils, which is related to its ability to establish associations with distinct microorganisms, mainly nitrogen-fixing bacteria (e.g. rhizobia) and vesicular-arbuscular mycorrhizal fungi. Cowpea tolerance to low fertility soils (Eloward and Hall 1987; Timko *et al*. 2007; Timko and Singh 2008) and a wide range of soil pH (Fery 1990), as well as the adaptation of cowpea to high temperatures and drought (Hall 2004), 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. The present review provides an overview of different issues about genomic and transcriptomic studies in cowpea, with an emphasis on studies related to genetic diversity and cowpea drought stress tolerance that could be useful with respect to integration in cowpea breeding programs.

1.1.3. Classification

The cowpea cultivated form obtained from the Antilles was first described by Linnaeus as *Dolichos unguiculatus* L., later being classified by Walpers as *Vigna unguiculata* (L.) Walp. (Pasquet 1998). 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 (Badiane *et al.* 2014) and was

subdivided into six sections, namely, *Vigna*, *Comosae*, *Macrodontae*, *Reticulatae*, *Liebrechtsia* and *Catiang* (Maxted *et al*. 2004). *Vigna unguiculata* (L.) Walp. includes annual cowpeas (ssp. *unguiculata*) and ten wild perennial subspecies (Coulibaly *et al*. 2002; Table 1.1.1). The subspecies *unguiculata* includes all the domesticated forms (var. *unguiculata*), as well as the wild and weedy forms [var. *spontanea* (Schweinf.) Pasquet] (Pasquet 1993; Coulibaly *et al*. 2002). The domesticated forms are subdivided into four cultivar-groups essentially based on seed and pod characters (Ng and Maréchal 1985; Coulibaly *et al*. 2002). 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 (Coulibaly *et al*. 2002). Pasquet (1998) also proposed the insertion of *melanophthalmus* (black-eyed pea) as another cultivar-group.

Table 1.1.1. Taxonomic classification of cowpea.

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

'Cowpea' is the *V. unguiculata* most popular worldwide name, although local names such as black-eyed beans, black-eyed peas, pink-eyes or southern peas (all used in the USA), 'frijol caupi' (Spanish speaking countries in America), 'lobia' (India), 'caupi' (Brazil), 'caupi'

and ‘carilla’ (Spain), ‘niebe’ (French speaking countries of Africa) and ‘feijao-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 approximately 5% outcrossing was reported in the cultivated varieties probably as a result of insect activities (Fery 1985; Badiane *et al*. 2014). Its growth habit could be prostate (trailing), semi-prostate, semi-erect, erect or climbing, depending not only mostly on genotype, but also on photoperiod and growth conditions, with the pattern of growth being determinate or indeterminate (IBPGR 1982; Timko *et al*. 2007). This crop is well adapted to a wide range of soil types from sands to heavy, including low fertility soils (Ehlers and Hall 1996). Plants grow in an extensive range of temperatures, with 28 °C the optimal temperature. Early flowering cowpea can produce a crop of dry grain in only 60 days, whereas longer season cowpeas may require more than 150 days to produce mature pods, depending on photoperiod (Timko *et al*. 2007).

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 (IBPGR 1982). 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 (Ige *et al*. 2011). Corollas can be purple, mauve–pink, yellow or white (IBPGR 1982). Each peduncle commonly develops two or three pods and pods differ in size, shape, colour and texture (Timko *et al*. 2007). They are cylindrical, although they could be straight, slightly curved, curved or coiled and, when they ripe, the colour can vary from yellow to brown or dark purple (IBPGR 1982). The sub-species/cultivar-group *Sesquipedalis* (more common in Asia) have very long green pods (40–100 cm) that are often used as green beans (or snap beans) (Timko *et al*. 2007), whereas the other groups have standard pods (10–25 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, as a result of the different pigmentations encircling the hilum (IBPGR 1982).

Environmental conditions, including photoperiod and growing conditions (temperature, rainfall, etc.), can also affect the plant height and morphology (Timko *et al*. 2007; Ehlers and Hall 1996). Cowpea root system is dense and well-developed (Pandey et al.

1984) 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 (Valenzuela and Smith 2002). These characteristics furnish cowpea plants with a high resistance to drought in comparison with other legumes.

1.1.4. Origin, domestication and distribution

Africa was suggested as the centre of origin of cowpea (Richard 1847). This assumption was not contested because wild cowpea plants have been found in tropical Africa and Madagascar (Steele 1976), where it was presumably domesticated subsequent to the Neolithic age (Vanderborght and Baudoin 2001). Pasquet (1999) 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 (Steele 1976; Vavilov 1926; Pasquet 2000), West Africa (Murdock 1959; Faris 1965; Rawal 1975; Vaillancourt and Weeden 1992; Ng 1995), and Eastern and Southern Africa (Baudoin and Maréchal 1985). Coulibaly *et al.* (2002), 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 (1988), West Africa appears to be the centre of diversity of cultivated forms. A 'diffuse' domestication in the African savanna after the dispersal of cereals was also hypothesized (Steele 1976; Garba and Pasquet 1998). This last hypothesis was presented by Harlan (1971), 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 (Ba *et al*. 2004). During the Neolithic period, the cowpea was first introduced into India, which was then considered a secondary centre of cowpea genetic diversity (Pan *et al*. 1982). 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* well as in America between the 16th and 17th centuries (AD) (Padulosi and Ng 1997). Although some reports suggest that cowpea has been cultivated in Europe at least since the 18th century BC and possibly from prehistoric times onward (Coulibaly *et al*. 2002; Tosti and Negri 2002), others suggest that it was only introduced in Europe around 300 BC, where it still remains as a minor crop in the southern part (Badiane *et al*. 2014). From Europe, more specifically from Portugal and Spain, this legume was exported in the 17th century to the New World (Fang *et al*. 2007; Badiane *et al*. 2014). Another important result was obtained by Fang *et al.* (2007) who provided evidence for the common origin of cowpea germplasm from Asia and North America different from the West Africa. However, such studies have mostly used breeding lines and, consequently, the introgression of extra regional germplasm could have occurred. Huynh *et al.* (2013), analysing a worldwide collection of cowpea landraces and African ancestral wild cowpeas by using more than 1200 single nucleotide polymorphism (SNP) markers, confirmed that accessions from Asia and Europe were more related to those from Western Africa, whereas accessions from Americas appeared to be more closely related to those from Eastern Africa.

1.1.5. Evaluation of genetic diversity

Cowpea has been referred as a worldwide crop with more prevalence in tropical areas, displaying a high phenotypic/morphological variability (Timko *et al*. 2007). Genetic diversity assessment is then useful for the preservation and utilization of germplasm resources, as well as for the improvement of varieties/cultivars (Tan *et al*. 2012). 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 agronomical traits (Pasquet 1998; Adewale *et al.* 2011; Stoilova and Pereira 2013; Cardona-Ayala *et al*. 2013; Egbadzor *et al*. 2013a; Egbadzor *et al*. 2014a). This characterization is followed by using a set of descriptors: (i) parameters related to plant morphology, such as growth habit, leaf type, flower colour, seed shape and colour and (ii) parameters related to 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 reasons explain 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, instead, reflect the interaction between genotype and environment (Magloire 2005).

The first biochemical markers to be used for genetic diversity analysis were the isozyme markers in the 1960s (Kumar *et al*. 2009). 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 (De La Vega 1993). Until the end of 1980s, 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 (1992) and Vaillancourt *et al.* (1993) 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 (Rao *et al*. 1992; Panella *et al*. 1993; Fotso *et al*. 1994; Odeigah and Osanyinpeiu 1996; Oppong-Konadu *et al*. 2005). Often, in these studies, the obtained results were not very conclusive as a result of a lack of domesticated cowpea and progenitor representative samples.

In comparison with morphological and biochemical markers, DNA molecular markers have a set of characteristics that make 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 (Weising *et al*. 1995; Kumar *et al*. 2009). DNA-based molecular markers have been extensively used in cowpea genetic diversity research, variety identification, phylogenetic analysis, gene mapping and resource classifications (Table 1.1.2). The first study using AFLP markers in cowpea was performed by Coulibaly *et al.* (2002), 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 investigated. It was shown that the wild cowpeas were more diverse than domesticated ones, and an Eastern African origin for the wild taxon was also suggested. This result was corroborated by Ba *et al.* (2004) using random amplified polymorphic DNA (RAPD) markers, and by Ogunkanmi *et al.* (2008) 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 (Fang *et al*. 2007) and RAPD markers (Zannou *et al*. 2008; Malviya *et al*. 2012; Prasanthi *et al*. 2012; Patil *et al*. 2013). In addition, RAPD markers were used to eliminate the putative duplicates of Senegal cowpea accessions in a germplasm bank and identify elite varieties (Fall *et al*. 2003). Currently, SSR is the most frequently used molecular marker in cowpea genetic diversity analyses, namely in cowpea landrace accessions from China, Africa and other Asian countries (Xu *et al*. 2007), Korea (Lee *et al*. 2009), Ghana (Asare *et al*. 2010), Southwestern Nigeria (Adetiloye *et al*. 2013), and Senegal (Badiane *et al*. 2012), 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, confirming the transferability of SSR markers between these two subspecies (Xu *et al*. 2010). In all of these studies, SSR markers also showed sufficient genetic variance that could be useful for improvement strategies in cowpea. SNP markers have gained an increasing importance because of their bi-allelic nature, higher frequency in the genome than SSRs and other markers, and their easily automated genotyping (Jones *et al*. 2007). In a study of the characterization of 113 cowpea accessions, comprising 108 from Ghana and five from abroad, 458 SNPs (out of 477) showed high polymorphism (Egbadzor *et al*. 2014b). These results suggest an unexpected high level of heterozygosity. The chip-based SNP detection technology is being widely used in plant genetic applications (Muñoz-Amatriaín *et al*. 2011; Ren *et al*. 2013; Xu *et al*. 2016). In cowpea, Illumina chip-based SNP detection platforms (GoldenGate and more recently iSelect; Illumina, San Diego, CA, USA) have been developed and are proving very useful for molecular characterization (Egbadzor *et al*. 2014b; Pottorff *et al*. 2014), genetic diversity analysis (Lucas *et al*. 2013; Xiong *et al*. 2016) and genetic mapping (Muchero *et al*. 2009; Lucas *et al*. 2011; Xu *et al*. 2011; María Muñoz-Amatriaín *et al*. 2017). 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 (Close *et al*. 2015).

Table 1.1.2. 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 parasitism resistance	Ouédraogo *et al.*, 2001
	V. unguiculata ssp. *unguiculata*	Phenetic organization and genetic diversity	Coulibaly *et al*., 2002
	V. unguiculata ssp. *spontanea*		
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Fang *et al*., 2007
	V. unguiculata ssp. *unguiculata*	Markers linked to cowpea golden mosaic virus	Rodrigues *et al*., 2012
RFLP	*V. unguiculata* ssp. *unguiculata Vigna radiata*	Markers linked to orthologous seed weight	Fatokun *et al*., 1992
	V. unguiculata ssp. *unguiculata*	Markers linked to aphid resistance gene	Myers *et al*., 1996
	V. unguiculata ssp. *unguiculata*	Diversity of indigenous bradyrhizobia	Krasova-Wade *et al*., 2003
	V. unguiculata ssp. *unguiculata*	Markers linked to genotypic and phenotypic responses to seedling-stage drought	Muchero *et al*., 2008
RAPD	*V. unguiculata* ssp. *unguiculata*	Genetic diversity	Fall *et al*., 2003
	V. unguiculata ssp. *unguiculata*	Genetic relatedness and gene flow	Nkongolo, 2003
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Ba *et al*., 2004
	V. unguiculata ssp. *spontanea*		
	Vigna unguiculata ssp. *unguiculata*	Genetic diversity	Zannou *et al*., 2008
	V. unguiculata ssp. *unguiculata* *Phaseolus vulgaris*	Genetic diversity and markers linked to cowpea resistance to pests weevil pests	Abdel-Sabour *et al*., 2010
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Malviya *et al*., 2012
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Prasanthi *et al*., 2012

	V. unguiculata ssp. *unguiculata*	Genetic diversity	Patil *et al*., 2013
SSR	*V. unguiculata* ssp. *unguiculata* *V. unguiculata* ssp. *dekindtiana var. pubescens*	Genetic diversity and relationships	*Li et al*., 2001
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Xu *et al*., 2007
	V. unguiculata ssp. *dekindtiana* *V. unguiculata* ssp. *ovata* *V. unguiculata* ssp. *kgalagadensis* *V. unguiculata* ssp. *rhomboidea* *V. unguiculata* ssp. *pubescens* *V. unguiculata* ssp. *mensensis* *V. unguiculata* ssp. *grandiflora* *V. unguiculata* ssp. *congolensis*	Genetic diversity	Ogunkanmi *et al*., 2008
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Lee *et al*., 2009
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Asare *et al*., 2010
	V. vexillata *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*	Genetic diversity and SSR transferability between *Vigna* species	Gupta and Gopalakrishna, 2010
	V. unguiculata ssp.	Genetic diversity of cowpea	Sawadogo *et al*., 2010

	unguiculata	cultivars resistant to *Striga gesnerioides*	
	V. unguiculata ssp. *sesquipedalis*	Genetic diversity and SSR transferability between sub-species	Xu *et al*., 2010
	V. unguiculata ssp. *unguiculata*		
	V. unguiculata ssp. *unguiculata*	Genetic distance and diversity	Adewale *et al*., 2011
	V. unguiculata ssp. *unguiculata*	Genetic map and identification of QTLs	Andargie *et al.*, 2011
	V. unguiculata ssp. *unguiculata*	Markers linked to Yellow Mosaic Virus Resistance genes	Gioi *et al*., 2012
	V unguiculata ssp. *unguiculata*	SSR transferability to other *Vigna* species	Bansal *et al*., 2012
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Badiane *et al*., 2012
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Adetiloye *et al.*, 2013
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Ali *et al.*, 2015
	V. unguiculata ssp. *unguiculata*	Consensus genetic linkage maps	Muchero *et al*., 2009
	V. unguiculata ssp. *unguiculata* *Glycine max*	Linkage mapping and synteny to other legumes	Lucas *et al*., 2011
	V. unguiculata ssp. *unguiculata*	Markers linked to resistance to foliar thrips	Lucas *et al*., 2012
SNP	*V. unguiculata* ssp. *sesquipedalis*	Genetic diversity and linkage disequilibrium	Xu *et al*., 2012
	V. unguiculata ssp. *unguiculata* *V. unguiculata* ssp. *dekindtiana*	Gene pool structure Phylogenetic relantioships	Huynh *et al*., 2013
	V. unguiculata ssp. *unguiculata*	Markers linked to seed size	Egbadzor *et al*., 2013b
	V. unguiculata ssp. *unguiculata*	Genetic diversity	Egbadzor *et al*., 2014b
	V. unguiculata ssp.	Genetic mapping and	Huynh *et al*., 2015

unguiculata	synteny of aphid resistance	
V. unguiculata ssp. *unguiculata*	Genetic diversity and population structure	Xiong *et al*. 2016
V. unguiculata ssp. *unguiculata* *V. unguiculata* ssp. *spontanea*	Consensus genetic map	Muñoz-Amatriaín *et al*. 2017
V. unguiculata ssp. *sesquipedalis*	Pod length QTLs	Xu *et al*. 2017

The combined use of different molecular markers could better assist the evaluation of genetic diversity. Diouf and Hilu (2005) used a combination of RAPD and SSR markers to assess genetic variability of local cowpea varieties and breeding lines from Senegal and identified 12 polymorphisms as a result of the broad genome coverage used. Combinations of AFLP and SAMPL (selectively amplified microsatellite polymorphic locus) markers (Tosti and Negri 2005), as well as AFLP and SSR markers (Gillaspie *et al*. 2005), were used to determine the genetic variation within and among closely related *V. unguiculata* accessions, whereas the combined use of RAPD and ISSR markers allowed the evaluation of genetic variations of seven *Vigna* species (El-hady *et al*. 2010). A combination of molecular and classical markers has been considered essential for making the results of genetic diversity more reasonable with respect to genetic cowpea breeding and the evaluation of germplasm resources (Tan *et al*. 2012). 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 seven accessions of a hybrid between cowpea (*V. unguiculata* ssp. *unguiculata*) and dwarf asparagus bean in Thailand (Tantasawat *et al*. 2010). Morphological characters were diverse among most accessions, although their exclusive use did not allowed a distinction between accessions. Indeed, ISSR markers showed higher efficiency for estimating the levels of genetic diversity and relationships among the two subspecies than SSR markers (Tantasawat *et al*. 2010). The combined use of morphological traits, RAPD and ISSR markers was also employed for discriminating landraces of cowpea scattered from all Algeria regions (Ghalmi *et al*. 2010), as well as for evaluating the genetic variability and relationships between two cowpea cultivars and nine elite genotypes (Gajera *et al*. 2014). Both studies showed that ISSR markers were better linked to morphological variation than RAPD markers.

1.1.5.1. 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 (Menendez *et al.* 1997; Ouédraogo *et al*. 2002; Muchero et al. 2009; Lucas *et al*. 2011; Andargie *et al*. 2011; Pottorff *et al*. 2012). A consensus genetic linkage map using expressed sequence tag-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) (Muchero *et al*. 2009). A significant macrosynteny with *Glycine max* and *Medicago truncatula* genomes was reported, as well as 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.* (2013). 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, as well as monitoring specific genes or genomic regions transmitted from parents to progeny (Tan *et al*. 2012). Using the recently developed Illumina iSelect genotyping assay for cowpea, Muñoz-Amatriaín *et al.* (2017) genotyped five biparental recombinant inbred lines (RIL) populations and developed a consensus genetic map containing over 37 000 SNPs mapped to approximately 3200 bins in 800 cM. These results are being used to genetically anchor an initial whole-genome shotgun assembly of the cowpea accession IT97K-499-35. To this assembly, sequences from approximately 4000 minimal tiling path bacterial artificial chromosomes (BAC) are being incorporated with the aim of increasing the number of anchored scaffolds and helping resolve the order within recombination bins.

The biotechnology based on such genetic maps and the use of DNA markers brings great hope to cowpea breeding because specific molecular markers could be used to select target traits with marker assisted selection (MAS) (Badiane *et al*. 2014). 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 (Egbadzor *et al*. 2013b). 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 (Muchero *et al*. 2008). The additional use of other high-density DNA markers in the genome could speed up the selection process in breeding programs even more. For breeding to resistance to the parasitic weed *Striga gesnerioides*,

SSR (Sawadogo *et al*. 2010) and AFLP (Ouédraogo *et al*. 2002; Boukar *et al*. 2004) markers have been used. Similarly, SNPs have been used to identify markers associated to cowpea resistance to foliar thrip (Lucas *et al*. 2012). The asparagus bean rust disease, caused by the fungus *Uromyces vignae*, was also associated with a specific AFLP marker that can now be effectively used for MAS (Li *et al*. 2007). Sequencing and analysis of the gene-rich hypomethylated portion of the cowpea genome was performed by Timko *et al.* (2008). More than 250 000 gene-space sequences reads 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.

1.1.6. 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 (Tester and Langridge 2010; Golldack *et al*. 2014). 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 (Singh and Matsui 2002). Mitra (2001) 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 as a result of low and erratic rainfall, is cowpea. Indeed, some studies noted cowpea to be one of the most tolerant crops to drought as a result of its capacity to grow in areas with no irrigation facilities and irregular rainfall (Ehlers and Hall 1996; D'Arcy-Lameta *et al*. 2006; Agbicodo *et al*. 2009; Cardona-Ayala and Jarma-Orozco 2013). This tolerance has been attributed to the three drought tolerance mechanisms (Agbicodo *et al*. 2009), 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 (Singh and Matsui 2002; Cardona-Ayala and Jarma-Orozco 2013). Because cowpea has the ability to tolerate severe drought conditions and displays a relatively small nuclear genome size (estimated at approximately 620 Mb), this legume has been

considered as an ideal model for studying the molecular mechanisms of drought tolerance in crops (Agbicodo *et al*. 2009).

1.1.6.1. 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 (Slabbert *et al*. 2004; Hayatu and Mukhtar 2010; Cardona-Ayala *et al*. 2013; Hayatu *et al*. 2014). The root system or rooting pattern are closely related to drought-tolerance mechanisms in legume crops (Pandey and Dhanasekar 2004; Matsui and Singh 2003). 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 (Matsui and Singh 2003). To examine cowpea drought tolerance ability, water potential, relative turgidity, diffusion pressure deficit, chlorophyll stability index measurements or carbon isotope discrimination are typically evaluated (Hall *et al*. 1990; Singh and Matsui 2002). However, most of these methods have the disadvantage of being slow, laborious, expensive and influenced by environmental conditions (Singh and Matsui 2002; Agbicodo *et al*. 2009). Slabbert *et al.* (2004) tested and proposed other methods that screen cowpea for drought tolerance, such as proline accumulation, 2,3,5-triphenyltetrazolium chloride assays, cell membrane stability, relative water content, leaf water potential, leaf area, chlorophyll *a* and *b* contents, chlorophyll fluorescence, carotenoids content, evaluation of anti-oxidative responses through enzyme activities determination [superoxide reductase, 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.

Because the complex regulatory processes of drought adaptation involves the control of water flux and cellular osmotic adjustments via the biosynthesis of osmoprotectants (Golldack *et al*. 2014), the determination of such compounds 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) (Zhu 2002; Farooq *et al.* 2009; Khan *et al.* 2015). However, these compounds do not accumulate in all plant species in sufficient amounts to avoid adverse effects of drought stress (Penna 2003; Farooq *et al*. 2009). Studies in drought

stress cowpea and osmoprotectants are still scarce. However, the application of chitosan in drought stress cowpea plants has been described to allow the maintenance of osmotic balance (Farouk *et al*. 2013).

Physiological changes related to 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 as a result of a decline of stomatal conductance that limits the carbon assimilation, as well as biochemical and photochemical adjustments (Chaves and Oliveira 2004; Pinheiro and Chaves 2011). 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 (Kutama *et al*. 2014). Under water stress conditions, a decrease in photosynthesis and stomatal conductance accompanied by an increase in the intrinsic water-use efficiency was detected in all genotypes, although differences between genotypes were found (Kutama *et al.* 2014). When cowpea genotypes differing in drought resistance were subjected to three distinct water stress conditions (unstressed, moderate and severe stressed), an increase in root biomass and a reduction in chlorophyll content were detected with water stress imposition (Hayatu and Mukhtar 2010).

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 (Finkelstein *et al*. 2002; Fujita *et al*. 2005). Kulkarni *et al.* (2000) 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 (Agbicodo *et al*. 2009). In cowpea, some studies have been developed aiming to understand the role of ABA in the drought tolerance (Iuchi *et al*. 2000; Costa *et al*. 2011).

Because 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 (Monteiro de Paula *et al.* 1993), which appears to be correlated to the inhibition of lipid biosynthesis and stimulation of lipolytic and peroxidative activities (El-Maarouf *et al*. 1999; Matos *et al*. 2001). The degradation of membrane lipids and the enzymatic antioxidant activity appears to be a useful method for evaluating the level of plant drought stress. However, data are still scarce in

cowpea (Sahsah *et al*. 1998; Matos *et al*. 2001; Slabbert *et al*. 2004; D'Arcy-Lameta *et al*. 2006; Contour-Ansel *et al*. 2006).

Agbidoco *et al.* (2009) 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 could be interesting with respect to evaluating and determining drought tolerance. Physiological, biochemical and agronomic responses to water deficit at the 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 affected the yield components with the exception of seed number per pod (Hamidou *et al*. 2007).

The knowledge transfer between plant species and cultivars should be taken with care because differences in drought tolerance were detected when evaluating distinct plant species or cultivars. For example, a comparison of physiological responses to drought between *V. unguiculata* and *Phaseolus vulgaris* demonstrated that both species significantly differ in the responses evaluated by leaf gas exchange parameters (Cruz de Carvalho *et al*. 1998).

1.1.6.2. 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 (Atkinson and Urwin 2012). Many cowpea drought-related genes have been deduced from previously recognized candidate genes for drought tolerance in other related species, and were subsequently confirmed by their differential expression in drought-stressed versus non-stressed cowpea plants. On the other hand, studies of 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 (Agbicodo *et al*. 2009).

Many cowpea genes are now recognized as being involved in drought responses (Table 1.1.3). Using a differential screening method, Iuchi *et al.* (1996a) 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 characterized further (*CPRD8*, *CPRD14*, *CPRD22* by Iuchi *et al*. 1996a and *CPRD12* and *CPRD46* by Iuchi *et al*. 1996b), followed by a description of two additional drought-inducible genes all from the same cowpea variety (*VuNCED1* and *VuABA1*) (Iuchi *et al*. 2000). *VuNCED1* encodes a 9-cis-epoxycarotenoid dioxygenase that catalyses a key step in ABA biosynthesis, whereas *VuABA1* encodes a zeaxanthin epoxidase (Iuchi *et al*. 2000) involved in another important key step of ABA biosynthesis. Indeed, zeaxanthin epoxidase has been reported as being required for resistance to osmotic and drought stress, ABA-dependent stomatal closure and regulation of the expression of stress-responsive genes (Seo and Koshiba 2002).

Table 1.1.3. 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*., 1996a
CPRD14	D83971	Response to dehydration stress	Iuchi *et al*., 1996a
CPRD22	D83972	Response to dehydration stress	Iuchi *et al*., 1996a
CPRD12	D88121	Response to dehydration stress	Iuchi *et al*., 1996b
CPRD46	D88122	Neoxanthin cleavage enzyme involved in ABA biosynthesis	Iuchi *et al*., 1996b
VuNCED1	AB030293	9-Cis-epoxycarotenoid dioxygenase involved in a key step of ABA biosynthesis	Iuchi *et al*., 2000
VuABA1	AB030295	Zeaxanthin epoxidase involved in early step of ABA biosynthesis	Iuchi *et al*., 2000
VuPLD1	U92656	Putative phospholipase D, a major lipid-degrading enzyme in plant	El-Maarouf *et al.*, 1999
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
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
VuPAT1	AF193067	Galactolipid acyl hydrolase involved in membrane degradation induced by drought stress	Matos *et al*., 2001
VuC1	AF278573	Protein inhibitor of cysteine proteinase belonging to the papain family	Diop *et al*., 2004
dtGR	DQ267474	Dual-targeted glutathione reductase, a key enzyme involved in detoxification of AOS	Contour-Ansel *et al*., 2006
cGR	DQ267475	Cytosolic glutathione reductase, a key enzyme involved in detoxification of AOS	Contour-Ansel *et al*., 2006
VucAPX	U61379	Cytosolic ascorbate peroxidase, a key enzyme involved in detoxification of AOS	D'Arcy-Lameta *et al*., 2006
VupAPX	AY466858	Peroxisomal ascorbate peroxidase, a key enzyme involved in detoxification of AOS	D'Arcy-Lameta *et al*., 2006
VusAPX	AY484493	Stromatic ascorbate peroxidase, a key enzyme involved in detoxification of AOS	D'Arcy-Lameta *et al*., 2006

VutAPX	AY484492	Thylakoidal ascorbate peroxidase, a key enzyme involved in detoxification of AOS	D'Arcy-Lameta *et al*., 2006
GST		Glutathione-S-transferase, a well-recognized stress-related gene	Gazendam and Oelofse 2007
PR-1		Pathogenesis-related-protein-1, a well-recognized stress-related gene	Gazendam and Oelofse 2007
VuNSR4	ABA55727.1	Digalactosildiacilglicerol sintase 1	Silva *et al*., 2012
VuNSR10	AAC49405.1	Kinase protein calcium dependent	Silva *et al*., 2012
VuNSR44	BAA13541.1 BAA12161.1	CPRD12 protein CPRD12 protein	Silva *et al*., 2012
VuNSR47	BAA12160.1	CPRD8 protein ("old yellow" enzyme)	Silva *et al*., 2012
VuNSR49	BAB11932.1	CPRD65 protein	Silva *et al*., 2012

According to the degradation of membrane lipids that occur under drought stress conditions (Monteiro de Paula *et al.* 1993), several other cowpea drought-related genes are recognized to be involved on lipid metabolism. El-Maarouf *et al.*(1999) 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, whereas they remained unchanged in a tolerant cultivar (El-Maarouf *et al*. 1999). From the leaves of the same cultivars, Matos *et al.* (2001) 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, whereas the tolerant exhibited lower levels of transcripts. These results suggest that drought stress in cowpea stimulates the hydrolysis of galactolipids, which are 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.* (2000). PAPs play a role in the enzymatic cascade that leads to membrane lipid degradation under environmental stresses or senescence (Sahsah *et al*. 1998). Marcel *et al.* (2000) 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, although it 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 via the use of protease-specific inhibitors (Diop et al. 2004) and cellular protection against oxidative damage through the regulation of anti-oxidant enzymes and free radical scavengers (Cruz de Carvalho 2008). 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 (Diop et al. 2004).

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*) GR from cowpea leaves was performed by Contour-Ansel *et al.* (2006). 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, although *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 (Contour-Ansel *et al*. 2006). The expression of other antioxidant enzyme genes (ascorbate peroxidases; APX) was also studied in the 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 (D'Arcy-Lameta *et al*. 2006). 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, whereas 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, compared to sensitive ones, again suggested 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 (D'Arcy-Lameta *et al*. 2006). 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

(Gazendam and Oelofse 2007). Silva *et al.* (2012) 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 (da Silva *et al*. 2012).

1.1.6.3. MicroRNA drought regulation

MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level through the recognition of target RNAs by almost 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 (Barrera-Figueroa *et al*. 2011; Khraiwesh *et al*. 2012). From two cowpea genotypes, one drought-tolerant and another drought-sensitive, 157 miRNAs were identified, 44 of which were drought-associated, with 30 being 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 a real-time-quantitative polymerase chain reaction (Shui *et al*. 2013). 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.

1.1.7. Conclusions

Global climate changes have an enormous impact on plant diversity patterns with significant current negative effects. In Europe, it is the Mediterranean countries where a higher impact of climate changes is 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 the use of

undervalued crop varieties. As a result of 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 establish symbiotic associations with distinct microorganisms (mainly rhizobia and mycorrhizal fungi) that turn it into 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 the responses to drought stress tolerance and also for use in future breeding programmes. DNA molecular markers have shown to be a good tool for germplasm evaluation and the selection of the most interesting drought stress/tolerant genotypes. Because 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 carried out. Integration of data from phenotype, biochemical and molecular characterization will help to clarify 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 that are more drought tolerant.

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1.1.8. References

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1.2. Thesis main objectives

The present work is included into the R&D project "Enhancing of legumes growing in Europe through sustainable cropping for protein supply for food and feed" (EUROLEGUME-FP7 nº 613781). The final aim of this project is the selection of grain legume lines, which will be integrated into a breeding program for obtaining more productive varieties with increased tolerance to drought. New varieties of cowpea, faba bean and pea (the main crops considered on the project) will contribute for a more sustainable Europe. The present work is focused on cowpea (*Vigna unguiculata* L. Walp.), which is an important grain legume, not only for its high protein content, but also for being extremely resilient to severe abiotic and biotic production constraints, such as heat, drought, low soil fertility, pests and diseases. This grain legume is mostly grown in dry environments, such as tropical Africa, Latin America and Southern Asia, where it constitutes a valuable source of protein in diets of millions of people. The increase of cowpea production and consumption can be important for European economy that currently exhibits a high deficit on plant protein.

This PhD thesis intends to increase the current knowledge needed for increasing cowpea production in Southern Europe. Two main specific aims are devised:

- to evaluate the genetic diversity of cowpea. To achieve this goal a set of Iberian Peninsula cowpea genotypes is characterized and evaluated using morphological and agronomical traits (**sub-chapter 2.1**). An important task for the increase of cowpea production in Southern Europe countries, including Portugal, is the selection of the best cowpea genotypes and their evaluation in different environments (**sub-chapter 2.2**). Using the same set of cowpea genotypes and also a worldwide cowpea collection, genetic diversity is also characterized by single nucleotide polymorphism (SNP) markers (**sub-chapter 2.3**). Crop breeding use different ways to introduce diversity to cropping systems, being important to optimize both agronomic value and the ability of plants to perform and live alongside one another. The different approaches for genetic diversity assessment allow an identification of the most suitable genotypes to sustainable and resilient farming systems.
- to evaluate cowpea drought responses and determine the most tolerant accessions. Cowpea responses to drought stress can be perceived at physiological, biochemical and transcriptomic levels. A global picture of drought responses in different cowpea accessions is aimed for determining the most useful assays to

screen the drought-tolerant genotypes in a worldwide cowpea collection (**sub-chapter 3.1**). Germination is the first plant stage affected by drought giving a good approach to screen drought tolerant genotypes (**sub-chapter 3.2**). These results will help the development of breeding programs for obtaining more resilient genotypes.

CHAPTER 2

Cowpea genetic diversity

Plant breeding, or crop genetic improvement, allows the production of new or improved varieties to be use by farmers. The main goals of plant breeding are the increasing of production yield, development of better varieties for new agricultural areas, the improvement of plant agronomic or quality characteristics, and/or the increasing of disease or pest resistances (Allard 1960). The predicted climate changing scenario is increasing the need for plant breeders to introduce new diversity into their programs, by accessing plant genetic resources (PGR) containing a range of different characteristics (Chapman *et al*. 2012). Indeed, PGR can be considered as the raw material for plant breeding, as well as the basis of food security and source of global energy. For these reasons, PGR preservation and characterization is mandatory for current and future demands (Nass *et al*. 2012). Landraces, in particular of grain legumes, can be conserved *ex situ* or *in situ* by farmers (on farm conservation). The *landrace* concept is difficult to define, but has been necessary for practical purposes. Casañas *et al*. (2017) proposed the following definition: *"Landraces are plant materials consisting of cultivated varieties that have evolved and may continue evolving, using conventional or modern breeding techniques, in traditional or new agricultural environments within a defined ecogeographical area and under the influence of local human culture."* Landraces, or traditional old varieties or even local varieties, have played a fundamental role in the history of crops, once they have evolved over time through the interaction between farmers and the environment (Villa *et al*. 2005). Besides good agronomic features, the consumer preference also have dictated landraces evolution. For example, depending on the world region, seed color and texture could be very important to consumers. Other important aspect is the short cooking time, mainly because less fuel can be used when cooking legume grains (Boukar *et al*. 2018). Therefore, landraces reflect the needs and preferences of local people, farmers and the agro-environment in which they were grown (Villa *et al*. 2005; Polegri and Negri 2010).

The key for success of any breeding program is the availability of genetic variation for desired traits. Efforts for the development of cowpea genetic resources are more recent than those developed for other crops (Boukar *et al*. 2018). Cowpea genetic diversity evaluation is a challenging topic for geneticists and breeders, mainly because the high phenotypic and morphological variability observed in this species (Timko *et al*. 2007). In last century, cowpea diversity has been estimated by measuring variation in qualitative (*e.g.* flower and seed color, growth habit) or quantitative (*e.g.* yield, pods and seed number) agronomic traits. A good

morphological characterization and agronomic evaluation allows a first selection of the best genotypes. The characterization and evaluation of 24 cowpea landraces from Iberian Peninsula, grown in three different environments (two in Portugal and one in Greece) is presented in **sub-chapter 2.1**. The environmental adaptation of the 12 most interesting cowpea genotypes is presented in **sub-chapter 2.2**, where results from adaptive trials (at three Iberian Peninsula locations: Vila Real, Elvas and Cartagena) in two consecutive years (2015 and 2016) are presented. The main disadvantage of using morphological and agronomical features for characterization is that it does not necessarily reflect the real genetic relationships between genotypes (Patil *et al*. 2013; Wamalwa *et al*. 2016). Furthermore, quantitative traits are strongly influenced by the environmental conditions as will be concluded in **sub-chapter 2.2**. To overcome these limitations, a molecular characterization using DNA markers has been an alternative for analysis of population structure and genetic diversity in plants (Arif *et al*. 2010; Sonah *et al*. 2013). In last years, single nucleotide polymorphism (SNP) markers have emerged as a powerful tool in genetic diversity studies, as compared to other markers (Xiong *et al*. 2016). With the recent advances of next generation sequencing (NGS) technologies, plant genotyping has been widely used for SNP discovery (Elshire *et al*. 2011; Sonah *et al*. 2013; Xiong *et al*. 2016). Recently, *Illumina Cowpea iSelect Consortium Array* has been developed and allowed to screen 51,128 SNPs from cowpea (Muñoz-Amatriaín *et al*. 2017). The genetic diversity and population structure of worldwide cowpea accessions, including a set from Iberian Peninsula, is presented in **sub-chapter 2.3**.

The results presented allow to conclude about that: (1) the morphological and agronomical characterization showed a high variability in Iberian Peninsula cowpea; (2) using the same traits were observed significant interactions among genotypes, locations and years presenting Elvas (Portugal) as the best location to grow cowpea; (3) the SNP marker identified different sub-populations being the genotypes grouped based on geographical origin and allowed to infer some hypothesis for the cowpea dispersion routes.

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2.1. Morphological and agronomical characterization

European cowpea landraces for a more sustainable agriculture system and novel foods

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2.1.1. Abstract

Genetic diversity is fundamental for breeding programs and consequently has an important role to obtain new varieties. To properly use the genetic diversity present in germplasm collections, a good knowledge of the agro-morphological traits of each accession is needed. The aim of this study was to explore the production capacity of 24 cowpea landraces from Southern Europe, through phenotypic characterization and evaluation in three different locations of Greece and Portugal.

Most qualitative parameters tested showed a high stability among the three locations. A wide difference was observed among the three locations with respect to number of days to flowering, ranging from 55 to 99 days. Quantitative traits showed a higher genotype × environment than genetic variance component. In general, an inverse relationship between σ^2ge/σ^2g ratio (where σ^2ge is genotype × genotype interaction and σ^2g is genotype impact) and heritability value was observed. Principal component analysis was able to group accessions based on their origin. The first two principal components explained 97.52% of variation, being the number of seeds per plant, plant height and seed protein content, the traits which contributed most to variability.

The results show that sufficient variation exists in different traits within landraces in the studied cowpea germplasm to pursue a breeding program. However, the quantitative traits shown a higher genotype x environment component.

2.1.2. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is a primarily self-pollinated species of the genus *Vigna*, a member of the Leguminosae family. Different areas have been proposed as cowpea domestication centers (Pasquet 2000; Coulibaly *et al.* 2002), although it is unquestionably of African origin (Steele 1976). Introduction of cowpea in Europe has been reported to occur throughout the eastern part of the Mediterranean Basin, as it was certainly cultivated by the Romans in the first century AD (Negri *et al.* 2000; Tosti and Negri 2005). This grain legume is cultivated in many tropical and subtropical regions of the world.6

Nowadays, cowpea is cultivated on a small scale in southern European countries, representing only 0.43% of the total cowpea seed production, amounting to 5.59 million tonnes in 2014 (FAOSTAT 2016). Cowpea is mainly used in the human diet but also as

forage for animal feeding. It is mainly cultivated for its dry grain, although in some regions young leaves, fresh pods and fresh seeds are also consumed (Singh *et al.* 2003), constituting a significant source of proteins, essential amino acids, minerals, vitamins and fiber (Timko *et al*. 2007; Boukar *et al.* 2011).

Agricultural productivity of food legumes, grown in semi-arid areas or drylands, e.g. the Mediterranean Basin, is usually characterized by instability, as it is influenced by several environmental constraints, such as water scarcity and extreme temperatures (Agbicodo *et al.* 2009; Fraire-velázquez and Balderas-Hernández 2013) that prevail in these areas (Daryanto *et al*. 2015). Tolerance to low water regimes and adaptation to high temperatures make cowpea an important crop for southern European countries; thus it is considered one of the most drought-tolerant crops (Hall 2004; Agbicodo *et al.* 2009). Furthermore, cowpea capacity to establish symbiosis with rhizobia and mycorrhizal fungi allows it to grow in low-fertility soils, reducing or even eliminating the need for application of inorganic fertilizers, thus resulting in a more environmentally sustainable culture as well as rendering it one of the soil fertility-restoring crops (Kwapata and Hall 1985; Timko *et al*. 2007).

Cowpea cultivation in southern Europe depends to an extent on a remarkable number of cowpea landraces that constitute a valuable genetic material for breeding programs (Tosti and Negri 2002; Lazaridi *et al.* 2017). They possess significant phenotypic variability and some have developed the capacity to tolerate biotic and abiotic stresses, and thus are used in agricultural systems with low inputs and high yield stability (Eagles and Lothrop 1993; Zeven 1998). Based on the landraces that still preserve high genetic variability in traits related to tolerance/resistance to certain abiotic and biotic factors, and high nutritional value, it is possible to establish a cowpea breeding strategy to obtain more productive and nutritious varieties. The implementation of these breeding programs will be of great importance for Europe, which is a major importer of grain legumes such as cowpea, 10 501 tonnes of dry cowpea having been imported in 2015 by the European Union (European Commission, online).

Availability, identification and characterization of plant genetic resources are fundamental to knowing the diversity present in the original material and the best way of undertaking a breeding program. Traditionally, the first step in studies of diversity and genetic relationships is to measure the variation in qualitative traits (such as growth habit and pattern, flower and seed color) and quantitative agronomic traits (such as number of pods per plant, number of seeds per plant and seed weight). Regarding cowpea, in recent years several studies

have been carried out on morphological and agronomical characterization (Negri *et al.* 2000; Adewale *et al*. 2011; Cardona-Ayala *et al*. 2013; Egbadzor *et al.* 2013; Stoilova and Pereira 2013; Egbadzor *et al.* 2014). In these studies a high level of variability between and even within cowpea landraces has been verified, which may be useful for breeding programs. However, a large amount of the European cowpea genetic material remains unexplored and unutilized by breeding programs. For this purpose, the main objective of this study was to explore, characterize and evaluate cowpea landraces originating from two southern European countries, grown in three different locations, aiming to enlarge the genetic diversity used in modern breeding programs.

2.1.3. Materials and Methods

2.1.3.1. Plant material and experimental design

Twenty-two landraces, one variety and a reference breeding line (IT97K-499-35) of *Vigna unguiculata* cv.-gr. *unguiculata* (Table 2.1.1) were subjected to agronomical and morphological characterization in three different locations in southern Europe: the Agricultural University of Athens (AUA), Athens, Greece (37° 59′ N, 23° 42′ E, 24 m); the National Institute for Agrarian and Veterinarian Research (INIAV), Elvas, Portugal (38° 53′ N, 07° 09′ W, 208 m); and the University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal (41° 17′ N, 07° 44′ W, 465 m), during spring–summer 2014. Sowing took place on 23May in AUA, on 29 April in INIAV and on 9 May in UTAD.

In AUA the soil was clay loam of pH (H2O) 7.7 and humus content of 6.3 g kg^{-1}. In INIAV, the soil was classified as sandy clay loam with a medium texture and presented 1.0 g kg^{-1} humus content, >200 mg kg^{-1} P_2O_5, >200 mg kg^{-1} K_2O_2 and pH(H_2O) 5.2. The soil in UTAD was classified as lime with a medium texture and presented 1.3 g kg^{-1} humus content, 91.0 mg kg^{-1} P_2O_5, 158.0 mg kg^{-1} K_2O_2 and pH (H_2O) 4.7. Before sowing, the experimental field was ploughed with a rotary tiller and supplied with mineral fertilizer 600 kg ha^{-1} NPK 11:15:15 in AUA, 250 kg ha^{-1} NPK 15:15:15 in INIAV and 5700 kg ha^{-1} limestone in UTAD.

Table 2.1.1. Collection code, geographical data and breeding status of the 24 cowpea accessions.

Country of origin	Code	Latitude	Longitude	Altitude (m)	Breeding type
Portugal					
	Cp4906	40°00′28′′N	8°27′04′′W	198	Landrace
	Cp5128	39°59′11′′N	7°26′39′′W	402	Landrace
	Cp5129	39°59′11′′N	7°26′39′′W	402	Landrace
	Cp5131	39°59′11′′N	7°26′39′′W	402	Landrace
	Cp5553	39°48′02′′N	8°06′03′′W	226	Landrace
	Cp5556	37°47′15′′N	7°43′32′′W	160	Landrace
	Cp5647	39°27′58′′N	7°56′14′′W	281	Landrace
	Cp5648	39°27′53′′N	8°02′44′′W	45	Landrace
	Vg50	40°51′15′′N	7°08′22′′W	523	Landrace
	Vg52	40°48′45′′N	7°23′26′′W	770	Landrace
	Vg56	41°44′38′′N	7°38′57′′W	673	Landrace
	Vg59	40°14′57′′N	7°17′22′′W	507	Landrace
	Vg60	40°22′00′′N	7°15′32′′W	633	Landrace
	Vg65	41°19′25′′N	7°28′04′′W	766	Landrace
	Vg67	41°17′52′′N	7°05′53′′W	247	Landrace
	Vg72	41°16′57′′N	6°35′06′′W	726	Landrace
	Vg73	41°27′19′′N	7°00′30′′W	750	Landrace
	Fradel				Variety
Spain					
	BGE022146	37°00′35′′N	3°00′26′′W	1082	Landrace
	BGE038474	36°31′47′′N	5°15′26′′W	225	Landrace
	BGE038477	36°36′51′′N	5°08′53′′W	769	Landrace
	BGE038478	36°37′37′′N	5°10′11′′W	622	Landrace
	BGE038479	36°37′37′′N	5°10′11′′W	622	Landrace
Nigeria					
	IT97K-499-35				Reference line

Twelve plants per accession were grown in a greenhouse for 2 weeks in AUA. The seedlings were then transplanted in the field and the plants were spaced at 50 cm from row to row and 20 cm apart within the row and drip irrigated. In INIAV and UTAD, 20 seeds per accession were directly sown in plots of 3.75 m^2 and plants were spaced at 75 cm from row to row and 25 cm apart within the row. In INIAV the accessions were drip irrigated, whereas in UTAD they were irrigated along grooves.

A randomized complete block experimental design (RCBD) was used in AUA with four replicates and three plants per replicate per accession. In INIAV and UTAD a completely

randomized experimental design was implemented and 12 plants of each accession were randomly selected. During the growing season, weeds were hand-controlled and incidences of pests and diseases were handled through chemical management in all locations.

2.1.3.2. Climate data

Altitude of locations ranged from 24 m (AUA) to 465 m (UTAD), and differed mainly regarding their average mean air temperature (°C) and precipitation (mm). The average maximum (*T*max) and minimum (*T*min) air temperature (°C) and total rainfall (mm) per month (from April to September) were recorded at weather stations located at each experimental location (Table 2.1.2).

Table 2.1.2. Temperature (ºC) and precipitation (mm) occurred in the three locations during cultivation period.

Location/ Month	Temperatures (ºC)			Precipitation (mm)
	Mean	Max	Min	
AUA				
April	15.2	26.9	9.1	39.0
May	17.1	32.5	12.7	2.0
June	21.5	38.8	16.8	10.6
July	24.7	36.6	21.6	0.0
August	24.2	38.6	21.9	0.0
September	24.5	33.8	15.7	20.8
INIAV				
April	16.3	22.7	9.9	90.0
May	19.7	28.1	11.4	23.8
June	19.2	26.9	11.6	1.5
July	24.9	34.5	15.3	4.3
August	25.0	34.7	15.3	0.0
September	22.5	29.5	15.5	80.4
UTAD				
April	13.9	19.9	8.9	44.7
May	14.8	21.6	8.8	28.5
June	17.5	24.4	11.9	26.5
July	20.6	27.8	14.5	29.3
August	20.3	27.9	14.2	0.4
September	17.8	24.0	13.6	89.3

2.1.3.3. Morphological and agronomical traits

A total number of 14 qualitative and quantitative traits were analyzed in the three experimental locations according to IBPGR descriptors (IBPGR 1982). Regarding qualitative traits, growth habit, flower color, seed color and shape, and eye color were recorded in all plants of each accession used in each location. Regarding quantitative traits studied, plant height (cm), first pod height (cm), number of pods, number of seeds and seed weight per plant (g) were recorded in 12 plants per accession. To analyze the number of days to flowering only the average for each accession was recorded. The average yield per accession per location was calculated (g m^{-2}), while 100-seed weight (g) was determined by weighing two random samples of each accession. Protein content (%) was determined by the Kjeldahl method (AOAC 1990) and calculated by multiplying the nitrogen content by 6.25.

2.1.3.4. Data analysis

All the traits were compared per accession across all and for each one of the three locations (AUA, INIAV and UTAD). Per accession and location, 12 plants were considered as replicates. The evaluation of qualitative traits was determined by the frequencies of each trait. Descriptive statistics per quantitative trait and location were obtained using the summary statistics procedure in SPSS program version 8.0 (IBM SPSS, Inc., Chicago, IL, USA). For each trait, the minimum, maximum, mean and standard deviation, and coefficients of variation (CV) were calculated.

To estimate variance components of traits, a complete linear mixed model was used in the analysis of all the quantitative traits within and across the accessions and locations using the restricted maximum likelihood (REML) algorithm of SPSS program version 8.0. The heritability of each quantitative trait was calculated using the following equation:

The heritability of each quantitative trait was calculated, using the following equation: $H^2 = (s_g^2) / [s_g^2 + (s_e^2/r)]$, where s_g^2 and s_e^2 represent the genetic and residual variance for each trait and r the number of replicates of each accession (Gitonga *et al.* 2014).

Pearson correlation coefficients between the different quantitative traits and locations were determined through SPSS program version 8.0. To quantify the variation size due to genotype × environment (location) interaction relative to main genotype variation, the quantitative parameters over locations were analyzed using a linear mixed model with the

REML procedure of SPSS program version 8.0. The genotypes and genotype × environment interaction (G × E) were considered as random effects and the locations as fixed effects.

The results of this mixed model quantify the size of the G ×E interaction relative to the genetic variance using the ratio $\sigma^2_{ge} / \sigma^2_g$ where σ^2_{ge} and σ^2_g represent the genotype × genotype interaction and the genotype impact, respectively. Principal component analysis (PCA) was performed using MVSP version 3.22 statistical software (Kovach 2010).

2.1.4. Results and Discussion

The environmental parameters or climatic data recorded in 2014 comparative to historical averages at the three locations (AUA, INIAV and UTAD) can be considered normal, suggesting that the data observed in this study reflect the plant performance in each location.

Qualitative traits are considered the most appropriate to determine a specific cultivar/variety because they are mostly genetically controlled, being independent from the environment. In this present study, the frequencies for each five qualitative traits studied were determined in regard to the three different locations (Table 2.1.3).

Table 2.1.3. Frequencies (%) of the five qualitative traits studied, presented separately in the three locations, for the 24 cowpea accessions.

Qualitative trait	Class	Frequencies (%)		
		AUA	**INIAV**	**UTAD**
Growth habit	Erect	47.40	16.67	86.90
	Semi-erect	12.30	79.17	13.10
	Intermediate	7.80	0.00	0.00
	Semi-prostate	32.50	4.16	0.00
Flower color	White	78.90	78.20	78.20
	Violet	9.20	21.80	21.20
	Mauve-pink	11.90	0.00	0.00
Seed color	Beige	11.50	17.40	13.10
	Brown	14.00	0.00	8.70
	Cream	63.00	78.30	73.90
	Other	11.50	4.30	4.30
Eye color	Eye absent	24.00	26.10	13.10
	Black	44.00	43.50	43.50
	Brown splash or gray	0.00	0.00	4.30
	Green	0.00	0.00	8.70
	Tan brown	24.50	30.40	30.40
	Other	7.50	0.00	0.00
Seed shape	Crowder	0.00	0.00	0.00
	Globose	4.00	4.30	13.10
	Kidney	67.50	82.60	56.50
	Ovoid	12.00	0.00	8.70
	Rhomboid	16.50	13.10	21.70

Growth habit presented some variation among locations, erect growth being the most common (47.4% and 86.9%, respectively) in AUA and UTAD, whereas in INIAV semi-erect (79.17%) was the most prevalent growth habit regarding the total accessions studied. For consumers and farmers, seed traits such as color seed and eye, seed size and seed coat are considered the most important traits of cowpea (Mustapha 2008; Egbadzor *et al.*, 2014). In all locations, seeds had a predominant cream color, kidney shape and black eye (Table 2.1.3), in accordance with consumer preferences (Stoilova and Pereira 2013). These findings are in contrast to the results obtained by Negri *et al*. (2000) and Egbadzor *et al*. (2013), who observed a higher variability in these two traits in cowpea accessions from Italy and Ghana.

Regarding the nine quantitative traits (Table 2.1.4), a high variability was observed in the number of days to flowering among the three locations. The average number of days to flowering was 52 (AUA), 65 (INIAV) and 99 (UTAD), with an average of 73.39 for the three

environments (Table 5). This differentiation could be explained by the different temperature range observed in the three locations and also the different sowing dates in each location (Table 2.1.2). The INIAV sowing date was earlier than AUA and UTAD because among the three locations INIAV is the warmest, so it is important to sow early (Table 2.1.2). In general, cowpea accessions with the higher and the lower values were concordant; namely, accession BGE038478 presented the latest flowering in all locations whereas accession Cp5131 presented the earliest one (Table 2.1.4). The beginning of flowering has been considered an important trait in genotype selection for cowpea improvement. Indeed, Silva *et al*. (2014) referred to its negative correlation with seed production. Moreover, accessions with earlier flowering dates would be more interesting because this way cowpea plants are more likely to escape high temperatures, long water stress periods and low relative humidity (Stoilova and Pereira 2013). In fact, Hamidou *et al.* (2007) verified that there is a higher drought susceptibility in the flowering stage than in the vegetative stage of cowpea.

Table 2.1.4. Quantitative traits average values obtained for the 24 cowpea accessions in each locations and F value and Tukey's test (significance level of 0.05) for the traits with replications.

	Plant height (cm)			**1st pod height (cm)**			**Nº of pods/plant**			**Nº of seeds/plant**			**Seed weight/plant (g)**			**100 seed weight (g)**		
	AUA	INIAV[a]	UTAD	AUA	INIAV	UTAD	AUA	INIAV	UTAD	AUA	INIAV	UTAD	AUA	INIAV	UTAD	AUA	INIAV[a]	UTAD
Cp4906	107.17	81.00	28.50	36.67	35.50	31.08	57.83	29.25	16.50	337.75	171.42	49.42	88.84	36.83	23.36	25.36	21.70	29.45
Cp5128	34.83	37.00	32.42	22.75	33.33	24.46	35.75	28.00	31.50	427.88	230.00	194.58	39.25	21.18	31.81	12.6	9.70	11.80
Cp5129	69.55	94.00	86.50	35.75	36.75	28.17	34.00	19.08	18.25	267.25	143.25	101.25	49.88	22.64	29.93	20.06	16.00	22.20
Cp5131	78.75	56.00	51.00	37.67	36.92	25.67	34.17	30.17	20.17	327.50	214.00	130.08	47.50	29.63	29.82	17.49	14.70	18.20
Cp5553	84.08	78.00	54.17	34.78	37.00	28.33	32.25	18.67	18.50	245.00	134.92	108.08	48.88	24.26	29.03	19.86	19.10	21.40
Cp5556	66.92	77.00	44.58	26.46	43.12	27.92	19.67	20.50	8.08	150.25	119.08	26.50	34.48	21.50	12.80	19.09	17.90	26.75
Cp5647	91.58	67.00	67.83	34.17	39.42	27.25	30.92	12.00	20.08	256.00	79.08	94.42	53.28	12.90	29.25	21.16	13.10	20.10
Cp5648	57.67	62.00	94.17	28.08	40.00	25.67	31.00	14.25	15.17	201.50	100.17	85.92	41.09	18.35	21.78	22.25	17.20	21.00
Vg50	103.17	66.00	47.67	39.58	34.58	24.42	29.00	17.17	12.67	221.50	120.25	48.67	50.88	20.26	16.36	21.99	15.90	24.85
Vg52	77.17	70.00	84.92	33.33	36.92	28.08	35.17	11.67	17.67	349.75	70.92	94.92	64.50	13.51	21.23	21.21	20.00	20.10
Vg56	132.5	76.00	89.50	42.50	38.67	29.08	66.64	18.83	12.08	361.38	122.83	51.92	68.50	23.39	20.69	20.51	20.30	21.80
Vg59	69.25	62.00	35.58	35.33	32.08	24.25	42.17	32.33	16.50	465.13	214.00	35.92	67.75	29.08	16.09	16.00	12.80	16.15
Vg60	92.17	61.00	35.42	36.96	36.92	17.92	57.08	21.17	15.25	574.25	146.67	68.00	85.25	23.46	23.48	18.33	15.30	18.15
Vg65	78.17	39.00	82.17	34.50	38.42	25.00	31.83	9.67	11.75	269.25	69.92	43.75	57.81	14.05	21.34	25.15	20.90	28.90
Vg67	73.25	67.00	131.08	42.75	38.25	21.50	40.00	12.92	8.17	314.38	88.33	40.58	83.00	20.28	15.43	23.95	22.80	30.85
Vg72	89.42	84.00	91.50	31.04	40.58	25.33	25.58	12.5	13.92	168.88	97.42	67.67	39.38	15.90	21.84	20.00	18.80	24.45
Vg73	57.63	48.00	113.42	33.26	38.92	28.42	40.83	19.25	18.83	291.58	155.50	129.75	45.08	21.50	31.73	16.89	15.50	20.95
Fradel	80.23	73.00	48.25	34.42	36.08	22.75	29.50	69.42	16.58	216.00	500.33	97.50	43.25	84.17	28.35	14.21	19.70	25.50
BGE022146	105.50	200.00	136.00	49.00	42.13	33.42	31.00	33.00	11.58	273.38	270.00	75.92	71.50	54.00	17.71	12.75	18.70	20.65
BGE038474	48.84	200.00	106.00	28.58	50.00	38.33	44.17	19.00	15.42	552.38	149.00	107.25	78.75	18.00	23.79	12.50	11.60	14.90
BGE038477	28.57	200.00	107.83	37.29	42.00	42.33	32.25	19.00	30.67	268.33	149.00	267.17	36.17	21.00	41.49	16.89	12.00	14.35

BGE038478	50.92	200.00	129.58	42.33	46.30	45.83	49.33	43.00	22.83	607.88	364.00	142.92	77.00	49.00	26.55	13.42	13.60	13.55
BGE038479	44.13	200.00	160.60	43.92	44.00	41.40	53.42	26.00	25.60	662.63	219.00	273.20	102.63	28.00	33.40	18.88	14.70	11.75
IT97K-499-35	35.89	66.00	33.38	33.82	35.00	24.13	94.22	36.00	18.38	906.22	284.00	216.38	169.67	52.00	22.73	12.20	18.50	10.25
F	5.17**	-	13.37**	2.08**	5.03**	8.73**	2.18**	12.70**	4.93**	3.76**	12.19**	12.73**	3.85**	12.49**	3.03**	7.73**	-	196.87**
Tukey$_{0.05}$	51.53	-	44.94	21.66	6.51	10.24	53.23	15.37	12.01	448.63	114.96	80.46	72.91	18.27	17.53	6.88	-	2.02

([a] for this location only had the average for the quantitative trait; ** significant at level of 0.01)

Table 2.1.4. (continuation)

	Days to flowering			**Yield (g m^{-2})**			**Protein (%)**		
	AUA	INIAV	UTAD	AUA	INIAV	UTAD	AUA	INIAV	UTAD
Cp4906	45	63	95	236.90	122.78	74.75	25.99	23.67	26.21
Cp5128	56	66	95	104.67	70.58	101.79	26.72	25.00	27.21
Cp5129	48	63	77	133.00	75.47	95.79	25.6	22.02	25.99
Cp5131	42	60	77	126.67	98.78	95.41	25.52	23.26	26.99
Cp5553	46	57	91	130.33	80.86	92.88	25.65	24.8	25.43
Cp5556	44	63	97	91.93	71.67	40.96	25.63	26.97	27.08
Cp5647	45	63	105	142.07	43.00	93.60	26.76	22.67	26.98
Cp5648	50	65	81	109.57	61.17	69.71	26.56	24.64	27.77
Vg50	45	63	109	135.67	67.53	52.35	24.12	20.2	26.14
Vg52	47	74	105	172.00	45.03	67.92	24.31	26.24	27.54
Vg56	45	63	102	182.67	77.97	66.21	24.68	24.55	26.03
Vg59	46	60	105	180.67	96.92	51.49	24.66	20.92	27.43
Vg60	44	60	81	227.33	78.19	75.12	24.87	21.31	24.35
Vg65	44	57	91	154.17	46.83	68.29	25.06	21.56	27.73
Vg67	47	57	81	221.33	67.61	49.36	25.34	20.98	27.62
Vg72	45	57	102	105.00	53.00	69.89	24.64	20.93	27.27
Vg73	61	57	91	180.33	71.67	101.52	24.37	20.75	26.41
Fradel	45	73	112	115.33	280.56	90.72	25.08	23.89	28.45
BGE022146	60	72	112	190.67	14.94	56.67	23.47	27.96	27.97
BGE038474	68	77	116	210.00	4.99	76.13	23.97	26.84	26.93
BGE038477	66	76	112	144.67	5.81	132.77	MD	29.34	29.51
BGE038478	71	76	123	273.67	13.64	115.79	27.16	27.98	29.48
BGE038479	68	78	119	205.33	7.64	84.96	28.07	29.34	31.19
IT97K-499-35	71	75	109	529.00	14.44	60.91	23.28	21.09	22.75
F									
Tukey$_{0.05}$									

([a] for this location only had the average for the quantitative trait; [**] significant at level of 0.01)

Variance analysis revealed significant differences, at a level of 1%, between accessions for six quantitative traits (plant height, first pod height, number pods per plant, number seeds per plant, seed weight and 100-seed weight (Table 2.1.4).

Plant height of the accessions fluctuated particularly in each tested location, ranging from 10 to 200 cm, with a mean value of 77.43 (Table 2.1.5). De Souza *et al.* (2007) previously reported similar maximum values for plant height in cowpea populations and a mean value of 164 cm, whereas a mean value of 113.7 cm was reported by Basaran *et al.* (2011). In comparison, Abayomi *et al*. (2008) reported a maximum plant height of 59.12 cm. A higher CV value was calculated for plant height (61.54%) than that reported by de Souza *et al.* (2007) indicating the high variability of this trait among the accessions tested in this study. The average value for the first pod height in the three environments was 33.93 cm. The extreme values of the three locations were observed in Cp5556 (4 cm) and BGE022146 (125 cm) accessions at the AUA location; at INIAV, the values ranged from 24 cm (Cp4906 and Vg 59 accessions) to 55 cm (BGE038478 accession), with an average of 38.12 cm; and at UTAD from 8 cm (Vg 59 accession) to 58 cm (BGE038474 and BGE038478 accessions), with an average of 28.53 cm (Table 2.1.5).

Table 2.1.5. Descriptive statistics for the nine quantitative traits studied, for each and all the three locations, for the 24 cowpea accessions.

Location	Trait	Min	Max	Mean	SD	CV (%)	H^2
AUA	Plant height (cm)	17.00	187.00	73.62	44.69	60.71	0.27
	1st pod height (cm)	4.00	125.00	35.64	14.89	41.78	0.10
	No of pods/plant	1.00	166.00	39.14	30.47	77.80	0.10
	No of seeds/plant	13.00	1454.00	362.56	313.01	86.33	0.27
	Seed weight/plant (g)	2.00	255.00	63.92	51.06	79.88	0.27
	100 seed weight (g)	5.00	33.30	18.55	5.57	30.05	0.49
	Days to flowering	42	71	52.04	10.04	19.29	
	Yield (g m^{-2})	91.93	529.00	179.29	88.59	49.41	
	Protein (%)	23.28	28.07	25.28	1.19	4.75	
INIAV	Plant height (cm)	37.46	200.00	94.22	56.88	60.36	MD
	1st pod height (cm)	24.00	55.00	38.12	5.75	15.08	0.29
	No of pods/plant	3.00	111.00	22.24	17.16	77.14	0.56
	No of seeds/plant	13.00	865.00	156.63	126.80	80.96	0.55
	Seed weight/plant (g)	2.40	140.00	25.47	20.28	79.61	0.55
	100 seed weight (g)	9.70	22.80	16.68	3.48	20.88	MD
	Days to flowering	57	78	65.62	7.40	11.28	
	Yield (g m^{-2})	4.99	280.56	65.46	56.05	85.52	
	Protein (%)	20.20	29.34	24.17	2.89	11.99	
UTAD	Plant height (cm)	10.00	200	77.43	40.03	62.31	0.51
	1st pod height (cm)	8.00	58	28.53	9.85	34.53	0.43
	No of pods/plant	1.00	58	17.12	10.38	60.63	0.25
	No of seeds/plant	4.00	548	100.52	84.84	84.39	0.50
	Seed weight/plant (g)	1.30	97.30	24.39	14.21	58.29	0.15
	100 seed weight (g)	10.00	31.00	20.33	5.73	28.16	0.99
	Days to flowering	77	123	99.50	13.61	13.68	
	Yield (g m^{-2})	40.96	132.77	78.54	22.48	28.63	
	Protein (%)	24.35	31.90	27.32	1.54	5.64	
Total	Plant height (cm)	10.00	200.00	76.26	46.93	61.54	0.15
	1st pod height (cm)	4.00	125.00	33.93	11.76	34.66	0.09
	No of pods/plant	1.00	166.00	26.56	23.49	88.29	0.10
	No of seeds/plant	4.00	1454.00	193.56	219.17	99.90	0.17
	Seed weight/plant (g)	1.00	255.00	36.09	25.62	98.70	0.12

100 seed weight (g)	5.00	33.30	14.33	9.26	64.62	0.54
Days to flowering	42	123	72.39	22.68	31.33	
Yield (g m^{-2})	4.99	529.00	107.76	79.67	73.91	
Protein (%)	20.20	31.90	25.52	2.38	9.32	

(Min - average minimum; Max - average maximum; Mean - average; SD - standard deviation; CV - coefficient of variation; H^2 - heritability; MD - missing data)

In modern agriculture, one of the most important characteristics in grain legumes is the first pod height. Plants with compact growth and a great distance of the first pod from the ground are highly desirable, allowing increased sowing density, facilitating mechanical harvesting and benefiting seed quality, since contact with soil and therefore rotting of pods and seeds are avoided. The importance of first pod height was also previously reported by Silva *et al.* (2014) who described a simple correlation between the first pod height and the number of seeds per plant.

High variability was presented for number of pods and seeds per plant. Specifically, number of pods per plant ranged from one (Fradel at AUA and Vg 67 at UTAD) to 166 (BGE038479 at AUA), with an average of 26.56 pods per plant; seeds per plant ranged from four (Cp5556 at UTAD) to 1454 (BGE038478 at AUA), with an average of 193.56 (Table 2.1.5). The mean number of pods per plant observed in this study, as well as the CV value, was higher than reported by de Souza *et al.* (2007) for Brazilian local cultivars and by Oliveira *et al*. (2015). Seed weight per plant was also characterized by high variability, ranging from 1 to 255 g. All three traits studied that are related to seed yield production presented high CV values, while CV values for number of pods and seeds per plant were higher than these reported by Ajayi *et al.* (2014) for cowpea breeding lines. Hundred-seed weight ranged from to 5 to 33.3 g, with an average of 18.52 g, which was slightly higher than that reported by Perrino *et al.* (1993) among cowpea landraces originating from the Mediterranean region.

Concerning yield, average values were calculated and for this reason it was not possible to perform statistical analysis. The average yield of the three locations was 107.76 g m^{-2}. In AUA, IT97K-499-35 had the highest yield (529 g m^{-2}) and Cp5128 the lowest (91.93 g m^{-2}), whereas in INIAV the yield varied between 4.99 g m^{-2} (BGE038474) and 280.56 g m^{-2} (Fradel). In UTAD yield ranged from 40.96 g m^{-2} (Cp5556) to 132.77 g m^{-2} (BGE038477). These results showed evidence of the good adaptation of some accessions to different environments, such as BGE038477 from Spain and Fradel from Portugal.

The parameters that presented higher heritability were different in the three locations: seed weight per plant (AUA), number of pods per plant (INIAV) and 100-seed weight (UTAD) (Table 2.1.5). The genetic variability transmitted from parents to their offspring is reflected by heritability (Mishra and Singh 2014). This parameter is very important because it indicates the possibility and extent to which improvement can change a trait by selection (Robinson *et al.* 1949; Mishra and Singh 2014). A high heritability alone is not sufficient to perform an efficient selection in advanced generations unless accompanied by a substantial amount of genetic advance (Jonhson *et al.* 1955; Mishra and Singh 2014). The different heritability observed could be explained by the behavior of the accessions in the different locations, allowing an understanding of how the environment affects these traits.

Regarding all three locations, the protein content varied between 20.20% (Vg50 in INIAV) and 31.90% (BGE038478 in UTAD), with an average of 25.69%. The lowest protein contents observed were 23.28% at AUA (IT97K-499-35), 20.20% at INIAV (Vg50) and 24.35% at UTAD (Vg60). BGE038478 showed the highest protein content in all three locations (28.07% at AUA, 29.34% at INIAV and 31.90% at UTAD). The values of protein content obtained are in agreement with the reference values that have been previously given for cowpea (Nielsen *et al.* 1993; Singh *et al.* 2002; Timko *et al.* 2007).

Correlation coefficients between the six quantitative traits and the three locations together are presented in Table 2.1.6. The number of pods per plant was correlated with the number of seeds per plant ($r = 0.813$, $P = 0.01$) and with the seed weight per plant ($r = 0.809$, $P = 0.01$). This allows us to infer that the selection to increase the number of pods per plant favors seed weight and, consequently, productivity. These results confirm those obtained by Mohammed *et al.* (2010), Stoilova and Pereira (2013) and Silva *et al*. (2014), who state that one of the most important components for seed production for cowpea is the number of pods per plant. Number of seeds per plant was negatively correlated with the 100-seed weight ($r = -0.144$, $P = 0.01$), which shows that selection for increased number of seeds can induce a reduction in the 100-seed weight.

Table 2.1.6. Estimates of variance components for genotypic variance and variance for genotype × environment and ratio of genotype × environment interaction variance to genetic variance for the five quantitative traits in 24 cowpea accessions.

Trait	Source of variance		
	Genotype (σ^2_g)	Genotype × environment (σ^2_{ge})	σ^2_{ge}/σ^2_g
Plant height	134	902.22	6.70
1st pod height	10.87	12.69	1.17
No of pods/plant	85.64	23.19	0.27
No of seeds/plant	5423.78	8104.40	1.49
Seed weight/plant	20.97	290.57	13.86

Four of the five quantitative traits (plant height, first pod height, number of seeds per plant and seed weight) revealed a higher G × E component than genetic variance component (Table 2.1.7). In general, an inverse relationship between $\sigma^2_{ge} / \sigma^2_g$ ratio and heritability value was observed.

Table 2.1.7. Pearson correlation coefficients for the six quantitative traits, in all the three locations, for the 24 cowpea accessions.

	Plant height	1st pod height	No of pods/plant	No of seeds/plant	Seed weight/plant	100 seed weight
Plant height	1					
1st pod height	0.274**	1				
No of pods/plant	0.052	0.263**	1			
No of seeds/plant	0.004	0.307**	0.813**	1		
Seed weight/plant	0.071	0.275**	0.809**	0.894**	1	
100 seed weight	0.189**	0.021	0.001	-0.144**	0.026	1

(** Correlation is significant at 0.01 level)

The first two principal components of PCA explained 97.52% (PC1=94.52% and PC2=3.00%) of the total variation (Fig. 2.1.1 and Table 2.1.8). The major trait that contributed to the first component separation was the number of seeds per plant (0.971), and to the second component plant height (−0.548) and protein content (0.790) (Table 2.1.8). PCA allowed the discrimination of cowpea accessions based on their country of origin. Portuguese accessions were grouped mainly together in the second and third quadrant, while the Fradel

variety and the reference line IT97K-499-35 were separated at higher distance (first quadrant). In addition, the Cp4906 accession, was separated from the other Portuguese accessions. This accession was the only one collected near the coast (Atlantic Ocean). Four of the five Spanish accessions were grouped in the fourth quadrant.

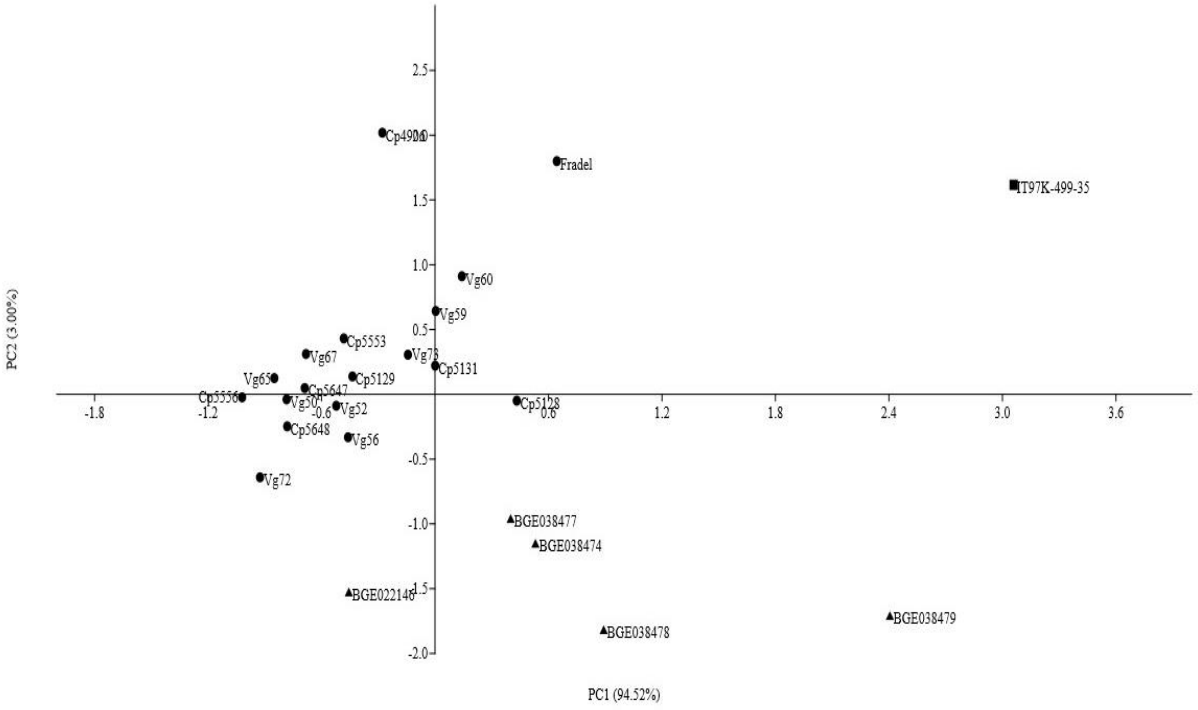

Figure 2.1.1. Principal component analysis (PCA) of cowpea accessions in all the three locations based on the eight quantitative traits measured (Circles, Portuguese origin; Triangles, Spanish origin; Square, Nigerian origin).

Table 2.1.8. Eigen values, factor scores and contribution of the first two principal axes (PC1, PC2) to the variation of the 24 cowpea accessions.

	PC1	PC2
Plant height	-0.056	-0.548
1st pod height	0.007	-0.102
No of pods/plant	0.076	0.056
No of seeds/plant	0.971	-0.198
Seed weight/plant	0.128	0.130
100 seed weight	-0.014	0.061
Yield	0.003	-0.038
Protein Content	0.180	0.790
Eigen value	14,845.234	471.433
Percentage (%)	94.518	3.002
Cumulative (%)	94.518	97.519

2.1.5. Conclusions

The present study highlights the high genetic diversity existing in the Iberian Peninsula cowpea genetic resources and useful knowledge about its breeding value. Seeds per plant is the trait that should be used primarily for plant selection. A clear distinction was observed between landraces and the reference samples, variety and breeding line. Moreover, the set of accessions with Spanish and Portuguese origin was discriminated in PCA, suggesting a specific gene pool structure.

G × E interaction, important yield components such as number of pods and seeds per plant and seed weight encompass variation between accessions. This variability reveals the potential of this germplasm for breeding programs to be conducted in different environments.

The accessions BGE038477 and BGE038478 from Spain and Cp5553 and Vg60 from Portugal have already been included in a cowpea breeding program.

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2.1.6. References

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2.2. Environmental adaptation

Genotype by environment interactions in cowpea (*Vigna unguiculata* L. Walp.) grown in the Iberian Peninsula

Martos-Fuentes M., Fernández J.A., Ochoa J., Carvalho M.*, Carnide V., Rosa E., Pereira G., Barcelos C., Bebeli P., Egea-Gilbert C. 2017. Genotype by environment interactions in cowpea (*Vigna unguiculata* L. Walp.) grown in the Iberian Peninsula. *Crop & Pasture Science*, 68: 924-931(doi: 10.1071/CP17071). JIF (2017) = 1.354; SJR (2017) Agronomy and Crop Science = Q1

*Carvalho M. contribution: UTAD field work, data analysis, material and methods and results writing

2.2.1. Abstract

The aim of this work was to determine the variance components and genetic and environmental stability of 12 cowpea genotypes at three locations (South-east of Spain: Cartagena, South and North of Portugal: Elvas and Vila Real, respectively) in the Iberian Peninsula in two consecutive years (2015 and 2016). The genotype, the environment and the genotype × environment interaction significantly influenced all the morphological and agronomical parameters evaluated. For both years, the highest yields were observed at Elvas, while Cartagena and Vila Real were the most suitable places to obtain crop precocity. Cartagena was the place where the filling of the seed was the fastest, probably due to the higher temperatures and radiation. The thermal time model (effective day-degrees) could be used to predict the period of cowpea development, therefore predict flowering and pod maturity date. Correlation analysis showed that days to flowering, days to maturity and the seed yield *vs* protein content exhibited negative correlations. The highest heritability was found for plant height and pod length at Cartagena and for 100-seed weigh at Elvas and Vila Real. In conclusion, the variations that exist in the studied accessions could give rise to a breeding programme to develop cowpea cultivars with interesting agronomic traits.

2.2.2. Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is originated in Southern Africa and belongs to the family *Fabaceae*, tribe *Phaseoleae* and genus *Vigna*, which comprises several species, subspecies and varieties depending on morphology and domestication (Padulosi and Ng 1997). Cultivated cowpea belongs to *V. unguiculata* spp. *unguiculata*, which contains the cultigroups *Unguiculata*, *Biflora*, *Sesquipedalis* and *Textilis* (Ng and Marechal 1985). This annual warm-season legume is one of the most widely adapted, versatile, and nutritious grain legumes (Ehlers and Hall 1997). During the 2010–2014 period, the world cowpea planting area was 58.1 million hectares and the production was 33.5 million tonnes. Africa has been responsible for 95.8% of worldwide cowpea production (FAOSTAT 2017). Nigeria and Niger are the largest producers with 3.4 and 1.6 million tonnes, respectively. In contrast, Europe is only responsible for 0.4% of worldwide cowpea production (FAOSTAT 2017). Now-a-days, cowpea is mainly

grown by subsistence farmers in west and central sub-Saharan Africa, but also is an important food source in the rest of Africa, Central and South America, South-east Asia and in the southern United States (Davis *et al*. 1991; Timko and Singh 2008). In addition, cowpea is being cultivated at a small scale in many parts of Southern Europe and countries around the Mediterranean Basin (Domínguez-Perles *et al*. 2015), providing these countries a considerable income through exports to Northern European and non-European countries (European Comission 2016). Like other grain legumes, cowpea has the capacity to establish association with nitrogen-fixing bacteria (like rhizobia) and vesicular-arbuscular mycorrhizal fungi that make this crop interesting for predicted climatic changes. Cowpea can be used for human food and for fodder livestock (Tarawali *et al*. 1997). For human food, dry grain is the most important part, but leaves and immature pods are also consumed. Dry grains provide a significant amount of dietary protein (18–35%), as well as a source of calories, vitamins, minerals and essential amino acids as lysine and tryptophan (Singh 2002). For all this proprieties, this is an attractive crop with which many research is being done to promote it and include it in diets, not only because of its protein content but also because other functional properties, such as chlorophylls, carotenoids and phenolic contents, and high antioxidant activity (Khalid *et al*. 2012; Campbell *et al*. 2016; Karapanos *et al*. 2017). However, the value of grain legumes as a source of nutrients depends on a plethora of factors, including genetic characteristics, agro-climatic conditions, and postharvest management (Gonçalves *et al*. 2016).

The environment plays a very important role in the development and growth of plants. The major driving force that pushes crop growth and development is temperature although there are other environmental factors that can modify the effect of temperature such as photosynthetically active radiation (PAR) or photoperiod. Locations, growing seasons, rainfall, may have positive or negative impacts on several plant species as well as in cowpea genotypes. The thermal time concept or the accumulation of temperature for a life cycle or a particular phase of plant development, in contrast to the chronological time, has been used frequently to study the cowpea development, with the advantage to be independent of location and time of sowing. Craufurd *et al*. (1997) have described the effects of photoperiod and temperature on several development stages. Thus, the base temperature for development of seed germination, seedling emergence, leaf appearance, and days from sowing to first flowering is 8-11ºC and the optimum temperature for most rapid reproductive development is close to 28ºC. In addition,

inclusion of radiation will allow describing development when temperature is not the only environmental variable affecting the process (Jones 2014). Thus, the 'effective degree-days' can be used to combine both temperature and radiation effects on plant development (Scaife *et al.* 1987). To our knowledge, no previous information exists on the effects of both temperature and radiation on cowpea development.

The association between the environment and the phenotypic expression of a genotype constitute the genotype (G) × environment (E) interaction, which determines if a genotype is widely adapted for an entire range of environmental conditions or separate genotypes must be selected for different sub-environments. Presence of the G × E interaction indicates that the phenotypic expression of one genotype might be superior to another genotype in one environment but inferior in a different environment (Falconer & Mackay 1996). Most of the studies in cowpea have been carried out on the genotypic variability and stability of some grain yield components (e.g. Akande 2007; Adewale *et al*. 2010; Shiringani and Shimelis 2011), showing generally significant G × E interactions. In addition, the protein content in seeds is also influenced by environmental and genotypic factors, being negatively correlated with yield (Oluwatosin 1997). Therefore, G × E should be taken into account in any breeding program.

Thus, the aim of this work was to determine the variance components and genetic and environmental stability of 12 selected cowpea genotypes at three locations of the Iberian Peninsula in two consecutive years. The results of this study may assist cowpea breeders in the manipulation of interested traits.

2.2.3. Material and methods

2.2.3.1. Plant material

Ten cowpea landraces (five from Portugal, three from Spain and two from Greece), one commercial variety from Portugal and one advanced line from Nigeria (Table 2.2.1) were used in three field experiments in 2015 and in 2016. The accessions were selected based on previously studies that were developed in the three locations where morphological and agronomical characteristics were evaluated. The agronomic characterization of the 12 genotypes was done at: Technical University of Cartagena

(UPCT), Cartagena, Spain (N 37º36'; W 00º 58'; 40 m) - field experiment 1; National Institute for Agrarian and Veterinarian Research (INIAV), Elvas, Portugal (N 38°53', W 07°09', 208 m) - field experiment 2; University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal (N 41°17'51", W 07°44'12", 465 m) - field experiment 3.

Table 2.2.1. Cowpea accessions, origin and breeding status

Accession	Origin	Status of accession
IT 97K-499-35	Nigeria	Advanced line
AUA1	Greece	Landrace
AUA2	Greece	Landrace
Cp 4877	Portugal	Landrace
Cp 5051	Portugal	Variety
Cp 5553	Portugal	Landrace
Vg 59	Portugal	Landrace
Vg 60	Portugal	Landrace
Vg 73	Portugal	Landrace
BGE038479	Spain	Landrace
BGE038474	Spain	Landrace
BGE038478	Spain	Landrace

2.2.3.2. Field experiment 1

Cultivars were planted on 29 May 2015 and 15 June 2016 in a randomized complete block design with four replications. One row per plot with 8-m length, 0.9-m row spacing and 7 m^2 were used. Seeds were sown by hand and seed rate was 10 seeds/m2. The topsoil (0–20 cm) was classified as clay loam with a medium texture in both growing seasons, and presented 1.97 g kg^{-1} organic matter, 78 mg kg^{-1} of P_2O_5, 354 mg kg^{-1} of K_2O_2 and pH(KCl) 8.4 in 2015 growing season, and 2.18 organic matter, 80.13 mg $kg^{-1}$of P_2O_5, 415.82 mg kg^{-1} of K_2O_2 and a pH(KCl) 8.3 in 2016. Before sowing, in both growing seasons, the experimental field was ploughed with a rotary tiller and fertilized with 30 kg ha^{-1} of ammonium nitrate, 170 kg ha^{-1} of potassium nitrate and 250 kg ha^{-1} of monoammonium phosphate. The trails were drip irrigated from the beginning of June until the end of September.

2.2.3.3. Field experiment 2

Cultivars were planted on 28 April 2015 and 24 May 2016 in a randomized complete block design with four replications. Two row plots with 3-m length, 0.6-m

row spacing and 3.6 m^2 were used. Seeds were sown by hand and seed rate was 11 seeds m^{-2}. The topsoil (0–20 cm) was classified as sandy clay loam with a medium texture in both growing seasons, and presented 1.3 g kg^{-1} organic matter, >200 mg kg^{-1} of P_2O_5, 153 mg kg^{-1} of K_2O_2 and pH (KCl) 6.9 in 2015 growing season, and 0.80 mg kg^{-1} organic matter, >200 mg kg^{-1} of P_2O_5, >200 mg kg^{-1} of K_2O_2 and pH (KCl) 6.4 in 2016. Before sowing, the experimental fields were ploughed with a rotary tiller and fertilized with 200 kg ha^{-1} of 15:15:15. The trials were drip irrigated from the beginning of May until the end of August.

2.2.3.4. Field experiment 3

Cultivars were planted on 11 May 2015 and 3 June 2016 in a randomized complete block design with four replications. Three row plots with 3-m length, 0.75-m row spacing and 6.7 m^2 were used. Seeds were sown by hand and seed rate was 11 seeds/m^2. The topsoil (0–20 cm) was classified as gleyic fluvisol with a medium texture in both growing seasons, and presented in 2015 1.29 g kg^{-1} organic matter, 36 mg kg^{-1} of P_2O_5, 103 mg kg^{-1} of K_2O_2 and a pH (KCl) 4.2, whereas in 2016 1.61 g kg^{-1} humus content, 44 mg kg^{-1} of P_2O_5, 11 mg kg^{-1} of K_2O_2 and a pH (KCl) 5.2. Before sowing in both growing seasons, the experimental field was ploughed with a rotary tiller and fertilized with 250 kg/ha of nitromagnesium 27 and 200 kg ha^{-1} of NPK (Ca-Mg-S) 8–12–12 (2–2–14). The trials were drip irrigated from the beginning of July until the end of August.

2.2.3.5. Climatic data and calculation of accumulated degree-days and effective degree-days

The mean daily air temperature, total rainfall (mm) and accumulated global radiation (MJ/m^2) from April to September for each experiment are presented in Table 2.2.2.

Table 2.2.2. Mean temperatures, precipitation and global radiation from April to September 2015 and 2016 in each location.

Environment/ Month	Year	Cartagena			Elvas			Vila Real		
		T (ºC)	R (mm)	Solar radiation (MJ/m^2)	T (ºC)	R (mm)	Solar radiation (MJ/m^2)	T (ºC)	R (mm)	Solar radiation (MJ/m^2)
April	2015	16.0	10.2	596.02	16.6	110.3	630.57	13.5	48.8	453.97
	2016	16.1	14.6	627.72	14.3	80.6	648.39	11.0	193.0	456.62
May	2015	20.2	0.0	825.38	22.0	2.8	813.55	17.5	69.6	713.86
	2016	18.6	3.0	791.06	17.2	119.7	717.96	14.2	124.4	536.18
June	2015	23.1	1.6	876.83	25.6	37.9	820.66	20.9	2.2	729.61
	2016	22.8	0.0	853.27	23.7	0.0	953.87	19.1	25.2	759.73
July	2015	27.2	0.6	852.9	26.5	0.0	847.19	22.5	0.4	781.33
	2016	25.4	0.0	825.15	28.5	0.1	956.20	23.8	0.2	821.62
August	2015	27.2	1.0	693.22	25.4	0.9	809.93	20.9	0.6	647.58
	2016	25.5	1.2	759.95	27.2	0.1	858.85	23.3	0.2	657.18
September	2015	22.8	72.6	519.29	22.1	32.8	604.26	17.4	1.2	489.99
	2016	23.7	25.0	587.09	24.0	0.0	671.23	19.6	28.4	515.56

Summations of heat units were determined based on base temperature using the coefficient of variation model (CV) to identify the accurate base temperature to adjust the method, according to Ochoa *et al*. (2011). The base temperatures tested ranged from 0ºC to 16ºC. The following methods were used:

Method 1. Standard degree-days method: $DD = \Sigma (T_m - T_b)$, where T_m and T_b are the daily mean and base temperatures respectively.

Method 2. Use of maximum instead mean temperature: $DD = \Sigma (T_M - T_b)$, where TM and Tb are the daily maximum and base temperatures respectively.

Method 3. The degree-days method modified by the effect of the daily photosynthetic radiation input or effective degree-days (EDD), calculated according to following equation: $EDD^{-1} = DD^{-1} + f\ PAR^{-1}$, where PAR is photosynthetically active radiation (MJ/m^2 day) and f is a constant that defines the relative importance of radiation and temperature (m^2/MJ).

The DD and EDD were calculated considering the climatic conditions from sowing to flowering and sowing to maturity.

2.2.3.6. Morphological and agronomical traits

Phenotypic data for days to flowering and maturation were collected when 50% of the plants begin to flower and have mature pods, respectively. Plant height, first pod height, pod length and width and number of seeds per pod were measured in 10 plants per plot randomly selected. Yield, adjusted to 12% moisture, and 100-seed weight were evaluated per plot. Protein content (AOAC 1990) was derived from the estimated nitrogen (N) content, which was determined by the Kjeldahl method (Bremmer 1960), by the following formula: protein content (%) = N content (%) × 6.25.

2.2.3.7. Data analysis

Analysis of variance (ANOVA) of the three factors (genotype, location and year) followed by the Tukey's test was performed for each parameter in each environment and in the assembly of the three environments using the IBM SPSS Statistics 20 software.

A complete linear mixed model was used to estimate variance components of parameters in the analysis of all the quantitative parameters within and across the accessions and locations using Restricted Maximum Likelihood (REML) algorithm of

SPSS program version 8.0. The heritability of each quantitative parameter was calculated for each environment using the following equation:

$$h^2 = V_g^2 / [V_g^2 + (V^2/r)]$$

where V_g^2 and V^2 represent genotypic and error variance for each parameter and r the number of replications. For the three environments, the heritabilities were calculated using the equation:

$$h^2 = V_g^2 / [V_g^2 + (V_{ge}^2/e) + (V^2/re)],$$

where V_{ge}^2 is the GxE interaction variance and e is the number of environments (Mendes-Moreira *et al*. 2015).

Pearson correlation coefficients between the different quantitative parameters and environments were determined through SPSS program version 8.0.

The principal components analysis (PCA) was performed using the MVSP program version 3.22.

2.2.4. Results and discussion

The plant genetic resources collections provide genetic variants, genes or genotypes that allow breeders to respond to new challenges based on systems of high production, high nutritional quality and disease and environmental resistance/tolerance. In the present study, we evaluated 12 cowpea accessions growing in three locations in the Iberian Peninsula (South-east of Spain: Cartagena, South of Portugal: Elvas and North of Portugal: Vila Real) during 2 years (2015, 2016) to identify morphological and agronomical parameters and the interactions among genotypes, environment and year.

In general, Vila Real registered the lowest temperatures and the lowest solar radiation. It is worth to highlights that the rainfall in Vila Real was 4-fold in 2016 than in 2015, whereas Cartagena had the driest conditions during the studied period (Table 2.2.2).

ANOVA to determine the effects of genotype, environment, year (Y) and their reciprocal interactions (G × E; G × Y; G × E; G × E × Y) on 10 morphological and agronomical parameters showed that all the factors had a high influence on the majority of the parameters (Table 2.2.3). These findings are according to those obtained by Shimelis and Shiringani (2010), who showed significant interactions among genotypes, locations and planting dates in cowpea. The genotype and the environment significantly

influenced all the parameters evaluated. Year effect was also an important factor affecting all parameter except first pod height, pod length and number of seed per pod. The G × E interaction was significant for all parameter, but G ×Y interaction was only significant for days to flowering and to maturity, first pod height, seed yield and number of seeds per pod. The E ×Y interaction affected all parameters, except first pod height and pod width.

Table 2.2.3. Analysis of variance for the 10 morphological and agronomical parameters evaluated in 12 cowpea accessions at 3 environments (Cartagena, Elvas, Vila Real) during 2 years (2015, 2016).

Paramaters	**ANOVA**						
	G	**E**	**Y**	**GxE**	**GxY**	**ExY**	**GxExY**
Days to flowering	***	***	***	***	***	***	***
Days to maturity	***	***	***	***	***	***	***
Plant height	***	***	***	***	n.s.	***	*
First pod height	***	*	n.s.	***	**	n.s.	*
Seed yield	***	***	***	***	**	***	***
100-Seed Weight	***	***	***	***	n.s.	**	n.s.
Pod length	***	**	n.s.	***	n.s.	**	n.s.
Pod width	***	***	***	***	n.s.	n.s.	*
Number of seeds / pod	***	***	n.s.	***	*	**	***
Protein content	***	*	***	***	n.s.	***	n.s.

(n.s. – no significant; * - significant at P<0.05; ** - significant at P<0.01; *** - significant at P<0.001)

Finally, the G × E × Y interaction was significant for all parameters, except 100-seed weight, pod length and protein content (Table 2.2.3). This high variability among the cowpea accessions indicates their utility in breeding programs.

The duration of the periods sowing to flowering and sowing to maturation were affected by the three factors and their interactions. In Cartagena, the days from sowing to maturity were the shortest, whereas in Elvas were the longest in both years (Table 2.2.4). Also in Cartagena the time from flowering to maturity was the shortest in both years, probably due to the effects of high temperature and radiation in this period (Tables 2.2.2 and 2.2.4).

Table 2.2.4. Means and standard deviation of the 10 morphological and agronomical parameters evaluated in 12 cowpea accessions at 3 environments (Cartagena, Elvas, Vila Real) during 2 years (2015, 2016).

Parameters	2015			2016		
	Cartagena	Elvas	Vila Real	Cartagena	Elvas	Vila Real
Days to flowering	75.58±10.34 a	66.44±6.28 c	70.00±9.21 b	59.21±9.13 b	67.94±13.54 a	69.98±3.56 a
Days to maturity	86.42±10.08 c	101.29±4.60 a	89.04±9.01 b	69.58±8.88 b	89.27±11.53 a	89.25±3.9 a
Plant height (cm)	212.47±63.26 a	123.87±61.36 b	57.07±32.58 c	212.72±62.33 a	146.38±60.67 b	80.05±37.39 c
First pod height (cm)	37.48±7.54 a	38.70±9.15 a	39.22±10.55 a	38.88±7.24 ab	36.97±4.78 b	41.84±8.59 a
Seed yield (g/m^2)	89.84±33.24 b	197.69±103.21 a	95.28±43.39 b	102.43±44.18 b	312.06±122.89 a	65.68±26.56 b
100-Seed Weight (g)	15.91±2.26 c	17.27±4.52 b	18.63±5.39 a	15.90±2.32 c	17.92±5.10 b	19.93±5.43 a
Pod length (cm)	17.16±3.75 a	16.94±2.82 a	16.63±1.81 a	17.10±3.89 a	16.38±3.21 b	17.14±1.94 a
Pod width (cm)	0.88±0.09 a	0.78±0.08 b	0.46±0.09 c	0.89±0.08 a	0.80±0.10 b	0.47±0.09 c
Number of seeds/pod	11.60±1.26 a	11.63±1.03 a	11.19±1.13 a	11.88±0.92 a	10.87±1.02 b	11.30±1.17 b
Protein content (%)	21.71±2.52 a	21.69±1.78 a	22.41±0.98 a	23.88±2.26 a	22.44±1.14 b	22.39±1.15 b

(For each year, means followed by the same letter in the row are not significantly different at the 0.05 level using Tukey test, n =4)

The analysis of the three methods showed the least CV was obtained with Method 3 (Table 2.2.5), demonstrating that PAR had an important effect on the duration of crop cycles in all accessions. The best fit for *f* ranged from 0.11 to 0.12. The accurate base temperature for all methods and accessions ranged from 2ºC to 14ºC, varying in some accessions for each calculation method and period. This temperature range differed to that proposed by Craufurd *et al*. (1997), who fixed 8-11°C for development of cowpea cultivated in Nigeria. An explanation of our different findings could be due to the base temperature drops with the increase of the daily thermal amplitude (Bonhomme 2000), higher in our conditions than in Nigeria.

Table 2.2.5. The base temperature for each cowpea accession and over growing periods incorporating PAR radiation.

Accession	Tbase (ºC)		*f*		CV (%)	
	S-F	S-M	S-F	S-M	S-F	S-M
IT97K-499-35	5	5	0.12	0.11	10.61	12.80
AUA1	2	2	0.12	0.11	11.18	07.80
AUA2	2	9	0.12	0.11	06.27	10.24
Vg 59	2	2	0.12	0.11	20.39	13.07
Vg 60	10	10	0.12	0.11	17.83	12.85
Vg 70	2	2	0.12	0.11	10.87	10.28
Cp 4487	2	2	0.12	0.11	10.33	09.80
Cp 5051	14	14	0.12	0.11	09.73	12.20
Cp 5553	2	2	0.12	0.12	10.83	09.49
BGE038479	7	11	0.12	0.12	04.97	04.83
BGE038474	12	12	0.12	0.11	10.59	02.25
BGE038478	2	2	0.12	0.11	10.94	08.63

(Tbase is the base temperature. *f* is a constant that defines the relative importance of radiation and temperature in the Method 3 (EDD calculation) as described before. CV is the coefficient of variation expressed as a percentage. S-F is the growing period from sowing to 50% of flowering. S-M is the growing period from sowing to maturity of pods)

The seed yield was also affected by the three factors and their interactions (Table 2.2.3). For both years, the highest yields were observed in the trials located in Elvas (Table 2.2.4). In the second year (2016), the seed yield average increased in Cartagena and Elvas, while decreased in Vila Real (data not shown). At Cartagena, the most productive accessions were BGE038474 and IT97K-499–35 in 2015 and BGE038474 in 2016 (Table 2.2.6). In this location, the yield ranged from 52 to 165.4 g m^{-2} and from 63.8 to 226.5 g m^{-2} in 2015 and in 2016, respectively. At Elvas, Cp 5051 and Vg73 were the most productive in 2015, whereas in 2016 the most productive were Cp5553

and Vg73. The yield ranged from 35.98 to 329.6 g m^{-2} in 2015 and from 152.32 to 514.4 g m^{-2} in 2016. The commercial variety Cp 5051 revealed to be one of the well adapted accessions to this environment, this result could be expected due to this variety was selected at the INIAV Breeding Station in Elvas. Finally, in the first year, the most promising accessions in Vila Real were Cp 5553, Vg 60 and Vg 73, whereas in 2016 the most productive was AUA1. The seed yield varied from 34.3 to 167.2 and from 39.4 to 123.0 g m^{-2} in 2015 and 2016, respectively. In general, the most productive accessions in each location were those that originally came from their own country, due to they are better adapted to their environmental conditions.

Table 2.2.6. Seed yield (g m^{-2}) for the 12 cowpea accessions evaluated at three environments (Cartagena, Elvas, Vila Real) during 2 years (2015, 2016).

Acessions	2015			2016		
	Cartagena	**Elvas**	**Vila Real**	**Cartagena**	**Elvas**	**Vila Real**
IT97K-499-35	142.43±26.51 a	247.17±93.29 abc	49.25±20.91 b	130.02±45.87 b	152.32±20.68 d	40.05±24.21 b
AUA1	52.00±13.89 c	84.14±17.41 de	101.05±55.20 ab	86.65±28.95 b	263.75±99.86 cd	123.03±47.77 a
AUA2	94.40±13.22 b	258.20±56.64 abc	76.73±28.21 ab	86.77±26.71 b	292.35±55.32 bcd	45.90±27.41 ab
Cp 4877	64.98±11.41 bc	169.89±37.26 bcd	89.50±31.05 ab	88.98±18.69 b	237.05±46.19 cd	39.35±16.62 b
Cp 5051	77.15±18.93 bc	329.56±45.06 a	79.45±17.04 ab	82.65±39.32 b	310.90±80.93 bcd	46.53±33.97 ab
Cp 5553	97.53±26.03 b	279.93±81.56 ab	167.15±71.96 a	122.23±40.51 b	506.82±91.66 a	82.58±51.01 ab
Vg 59	67.85±12.81 bc	231.72±41.56 abc	57.10±14.26 b	70.20±13.59 b	465.75±137.68 ab	57.05±22.51 ab
Vg 60	64.53±11.09 bc	257.20±58.07 abc	153.58±58.14 a	83.58±9.88 b	352.35±53.57 abc	103.80±9.51 ab
Vg 73	75.20±4.38 bc	310.67±95.24 a	157.78±38.44 a	63.83±17.34 b	514.37±85.65 a	61.80±13.58 ab
BGE038479	83.55±9.77 bc	40.66±2.36 de	34.33±15.44 b	73.43±20.33 b	197.82±41.13 cd	44.40±15.05 ab
BGE038474	165.35±1.11 a	35.98±9.42 e	78.68±27.45 ab	226.45±60.19 a	217.12±43.74 cd	71.65±31.79 ab
BGE038478	91.23±2.66 b	127.12±18.31 cde	98.75±12.80 ab	114.38±15.77 b	234.11±4.71 cd c	72.18±51.72 ab

(Means followed by the same letter in the column for each year are not significantly different at the 0.05 level using Tukey test, n=4)

The highest plant height and pod width were observed in Cartagena in both years (Table 2.2.4). In 2015, the first pod height, pod length and number of seeds per pod did not differ among the trial places. Vila Real was the location in which the seeds reached the highest 100-seed weight in both years. The seed size, measured as 100-seed weight, is one of the most important parameter for the consumer's preference.

As regards protein content, it was influenced by genotype, environment and their interaction (Table 2.2.3), in agreement with the results obtained by Oluwatosin (1997) with 15 cowpea cultivars grown in three locations in Nigeria and Ravelombola *et al*. (2016) who grown 11 cowpea breeding lines in three locations in Arkansas. The highest percentage was found in Cartagena in 2016 (~24% in average) (Table 2.2.4). The values of protein content obtained in this study are in agreement with the results found in literature (Singh 2002; Timko *et al*. 2007).

In general, correlation coefficients between the 10 parameters in the three environments and 2 years were not too high (Table 2.2.7). The highest correlation coefficient was between days to flowering and days to maturity (r = 0.737, P = 0.01) and between plant height and pod width (r = 0.488, P = 0.01). The correlation between days to flowering and days to maturity was expected because they are closer in the plant development. Plant height and 100-seed weight showed the highest negative correlation (r = –0.360, P = 0.01) (Table 2.2.7), which shows that selection for the increase of plant height can induce a reduction in the 100-seed weight. There was also a negative correlation between the beginning of flowering and seed production in 2016 as it was reported by Silva *et al*. (2014). The seed yield and protein content exhibited negative correlations, which is agreement to the results obtained by Oluwatosin (1997) in cowpea and by Simmonds (1995) in cereals, and consequently indicates some restrictions in breeding alongside for high-yielding and high-protein genotypes. For the first pod height and seed yield, a positive correlation was registered. And for number of seeds per pod and 100-seed weight it was negative in agreement with the result obtained by Silva *et al*. (2014). The correlation between pod length and 100-seed weight was positive as the results obtained by Peksen and Artik (2004).

Table 2.2.7. Pearson correlation coefficients for 10 morphological and agronomical parameters for 12 cowpea accessions in the three environments (Cartagena, Elvas, Vila Real) and two years (2015, 2016).

	Days to flowering	**Days to maturity**	**Plant height**	**First pod height**	**Seed yield**	**100 Seed Weight**	**Pod length**	**Pod width**	**Number seeds/pod**	**Protein content**
Days to flowering	1									
Days to maturity	0.737**	1								
Plant height	0.089	-0.178**	1							
First pod height	0.118*	0.084	0.210**	1						
Seed yield	-0.277**	0.018	-0.006	0.029	1					
100 Seed weight	-0.142*	-0.031	-0.360**	-0.092	0.092	1				
Pod length	-0.220**	-0.231**	0.167**	0.186**	-0.033	0.176**	1			
Pod width	-0.150*	-0.234**	0.488**	-0.149*	0.240**	0.077	0.055	1		
Number of seeds/pod	-0.108	-0.116*	0.160**	0.142*	0.076	-0.216**	0.227**	0.047	1	
Protein content	-0.148*	-0.255**	0.115	0.105	-0.172**	-0.106	0.196**	0.025	0.081	1

(* - Correlation significant at P<0.05; ** - Correlation significant at P<0.01)

Heritability reflects the genetic variability that is transmitted from parents to their offspring (Robinson *et al*. 1949). Heritability, in broad-sense, estimates across environments, ranged from 0.29 for seed yield to 0.91 for pod width (Table 2.2.8). In general, it was higher at the Cartagena than at the other environments, with the exception of days to flowering, days to maturity, 100-seed weight and number of seeds per pod. The parameters plant height and pod length had the highest heritability at UPCT (0.99), whereas at Elvas and Vila Real was 100-seed weight that had the highest heritability (0.99) (Table 2.2.8). A hundred had high values of heritability in the three environments (0.94 at UPCT, 0.99 at Elvas and 0.99 at Vila Real) and across the three environments (0.89). These values are very close to the ones obtained in other studies with cowpea, which were always higher than 0.83 (Drabo *et al*. 1984; Omoigui *et al*. 2006; Manggoel *et al*. 2012; Egbadzor *et al*. 2013). These parameters with high heritabilities can be used in future breeding programs and for further quantitative genetic studies. However, it is important to refer that a high heritability alone is not enough to perform an efficient selection in advanced generations unless that it is accompanied by substantial genetic gains (Johnson *et al*. 1955; Mishra and Singh 2014). In the three environments (Cartagena*Elvas*Vila Real), the lowest values of heritability were estimated in seed yield (0.29) and protein content (0.53) (Table 8). The days to maturity (0.79), first pod height (0.52) and protein content (0.36) were the parameters with lowest values of heritability in Cartagena, Elvas and Vila Real, respectively. The low seed yield heritability was also reported by Omoigui *et al*. (2006) in cowpea. The value obtained in protein content is in agreement with the value reported by Ravelombola *et al*. (2016), who estimated a protein content of 0.58, and pointed out that this parameter can be inherited and can be selected for in the progeny.

Table 2.2.8. Heritability for the 10 morphological and agronomical parameters evaluated for Cartagena, Elvas and Vila Real and across the three environments in 12 cowpea accessions.

	Cartagena	**Elvas**	**Vila Real**	**Cartagena*Elvas*Vila Real**
Days to flowering	0.80	0.94	0.80	0.59
Days to maturity	0.79	0.84	0.77	0.60
Plant height (cm)	0.99	0.96	0.90	0.78
First pod height (cm)	0.98	0.52	0.92	0.77
Seed yield (g/m^2)	0.93	0.82	0.78	0.29
100 Seed Weight (g)	0.94	0.99	0.99	0.89
Pod length (cm)	0.99	0.97	0.93	0.81
Pod width (cm)	0.98	0.97	0.97	0.91
Number of seeds/pod	0.84	0.64	0.88	0.65
Protein content (%)	0.89	0.65	0.36	0.53

PCA of the 12 cowpea accessions in three different environments in two seasons is presented in Fig. 1. The first two principal components (PC) explained 98.59% (PC1 = 59.75 and PC2 = 38.84) of total variation. In PC1, the main contributing parameter was yield (0.98) and in PC2 plant height (0.98) (Table 2.2.9). Manggoel and Uguru (2011) and Doumbia *et al*. (2013) also obtained in their studies that yield and plant height were parameters that contribute to the divergence between accessions. In addition, they found another parameter such as number of peduncles and flowers per plant, the days to flowering and days to maturity, which also contributed to the divergence, although some of them were not analyzed in the present study. The accessions characterized at Vila Real were grouped principally in the third quadrant, those characterized in Cartagena were mainly distributed in the second quadrant and the accessions at Elvas were dispersed for the four quadrants, although the majority were in first and fourth ones (Fig. 2.2.1).

Table 2.2.9. Eigen value, factor scores and contribution of the first two principal component axis to variation in the 10 morphological and agronomical parameters of 12 cowpea accessions.

	Axis 1	**Axis 2**
Eigenvalues	9456.529	6147.711
Percentage	59.747	38.842
Cumulative percentage	59.747	98.589
Days to flowering	-0.034	0.001
Days to maturity	0.027	-0.022
Plant height	-0.201	0.979
First pod height	-0.009	0.018
Seed yield	0.978	0.202
100 Seed Weight	0.008	-0.021
Pod length	-0.003	0.006
Pod width	0	0
Number of seeds/pod	0	0.003
Protein content	-0.005	0.002

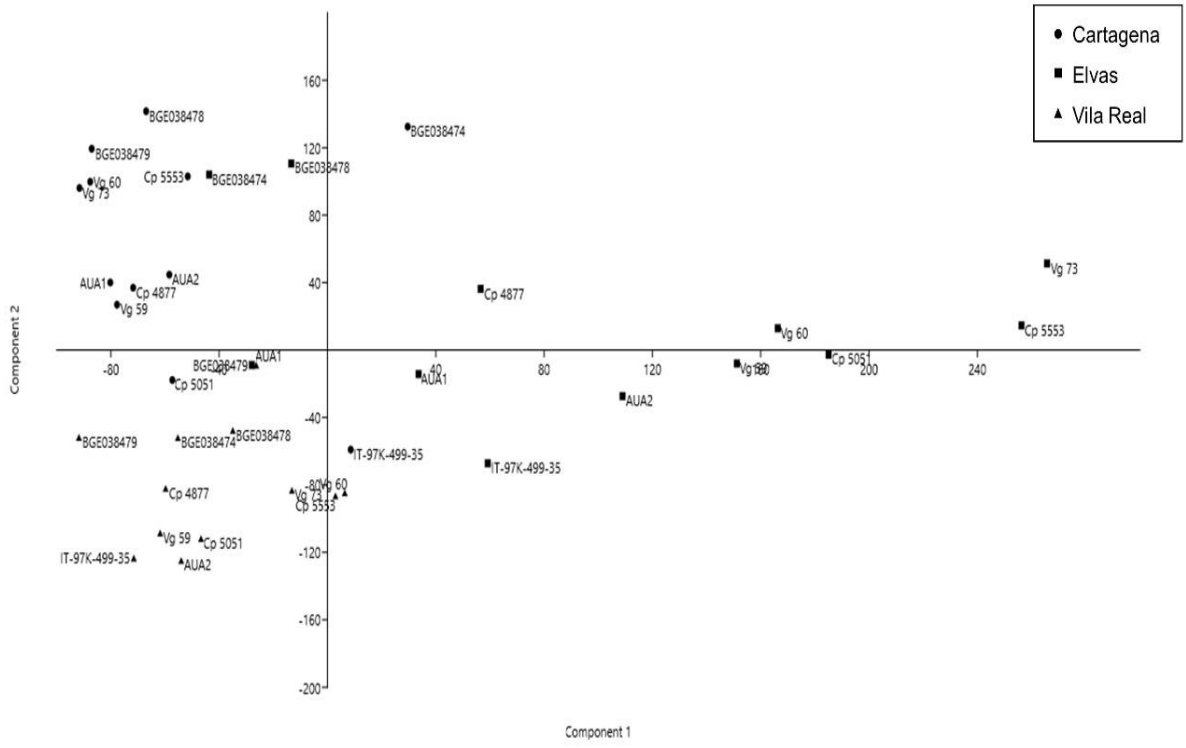

Figure 2.2.1. Principal component analysis of 12 cowpea accessions (average of 2 years) and the three environments based on 10 quantitative traits. The data are the mean of 2 years.

2.2.5. Conclusions

The results indicate the existence of significant interactions among genotypes, locations and years, providing a useful knowledge about the breeding value of the genetic resources studied. INIAV could be the best place to grow these accessions because of the highest yield obtained. However, if we are looking for precocity, Cartagena and Vila Real are the most suitable places. Cartagena was the place where the filling of the seed was the fastest, probably due to the higher temperatures and radiation. The thermal time model (EDD) could be used to predict the period of cowpea development, therefore predict flowering and pod maturity dates, an important issue in harvest logistic and marketing strategies.

Acknowledgments

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2.3. Molecular characterization

Genetic diversity and structure of Iberian Peninsula cowpeas compared to worldwide cowpea accession using high density SNP markers

Carvalho M.[*], Muñoz-Amatriaín M., Castro I., Lino-Neto T., Matos M., Egea-Cortines M., Rosa E., Close T.J., Carnide V. 2017. Genetic diversity and structure of Iberian Peninsula cowpeas as compared to world-wide cowpea accessions using high density SNP markers. *BMC Genomics*, 18:891(doi: 10.1186/s12864-017-4295-0). JIF (2017) = 3.730; SJR (2017) Genetics = Q1.

[*]Carvalho M. contribution: conducted field and lab experiment, data analysis and manuscript writing

2.3.1. Abstract

Cowpea (*Vigna unguiculata* L. Walp) is an important legume crop due to its high protein content, adaptation to heat and drought and capacity to fix nitrogen. Europe has a deficit of cowpea production. Knowledge of genetic diversity among cowpea landraces is important for the preservation of local varieties and is the basis to obtain improved varieties. The aims of this study were to explore diversity and the genetic structure of a set of Iberian Peninsula cowpea accessions in comparison to a worldwide collection and to infer possible dispersion routes of cultivated cowpea.

The Illumina Cowpea iSelect Consortium Array containing 51,128 SNPs was used to genotype 96 cowpea accessions including 43 landraces and cultivars from the Iberian Peninsula, and 53 landraces collected worldwide. Four subpopulations were identified. Most Iberian Peninsula accessions clustered together with those from other southern European and northern African countries. Only one accession belonged to another subpopulation, while two accessions were 'admixed'. A lower genetic diversity level was found in the Iberian Peninsula accessions compared to worldwide cowpeas.

The genetic analyses performed in this study brought some insights into worldwide genetic diversity and structure and possible dispersion routes of cultivated cowpea. Also, it provided an in-depth analysis of genetic diversity in Iberian Peninsula cowpeas that will help guide crossing strategies in breeding programs.

2.3.2. Introduction

Cowpea (*Vigna unguiculata* L. Walp., 2n = 2x = 22) is a member of the Fabaceae family and one of the most important grain legumes growing in tropical and subtropical regions (Tan *et al*. 2012). Grain-type cowpea, also known as common cowpea or African cowpea belongs to subspecies *unguiculata* while vegetable cowpea, commonly known as asparagus bean or 'yardlong' bean, belongs to subspecies *sesquipedalis* (Xu *et al*. 2016). These two subspecies are differentiated mainly by their plant architecture, pod size and thickness, and end use (Timko *et al*.2007; Xu *et al.* 2010), but they both possess a high protein content (Timko *et al*. 2007; Singh *et al.* 2002). Other important characteristics of cowpea are the capacity to fix atmospheric nitrogen through symbiosis with root nodule

bacteria (Ehlers and Hall 1996), the ability to grow in low fertility soils (Eloward and Hall 1987), and the high tolerance to high temperatures and drought (Hall 2004). These attributes make cowpea a key crop in the context of global climate change and food security. In Southern Europe, namely the Iberian Peninsula, rainfall is projected to decrease while temperature is projected to increase (Kröner *et al*. 2017).

Cowpea is native to Africa (Richard 1847; Steele 1976) although the center of domestication is still uncertain. In the Neolithic period, cowpea was first introduced into India, which is now considered a secondary center of genetic diversity (Pant *et al*. 1982). Some reports suggest that cowpea has been cultivated in Europe at least since the eighteenth century BC and possibly since prehistoric times (Coulibaly *et al*. 2002; Tosti and Negri 2002), while others suggest that it was introduced in Europe around 300 BC, where it still remains as a minor crop in the southern part of the continent. These two scenarios are not mutually exclusive. From Europe, more specifically from Spain, it has been speculated that cowpea was exported in the seventeenth century to the New World (Purseglove 1968; Fang *et al*. 2007; Badiane *et al*. 2014).

Assessment of the genetic diversity within a crop's germplasm is fundamental for crop improvement and selection (Tan *et al*. 2012). Moreover, the utilization of landraces is valuable as they can contain favorable alleles for many agronomic traits (Sinha and Mishra 2013). Until now, Iberian Peninsula cowpeas, including landraces, have not been genetically characterized, which is a prerequisite for their full exploitation in breeding. Recently, an iSelect BeadArray which assays 51,128 SNPs has been developed for cowpea and used to generate a consensus genetic map containing 37,372 SNPs and to assess genetic diversity within West African breeding materials (Muñoz-Amatriaín *et al*. 2017), and to better understand the genetic basis underlying pod length variation (Xu *et al*. 2016).

Europe has a deficit of grain legumes, including cowpea. Imports into Europe were about 1.7 million tonnes worth 1.3 billion € in 2015 (CBI 2017). The recently developed Cowpea iSelect Consortium Array (Muñoz-Amatriaín *et al*. 2017) provides an opportunity to use this tool to understand diversity in Iberian Peninsula cowpea germplasm and to apply this knowledge to breeding varieties producing higher and stable yields in the hotter, drier summers of Southern Europe. The main objectives of this study were to: (1) understand genetic diversity and structure in a set of Iberian Peninsula cultivated cowpea accessions in comparison to a worldwide collection of cowpea accessions; and (2) infer possible dispersion

routes of cultivated cowpea, focusing on the contribution of the Iberian Peninsula cowpea germplasm.

2.3.3. Materials and methods

2.3.3.1. Plant material

A total of 96 cowpea accessions from twenty-four countries were used in this study. They included 33 accessions from Portugal, 10 accessions from Spain (for a total of 43 accessions representing the diversity of Iberian Peninsula germplasm), and 53 accessions from genebanks at the National Institute for Agrarian and Veterinarian Research (INIAV, Portugal), the National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA, Spain), the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany), the Botanic Garden Meise (Belgium), the University of Perugia (Italy), and the Brazilian Agricultural Research Corporation (EMBRAPA, Brazil). These 53 accessions were chosen to represent worldwide cowpea diversity (Additional File 2.3.1). From these 96 accession, 86 belonged to ssp. *unguiculata*, while 10 were part of the ssp. *sesquipedalis*.

Leaves from three individual plants of each accession were collected. Total genomic DNA from each plant was extracted from 50 mg of well-developed trifoliate leaves (two-weeks-old) with the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) using the Lysis Buffer 1 (based on the CTAB method) and the standard protocol according to the manufacturer's instructions. DNA concentrations were measured using a NanoDrop 1000 (Invitrogen, California, USA). In order to verify DNA integrity, 2 µL of DNA were subjected to gel electrophoresis on 1.0% (w/v) agarose gel, stained with ethidium bromide. Equal amounts of the three DNA samples of each accession were bulked for genotyping to get a better estimation of diversity within each accession/bulk.

2.3.3.2. SNP genotyping and data curation

The 96 accessions were genotyped with the Illumina Cowpea iSelect Consortium Array containing 51,128 SNPs (Muñoz-Amatriaín *et al*. 2017) at the University of Southern California Molecular Genomics Core Facility (Los Angeles, CA, USA). SNPs included in this

iSelect array were discovered in a panel of 37 phenotypically and genetically diverse accessions of cultivated cowpea from 12 countries in Africa, China and the USA, and included four accessions of ssp. s*esquipedalis* (Muñoz-Amatriaín *et al*. 2017). SNP calling was performed in GenomeStudio v.2011.1 software (Illumina Inc., San Diego, CA, USA) using the same cluster file as in Muñoz-Amatriaín *et al*. (2017). Quality control filters were applied to both SNPs and samples: first, SNPs with missing data and/or heterozygous calls in >20% accessions were eliminated; second, accessions with >20% missing SNP calls (which may be indicative of poor DNA quality) and/or >20% heterozygous calls were removed from further analysis. The 20% heterozygosity threshold was chosen based on outcrossing rates from 1-15% reported for cultivated cowpea (Duke 1981; Pasquet 1998; Timko *et al*. 2007). In addition, SNPs were used to identify potentially identical individuals in the collection by performing pair-wise comparisons.

2.3.3.3. Population structure and genetic diversity analyses

Population structure was estimated using the Bayesian model-based approach implemented in the software STRUCTURE v2.3.4 (Pritchard *et al*. 2000) and by Principal Component Analysis (PCA) in TASSEL v.5 (Bradbury *et al*. 2007) using SNPs with a minor allele frequency (MAF) >0.05. To identify the most likely number of subpopulations, STRUCTURE was run for each hypothetical number of subpopulations (*K*) between 1 and 8 using a burn-in period of 5,000 iterations and a run length of 5,000 Monte Carlo Markov Chain (MCMC) iterations. LnP(D) and Δ*K* values (Evanno *et al*. 2005) were plotted with Structure Harvester (Earl and von Holdt 2012). After estimating the best *K*, a new run using a burn-in period of 100,000 and 100,000 MCMC was performed to assign accessions to subpopulations. Those accessions with a membership probability lower than 0.70 of belonging to one subpopulation were assigned to an 'admixed' group.

Principal Component Analysis (PCA) was conducted in TASSEL v.5 (Bradbury *et al*. 2007) on the same dataset and plotted using TIBCO Spotfire® 6.5.0.

A neighbor-joining (NJ) tree was generated based on Manhattan distances using the R package "Phyclust" (Chen 2011).

Expected heterozygosity (*He*) and polymorphism information content (PIC) (Botsein *et al*. 1980) were calculated for all *V. unguiculata* ssp. *unguiculat*a accessions and then

separately for Iberian Peninsula accessions and for the worldwide set of accessions as in Muñoz-Amatriaín *et al*. (2017).

SNP data were used to generate a similarity matrix between *V. unguiculata* ssp. *unguiculat*a accessions from Iberian Peninsula based on simple matching coefficient (number of common SNP alleles divided by the total number of SNPs).

2.3.4. Results

2.3.4.1. SNP genotyping and data curation

A high-density genotyping array containing 51,128 SNPs (Muñoz-Amatriaín *et al*. 2017) was used to genetically characterize 43 landraces and cultivars from the Iberian Peninsula and 53 landraces collected worldwide for a total of 96 cowpea accessions. After SNP calling using GenomeStudio software (Illumina Inc., San Diego, CA, USA), quality control (QC) filtering was applied to both SNPs and accessions with the goal of removing SNPs with low performance accuracy, and accessions that failed in the SNP assay and/or were highly heterozygous (see Methods). Five accessions were eliminated, one of them (Ac61) because of its high percentage of missing calls (40%) indicating poor DNA quality, and the remaining four (Ac45, Ac46, Ac65 and Ac79) because they had high levels of "heterozygosity" (because DNAs were mixed from three plants, the apparent heterozygosity may have an alternative explanation of high heterogeneity between individuals), ranging from 22% to 33% heterozygous calls. These percentages exceeded the expected genetic variability within a cowpea landrace, where outcrossing rates from < 1% to a maximum of 15% have been reported (Duke 1981; Pasquet 1998; Timko *et al*. 2007). The remaining 91 accessions had percentages of heterozygosity from 0-16%, with an average of 2.7% heterozygosity.

A total of 44,056 good-quality polymorphic SNPs and 91 samples were used for further analysis. Pairwise SNP comparisons among accessions showed that Ac39 and Ac43 were potentially duplicates (100% similar SNP calls). These two accessions are members of ssp. *sesquipedalis* that were obtained from the National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA, Spain) genebank. This identity was also apparent at the phenotypic level (e.g. samples had the same growth habit, leaf type, flower color, seed color and shape, and hilum color).

2.3.4.2. Genetic diversity and structure in the whole population

Genetic structure in the entire population of 91 accessions was evaluated using STRUCTURE v.2.3.5 (Evanno *et al*. 2005), principal component analysis (PCA) in TASSEL V.5.0 (Bradbury *et al*. 2007) and a Neighbor-Joining (NJ) tree generated with "Phyclust" (Chen 2011).

Using STRUCTURE, the estimated log probability of the data for each given population (*K*), from 1 to 8, reached a maximum at $K = 4$ (Additional files 2.3.2 and 2.3.3). In addition, Evanno's ΔK also showed the highest value at $K = 4$ (Additional files 2.3.2 and 2.3.3). These results indicated that the most likely number of subpopulations in this dataset is four. A new run was performed at $K=4$ to assign accessions to subpopulations. Accessions with membership probability lower than 0.70 of belonging to one subpopulation were assigned to an 'admixed' group (Additional file 2.3.4). Subpopulation 1 included nine accessions, all of them members of ssp. *sesquipedalis*. All other subpopulations (2, 3, and 4) consisted of ssp. *unguiculata* accessions (Fig. 2.3.1; Additional file 2.34). Subpopulation 2 (41 accessions) included accessions from southern Europe, North Africa and Cuba; subpopulation 3 (13 accessions) included accessions from countries in South and Southeast Africa, South America and Asia; and subpopulation 4 (4 accessions) was composed of only West African accessions (Fig. 2.3.1; Additional file 2.3.4). The remaining 24 accessions were 'admixed'.

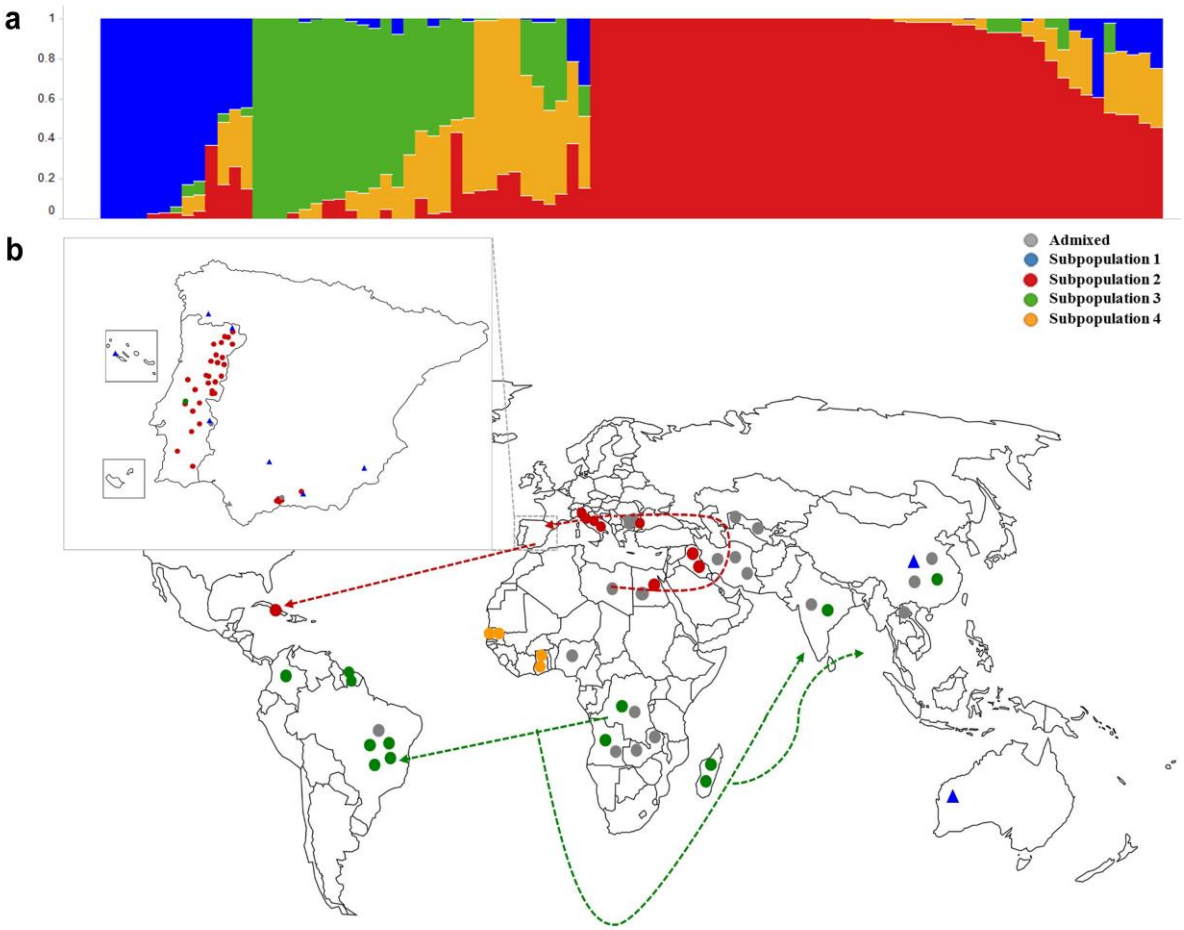

Figure 2.3.1. Population structure for 91 cowpea accessions. (a) Plot of ancestry estimates for K=4; (b) geographical distribution and population structure of accessions used in this study, and inferred cowpea dispersion routes. Exact locations are provided for Iberian Peninsula accessions. For genebank accessions, coordinates were slightly adjusted in cases where latitude and longitude were identical to allow a visualization of all samples in the study. Each color represents a subpopulation as inferred by STRUCTURE (blue = subpopulation 1; red = subpopulation 2; green = subpopulation 3; orange = subpopulation 4), with 'grey' being used for the 'admixed' group (membership coefficient<0.7). Shapes are used to distinguish the two subspecies of *Vigna unguiculata* used in this study, with circles representing ssp. *unguiculata* accessions and triangles indicating ssp. *sesquipedalis* accessions.

This four major subpopulations were also distinguished by PCA (Fig. 2.3.2, upper plots): PC1 clearly separated subpopulations 2 and 3, while PC2 separated ssp. *sesquipedalis* accessions belonging to subpopulation 1 from the ssp. *unguiculata* ones. Subpopulation 4 was separated from the rest in PC3 (Fig. 2.3.2, upper plots). The NJ tree showed accessions clustered by subpopulation membership, supporting results from both STRUCTURE and PCA (Fig. 2.3.3).

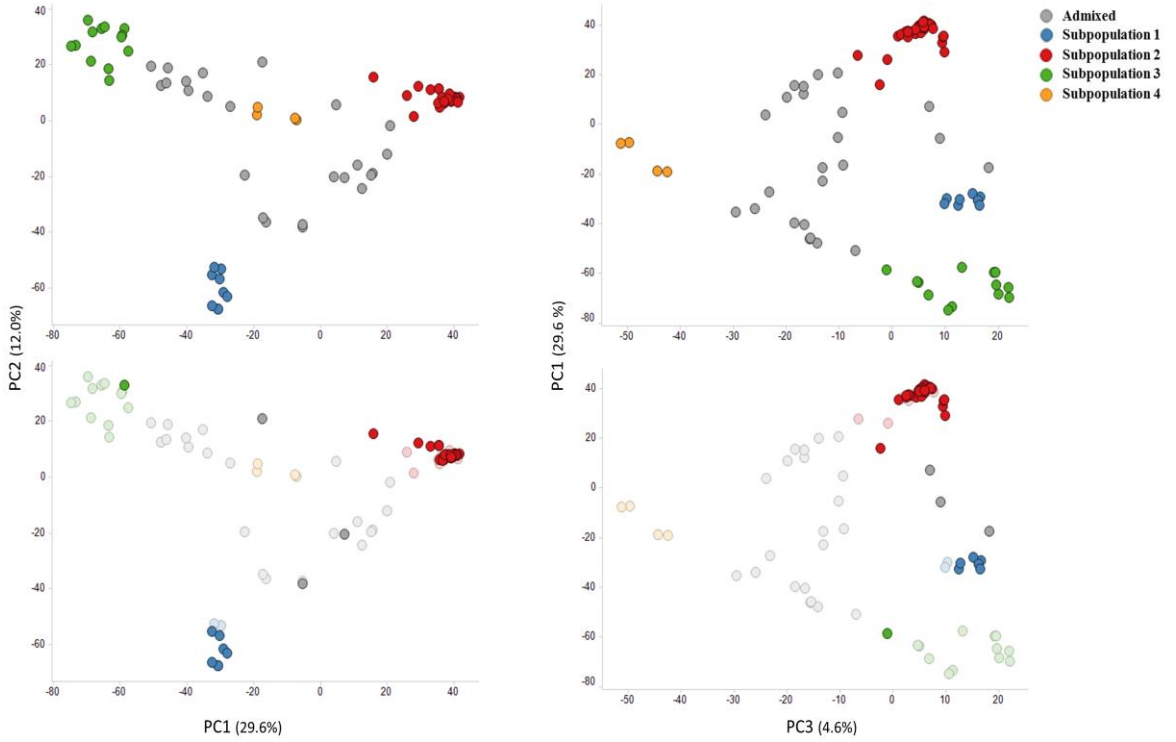

Figure 2.3.2. Principal component analysis of cowpea accessions used in this study. The accessions are colored by subpopulation membership (*K*=4). Upper plots display all accessions, while the lower plots highlight only cowpea accessions from Iberia Peninsula.

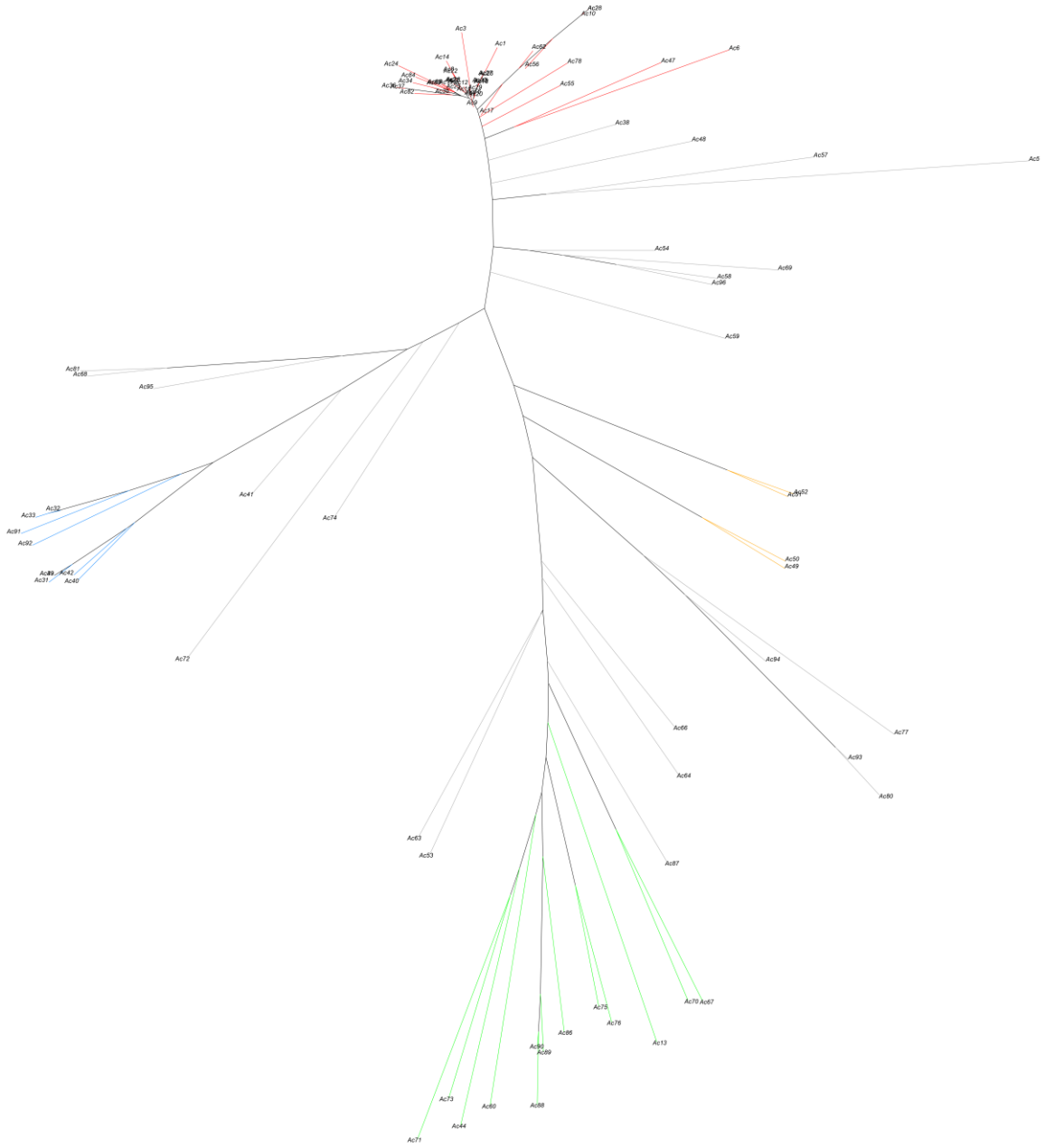

Figure 2.3.3. Neighbor-joining tree of 91 cowpea accessions with colors representing subpopulation membership (blue = subpopulation 1; red = subpopulation 2; green = subpopulation 3; orange = subpopulation 4; and grey = admixed).

PIC and *He* were calculated for the entire population and separately for each subpopulation (Table 2.3.1). Considering the whole dataset, the average PIC and *He* were 0.22 and 0.26, respectively. Average PIC values ranged from 0.07 in subpopulation 2 to 0.18 in subpopulation 3, while average *He* ranged from 0.09 to 0.23 in subpopulations 2 and 3, respectively (Table 2.3.1). This indicates that subpopulation 3 is the most diverse genetically, while subpopulation 2 appeared the least diverse, even though it contained the highest number of accessions (Table 2.3.1).

Table 2.3.1. Polymorphism information content (PIC) and expected heterozygosity (*He*) calculated for the entire population and for each subpopulation.

Data set	Nº accessions	Nº countries	PIC	*He*
All accessions	91	24	0.22	0.26
Subpopulation 1	9	4	0.12	0.14
Subpopulation 2	41	7	0.07	0.09
Subpopulation 3	12	8	0.18	0.23
Subpopulation 4	4	2	0.12	0.15

The geographical distribution of accessions together with their subpopulation membership allowed inference of possible dispersion routes (Fig. 2.3.1). The similarity between European and northern African accessions seems to indicate that cowpeas were brought by Arabs to Europe. The accession from Cuba may have been brought by Spanish navigators because Cuba was a Spanish colony and consequently commercial exchanges were frequent. The accessions from South America and Asia belonged to the same subpopulation as those from South/East Africa (Fig. 2.3.1). It is possible that these were brought from that region in Africa to Asia and South America during the discovery period, when Portuguese had an important role in commercial routes in the southern hemisphere. If so, Iberian Peninsula people may have had an important role in the distribution of cowpea from Africa and Europe to other parts of the world.

2.3.4.3. Genetic structure and diversity of Iberian Peninsula accessions from subspecies *unguiculata*

Genetic structure and diversity were explored for 35 Iberian Peninsula accessions belonging to ssp. *unguiculata* compared to 46 worldwide ssp. *unguiculata* accessions. Due to the low number of ssp. *sesquipedalis* accessions in the dataset (10 in total) and the fact that grain-type cowpea (ssp. *unguiculata*) is the most cultivated and consumed in Europe, ssp. *sesquipedalis* accessions were not included in these analyses. Most of the 35 *V. unguiculata* ssp. *unguiculata* accessions from the Iberian Peninsula belonged to subpopulation 2, together with other Genebank accessions from Europe (Fig. 2.3.2, lower plots; Additional file 2.3.4). Only two accessions from Portugal (Ac5 and Ac13) and one accession from Spain (Ac38) did not belong to this subpopulation: Ac13 belonged to subpopulation 3, while accessions Ac5 and Ac38 were considered admixed (estimated proportion of subpopulation 2 = 0.43 and 0.61, respectively). These three accessions would then likely contain unique alleles not present in

any other Iberian Peninsula accession studied. An examination of the SNP data from all 35 Iberian Peninsula accessions showed that, of all polymorphic SNPs (29,550) in the Iberian Peninsula dataset, 4,777 were contributed only by Ac13 (16.2%). These unique alleles from Ac13 were distributed all over the linkage groups (LGs; Additional file 2.3.5). As expected, Ac5 and Ac38 contained a lower number of unique alleles, 1,849 (6.3%) for Ac5 and 534 (1.8%) for Ac38. Unique alleles from Ac5 were found in all cowpea chromosomes, while those from Ac38 were mainly present on the pericentromeric region of LG3 and LG11, and towards the distal end of LG8 (Additional file 2.3.5).

PIC and *He* were calculated for the entire set of 81 *V. unguiculata* ssp. *unguiculata* accessions, and then separately for Iberian Peninsula accessions and for those from other countries (Table 2.3.2). Considering the ssp. *unguiculata* whole dataset, average PIC and *He* were 0.21 and 0.25, respectively. PIC and *He* values were quite different between accessions from the Iberian Peninsula (0.09 and 0.10, respectively) and those from the worldwide collection (0.25 and 0.31, respectively). This indicates that genetic diversity in Iberian Peninsula ssp. *unguiculata* accessions is low compared to the diversity available in the worldwide sample of cultivated cowpeas. To better understand and compare accessions from the Iberian Peninsula at the genetic level, similarity matrix was generated based on comparisons between all 35 accessions (Additional file 2.3.6). From this it was apparent that Ac5, Ac13, and Ac38 had the lowest similarity indexes with the rest of the Iberian Peninsula accessions. This was expected since they had the lowest genomic ancestry proportions of subpopulation 2, to which all other Iberian Peninsula accessions belong (Additional file 2.3.4). The other 32 accessions were very similar to each other, with percentages of similarity ranging from 77.0% to 99.9%.

Table 2.3.2. Polymorphism information content (PIC) and expected heterozygosity (*He*) calculated for *V. unguiculata* ssp. *unguiculata* accessions.

Data set	**Nº accessions**	**Nº countries**	**PIC**	***He***
All *V. unguiculata* ssp. *unguiculata* accessions	81	23	0.21	0.25
Iberian Peninsula accessions	35	2	0.09	0.10
Accessions from other countries	46	21	0.25	0.31

2.3.5. Discussion

Genetic characterization of germplasm resources is essential for conservation and the sustainable use of their diversity (Govidaraj *et al*. 2015). In recent years, several studies have characterized cowpea germplasm mainly from Africa and Asia (Coulibaly *et al*. 2002; Ba *et al*. 2004; Lee et al. 2009; Asare et al. 2010; Badiane *et al.* 2012). However, there have been no studies exploring in depth the genetic diversity of southern European cowpeas.

In this study, high-density SNP genotyping using the Cowpea iSelect Consortium Array (Muñoz-Amatriaín *et al.* 2017) has provided a means to study population structure and genetic diversity in a set of 91 worldwide cowpea accessions, with a special focus on 43 accessions from the Iberian Peninsula. A high proportion of the SNPs assayed by the array were polymorphic in the dataset (44,056 of 51,128; 86%). Also PIC and *He* values obtained from the entire population are similar to those reported by Huynh et al. (2013) and Muñoz-Amatriaín *et al.* (2017) using a larger dataset, indicating that the selection of worldwide accessions in the present work provides a good representation of the diversity in cultivated cowpea.

The SNP genotyping of these accessions enabled identification of one apparent duplication: Ac39 and Ac43, which are members of the subspecies *sesquipedalis*. These were provided by the National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA, Spain) genebank, and their passport information is limited. Ac39 and Ac43 are both from Spain, but from two different regions: Ac1 is from Cordoba (Andalucia region, south of Spain) and Ac43 from Ourense (Galicia region, north of Spain). A common cause of redundant accessions is the unwitting submission of the same accession to the genebank, then generating more than one name or designator. Identifying these redundant accessions is not possible using phenotype data alone (Muñoz-Amatriaín *et al*. 2014). Duplicated accessions do not contribute to genetic diversity of collections while generating unnecessary and additional costs to genebank (Spooner *et al*. 2005).

The population structure analysis assigned the 91 accession to four subpopulations. In agreement with the results of Huynh *et al*. (2013) and Xiong *et al*. (2016), two of the subpopulations identified (subpopulation 3 and subpopulation 4) corresponded to the East/South Africa and the West Africa gene pools, respectively. In addition to those two genetic clusters, our study identified two more subpopulations composed of North Africa and South Europe accessions (subpopulation 2) and *V. unguiculata* ssp. *sesquipedalis* accessions

(subpopulation 1). The aforementioned studies may not have identified those two populations because of a lack of accessions from these regions.

The geographic distribution of the accessions from the three ssp. *unguiculata* subpopulations enabled inference of possible dispersion routes of domesticated cowpea (Fig. 2.3.1). It has been reported that some Iberian Peninsula crops were introduced in Europe through the "Arab corridor" (Saúco and Cubero 2011). Our study is consistent with the idea that cowpea was one of the crops brought by Arabs from North Africa to Europe in ancient times. From the end of the 15th century until the middle of the 17th century, Portugal and Spain, which form the Iberian Peninsula, had an important role in the great discovery period. Saúco and Cubero (2011) described how powers from the Iberian Peninsula had an important contribution to the exchange and acclimatization of new and old world crops, including cowpea, due to exploration voyages and commercial routes established by them. This information together with the genetic data from this study seems to indicate that the accession from Cuba (Ac62) belonging to subpopulation 2 may have been brought by the Spaniards. This island was discovered in 1492 by Christopher Columbus and belonged to Spain until 1898, so it seems plausible that the Spaniards introduced this crop to Cuba. On the other hand, Portuguese sailors explored and dominated the Southern hemisphere including South America (more specifically Brazil), Southern Africa (Angola, Guinea Bissau, Mozambique) and India. They established direct contact between Europe, South America and India, and later with Southeast Asia and China (Saúco and Cubero 2011). Since subpopulation 3 includes accessions from all these regions, it is possible that slaves being transported in Portuguese ships crossing the Atlantic Ocean were the ones who introduced cowpea cultivation into Brazil. Additional cowpea introduction into India and later China may also have occurred through the Portuguese sea routes as well.

Cowpea genetic diversity among countries and regions can be affected by environmental factors and customs of cowpea consumption (Xiong *et al*. 2016). In the Iberian Peninsula, cowpea is a minor crop, mostly based on cultivation of landraces. These landraces reflect the cultural identity of local people and are reservoirs of diversity for breeding improvement. Given the narrow genetic base found in this study for most of the Iberian Peninsula cowpea, introduction of additional diversity into the Iberian Peninsula genepool seems sensible to keep increasing yields under changing climatic conditions (Govindaraj *et al*. 2015). Three of the accessions belonging to the Iberian Peninsula were more diverse than the rest: Ac13 was the most different from the others and had mostly subpopulation 3 ancestry,

while Ac5 and Ac38 had admixed ancestry from subpopulations 2 and 3, and subpopulations 1 and 2, respectively (Additional file 2.3.4). Ac5 is a variety developed by breeders at INIAV-Elvas (Portugal) and Ac38 is a landrace from Spain. Given its proportion of ancestry from subpopulation 3 (0.50), Ac5 may have resulted from crosses between accessions from the Iberian Peninsula and South/East African materials. Although Ac38 is morphologically similar to other ssp. *unguiculata* accessions, its genome has an estimated proportion of subpopulation 1 ancestry of 0.39 (Additional file 2.3.4). This accession could be the result of intentional crosses between the two cultivar-groups. The introduction of Ac13, a member of subpopulation 3, into Portugal could have occurred in the 70's. During that time, Portuguese living in Angola, Guinea and Mozambique returned to Portugal and could have brought that cowpea landrace with them. It is also possible that during the great discover period navigators brought that accession from Africa, Asia or South America (Brazil). The aforementioned accessions Ac5, Ac13 and Ac38 can be very useful for breeding programs as they can bring additional genetic diversity without compromising adaptation to the environment.

2.3.6. Conclusions

Higher cowpea production is needed in Europe to meet demand, and only Southern European countries possess climatic conditions that are favorable for growing this legume crop. Here we have genetically characterized a geographically diverse set of cowpeas that are cultivated in the Iberian Peninsula using a high-density genotyping array, and we have compared them to cowpea accessions collected worldwide. Our study identified four subpopulations in the whole dataset, with most Iberian Peninsula accessions of ssp. *unguiculata* belonging to the same subpopulation and having lower levels of genetic diversity than worldwide cowpea accessions. However, we identified one Iberian Peninsula landrace with ancestry from another subpopulation and two accessions having admixture of different subpopulations. These three accessions may be used to incorporate new genetic diversity into breeding programs without compromising adaptation. Possible dispersion routes of cultivated cowpea have been also inferred using the SNP data combined with passport information. In the future, favorable alleles for simple and complex traits could be mined from these accessions via genome-wide association studies.

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2.3.7. References

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2.3.7. Additional files

Additional file 2.3.1. Information on cowpea accessions used in this study.

Accession	Taxon	Type of material	Donor Institution	Origin Country
Ac1	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac2	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac3	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac4	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac5	*Vigna unguiculata ssp. unguiculata*	Cultivar	INIAV, Portugal	Portugal
Ac6	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac7	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac8	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac9	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac10	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac11	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac12	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac13	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac14	*Vigna unguiculata ssp. unguiculata*	Landrace	INIAV, Portugal	Portugal
Ac15	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac16	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac17	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac18	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac19	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac20	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac21	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac22	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac23	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac24	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac25	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal

Ac26	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac27	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac28	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac29	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac30	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Portugal
Ac31	*Vigna unguiculata ssp. sesquipedalis*	Landrace	UTAD, Portugal	Portugal
Ac32	*Vigna unguiculata ssp. sesquipedalis*	Landrace	UTAD, Portugal	Portugal
Ac33	*Vigna unguiculata ssp. sesquipedalis*	Landrace	CRF-INIA, Spain	Portugal
Ac34	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Spain
Ac35	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Spain
Ac36	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Spain
Ac37	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Spain
Ac38	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Spain
Ac39	*Vigna unguiculata ssp. sesquipedalis*	Landrace	CRF-INIA, Spain	Spain
Ac40	*Vigna unguiculata ssp. sesquipedalis*	Landrace	CRF-INIA, Spain	Spain
Ac41	*Vigna unguiculata ssp. sesquipedalis*	Landrace	CRF-INIA, Spain	Spain
Ac42	*Vigna unguiculata ssp. sesquipedalis*	Landrace	CRF-INIA, Spain	Spain
Ac43	*Vigna unguiculata ssp. sesquipedalis*	Landrace	CRF-INIA, Spain	Spain
Ac44	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Angola
Ac45	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Benim
Ac46	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Benim
Ac47	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Egypt
Ac48	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Egypt
Ac49	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Ghana
Ac50	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Ghana
Ac51	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Senegal
Ac52	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Senegal
Ac53	*Vigna unguiculata ssp. unguiculata*	Cultivar	IPK Gatersleben, Germany	Zambia
Ac54	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Iran

Ac55	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Iraq
Ac56	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Iraq
Ac57	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Libyan Arab Jamahiriya
Ac58	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Uzbekistan
Ac59	*Vigna unguiculata ssp. unguiculata*	Hybrid	IPK Gatersleben, Germany	Uzbekistan
Ac60	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Colombia
Ac61	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Colombia
Ac62	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Cuba
Ac63	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Angola
Ac64	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Zambia
Ac65	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Congo
Ac66	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Congo
Ac67	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Madagascar
Ac68	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	China
Ac69	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Iran
Ac70	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Madagascar
Ac71	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	China
Ac72	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Lao People's Democratic Republic
Ac73	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	India
Ac74	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	India
Ac75	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Surinam
Ac76	*Vigna unguiculata ssp. unguiculata*	Landrace	Botanic Garden Meise, Belgium	Surinam
Ac77	*Vigna unguiculata ssp. unguiculata*	Cultivar	University of California Riverside, USA	Nigeria
Ac78	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Bulgaria
Ac79	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Bulgaria
Ac80	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Bulgaria
Ac81	*Vigna unguiculata ssp. unguiculata*	Landrace	CRF-INIA, Spain	Bulgaria
Ac82	*Vigna unguiculata ssp. unguiculata*	Landrace	Università degli Studi di Perugia, Perugia, Italy	Italy
Ac83	*Vigna unguiculata ssp. unguiculata*	Landrace	Università degli Studi di Perugia, Perugia, Italy	Italy

Ac84	*Vigna unguiculata ssp. unguiculata*	Landrace	Università degli Studi di Perugia, Perugia, Italy	Italy
Ac85	*Vigna unguiculata ssp. unguiculata*	Landrace	Università degli Studi di Perugia, Perugia, Italy	Italy
Ac86	*Vigna unguiculata ssp. unguiculata*	Breeding	EMBRAPA, Brazil	Brazil
Ac87	*Vigna unguiculata ssp. unguiculata*	Cultivar	EMBRAPA, Brazil	Brazil
Ac88	*Vigna unguiculata ssp. unguiculata*	Cultivar	EMBRAPA, Brazil	Brazil
Ac89	*Vigna unguiculata ssp. unguiculata*	Cultivar	EMBRAPA, Brazil	Brazil
Ac90	*Vigna unguiculata ssp. unguiculata*	Cultivar	EMBRAPA, Brazil	Brazil
Ac91	*Vigna unguiculata ssp. sesquipedalis*	Cultivar	Botanic Garden Meise, Belgium	Australia
Ac92	*Vigna unguiculata ssp. sesquipedalis*	Cultivar	Botanic Garden Meise, Belgium	China
Ac93	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Bulgaria
Ac94	*Vigna unguiculata ssp. unguiculata*	Landrace	UTAD, Portugal	Bulgaria
Ac95	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	China
Ac96	*Vigna unguiculata ssp. unguiculata*	Landrace	IPK Gatersleben, Germany	Iran

(**INIAV** - National Institute for Agrarian and Veterinarian Research, Portugal; **UTAD** -University of Trás-os-Montes and Alto Douro, Portugal; **CRF-INIA** - National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research, Spain; **IPK** - Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany; **EMBRAPA** – Brazilian Agricultural Research Corporation, Brazil)

Additional file 2.3.2. Raw STRUCTURE output for all runs (1st table) and Δ*K* calculations for each *K* (2nd table).

1st Table

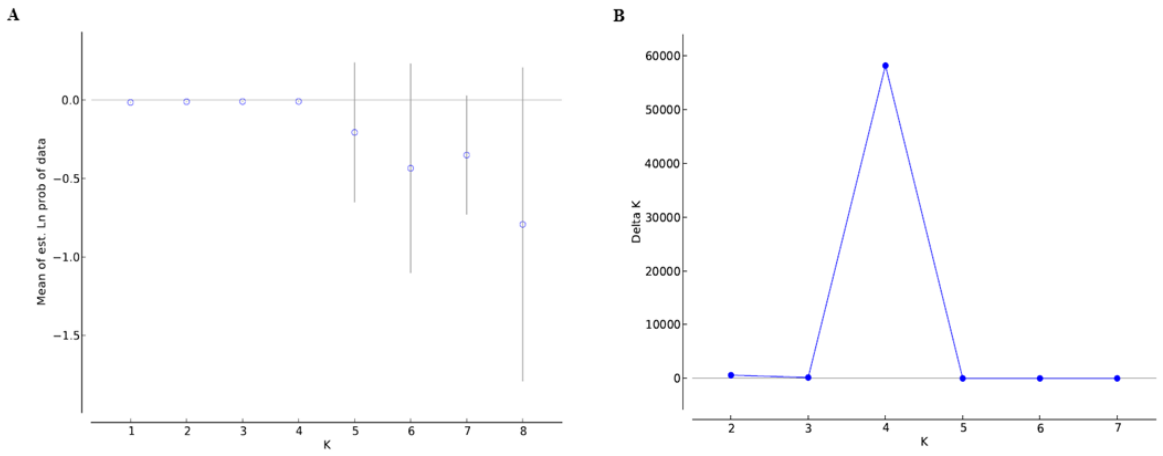
File name	Run #	K	Est. Ln prob. of data	Mean value of Ln likelihood	Variance of Ln likelihood
Results5000_5000_run_4_f	4	1	-1504429.7	-1495625	17609.4
Results5000_5000_run_3_f	3	1	-1503985.8	-1495623.5	16724.5
Results5000_5000_run_1_f	1	1	-1504546.4	-1495623.2	17846.4
Results5000_5000_run_5_f	5	1	-1504185.3	-1495621.4	17127.9
Results5000_5000_run_2_f	2	1	-1504375.6	-1495622.2	17506.9
Results5000_5000_run_8_f	8	2	-1064725.3	-1051478.1	26494.4
Results5000_5000_run_6_f	6	2	-1064596.2	-1051467	26258.4
Results5000_5000_run_10_f	10	2	-1064834.1	-1051460.6	26747
Results5000_5000_run_9_f	9	2	-1064782.6	-1051539.9	26485.3
Results5000_5000_run_7_f	7	2	-1065756.2	-1052897.2	25718
Results5000_5000_run_12_f	12	3	-902676	-883986.7	37378.5
Results5000_5000_run_11_f	11	3	-904074.1	-884943.9	38260.3
Results5000_5000_run_14_f	14	3	-902248.2	-884970.4	34555.5
Results5000_5000_run_13_f	13	3	-902636.1	-885000.5	35271.1
Results5000_5000_run_15_f	15	3	-902894	-883977.2	37833.7
Results5000_5000_run_19_f	19	4	-854261.2	-830219.5	48083.3
Results5000_5000_run_20_f	20	4	-854169.3	-830220.4	47897.7
Results5000_5000_run_18_f	18	4	-853577.2	-830022.6	47109.1
Results5000_5000_run_17_f	17	4	-854142.1	-830176.2	47931.8
Results5000_5000_run_16_f	16	4	-853571.1	-830189.4	46763.4
Results5000_5000_run_23_f	23	5	-827059.3	-795944.4	62229.7
Results5000_5000_run_25_f	25	5	-864988	-795646.7	138682.5
Results5000_5000_run_24_f	24	5	-811106.5	-787247.7	47717.6
Results5000_5000_run_21_f	21	5	-831728.7	-787282.8	88891.8
Results5000_5000_run_22_f	22	5	-99778024	-811673.4	197932701.2
Results5000_5000_run_27_f	27	6	-62394034.4	-780542.3	123226984.1
Results5000_5000_run_26_f	26	6	-827366.3	-755845.1	143042.4
Results5000_5000_run_29_f	29	6	-857882.5	-753633.2	208498.7
Results5000_5000_run_30_f	30	6	-152399658.7	-762643.8	303274029.7
Results5000_5000_run_28_f	28	6	-942860.2	-753808.1	378104.3
Results5000_5000_run_35_f	35	7	-817595.5	-749475.4	136240.3
Results5000_5000_run_31_f	31	7	-67805785.7	-756839.2	134097893.1
Results5000_5000_run_33_f	33	7	-24407188.4	-756112.8	47302151.3
Results5000_5000_run_34_f	34	7	-81360274.4	-759241.7	161202065.5
Results5000_5000_run_32_f	32	7	-902342.9	-750119.9	304446
Results5000_5000_run_40_f	40	8	-1136558.6	-752042.9	769031.5
Results5000_5000_run_36_f	36	8	-93383069.1	-752730.9	185260676.4
Results5000_5000_run_37_f	37	8	-243137937.8	-787214.2	484701447.1

Results5000_5000_run_39_f	39	8	-57611809.8	-741380.4	113740858.8
Results5000_5000_run_38_f	38	8	-924530.7	-750949.1	347163.3

2nd Table

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-1504304.56	220.807706	—	—	—
2	5	-1064938.88	465.419238	439365.68	277332.48	595.876701
3	5	-902905.68	693.370022	162033.2	113071.7	163.075553
4	5	-853944.18	340.667003	48961.5	19817598.6	58172.9326
5	5	-20622581.3	44249242	-19768637.1	3093142	0.069903
6	5	-43484360.4	66457755.3	-22861779.1	31287502.2	0.470788
7	5	-35058637.4	37645624.2	8425723.04	52605866.9	1.397397
8	5	-79238781.2	99704312	-44180143.8	—	—

Additional file 2.3.3. Exploration of the optimal number of subpopulations (*K*) in the entire dataset. Plots were generated with Structure Harvester [26]. (A) Estimated log probability of the data for each *K* between 1 and 8. (B) Δ*K* values as a function of *K*.



Additional file 2.3.4. Genetic structure information on the 91 accessions. The estimated membership of each accession in the four subpopulations is shown, as well as the PCA coordinates.

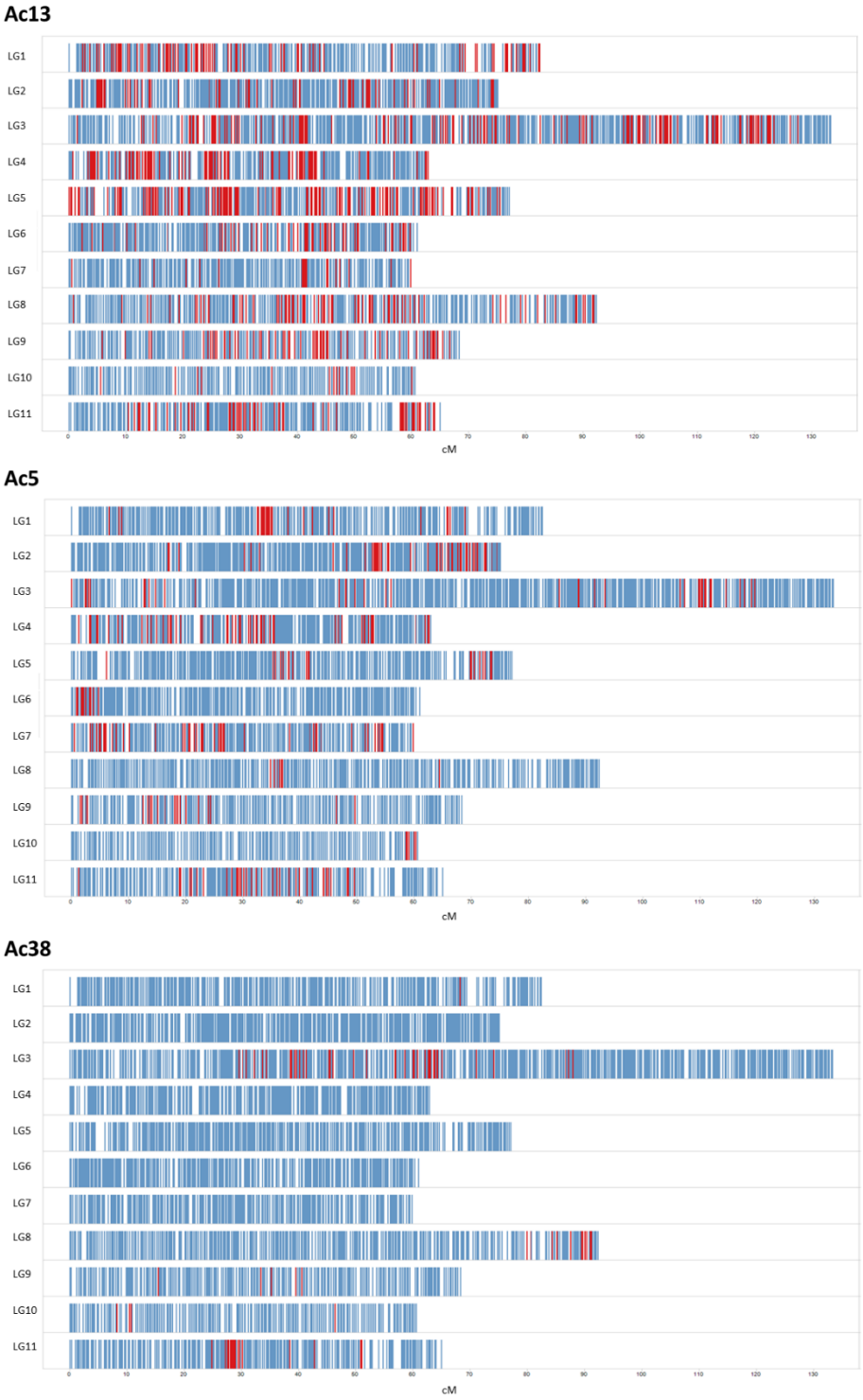
Accession number	Country of origin	Subspecies	STRUCTURE				PCA		
			Subpop. 1	Subpop. 2	Subpop. 3	Subpop. 4	PC1	PC2	PC3
Ac1	Portugal	*unguiculata*	0.000	0.997	0.000	0.003	41.594	8.339	5.917
Ac2	Portugal	*unguiculata*	0.000	0.934	0.066	0.000	35.342	11.305	9.635
Ac3	Portugal	*unguiculata*	0.001	0.985	0.000	0.014	39.609	7.629	4.953
Ac4	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	39.575	8.057	5.257
Ac5	Portugal	*unguiculata*	0.000	0.432	0.503	0.065	-17.375	21.023	18.200
Ac6	Portugal	*unguiculata*	0.000	0.707	0.153	0.140	15.916	15.636	-2.380
Ac7	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	39.031	6.953	5.863
Ac8	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	38.879	8.103	4.982
Ac9	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	39.163	7.253	5.971
Ac10	Portugal	*unguiculata*	0.000	0.931	0.069	0.000	32.922	11.150	9.406
Ac11	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	39.385	7.624	5.436
Ac12	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	39.569	7.209	5.406
Ac13	Portugal	*unguiculata*	0.000	0.048	0.777	0.176	-58.624	32.832	-1.029
Ac14	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	38.816	7.582	4.894
Ac15	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	40.270	8.449	6.165
Ac16	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	38.159	8.005	5.928
Ac17	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	29.287	12.380	9.828
Ac18	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	40.041	8.097	7.468
Ac19	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	36.492	6.520	4.417
Ac20	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	40.226	8.372	6.320
Ac21	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	39.346	7.808	5.339
Ac22	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	38.062	7.532	5.383
Ac23	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	38.631	7.899	5.125
Ac24	Portugal	*unguiculata*	0.000	0.950	0.000	0.050	35.573	6.387	1.139
Ac25	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	36.764	7.561	5.337

Ac26	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	40.829	7.892	6.433
Ac27	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	40.486	7.958	6.949
Ac28	Portugal	*unguiculata*	0.000	0.934	0.066	0.000	35.474	11.303	9.688
Ac29	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	40.212	7.904	7.153
Ac30	Portugal	*unguiculata*	0.000	1.000	0.000	0.000	40.376	8.027	5.435
Ac31	Portugal	*sesquipedalis*	1.000	0.000	0.000	0.000	-32.490	-66.203	16.404
Ac32	Portugal	*sesquipedalis*	1.000	0.000	0.000	0.000	-32.603	-55.193	12.406
Ac33	Portugal	*sesquipedalis*	0.939	0.028	0.032	0.000	-30.232	-56.735	12.728
Ac34	Spain	*unguiculata*	0.001	0.969	0.000	0.029	37.089	6.258	3.271
Ac35	Spain	*unguiculata*	0.000	1.000	0.000	0.000	36.511	5.831	2.600
Ac36	Spain	*unguiculata*	0.000	0.982	0.000	0.018	37.507	8.076	2.932
Ac37	Spain	*unguiculata*	0.000	0.986	0.000	0.014	37.250	7.970	2.518
Ac38	Spain	*unguiculata*	0.391	0.609	0.000	0.000	7.094	-20.371	7.034
Ac39	Spain	*sesquipedalis*	1.000	0.000	0.000	0.000	-30.600	-67.515	16.233
Ac40	Spain	*sesquipedalis*	0.975	0.025	0.000	0.000	-29.148	-61.382	16.604
Ac41	Spain	*sesquipedalis*	0.630	0.365	0.005	0.000	-5.425	-38.102	9.001
Ac42	Spain	*sesquipedalis*	0.969	0.031	0.000	0.000	-27.964	-63.118	15.179
Ac43	Spain	*sesquipedalis*	1.000	0.000	0.000	0.000	-30.600	-67.522	16.233
Ac44	Angola	*unguiculata*	0.016	0.000	0.937	0.047	-73.260	26.851	11.318
Ac45	Egypt	*unguiculata*	0.001	0.791	0.046	0.163	25.958	9.083	-0.918
Ac46	Egypt	*unguiculata*	0.061	0.656	0.000	0.283	20.808	-1.731	-10.259
Ac47	Ghana	*unguiculata*	0.001	0.143	0.007	0.849	-19.110	2.121	-42.522
Ac48	Ghana	*unguiculata*	0.000	0.144	0.009	0.847	-18.808	4.731	-44.348
Ac49	Senegal	*unguiculata*	0.000	0.235	0.000	0.765	-7.180	0.349	-49.629
Ac50	Senegal	*unguiculata*	0.000	0.222	0.000	0.778	-7.756	0.849	-51.228
Ac51	Zambia	*unguiculata*	0.035	0.026	0.551	0.387	-47.667	12.446	-14.158
Ac52	Iran	*unguiculata*	0.098	0.622	0.000	0.280	19.952	-12.131	-13.969
Ac53	Iraq	*unguiculata*	0.000	0.891	0.000	0.109	27.869	1.598	-6.584
Ac54	Iraq	*unguiculata*	0.000	1.000	0.000	0.000	38.686	9.459	7.705
Ac55	Lybian	*unguiculata*	0.022	0.530	0.149	0.299	4.711	5.573	-9.361

Ac56	Uzbekistan	*unguiculata*	0.161	0.524	0.000	0.314	15.548	-19.031	-18.502
Ac57	Uzbekistan	*unguiculata*	0.169	0.477	0.000	0.354	11.025	-15.944	-19.823
Ac58	Colombia	*unguiculata*	0.001	0.031	0.967	0.001	-68.305	31.787	20.051
Ac59	Cuba	*unguiculata*	0.000	1.000	0.000	0.000	41.215	6.675	5.806
Ac60	Angola	*unguiculata*	0.003	0.034	0.530	0.434	-46.154	13.479	-15.526
Ac61	Zambia	*unguiculata*	0.012	0.070	0.425	0.494	-39.584	10.735	-18.437
Ac62	Congo	*unguiculata*	0.015	0.096	0.320	0.569	-33.968	8.634	-25.935
Ac63	Madagascar	*unguiculata*	0.045	0.000	0.799	0.156	-63.571	18.557	4.956
Ac64	China	*unguiculata*	0.475	0.171	0.042	0.312	-16.314	-36.399	-9.186
Ac65	Iran	*unguiculata*	0.245	0.457	0.000	0.298	12.307	-24.415	-16.750
Ac66	Madagascar	*unguiculata*	0.077	0.000	0.763	0.159	-63.271	14.347	4.675
Ac67	China	*unguiculata*	0.003	0.000	0.922	0.076	-74.662	26.535	10.588
Ac68	Lao	*unguiculata*	0.333	0.154	0.153	0.361	-22.602	-19.452	-13.189
Ac69	India	*unguiculata*	0.031	0.000	0.840	0.129	-68.689	21.137	6.884
Ac70	India	*unguiculata*	0.211	0.375	0.000	0.414	3.961	-20.162	-23.921
Ac71	Surinam	*unguiculata*	0.011	0.041	0.853	0.096	-57.633	24.836	13.137
Ac72	Surinam	*unguiculata*	0.000	0.088	0.907	0.004	-59.353	30.467	19.254
Ac73	Nigeria	*unguiculata*	0.001	0.124	0.410	0.466	-35.118	17.107	-29.565
Ac74	Bulgaria	*unguiculata*	0.011	0.915	0.000	0.074	35.781	4.781	2.791
Ac75	Bulgaria	*unguiculata*	0.002	0.102	0.557	0.340	-45.622	18.839	-15.381
Ac76	Bulgaria	*unguiculata*	0.444	0.149	0.042	0.364	-17.230	-34.875	-13.075
Ac77	Italy	*unguiculata*	0.000	0.971	0.000	0.029	36.254	8.398	1.715
Ac78	Italy	*unguiculata*	0.000	0.986	0.000	0.014	35.305	6.232	2.956
Ac79	Italy	*unguiculata*	0.001	0.988	0.001	0.011	38.188	7.809	4.336
Ac80	Italy	*unguiculata*	0.000	0.996	0.000	0.004	37.128	6.990	2.901
Ac81	Brazil	*unguiculata*	0.000	0.096	0.902	0.001	-59.642	30.097	19.437
Ac82	Brazil	*unguiculata*	0.000	0.000	0.679	0.321	-50.735	19.530	-7.002
Ac83	Brazil	*unguiculata*	0.000	0.000	1.000	0.000	-69.675	35.952	22.080
Ac84	Brazil	*unguiculata*	0.000	0.000	1.000	0.000	-65.601	33.094	21.914
Ac85	Brazil	*unguiculata*	0.000	0.000	1.000	0.000	-64.620	33.459	19.590

Ac86	Australia	*sesquipedalis*	0.811	0.038	0.069	0.082	-29.716	-53.031	10.307
Ac87	China	*sesquipedalis*	0.826	0.015	0.069	0.098	-31.804	-52.573	9.897
Ac88	Bulgaria	*unguiculata*	0.014	0.129	0.484	0.374	-40.164	14.014	-16.645
Ac89	Bulgaria	*unguiculata*	0.002	0.116	0.280	0.602	-27.102	5.180	-23.155
Ac90	China	*unguiculata*	0.453	0.263	0.000	0.284	-5.345	-37.270	-10.314
Ac91	Iran	*unguiculata*	0.179	0.523	0.000	0.299	15.144	-19.502	-16.679

Additional file 5 - Genomic location of unique alleles in Ac13, Ac5 and Ac38 on cowpea linkage groups (LGs). Genomic regions colored in red contain unique alleles in the corresponding accession, while regions containing non-unique alleles are represented in blue. For the figure, one marker per locus was kept, giving priority to unique alleles over non-unique ones. In white are represented regions lacking mapped SNPs. LG number and cM positions are based on the cowpea consensus genetic map available from Muñoz-Amatriaín et al. (2017).



Additional file 6 - Matrix showing genetic pair-wise similarity values for Iberian Peninsula accessions.

	Ac1	Ac2	Ac3	Ac4	Ac5	Ac6	Ac7	Ac8	Ac9	Ac10	Ac11	Ac12	Ac13	Ac14	Ac15	Ac16	Ac17	Ac18	Ac19	Ac20	Ac21	Ac22	Ac23	Ac24	Ac25	Ac26	Ac27	Ac28	Ac29	Ac30	Ac34	Ac35	Ac36	Ac37	Ac38
Ac1																																			
Ac2	0.9279																																		
Ac3	0.9622	0.9183																																	
Ac4	0.9298	0.8858	0.9265																																
Ac5	0.7539	0.7789	0.7495	0.7215																															
Ac6	0.8605	0.8332	0.8696	0.8349	0.7093																														
Ac7	0.9168	0.8897	0.9222	0.9306	0.7315	0.8421																													
Ac8	0.9123	0.8864	0.919	0.9218	0.7316	0.8419	0.9443																												
Ac9	0.933	0.8931	0.9272	0.9498	0.7317	0.8416	0.9396	0.9298																											
Ac10	0.911	0.9756	0.9018	0.8741	0.759	0.8178	0.8771	0.8742	0.8827																										
Ac11	0.9223	0.8924	0.9278	0.9367	0.7233	0.8441	0.9364	0.9383	0.9425	0.882																									
Ac12	0.9253	0.8922	0.9266	0.9355	0.7264	0.8429	0.9333	0.9336	0.9506	0.8829	0.9519																								
Ac13	0.6269	0.6288	0.6342	0.6047	0.6842	0.6708	0.6055	0.6105	0.6033	0.6173	0.6078	0.6036																							
Ac14	0.934	0.9049	0.937	0.9206	0.7415	0.8614	0.9341	0.9454	0.9294	0.8907	0.9386	0.928	0.6273																						
Ac15	0.9553	0.9093	0.943	0.9453	0.7402	0.8509	0.9237	0.915	0.9478	0.8979	0.9315	0.9344	0.6168	0.9281																					
Ac16	0.932	0.8905	0.9219	0.9463	0.735	0.8475	0.9338	0.9248	0.9497	0.8804	0.9341	0.9352	0.6141	0.9248	0.9489																				
Ac17	0.8442	0.8471	0.8342	0.8467	0.6884	0.7697	0.8385	0.831	0.859	0.8401	0.8466	0.8456	0.5641	0.8392	0.8554	0.8513																			
Ac18	0.9462	0.8984	0.9279	0.9272	0.7369	0.8429	0.9208	0.9209	0.9471	0.888	0.9287	0.9364	0.6062	0.9201	0.9496	0.9455	0.8451																		
Ac19	0.9098	0.8816	0.916	0.9134	0.7237	0.8435	0.9325	0.9352	0.9366	0.8704	0.934	0.9389	0.6059	0.9313	0.9185	0.9221	0.8387	0.9188																	
Ac20	0.943	0.9007	0.9327	0.9467	0.7373	0.8428	0.9383	0.9313	0.9614	0.8899	0.9441	0.9436	0.6115	0.9354	0.9553	0.9525	0.8581	0.9506	0.932																
Ac21	0.9158	0.8907	0.9205	0.9291	0.7352	0.8446	0.952	0.9578	0.9415	0.8766	0.9435	0.9379	0.6089	0.943	0.925	0.9292	0.8405	0.9205	0.9365	0.9404															
Ac22	0.9238	0.8932	0.9257	0.9234	0.7392	0.8494	0.9459	0.9482	0.9378	0.8802	0.9375	0.9388	0.6173	0.9561	0.9262	0.93	0.8377	0.9235	0.9442	0.9388	0.945													
Ac23	0.914	0.8873	0.9159	0.9269	0.7283	0.8438	0.9474	0.9549	0.9384	0.8749	0.9437	0.9382	0.6076	0.9487	0.9199	0.9262	0.8403	0.9193	0.9336	0.9365	0.9619	0.9448													
Ac24	0.915	0.8823	0.9181	0.9021	0.728	0.8396	0.9091	0.9133	0.9168	0.8686	0.913	0.9132	0.6157	0.9092	0.9123	0.9051	0.8232	0.9113	0.9112	0.9148	0.9131	0.9174	0.9096												
Ac25	0.9042	0.8772	0.9105	0.9241	0.7234	0.8409	0.9454	0.9512	0.9366	0.8662	0.9385	0.9324	0.6064	0.9391	0.9172	0.9282	0.8372	0.9223	0.9295	0.9321	0.9543	0.9468	0.9546	0.9091											
Ac26	0.9593	0.9039	0.9362	0.9394	0.7381	0.8438	0.9263	0.9218	0.953	0.8906	0.9284	0.936	0.6114	0.9254	0.9519	0.9461	0.8495	0.9517	0.9234	0.957	0.9262	0.9283	0.9274	0.9166	0.9172									
Ac27	0.9687	0.9186	0.9473	0.9365	0.7498	0.8549	0.9297	0.9299	0.9536	0.9044	0.9331	0.9415	0.6207	0.9318	0.9668	0.9488	0.852	0.9647	0.9278	0.9627	0.9359	0.9333	0.9301	0.9286	0.9238	0.9707								
Ac28	0.9285	0.9993	0.9189	0.8864	0.7794	0.8337	0.8903	0.887	0.8938	0.9763	0.8929	0.8923	0.6291	0.9055	0.91	0.8911	0.8479	0.8991	0.8823	0.9015	0.8914	0.8937	0.888	0.8828	0.8778	0.9045	0.9193							
Ac29	0.9406	0.8988	0.9286	0.9469	0.7405	0.8401	0.9295	0.9223	0.958	0.8894	0.9371	0.9408	0.6094	0.9282	0.9568	0.9502	0.8557	0.9475	0.9272	0.9636	0.929	0.9355	0.9275	0.9117	0.9234	0.9556	0.9635	0.8993						
Ac30	0.9496	0.9037	0.9332	0.9354	0.7355	0.8447	0.922	0.9139	0.9452	0.89	0.93	0.9323	0.615	0.9281	0.9589	0.945	0.855	0.9394	0.9218	0.9545	0.9198	0.9313	0.9186	0.9101	0.9127	0.9544	0.959	0.9044	0.9557					
Ac34	0.9261	0.8968	0.9318	0.9177	0.7344	0.8466	0.9279	0.9236	0.9256	0.8837	0.9249	0.9206	0.6179	0.9231	0.9199	0.9228	0.8294	0.9213	0.9208	0.9292	0.9292	0.9283	0.929	0.9187	0.9204	0.9317	0.9382	0.8975	0.9291	0.9225				
Ac35	0.9033	0.8793	0.9076	0.9139	0.7174	0.833	0.929	0.924	0.9203	0.869	0.9211	0.9194	0.6003	0.9132	0.9096	0.9167	0.83	0.9083	0.9222	0.9206	0.926	0.9225	0.9258	0.9156	0.9224	0.9126	0.9182	0.8801	0.9172	0.9117	0.9445			
Ac36	0.9246	0.8936	0.9256	0.9099	0.7394	0.8499	0.9188	0.9259	0.9233	0.8765	0.9147	0.9155	0.6269	0.9216	0.9162	0.9157	0.8231	0.9137	0.9214	0.9247	0.9256	0.9311	0.9204	0.9153	0.9156	0.9244	0.9343	0.8942	0.9216	0.9167	0.9258	0.9242		
Ac37	0.9253	0.8955	0.9279	0.9188	0.7381	0.8524	0.9163	0.9279	0.9261	0.8793	0.9192	0.9199	0.6258	0.9228	0.9206	0.9193	0.8257	0.9189	0.9204	0.929	0.923	0.9273	0.9202	0.9187	0.9146	0.9254	0.9369	0.8962	0.9281	0.9216	0.9241	0.9275	0.9727	
Ac38	0.7674	0.7474	0.7702	0.7711	0.6365	0.7195	0.7747	0.7805	0.7802	0.7463	0.7808	0.7753	0.5598	0.7743	0.7725	0.7747	0.7077	0.7628	0.7852	0.7821	0.7837	0.7834	0.7753	0.7704	0.7784	0.7714	0.7779	0.7479	0.7809	0.7749	0.7745	0.7871	0.7959	0.8028

CHAPTER 3

Cowpea drought stress responses

Climate change predictions point to an increase of extreme events in the next years, such as long drought periods and increased precipitation in others (Thornton *et al*. 2014). As water stress (drought) is one the most severe environmental/abiotic stresses, global climate change will affect the plant development and crop yields (Vadez *et al*. 2012; Fang and Xiong 2015). The search and identification of crops and varieties with a higher tolerance to drought is extremely important and urgent for obtaining higher and steadier yields (Watanabe *et al*. 1997). Legumes farming, and particularly cowpea, can be a crucial strategy to make the agriculture more sustainable and mitigate the effects of climate alterations (Jensen *et al*. 2012). Cowpea is considered as one of the most tolerant legume crops to drought (Agbicodo *et al*. 2009), also displaying the ability of fixing atmospheric nitrogen (as other legumes) thus allowing a reduction on greenhouse gases (GHG) emission produced by livestock (Stagnari *et al*. 2017).

Drought is a complex trait and plant responses occur at different levels (Agbicodo *et al*. 2009; Fang and Xiong 2015). Individual responses include alterations on plant physiology, biochemical metabolism (metabolic pathways) and gene expression. Several plant outcomes could result from these responses, such as drought avoidance, drought escape and drought tolerance (Mitra 2001). One of major mechanisms used to cope drought stress by plants is the drought tolerance. This mechanism is defined by the ability of plants to sustain a certain level of physiological activities under severe drought stress conditions through the regulation of thousands of genes and series of metabolic pathways to reduce or repair the resulting stress damage (Mitra 2001; Fang and Xiong 2015). Understanding natural drought tolerance mechanisms is a key step for the selection of plants with improved tolerance (Ashraf 2010). To obtain drought tolerant varieties through plant breeding programs, the identification of methods to evaluate tolerance levels in germplasm is fundamental. These methods will be used for crossing and/or selecting segregated populations (Watanabe *et al*. 1997). In the last years, some studies had been developed to evaluate the drought tolerance *V. unguiculata* varieties using methods based on morphological, physiological, biochemical and molecular aspects (Matsui and Singh 2003; Hayatu and Mukhtar 2010; Hayatu *et al*. 2014; Kutama *et al*. 2014). Until now, the most suitable and informative methodology for a good evaluation of drought tolerance is not clear. In this work, to improve the knowledge and understanding of cowpea drought tolerance, different methods (physiological, biochemical and molecular) were evaluated using four cowpea genotypes (two Portuguese and two international references) and

three drought stress conditions (moderate and severe stress and control). The results presented in **sub-chapter 3.1** suggest that stomatal conductance and biochemical markers (free proline and anthocyanins contents) are the most suitable parameters for screening cowpea genotypes.

The capacity of germination under drought stress conditions is also an interesting feature to be screened for obtaining more sustainable varieties, mainly because germination is a key step for crop propagation (Ravelombola *et al.* 2017). Water deficit can also strongly affects the seedling emergence and plant establishment (Kaya *et al.* 2006; Yan 2015). The screen of tolerant genotypes during germination is an easily applied, low-cost and effective approach (Ashraf and Foolad 2007). Furthermore, this methodology is particularly important when the screen of a large amount of genotypes is necessary in a short period. Using the previous knowledge about methodologies to evaluate drought tolerance (free proline content; sub-chapter 3.1), the screening of 58 worldwide cowpea genotypes (some of them also used in chapter 2) was performed at a seedling stage, using polyethylene glycol 6000 (PEG-6000). In **sub-chapter 3.2**, seven cowpea genotypes are suggested to be the most drought tolerant. These results can be useful in future breeding programs to mitigate climate change.

The results presented in this chapter allowed a better understanding of cowpea drought stress responses, suggesting the most effective methodologies for discriminating cowpea genotypes drought tolerance. Beyond their further use on future breeding programs, the selected methods were used for surveying a worldwide cowpea collection, resulting in the suggestion of the five most tolerant cowpea genotypes.

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3.1. Evaluation of drought stress responses

Evaluating stress responses in cowpea under drought stress

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*Carvalho M. contribution: conducted greenhouse and lab experiments, data analysis and manuscript writing

3.1.1. Abstract

Drought impact on plants is an increasing concern under the climate change scenario. Cowpea (*Vigna unguiculata* L. Walp.) is considered as one of the most tolerant legume crops to drought, being the search for the best well-adapted genotypes crucial to face the future challenges. Different approaches have been used for differentiating plant responses to drought stress. Plants of four cowpea genotypes were submitted to three watering regimens (a severe and moderate drought stress, and well-watered control) during 15 days, and several physiological, biochemical and molecular parameters were evaluated. Stressed plants revealed commonly-described drought stress characteristics, but not all assayed parameters were useful for discriminating plants with different drought severities or genotypes. The analyses which have contributed most to genotype discrimination were those related with stomatal function, and biochemical markers such as proline and anthocyanin contents. Antioxidant enzymes activities and related genes expression did not differed among genotypes or upon drought stress treatments, suggesting that scavenging enzymes are not involved in the differential ability of cowpea plants to survive under drought stress. This information will be useful to evaluate and use genetic resources, as well as design strategies for breeding cowpea resistance to drought stress.

3.1.2. Introduction

Cowpea (*Vigna unguiculata* L. Walp.) belongs to the Leguminosae (or Fabaceae) family, which stand for their capacity to fix atmospheric nitrogen through the symbiotic relationship with soil bacteria. Cowpea, native from Africa, has been widely cultivated in tropical and subtropical regions (Timko *et al*. 2007). This legume crop is particularly featured by the high protein content, reasonable adaptation to low fertility soils and as well to high temperatures and drought (Timko *et al*. 2007; Agbicodo *et al*. 2009). Altogether, these features make cowpea a key crop in the context of global climate change and food security. A general temperature increase and rainfall decrease is projected for Europe, where the Mediterranean countries are expected to suffer the major climate change effects (Kröner *et al*. 2017). Water deficit is one of the most serious challenge under climate change and one of the most important abiotic stresses that negatively affects crop plants production (Cruz de

Carvalho 2008; Agbicodo *et al*. 2009). Cowpea has been referred as one of the most tolerant legume crops to drought (Agbicodo *et al*. 2009; Merwad *et al*. 2018).

Responses to drought are complex and different mechanisms have been developed by plants to adapt and survive during drought periods (Cruz de Carvalho 2008; Merwad *et al*. 2018; Carvalho *et al*. 2017). Drought stressed plants reveal several morphological, physiological, biochemical, and molecular changes that adversely affect their development, growth and productivity (Hayatu *et al*. 2014; Toscano *et al*. 2016). One of the first physiological responses is a decrease in chlorophyll content, photosynthesis rate and transpiration (Mafakheri *et al*. 2010; Singh and Reddy 2011; Kutama *et al*. 2014). Drought responses also include increased peroxidation of lipid membranes and accumulation of reactive oxygen species (ROS). Plants developed strategies to balance ROS production, many of which involve antioxidant enzyme activities. ROS scavenging enzymes, such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione peroxidase, comprise a complex enzymatic system that minimizes the effects of oxidative stress (Toscano *et al*. 2016). Higher antioxidant enzymes activity can contribute for a drought tolerance by increasing the protection capacity against oxidative damage. To our knowledge, few studies on the response of these enzymes on cowpea under drought conditions have been performed. Another plant protection tool against mild drought stress includes the accumulation of osmolytes, being proline one of the most common in drought stressed plants (Mafakheri *et al*. 2010). Proline accumulation is commonly considered as part of the stress signalling and influences adaptive plant responses. For the identification of genes, pathways and processes controlling cowpea responses to drought stress, several genomic and genetic approaches have been developed (Chaves *et al*. 2003). Several cowpea drought-related genes have been identified, some of which involved in antioxidant metabolism (reviewed by Carvalho *et al*. 2017).

The understanding of physiological, biochemical or genetic mechanisms underlying cowpea drought tolerance has been pursued by several authors, but to date there's no comprehensive and integrated studies considering the three of cowpea drought tolerance. Our work intends to present the foreknowledge about cowpea drought responses, aiming to answer the following questions: i) how is cowpea physiology affected by drought conditions and which are the best physiological parameters to discriminate genotypes under drought stress in cowpea? ii) what are the biochemical and molecular signatures of cowpea drought responses and how they can be used for cowpea genotypes fingerprint? iii) what are the main antioxidant enzymes involved in cowpea drought stress responses? iv) which are the best parameters for differentiating cowpea drought susceptibility/tolerance of different genotypes?

Thus, two Portuguese cowpea genotypes and two drought-susceptible controls (previously described by Hamidou et al. 2007 and Muchero et al. 2008), were used and submitted to three different water regimes. The physiological and biochemical responses of cowpea plants were monitored, as well as the gene expression profiling of drought stress-related genes, for comparing drought stress responses.

3.1.3. Materials and Methods

3.1.3.1. Plant material and experimental design

Two Portuguese genotypes, a commercial variety (Cp5051) and a Northern landrace (Vg50), as well as two control genotypes with described drought responses (Bambey 21 and CB46), were selected for this work. Bambey 21 from Senegal is highly susceptible to drought, whereas California Blackeye 46 (CB46) from University of California, Davis (USA) is moderately susceptible (Hamidou *et al*. 2007; Muchero *et al*. 2008).

Plant growth (including drought imposition) was performed in a glasshouse at the University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal (41º 17' N, 07º 44' W, 465 m), from June to July 2016. During this period, the average temperature recorded was 28.3 ºC ranging from 18.1 ºC to 42.0 ºC (Additional file 3.1.1). Plants were grown under natural photoperiod. Pots of 12-L were filled with identical volumes of a mixture of soil/sand/peat (2:1:1, v/v/v). All pots were watered up to field capacity (FC) one day before sowing, allowing the draining of water excess. Four seeds selected by size and form were sown in each pot. After two weeks, two well-developed seedlings of similar size were kept in each pot, and the other two were removed carefully by hand. Each genotype was sown in six pots (replicates). Pots were regularly watered to keep the soil at 75% of FC during the first 30 days after sowing. Following this period, plants were submitted to three watering treatments for 15 days: i) control maintaining the pot mixture at a minimum of 75% of FC; ii) moderate water stress with the pot mixture kept at 25% of FC; and iii) severe water stress by withholding irrigation considered 0% FC. Pots were completely randomized between the four genotypes and three watering regimens (75%, 25% and 0% of FC). In total, 144 plants (4 genotypes x 3 watering regimens x 6 pots x 2 plants) were used. For physiological evaluation, measurements were done during the morning (09:00-11:00) at four distinct periods (1, 10 and 15 days after imposing water stress), in leaves of one plant of each pot. To perform the

biochemical analysis, young full expanded leaves were harvested following the 15 days of water stress treatments and immediately frozen in liquid nitrogen. For molecular analysis (RNA extraction), young leaves were also harvested following the 15 days of water stress treatments and immediately frozen in liquid nitrogen. Leaf samples were individually ground to a fine powder using liquid nitrogen and 60 mg aliquots were maintained at -80 ºC up to their use.

3.1.3.2. Measurement of gas exchange and chlorophyll *a* fluorescence

Gas exchange parameters were determined in fully expanded leaves with an infrared portable gas exchange analyzer (*LC pro+*, ADC, Hoddesdon, UK). Stomatal conductance (g_s), net CO_2 assimilation rate (A) and intrinsic water use efficiency (A/g_s) were estimated according to the equations described by von Caemmerer and Farquhar (1981). Chlorophyll *a* fluorescence features were obtained *in situ*, in the same period of gas exchange measurements, using a pulse-amplitude-modulated fluorimeter (FM2, Hansatech Instruments, Norkfolk, UK). Maximum quantum efficiency of photosystem II was calculated as $F_v/F_m = (F_m - F_0)/F_m$. The fluorescence signal from 30 min dark-adapted leaves was measured when all reaction centers were open, by using a low intensity pulsed measuring light source (F_0). During a pulse saturating light [0.7 s pulse of 15,000 μmol photons $m^{-2}s^{-1}$ of white light], when all reactions centers were closed, a second fluorescence signal was measured (F_m). Following F_v/F_m estimation, after a 20 s exposure to actinic light [1,500 μmol photons $m^{-2}s^{-1}$], light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light [15,000 μmol photons $m^{-2}s^{-1}$] for 0.7 s to establish F_m'. The sample was then shaded for 5 s with a far-red light source to determine F_0'. From these measurements, different fluorescence attributes were calculated, according to Bilger and Schreiber (1986) and Genty *et al.* (1989): the photochemical quenching [$qP = (F_m' - F_s)/(F_m' - F_0')$] and the efficiency of electron transport, as a measure of the quantum effective efficiency of PSII [$\Phi_{PSII} = \Delta F/F_m' = (F_m' - F_s)/F_m'$]. The photosynthetic electron transport rate was estimated as $ETR = (\Delta F/F_m') \times PPFD \times 0.5 \times 0.84$ (Marinari *et al*. 2007), where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 is the factor that assumes equal distribution of energy between both photosystems, and 0.84 the used leaf absorbance as the most common value for C_3 plants (Bilger and Schreiber 1986).

3.1.3.3. Determination of biochemical markers

Free proline content was measured according to Bates (1973), with some modifications. Tissue (60 mg) was homogenized in 1.5 mL of 3 % (w/v) sulphosalicylic acid and centrifuged at 12,000 *g* for 15 min. Equal volumes of acid-ninhydrin and glacial acetic acid (0.4 mL) were mixed with 0.1 mL of supernatant and the resulting mixture was heated on a boiling water bath for 1 h. Toluene (0.8 mL) was added to the mixture and the toluene phase absorbance was read at 520 nm. Free proline content was estimated by referring to a standard curve of L-proline and expressed as µg proline/mg of protein.

Lipid peroxidation was determined through the quantification of malondialdehyde (MDA) content by thiobarbituric acid method, as described by Loreto and Velikova (2001), with some modifications. Tissue (60 mg) was homogenized in 0.1 % (w/v) of trichloroacetic acid (TCA) and centrifuged at 12,000 *g* for 15 min. The supernatant (0.25 mL) was mixed with 1 mL of 20 % TCA containing 0.5 % thiobarbituric acid (TBA) and was incubated to 95 ºC, in a water bath, for 30 min. The reaction was stopped by an ice bath and samples were centrifuged at 10,000 *g* for 5 min. The absorbance of supernatant was read at 532 nm and 600 nm. The concentration of MDA was calculated by subtracting the A_{532} to A_{260} and using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The hydrogen peroxide (H_2O_2) content was determined using the plant extracts prepared for lipid peroxidation determination and the method described by Loreto and Velikova (2001), with some volume modifications. A supernatant aliquot (0.5 mL) was added to 0.5 mL of 10 mM potassium phosphate buffer (KH_2PO_4, pH 7.0) and 1 mL of 1 M of potassium iodide (KI). The absorbance was measured at 390 nm and the H_2O_2 content was extrapolated through a standard calibration curve, previously made using solutions with known H_2O_2 concentrations.

The relative anthocyanin content was determined according to Kant *et al*. (2006). Leaf tissue (60 mg) was homogenized in 1 mL of methanol (acidified with 1% HCl) and incubated overnight. After adding 0.7 mL of distilled water and 1.75 mL of chloroform, the extract was centrifuged at 4,000 *g* for 2 min. The relative anthocyanins amount was calculated by subtracting the absorbance readings at A_{657} to A_{530} of the aqueous phase.

Chlorophylls (*a* + *b*) were quantified according to Arnon (1949). Leaf tissue (60 mg) was homogenized in 10 mL of aqueous acetone (80%, v/v), incubated overnight at 4ºC in the dark, and chlorophyll extracts were used for absorbance readings (A_{663} to A_{645}).

The protein content was determined using the Bradford's method (Bradford 1976), using BSA as standard. Protein concentration values were used to normalize all biochemical results. For each biochemical quantification, six leaf samples of each genotype and condition were used, and were independently repeated three times (n = 18). Spectrophotometric measures were done in an *Evolution 201 series UV-Visible Spectrophotometer* (ThermoScientific, Waltham, USA).

3.1.3.4. Measurement of antioxidant enzyme activities

Superoxide dismutase (SOD) and guaiacol peroxidase (POX) activities were determined according to Cavalcanti *et al.* (2004), with some modifications. Leaves (60 mg) was homogenized in 1 mL of 100 mM of potassium phosphate buffer (KH_2PO_4, pH 6.8) containing 0.1 mM EDTA and centrifuged at 12,000 *g* for 15 min at 4ºC. An enzymatic extract aliquot (20 µL) was added to 150 µL of 50 mM of potassium phosphate buffer (KH_2PO_4, pH 7.8) containing 13 mM L-methionine and 100 µM EDTA. SOD activity was determined by adding 15 µL of 75 µM NBT and 15 µL of 2 µM riboflavin. The reaction was incubated under a 30W fluorescent lamp at RT, during 5 min, after which the absorbance was measured at 560 nm. One SOD unit was the amount of enzyme required to inhibit 50% the NBT photoreduction, in comparison with blank (tubes without plant extract). POX activity was determined by adding the same enzymatic extract (10 µL) to 140 µL of 50 mM of potassium phosphate buffer (KH_2PO_4, pH 7.8) containing 20 mM guaiacol and 20 mM H_2O_2. The reaction was incubated for 30 min at 30ºC, being stopped by adding 50 µL of 5% (v/v) H_2SO_4. The absorbance was measured at 480 nm. One POX unit was defined as the change of 1.0 absorbance unit per ml enzymatic extract. Catalase (CAT) activity was measured using the same enzymatic extract and the protocol proposed by Aebi (1983). A mix with 120 µL of potassium phosphate buffer (KH_2PO_4, 50 mM, pH 7.0) and 10 µL enzymatic extract was stabilized for 5 min at 25ºC. After adding 70 µL of 0.2% (v/v) H_2O_2, the decomposition of H_2O_2 was followed at 240 nm. Enzyme activity was calculated using the molar extinction coefficient of H_2O_2 0.0394 $mM^{-1}cm^{-1}$. One CAT unit is defined as the amount of enzyme causing the decomposition of 1 µmol of H_2O_2 per minute, at 25 ºC.

Ascorbate peroxidase (APX) and glutathione reductase (GR) activities were determined following the method by Murshed *et al.* (2008), with some modifications. Enzymatic extract was prepared using tissue plant (60 mg) and 1 mL of 50 mM of MES/KOH buffer (pH 6.0), containing 40 mM KCl, 2 mM $CaCl_2$ and 1 mM L-ascorbic acid (AsA), and

centrifuged at 12,000 *g* for 10 min at 4ºC. For APX activity estimation, an enzymatic extract aliquot (10 µL) was added to 185 µL of 50 mM of potassium phosphate buffer (KH_2PO_4, pH 7.0) containing 1 mM AsA. After being shaken during 5 seg, the reaction mixture absorbance was followed at 290 nm for 3 min at 25ºC, to determine nonspecific ascorbate degradation. APX activity was started by adding 20 µL of 50 mM of H_2O_2 and determined by following the absorbance at 290 nm for 5 min at 25ºC. The APX specific activity was calculated using the 2.8 mM^{-1} cm^{-1} extinction coefficient, being one unit defined as the amount of enzyme that oxidizes 1 µmol of ascorbate per min. For determining GR activity, an enzymatic extract aliquot (10 µL) was added to 150 µL of 50 mM of HEPES buffer (pH 8.0) containing 0.5 mM of EDTA and 20 µL of 20 mM GSSG. Supernatant was shaken during 5 sec and the absorbance at 340 nm was measured for 3 min at 25ºC to determine nonspecific NADH oxidase activity. GR activity was started by adding 20 µL of 0.05 mM of NADPH and determined by following the absorbance at 340 nm for 5 min at 25ºC. GR specific activity was calculated from the 6.22 mM^{-1} cm^{-1} extinction coefficient. One unit was defined as the amount of enzyme that will reduce 1 nmol of GSSG per min. All enzyme activities were expressed in U/mg of protein.

All enzymatic assays were performed using freshly prepared extracts, maintained on ice until analysis. For each antioxidant enzyme measurement, six leaf samples of each genotype and condition were used and independently repeated for three times (n = 18). The microplate reader used for all readings was the *PowerWave XS2* (BioTek Instruments, Inc., Winooski, USA), equipped with an internal temperature incubator and a shaker for kinetic analysis.

3.1.3.5. Total RNA extraction and reverse transcription

Leaf samples were harvested in four plants of different replicates of each genotype/condition, following 15 days of stress imposition, being immediately grounded to a fine powder with liquid nitrogen. Total RNA was extracted using the *NucleoSpin RNA Plant kit* (Macherey-Nagel, Düren, Germany), as described by the manufacturer. RNA integrity and DNA contamination were assessed in a 1% (w/v) agarose gel, while RNA concentration and quality were estimated using the A_{260}/A_{280} ratio with the spectrophotometer *Powerwave XS2* (BioTek Instruments, Inc., Winooski, USA). The cDNA was synthesised from 1000 ng of total RNA using *High Capacity cDNA Reverse Transcription kit* (Applied Biosystems, Foster

City, USA), according to manufacturer's protocol. The resulting cDNA was diluted to 1:10 and stored at -20°C.

3.1.3.6. Gene expression analysis

A total of thirteen genes, including drought and oxidative stress-related genes, as well as three reference genes were studied (Table 3.1.1). Specific primers for drought or oxidative stress-related genes were designed based on sequences available in the NCBI database and/or described in previous studies (accession numbers provided in Table 3.1.1). Primer pairs were designed using *Primer plus 3* program, considering the following criteria: 20-25 bp of primer size, GC content of 45-60% and melting temperature (T_m) around 60-62 ºC. Primers were checked by *OligoCalc* program. Gene expression was firstly tested by semi-quantitative PCR using the *Taq PCR Master mix kit* (Qiagen, Hilden, Germany). cDNA amplifications were carried out in a *BioRad T100 Thermal Cycler* (BioRad, Hercules, USA) and amplicons were separated by electrophoresis on agarose gels (1.7%, w/v), running at 90 V for 75 min, and stained in a ethidium bromide solution. Gels were visualized using the *Molecular Image Gel-Doc^{TM} XR^{+}* with *Image LabTM Software* (BioRad, Hercules, USA). Semi-quantitative PCR analyses were independently repeated three times. The expression of each gene was evaluated at the linearity phase of the amplification reaction by the previous comparison of corresponding PCR products at different cycles. Different expression levels were determined according to the amplification intensity. Two differentially expressed genes were further studied by quantitative real-time PCR (qPCR), using a *StepOnePlus Real Time PCR system* (Applied Biosystems, Foster City, USA). qPCR amplifications were performed in triplicate and analyzed using *StepOnePlus Real Time PCR software* (Applied Biosystems, Foster City, USA). Only threshold quantification cycle (Ct) values, leading to a Ct mean with a standard deviation below 0.5, were considered. Mean PCR efficiency per gene was estimated using standards curves based on ten-fold dilutions of corresponding cDNA mixture of all the samples (in triplicate). The efficiency values varied from 96 to 108% for reference and target genes. Gene expression final values were normalized using the mRNA levels of the each genotype control treatment. The expression values were normalized by the average expression of reference genes, according to Pfaffl (2001). The $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001) was used to calculate relative mRNA levels of genes, using *VuEF1-α* and *VuPp2A* as reference genes for normalization.

Table 3.1.1. Descriptions of all drought and oxidative-related cowpea genes and the housekeeping genes used as reference, used for expression analyses.

Type	Gene	Genebank accession	Reference	Gene function	Primers sequences (5'3')	Amplicon size (bp)	Ta (ºC)
Housekeeping genes	*VuEF1-α*	XP_003553292	Weiss *et al.* (2018)	Elongation factor 1-alpha	F: GCCTGGTATGGTGGTGACTT R: GCGAACTTCACTGCAATGTG	280	60
	VuPp2A	AT1G13320	Silva *et al.* (2015)	Regulatory subunit of phosphatase 2A protein	F: CATTGTTGAGCTTGCTGAGG R: GAGCACCAAGCTTGTCATCA	150	60
	VuSkip 16	NP_001242370	Weiss *et al*. (2018)	ASK-interacting protein 16	F: ACAGCCGTTGAACAAAAAGG R: GTGGCTTCTTCGTCCACACT	300	60
Drought-related genes	*VuCPRD14*	D83971	This work	Response to dehydration stress	F: GTACCCAACATTGCAACTTC R: ACAGTATCCTTGATGCTCAC	150	57
	VuCPRD22	D83972	Muchero *et al.* (2010)	Response to dehydration stress	F: CAAGTTACCAGAAGCAGTAC R: CCACATTTACACGACAAGAC	900	57
	VuCPRD65	AB030293	This work	9-Cis-epoxycarotenoid dioxygenase 1	F: CCCTTCAAAGACCTACCTTCC R: GGATGTGGATGTGGATGTTG	150	60
	VusHsp17.7	EF514500	Silva *et al*. (2015)	Small heat shock protein 17.7 KDa	F: GGACGAAGGAGAAGGAGGAC R: TCCTCCTTGGGAACAGTGAC	150	60
	VuNced1	AB030293	Silva *et al*. (2015)	9-Cis-epoxycarotenoid dioxygenase 1	F: CGAAGACGATTTACCCTACCAC R: GAGGTAAGGCTTCTGAATGACG	180	55
Oxidative-related genes	*VucGR*	DQ267475	This work	Cytosolic glutathione reductase	F: GGGATGGGTTCTGAAGTTGA R: ATTCCCCTGCCTTCAAGATT	120	60
	VuPAP-α	AF165891	This work	Putative phosphatidate phosphatase	F: AAGGGGTCGTAAAGGAAGGA R: TTTTGCAACATGACCTCTGC	130	60
	VuPAP-β	AF171230	This work	Putative phosphatidate phosphatase	F: CTCTTGGTCCTTTGCTGGTC R: CCACGAGGATCGGTAAGAAA	150	60
	VuPLD1	U92656	This work	Putative phospholipase D	F: GCTCATAGGTGTTGGGAGGA R: GCCGCCTAGAATCCCTTATC	150	60
	VusAPX	AY484493	This work	Stromatic ascorbate peroxidase	F: GCTTCTCCAGCCAATCAAAG R: CTTCGGGACATTGTTCAGGT	150	60

3.1.3.7. Statistical data analysis

Data from physiological and biochemical measurements were presented as the mean of four to six independent experiments with the respective SE bars. Data from gene expression analyses were presented as the mean of four independent experiments with the respective SE bars. Differences between means were analysed with one-way ANOVA followed by Tukey's test ($p < 0.05$ considered as significant) or with two-way ANOVA followed by Bonferroni test ($p < 0.05$ considered as significant), using *IBM SPSS Statistics* version 20 software (IBM SPSS, Inc., Chicago, USA). Principal component analysis (PCA) was performed using *Past* version 3.19 statistical software (Hammer *et al*. 2001), using values normalized into percentage taking into account the maximum value obtained for each assay/test.

3.1.4. Results and Discussion

The responses of four cowpea genotypes to drought stress were evaluated at physiological, biochemical and molecular levels. For the physiological evaluation, the stomatal function and photosynthetic capacity were determined; the biochemical evaluation was assessed by stress markers and antioxidant enzymes; and at molecular level, the expression of drought candidate genes was determined. These evaluations will contribute to the understanding of drought tolerance mechanisms and elucidate on the most appropriate methodologies to discriminate different drought tolerance levels in cowpea genotypes.

3.1.4.1. Physiological responses of cowpea under drought stress

Stomatal function and photosynthetic capacity have been considered good indicators of plant response to water deficit being both non-invasive procedures. The effects of drought stress, were examined measuring several parameters related to physiological responses in four cowpea genotypes during the experiment course on control (75% FC) and severe (0% FC) stress conditions. These two treatments represented the extreme conditions used in the study. Table 3.1.2 shows the effects of water restrictions in different parameters, such as stomatal conductance (g_s), net CO_2 assimilation (A) and intrinsic water use efficiency (A/g_s) in day 1, day 10 and day 15 after stress imposition. In general, no significant differences between genotypes were found concerning these parameters. When considering severe stress

imposition, g_s and A showed significant differences over time ($p < 0.001$). As a consequence, the difference between treatments (75% and 0% FC) increased during time, becoming significantly different ($p < 0.001$) after 10 days of water privation: the average g_s was significantly reduced by 83% (day 10) and 92% (day 15), and the average A significantly reduced by 67% (day 10) and 97% (day 15). Significant decreases in g_s and A parameters have been considered as an evidence of stomatal limitation due to drought stress induction (Anjum et al. 2011; Munjonji et al. 2018) and have already been reported as a cowpea response to drought (Singh and Raja Reddy 2011; Kutama *et al*. 2014). In addition, significant decrease of g_s in drought treatments suggested an efficient adaptive transpiration control (Hessini et al. 2008). During drought imposition (0% FC), intrinsic water use efficiency (evaluated by A/g_s) also decreased for all four genotypes. However, a transient stomatal regulation led to a slight increase of this parameter in the first 10 days upon stress imposition. This suggests an adjustment to water loss through transpiration and absorption of CO_2 (Wu and Bao 2011). The distinct genotypes revealed significantly different water use efficiencies after 15 days of drought imposition ($p < 0.01$), but not on stomatal conductance or net CO_2 assimilation. The susceptible Bambey 21 and Vg50 genotypes revealed a lower efficiency of water use efficiency than the moderately susceptible CB46 and Cp5051 genotypes. Plants capacity to establish efficient rooting system may be involved in different drought tolerance responses (Agbicodo *et al*. 2009) namely plants stomatal function (Nahar *et al*. 2015). Accordingly, Munjonjii *et al*. (2018) observed that genotypes with well-developed root system maintain relatively higher g_s and A values than genotypes with limited root system and lower g_s and A values, indicating a lower drought tolerance.

Table 3.1.2. Effects of water restriction in three different periods on stomatal function of four cowpea genotypes (n = 6 per genotype/condition). Significant differences were evaluated by one-way ANOVA (followed by the Tukey test) or two-way ANOVA (followed by Tukey test).

Treatment / Genotype	**gs (mmolm^{-2}s^{-1})**				**A (µmolm^{-2}s^{-1})**				**A/gs (µmolmol^{-1})**			
	Day1	Day10	Day15	**p value (day)**	Day1	Day10	Day15	**p value (day)**	Day1	Day10	Day15	**p value (day)**
Control												
Cp5051	378.33	391.60	295.93	n.s.	21.81	22.60	19.43	n.s.	50.43	57.16	67.78	*0.027*
Vg50	274.63	300.25	297.45	n.s.	15.84	20.77	22.78	**0.010**	68.21	62.05	79.62	***<0.001***
Bambey 21	342.64	392.33	262.14	*0.042*	19.78	20.37	20.76	n.s.	51.78	58.51	72.87	**0.006**
CB46	288.47	405.53	292.45	*0.044*	18.52	19.64	19.57	n.s.	66.27	56.03	70.79	n.s.
p value (genotype)	n.s.	n.s.	n.s.		**0.010**	n.s.	n.s.		n.s.	n.s.	n.s.	
0% of FC												
Cp5051	244.36	61.63	15.64	***<0.001***	19.33	8.95	0.804	***<0.001***	71.62	102.59	51.32	***<0.001***
Vg50	273.46	30.73	19.72	***<0.001***	16.84	3.88	0.534	***<0.001***	62.81	82.03	26.89	***<0.001***
Bambey 21	352.48	41.58	23.02	***<0.001***	19.25	4.24	0.529	***<0.001***	50.46	97.22	26.43	***<0.001***
CB46	260.03	47.51	16.02	***<0.001***	19.76	7.61	0.802	***<0.001***	81.14	97.96	51.53	*0.012*
p value (genotype)	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.		n.s.	n.s.	*0.017*	
p value (treatment)	n.s.	***<0.001***	***<0.001***		n.s.	***<0.001***	***<0.001***		n.s.	***<0.001***	***<0.001***	
p value (treatment x genotype)	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.		n.s.	*0.026*	***<0.001***	

(A - net CO_2 assimilation; A/g_s-intrinsic water use efficiency; FC - field capacity . ***Bold/italics*** - indicates significant differences at level $p < 0.001$; **bold** - significant differences at level $p < 0.01$; *italics* - indicates significant differences at level $p < 0.05$; n.s. - no significant differences)

To examine the effects of water restriction on photochemical reactions of cowpea genotypes, maximum efficiency of PSII photochemistry (as revealed by F_v/F_m ratio), effective quantum efficiency of photosystem II (Φ_{PSII}), apparent electron transport rate (ETR) and photochemical fluorescence quenching (qP) parameters related to chlorophyll *a* fluorescence were measured and analyzed (Table 3). A reduction of F_v/F_m ratio was observed in all genotypes under drought treatment (0% FC) during the experiment. As control plants did not reveal significant changes on this parameter, differences between both water regimens became significant after 10 days ($p < 0.01$) and were further amplified by drought persistence (15 days, $p < 0.001$). This can be explained by the drought stress affects PSII efficiency leading to an F_v/F_m decrease, as was observed by Singh and Reddy (2011). Maximum reduction of F_v/F_m ratio was observed in Bambey 21 genotype and the minimum reduction was in CB46 genotype. A similar behavior was obtained when determining the Φ_{PSII} and ETR. Both parameters declined after 10 days of water privation, while control plants revealed less significant changes on parameters levels that could be explained by the plant development or genotype features. As for F_v/F_m ratio, in the same period, differences on Φ_{PSII} or ETR among genotypes were not significant. Although exhibiting a similar trend, the qP was the only parameter in which significant differences were observed between genotypes. Differences were detected both in control (75% FC) and stressed plants (0% FC) after 15 assay days, revealing that genotypes could present differences on their chlorophyll fluorescence during development. The moderately susceptible CB46 and Cp5051 genotypes revealed a higher qP, indicating that more energy was quenched to the primary photochemical reactions leading to a more efficient photosynthetic process (Krause and Weis 1991). Vg50 genotype revealed an even lower qP value than the susceptible Bambey 21 genotype.

Table 3.1.3. Effects of water restriction in three different periods on photosynthetic efficiency of four cowpea genotypes (n = 6 per genotype/condition). Significant differences were evaluated by one-way ANOVA (followed by the Tukey test) or two-way ANOVA (followed by Tukey test).

Treatment Genotype	**Fv/Fm**				**Φ_{PSII}**				**qP**				**ETR**			
	Day1	Day10	Day 15	**p value (day)**	Day1	Day10	Day15	**p value (day)**	Day1	Day10	Day15	**p value (day)**	Day1	Day10	Day15	**p value (day)**
Control																
Cp5051	0.878	0.885	0.882	n.s.	0.589	0.697	0.700	*0.026*	0.823	0.897	0.889	***0.001***	370.99	439.15	441.03	*0.026*
Vg50	0.883	0.874	0.888	n.s.	0.626	0.683	0.698	*0.024*	0.824	0.889	0.909	*0.014*	394.66	430.38	439.46	*0.024*
Bambey 21	0.879	0.880	0.882	n.s.	0.641	0.713	0.669	*0.028*	0.844	0.908	0.878	n.s.	403.51	449.20	421.60	*0.028*
CB46	0.875	0.885	0.889	n.s.	0.586	0.695	0.703	**0.002**	0.795	0.887	0.931	**0.003**	368.98	437.86	443.15	**0.002**
p value (genotype)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	*0.036*		n.s.	n.s.	n.s.	
0% of FC																
Cp5051	0.883	0.871	0.775	***<0.001***	0.675	0.523	0.398	***<0.001***	0.861	0.837	0.647	***<0.001***	425.34	368.68	251.00	**0.003**
Vg50	0.884	0.836	0.782	*0.012*	0.638	0.518	0.320	***<0.001***	0.857	0.745	0.481	***<0.001***	402.16	326.48	201.39	***<0.001***
Bambey 21	0.887	0.865	0.799	***<0.001***	0.635	0.553	0.332	***<0.001***	0.828	0.774	0.536	***<0.001***	400.34	348.14	209.31	***<0.001***
CB46	0.877	0.826	0.758	*0.026*	0.539	0.571	0.326	**0.003**	0.778	0.807	0.556	***<0.001***	339.55	359.51	205.29	**0.003**
p value (genotype)	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.		n.s.	n.s.	**0.008**		n.s.	n.s.	n.s.	
p value (treatment)	n.s.	**0.005**	***<0.001***		n.s.	***<0.001***	***<0.001***		n.s.	***<0.001***	***<0.001***		n.s.	***< 0.001***	***< 0.001***	
p value (treatment x genotype)	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.		n.s.	n.s.	***<0.001***		n.s.	n.s.	n.s.	

(F_v/F_m – Maximum quantum efficiency of photosystem II; Φ_{PSII} - effective quantum efficiency of photosystem II; qP - photochemical fluorescence quenching; ETR - apparent electron transport rate; FC - field capacity. ***Bold/italics*** - indicates significant differences at level $p < 0.001$; **bold** - indicates significant differences at level $p < 0.01$; *italics* - indicates significant differences at level $p < 0.05$; n.s. - no significant differences)

Figure 3.1.1 shows how the chlorophyll content of all cowpea genotypes evolved at the three different water regimens. The chlorophyll content determination revealed a significant reduction under drought stress conditions, being the mean 46% at moderate stress and 68% at severe stress conditions. Chlorophylls did not vary significantly between days 1 and 15 under control conditions (data not show). The lowest levels of chlorophylls were observed in severely stressed plants (0% FC) after 15 assay days ($p < 0.05$, in comparison to 75% FC). This decrease could be explained by changes in chloroplast structure or biosynthesis inhibition of chlorophyll or its precursors (Nahar *et al*. 2015). Previous studies indicated that drought tolerant genotypes were able to maintain a higher chlorophyll content than susceptible genotypes under drought conditions (Siddiqui *et al*. 2015). Cp5051 and CB46 genotypes seemed to be the most affected by drought stress (0% FC) and presented lower values of chlorophylls content (a reduction of 79% and 76%, respectively); but differences were not statistically significant among all genotypes.

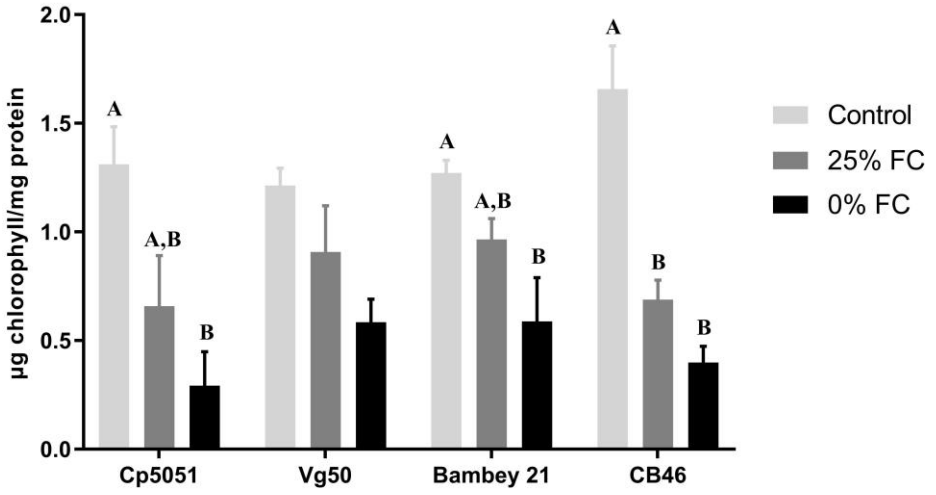

Figure 3.1.1. Chlorophyll content after 15 days of drought stress in four cowpea genotypes. Values represent mean ± SEM (n=6). Different uppercase letters indicate significant differences between treatments within the same genotype (one-way ANOVA followed by Tukey's test at $p < 0.05$). FC - field capacity.

The reduction of photosynthetic parameters is commonly observed under stress situations and have been reported for different plant species (reviewed by Gururani *et al*. 2015), being the decrease of all evaluated parameters (F_v/F_m ratio, Φ_{PSII}, qP and ETR) and chlorophyll content with drought imposition observed in other studies (Souza *et al*. 2004; Singh and Reddy 2011). Altogether, the obtained results revealed that cowpea plants evidenced symptoms of drought stress after 10 days of water withholding (0% FC), which

were further enhanced by drought persistence. The evaluation of gas exchange and chlorophyll *a* fluorescence parameters also suggested that Bambey 21 and Vg50 were the most affected genotypes under drought stress conditions.

3.1.4.2. Biochemical responses of cowpea under drought stress

Plants display a set of biochemical responses when exposed to different stress situations, such as: (1) accumulation of compatible solutes, mainly in those plants exposed to water stress (Anjum *et al*. 2011), (2) production of anthocyanins (Kovinich *et al.* 2015), (3) increasing of lipid peroxidation processes (Anjum *et al*. 2011), and (4) alterations on hydrogen peroxide (H_2O_2) content (Zhou *et al*. 2006). Figure 3.1.2 presents the evaluation of proline (3.1.2A), anthocyanins (3.1.2B), lipid peroxidation (MDA) (3.1.2C) and H_2O_2 (3.1.2D) contents. After 15 days of moderate (25% FC) and severe (0% FC) drought stress and control (75% FC). Leaf proline contents increased significantly throughout the drought stress period (Fig. 2A) in all genotypes, which is in agreement with other studies (Cavalcanti *et al*. 2004; Singh and Reddy 2011; Merwad *et al.* 2018). The four genotypes presented differences on proline accumulation, which suggests differences in their drought tolerance since higher proline accumulation under stress conditions has been correlated with stress-tolerance plants (Anjum *et al.* 2011; Toscano *et al*. 2016). Vg50 genotype presented the highest proline content under severe drought imposition (6.4 µg proline/mg protein), more than two-fold when compared with other genotypes under study. Other well-studied plant response to stress conditions is the production of anthocyanins, which is often correlated with enhanced stress tolerance (Kovinich *et al*. 2015). The amount of leaf anthocyanins in all genotypes increased in response to drought stress imposition compared to the control plants (Fig. 3.1.2B), which agrees with studies on other crops (Efeoğlu *et al*. 2009; Kovinich *et al*. 2015). Nevertheless, results also point to a greater response of Bambey 21 genotype to drought, due to the production of higher amounts of anthocyanins when compared to other genotypes. For other hand, Cp5051 genotype presented the lowest values, suggesting that this genotype is less affected by drought condition.

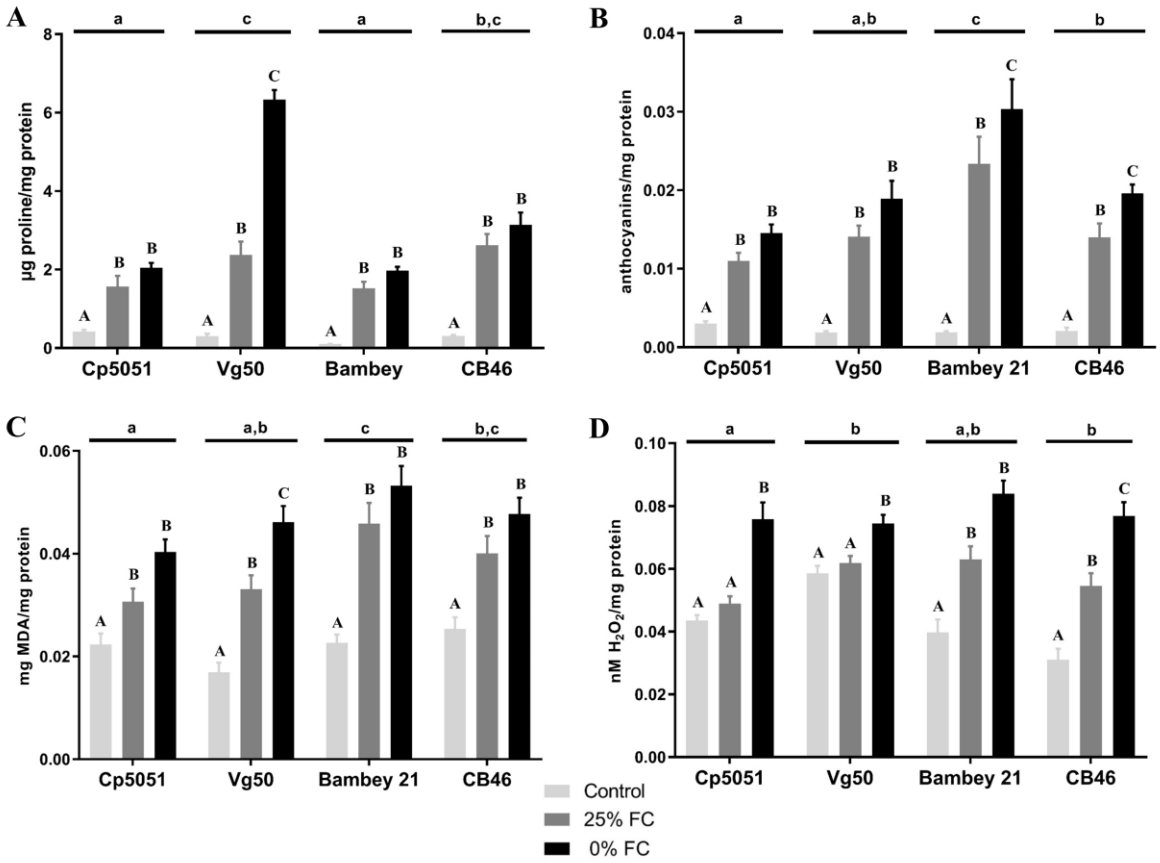

Figure 3.1.2. Biochemical stress markers responses after 15 days of drought stress in four cowpea genotypes. A – Proline; B - anthocyanins; C – MDA; D - H_2O_2; FC - field capacity. Values represent mean ± SEM (n=6). Different uppercase letters indicate significant differences between treatments within the same genotype (one-way ANOVA followed by Tukey's test at $p < 0.05$). Different lowercase letters indicate significant differences between genotypes in every water regimen (two-way ANOVA followed by Bonferroni test at $p < 0.05$).

One of the predicted consequences of stress is an increase of lipid peroxidation, mainly due to an overproduction of H_2O_2 (Anjum *et al*. 2011), as was detected for all drought stressed plants (25 and 0% FC) in comparison to control plants (75% FC, Fig. 2C). Susceptible plants suffering from pronounced stress are described to present higher levels of MDA than more tolerant plants (Bacelar *et al*. 2006). Therefore, the higher level of lipid peroxidation in Bambey 21 suggests a higher sensitivity of this genotype to drought stress, when compared to other genotypes. In contrast, Cp5051 and Vg50 genotypes competed for the lowest MDA accumulation values, revealing a better protection mechanism against oxidative damage. These results are in agreement with H_2O_2 production levels (shown in Fig. 3.1.2D), which were more pronounced in Bambey 21 and CB46 genotypes (displaying 2.6-fold higher production under severe stress than control).

Although it is consensual that varieties/genotypes sensitive to water stress accumulate higher amounts of H_2O_2 (Chakraborty and Pradhan 2012), our results suggest that non-stress plant levels should be taken into account on plant tolerance. The higher levels of H_2O_2 in Vg50 on well-watered plants (75% FC) could play a determinant role for its higher drought-tolerance, due to the dual role of H_2O_2 production.

From all studied plant responses, Bambey 21 was the genotype that presented the highest levels of biochemical stress indicators, such as MDA and anthocyanins, and the lowest amounts of the protective proline. These results are in agreement with other studies that referred Bambey 21 as a drought susceptible genotype using other methodologies (Hamidou *et al*. 2007; Muchero *et al.* 2008).

In this work, we detected a H_2O_2 overproduction with drought stress intensity. This reactive oxygen species (ROS) plays different functions in plants: at higher concentrations causes oxidative damage and at lower concentrations initiates cell signaling (reviewed by Hossain *et al*. 2015). Plants display defensive mechanisms and biochemical strategies that prevent damage caused by ROS. Their enzymatic defenses include many antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), guaiacol phenol peroxidase (POX), ascorbate peroxidase (APX), and glutathione reductase (GR), which together control ROS levels at adequate concentrations for cell function. Therefore, the study of oxidative stress and enzymes involved in ROS can be fundamental for understanding the mechanisms that allow plants to adapt and survive during drought stress periods (Cruz de Carvalho 2008) and at same time to identify the best adapted genotypes to stressful conditions. Figure 3.1.3 gives an overview of the effects of droughts stress imposition (0% and 25% FC) comparatively to control (75% FC) in the four cowpea genotypes through SOD (3.1.3A), CAT (3.1.3B), POX (3.1.3C), APX (3.1.3D) and GR (3.1.3E) activities. Superoxide dismutase (SOD) has been considered the first defense line against the accumulation of ROS under drought (You and Chan 2015), but is also responsible for H_2O_2 production through superoxide dismutation. Results obtained revealed a general trend for SOD activity increase with drought, especially at 25% of FC, severity, which is in agreement with other results obtained in cowpea under drought stress (Merwad *et al*. 2018). However, significant changes were only detected in the Cp5051 genotype (Fig. 3.1.3A). Several enzymatic pathways are responsible for scavenging H_2O_2, such as catalases (CAT) or the ascorbate-glutathione cycle that combines several enzyme activities, including ascorbate peroxidase (APX) and glutathione reductase (GR) (Anjum *et al*. 2016). Guaiacol phenol peroxidase (POX) also

protects cells against the destructive influence of H_2O_2, decomposing it through the oxidation of phenolic and endiolic co-substrates (van Doorn and Ketsa 2014). In our work, well-watered plants presented higher values of CAT activity than plants under stress, except in Cp5051 genotype, although significant differences were only registered for Bambey 21 genotype (Fig. 3.1.3B). CAT activity results contrast with the other assayed H_2O_2 detoxifying enzymes also because significant differences were detected among well-watered genotype plants ($p < 0.05$, results not shown). These results suggest that the main enzymatic process that controls the damaging H_2O_2 produced by stressful conditions does not involve catalase, but other detoxifying pathways. Guaiacol peroxidases (POX) presented a significant increase after stress imposition, except for Vg50 genotype (Fig. 3.1.3C). Ascorbate–glutathione pathway enzymes (APX, GR) also presented an increasing trend with drought intensity, although significant increases were only detected for CB46 and Cp5051 genotypes, respectively ($p < 0.05$, Fig. 3.1.3D,E).

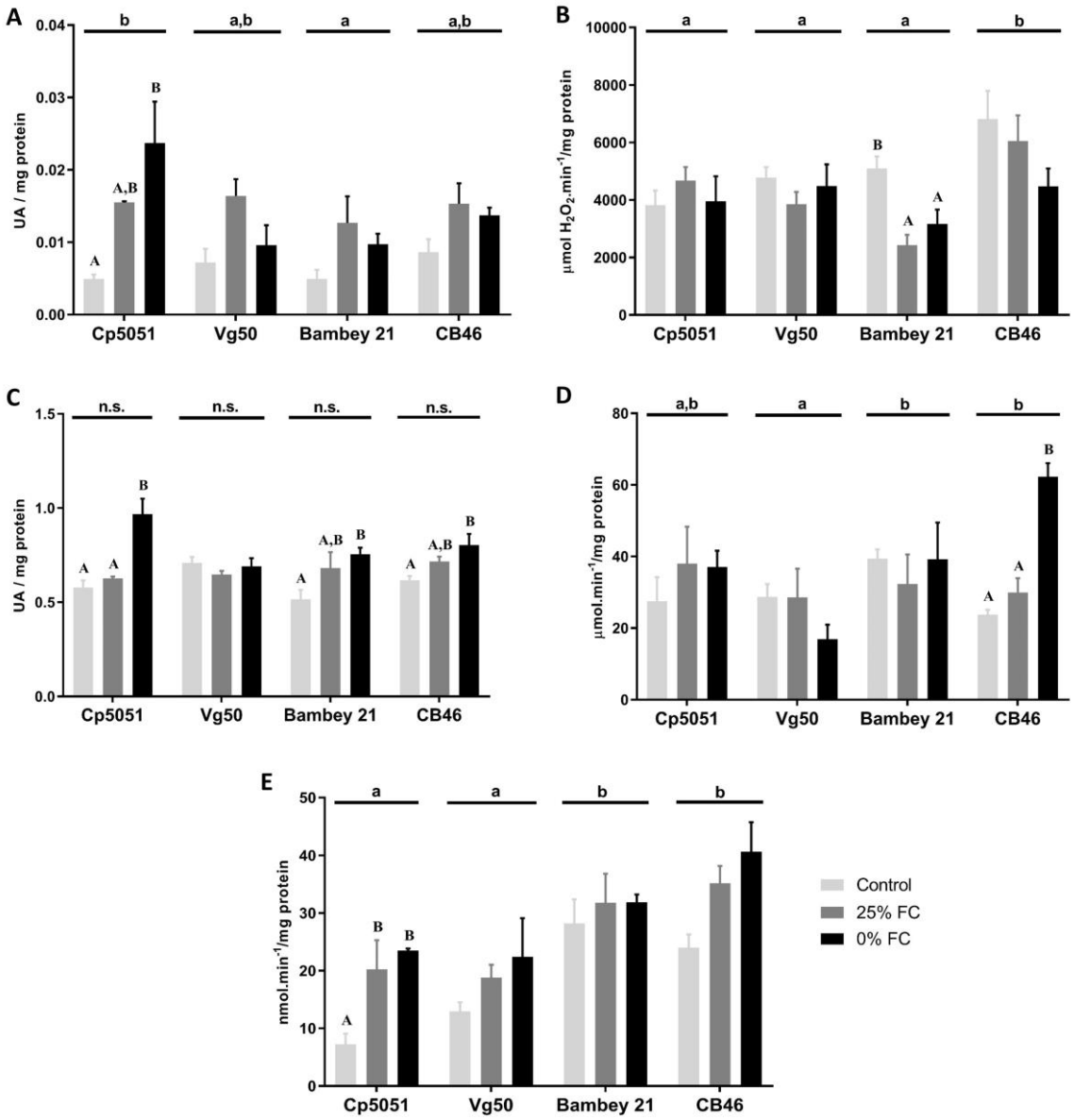

Figure 3.1.3. Antioxidant enzyme responses after 15 days of drought stress in four cowpea genotypes. A - superoxide dismutase; B - catalase; C - guaiacol peroxidase; D - ascorbate peroxidase; E - glutathione reductase; FC - field capacity. Values represent mean ± SEM (n=6). Different uppercase letters indicate significant differences between treatments within the same genotype (one-way ANOVA followed by Tukey's test at $p < 0.05$). Different lowercase letters indicate significant differences between genotypes in every water regimen (two-way ANOVA followed by Bonferroni test at $p < 0.05$). No lettering or "n.s." indicate non-significant differences.

Previous studies on oxidative stress and antioxidant enzymes activities in response to drought showed inconsistent and contradictory results. Cavalcanti *et al*. (2004) detected a CAT activity decrease after drought imposition (approximately 2-fold decrease), while Nair *et al*. (2008) and Merwad *et al*. (2018) verified an increase of CAT activity with increasing

water stress (1.8-fold and 2-fold increase, respectively). Different effects of water stress on APX and GR activities were also detected in different cowpea cultivars (D'Arcy-Lameta *et al*. 2006; Contour-Ansel *et al*. 2006). The registered differences in antioxidant enzymes activities can be associated to the distinct drought tolerance levels of cowpea genotypes, as well as to distinct stress imposition methodologies. Furthermore, ROS accumulation and antioxidant enzymes upregulation are considered to be directly related/dependent on plant species, plant genotype, degree of plant tolerance, stress level, stress duration, plant development (Contour-Ansel *et al*. 2006; D'Arcy-Lameta *et al*. 2006; Harb *et al*. 2015).

3.1.4.3. Gene-expression profiling of cowpea plants under drought stress

Transcriptomic studies have also been considered and integrated with biochemical and physiological responses as a whole in studying plant responses to multiple abiotic or biotic stresses through the identification of genes, pathways and processes. Many cowpea genes have been identified as being involved in drought and oxidative responses (reviewed by Carvalho *et al*. 2017). To have a complete picture of plant stress responses is also important to analyze the genes expression.

Based on previous studies, a total of thirteen genes with different functions were selected for gene expression analysis under drought stress conditions in four cowpea genotypes (Table 3.1.1) and their gene expression profiling was evaluated by semi-quantitative RT-PCR. All amplifications resulted in a single amplicon of the expected length (Table 3.1.1). From this set of genes, five are drought stress-related (DG) and their expression was evaluated (Fig. 3.1.4). Figure 4A presents the profiling by semi-quantitative RT-PCR of the five DG genes in the two drought stress conditions (0 and 25 % FC) compared to control (75% FC). A differential gene expression was observed for DG genes under water stress treatments, which mostly revealed a higher expression with drought stress intensification. Most of DG genes also exhibited a low expression level in plants without water limitation (75% FC; results not show). Differences among genotypes were detected, although without a specific genotype expression pattern. Nevertheless, Cp5051 and Vg50 genotypes revealed a higher transcriptional response to drought severity.

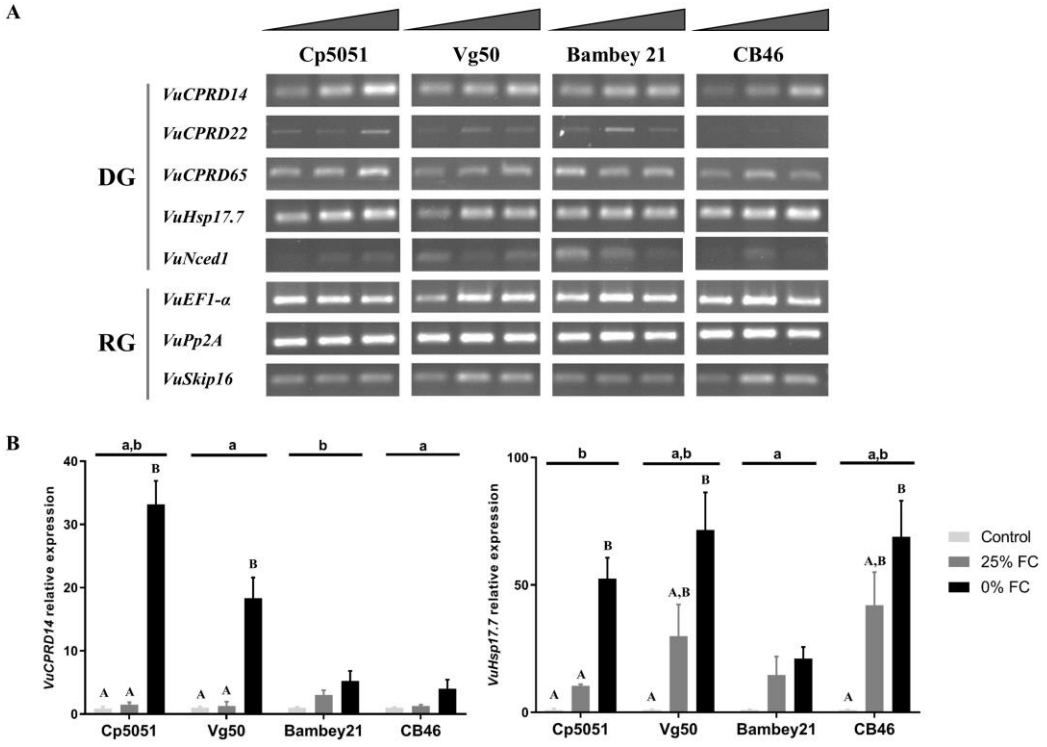

Figure 3.1.4. Gene expression after 15 days of drought stress in four cowpea genotypes. (A) Gene expression profile by semi-quantitative RT-PCR of five genes related to drought stress (DG) and three reference genes (RG). Stress severity is indicated by a triangle, from control (75% of FC) to severe stress (0% of FC). (B) Effect of the same drought stress treatments in the expression of two drought-related genes (*VuCPRD14* and *VuHsp17.7*), as evaluated by qPCR. Values are the mean ± SEM (n = 4). Different uppercase letters indicate significant differences between treatments within the same genotype (one-way ANOVA followed by Tukey's test at $p < 0.05$). Different lowercase letters indicate significant differences between genotypes in every water regimen (two-way ANOVA followed by Bonferroni test at $p < 0.05$). No lettering indicates non-significant differences. FC - field capacity.

Five oxidative stress related genes (OG), previously described (Additional file 3.1.2), were also evaluated by semi-quantitative RT-PCR. The results did not reveal significant differences between genotypes and drought stress treatments, which is in agreement with antioxidant enzymes activity results; however, in previous studies these OGs were reported to be highly expressed in susceptible cowpea genotypes comparatively to tolerant ones. Some studies refer that APX and GR activities are much variable depending on water stress level and plant tolerance degree (Contour-Ansel *et al*. 2006; D'Arcy-Lameta *et al*. 2006).

Based on semi quantitative RT-PCR genes profiling, two drought related genes, *VuCPRD14* and *VuHsp17.7*, were selected for further expression analysis studies. *VuEF1-α* and *VuPp2A* were used as reference genes due to their stability under drought stress conditions (Da Silva *et al*. 2015; Zegaoui *et al*. 2017; Weiss *et al*. 2018). In general, both DGs

exhibited a low expression level in plants without water limitation (75% FC; results not show). Figure 3.1.4B shows the effects of drought stress treatments in the expression of two DG genes of four cowpea genotypes. Gene expression analysis by qPCR revealed that both DG genes were mostly expressed at 0 and 25% of FC treatments (Fig. 3.1.4B), being clear their association with cowpea drought response. Both genes have been described as being involved in abiotic stress responses and were previously reported their up-regulation in cowpea leaves under drought stress (Iuchi *et al*. 1996; Simoes-Araujo *et al*. 2008; Da Silva *et al*. 2015). *VuCPRD14* gene expression was significantly higher ($p < 0.05$) in Cp5051 genotype. *VuHsp17.7* gene was predominantly expressed under severe (0% FC) and moderate drought stress (25% FC), with significant differences in the genotypes Cp5051 and Vg50, with drought stress intensity. High expression levels of *VuCPRD14* and *VuHsp17.7* drought-related genes detected under severe drought conditions also indicate Cp5051 genotype as the most drought-tolerant.

3.1.4.4. Physiological, biochemical and transcriptional fingerprinting of cowpea under drought stress

In the last years, many methods for evaluating and discriminating cowpea genotype drought tolerance have been developed (Cavalcanti *et al*. 2004; Singh and Reddy 2011; Merwad *et al*. 2018). In order to compare different procedures, cowpea plants from distinct genotypes were subjected to the same drought conditions and assayed for different physiological, biochemical and molecular approaches. The inconsistent outcomes of these assays led us to search for the best approaches for identifying (sometimes subtle) different drought responses. To further understand the quantitative relations between each parameter and determine their contribution to genotypes drought tolerance, principal component analysis (PCA) performed for each set of parameters under study (Fig. 3.1.5). Physiological parameters have been frequently used for selecting water stress tolerant cowpea genotypes (Singh and Reddy 2011; Kutama *et al*. 2014) and can be indicators of the crops responses to drought stress (Zu *et al*. 2017). A clear discrimination of stress severity is obtained when using all assayed physiological parameters (gas exchange and photosynthetic parameters; Fig. 3.1.5A). The first two principal components of PCA explained 97.4% (PC1 = 83.8% and PC2 = 13.6%) of total variation. All well-watered control plants clustered together with plants with only one day of stress imposition, revealing that these are not suffering from water privation.

However, as drought proceeds, a clear discrimination of plant physiological parameters is observed. From all physiological parameters, those related with stomatal function (A, g_s and A/g_s) contributed the most for this discrimination. PCA also suggested Cp5051 followed by CB46, as the cowpea genotypes less affected by drought.

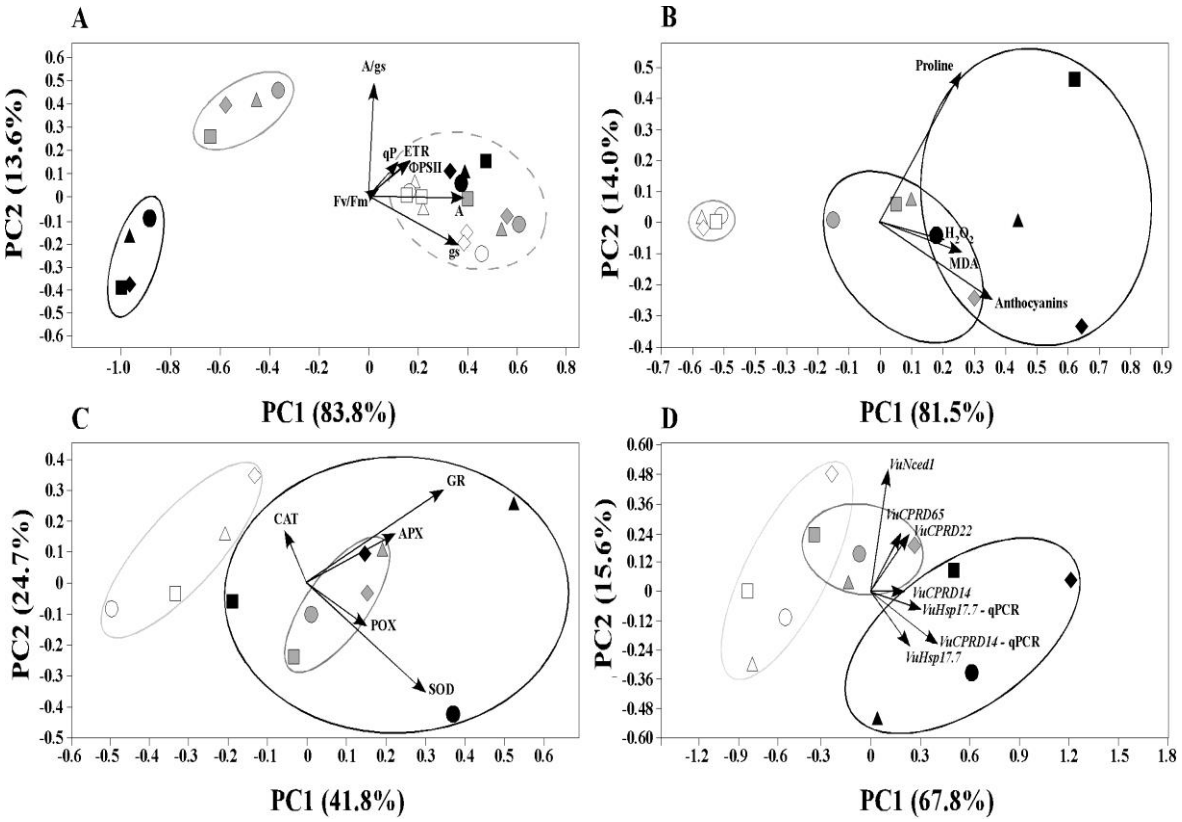

Figure 3.1.5. Principal component analysis for discriminating drought responses and genotype differences. (A) Gas-exchange and photosynthetic parameters (gs, A, A/gs, F_v/F_m, Φ_{PSII}, qP and ETR). (B) Biochemical markers of drought stress (proline, anthocyanin, MDA and H_2O_2 contents). (C) Antioxidant enzymes activity (CAT, SOD, POX, APX and GR activities). (D) Expression of drought-related genes using the expression data obtained from RT-PCR and qPCR of five drought-related genes (*VuCPRD14*, *VuCPRD22*, *VuCPRD65*, *VuHsp17.7* and *VuNced1*). For (A), cowpea genotypes were exposed to two different water regimens (0% FC and 75% FC) and parameters were determined in three different days. Each day corresponds to a different color (white – day 1; gray – day 10; black – day 15). For (B to D), plants from different cowpea genotypes were exposed to three different water regimens (white - well-watered; gray – moderate drought stress; black - severe drought stress) and parameters were determined after 15 days of stress imposition. Genotypes correspond to different shapes (Cp5051 - circle; Vg50 - square; Bambey 21 – diamond; CB46 – triangle). Arrows indicate eigenvectors representing the strength (given by the length of the vector) and direction of the parameter correlation relative to the first two principal components (PC1 and PC2).

Regarding the proline, anthocyanins, MDA and H_2O_2 contents, a clear discrimination of plants under drought stress imposition was also obtained (Fig. 3.1.5B). The first two principal component of PCA explained 95.5% (PC1 = 81.5% and PC2 = 14.0%) of total

variation. Plants in control conditions (75% FC) clustered more proximately than in drought stress, which may be explained to the differential genotype*biochemical parameter response in each stress situation. Anthocyanin and proline contents were the two parameters that most contributed for this discrimination among stressed plants. Although not so evident in physiological evaluation, the analysis also suggested Cp5051 as the less affected genotype by drought, always clustering closer to control plants. The use of several oxidative stress markers has been recurrently used for studying plant responses to drought stress (Zhou *et al*. 2006; Anjum *et al*. 2011; Toscano *et al*. 2016), as well as the evaluation of antioxidant enzyme activities (Cavalcanti *et al*. 2004; Singh and Reddy 2011; Anjum et al. 2016; Toscano *et al*. 2016; Merwad *et al*. 2018). In this study, no distinct patterns of antioxidant enzyme activities were associated to genotype and/or severity drought imposition (Fig. 3.1.5C). The first two principal component of PCA explained 72.8% (PC1 = 48.1% and PC2 = 24.7%) of total variation. GR and SOD were the enzymes that mainly contributed for genotypes discrimination and control plants (75% FC) clustered in a separate group. Drought stress plants were randomly distributed and were not grouped in any distinct group, suggesting that antioxidant enzymes did not allow to evaluate differences between genotypes or even among different drought stress levels. Many genes are recognized as being involved in drought responses and have been used for discerning the drought responses of different cowpea genotypes. In our work, gene expression data (semi quantitative RT-PCR and qPCR results) allowed the discrimination of different drought stress treatments (Fig. 3.1.4A,B). The first two principal component of PCA explained 83.4% (PC1 = 67.8% and PC2 = 15.6%) of total variation (Fig. 3.1.5C). The expression of *VuCPRD14* (by semi quantitative RT-PCR) and *VuNced1* (by qPCR) were the most relevant for plant/drought stress discrimination. However, a similar discrimination was obtained when only considering semi-quantitative RT-PCR (Additional file 3.1.3), revealing that qPCR is not required for drought responses differentiation. Gene expression analysis did not allow identifying any relation between drought responses and genotypes.

3.1.5. Conclusions

Global warming has been an increasing problem, further enhanced by frequent drought stresses events, both in duration and intensity. The study and search for crops with some

drought tolerance is a pressing issue. Cowpea has several agronomic features that turn this crop naturally adapted to these constrains. For this reason, the knowledge of physiological, biochemical and transcriptional responses to drought stress is fundamental. This finding could be of importance when several cowpea genotypes need to be compared, simplifying survey protocols. A severe and moderate drought stress was imposed to four cowpea genotypes. Physiological, biochemical and gene-expressional behavior indicated that assayed plants were indeed under drought stress and genotypes revealed different trends. Bambey 21 and Vg50 genotypes revealed a low efficiency of water use, contrasting with the moderately susceptible CB46 and Cp5051 genotypes. On the other hand, Cp5051 genotype was the most drought resilient, as revealed by biochemical and molecular responses. Stomatal function, and stress indicators, such as proline and anthocyanin contents, were the most appropriate markers to discrimination cowpea genotypes under drought stress and therefore can be used in screening the tolerance to water deficit, simplifying survey protocols. They indicated that Cp5051 genotype is least affected by drought stress then Vg50. The scavenging enzymes (SOD, CAT, POX, APX and GR) were not implicated in the ability of cowpea plants to survive under higher levels of drought stress. Molecular analysis revealed that *VuCPRD14* and *VuHsp17.7* genes were the most relevant for cowpea drought stress discrimination and semi-quantitative RT-PCR allows the drought responses differentiation between genotypes. Cp5051 and Vg50 genotypes had never been studied. The stomatal function, and stress indicators (proline and anthocyanin contents) parameters used in this study At the end, this work provided useful and valuable information for screening of cowpea genotype better adapted to drought stress.

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3.1.6. References

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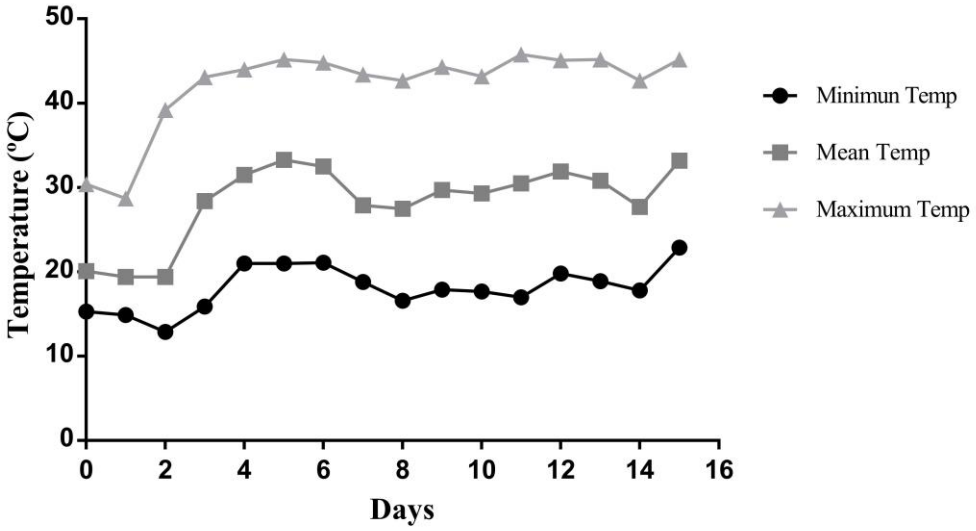
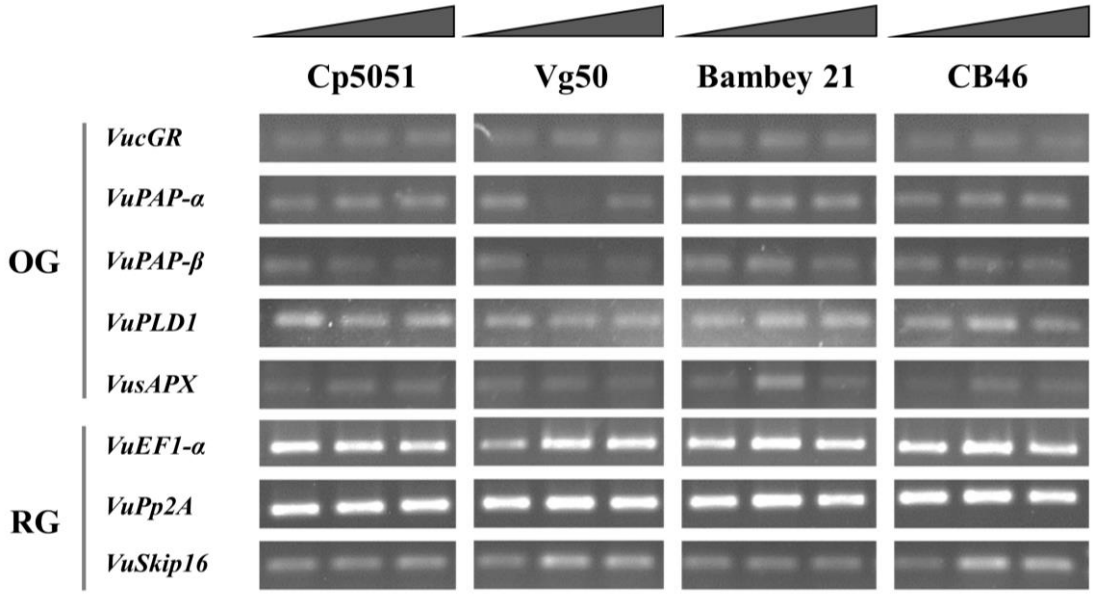
3.1.7. Additional files

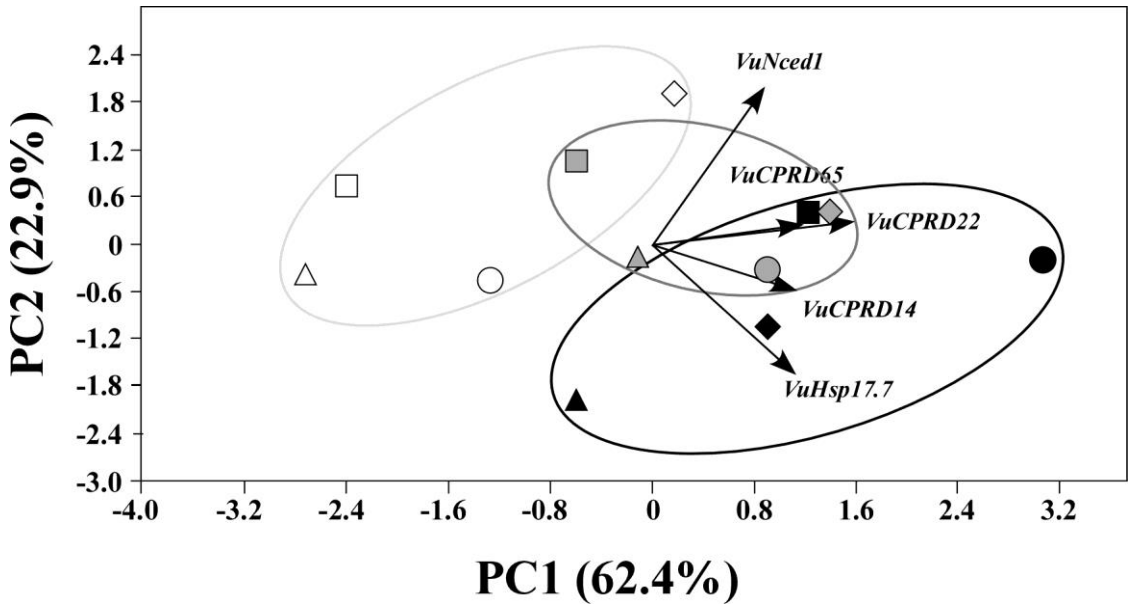
Additional file 3.1.1. Daily maximum, mean and minimum temperatures recorded during the 15 days of drought stress experiment.



Additional file 3.1.2. Gene expression of oxidative stress-related genes on cowpea genotype plants under drought stress conditions. OG - oxidative stress-related genes; RG - reference genes. Stress severity is indicated by a triangle, from control (75% of FC) to severe stress (0% of FC).



Additional file 3.1.3. Principal component analysis for discriminating drought responses and genotype differences using the expression of drought-related genes. Arrows indicate eigenvectors representing the strength and direction of the gene correlation relative to the first two principal components (PC1 and PC2). Genotypes correspond to different shapes: Cp5051 - circle; Vg50 - square; Bambey 21 - diamond; CB46 – triangle. Water regiments correspond to different colors: black - 0% FC; gray - 25% FC; white - 75% FC. Arrows indicate eigenvectors representing the strength and direction of the parameter correlation relative to the first two principal components (PC1 and PC2).



3.2. Screening of drought tolerant genotypes

Screening of worldwide cowpea collection to drought tolerant at a germination stage

Carvalho M., Matos M., Castro I., Monteiro E., Rosa E., Lino-Neto T., Carnide V. (2018). Screening of worldwide cowpea collection to drought tolerant at a germination stage. Scientia Horticulturae 247: 107-115 (doi: 10.1016/j.scienta.2018.11.082). JIF (2017) = 1.760; SJR (2017) Horticulture = Q1.

*Carvalho M. contribution: conducted lab experiments, data analysis and discussion, and manuscript

3.2.1. Abstract

Global warming has an increasing impact on the availability of water for agriculture. Crops tolerant to high temperatures and drought, such as cowpea (*Vigna unguiculata* L. Walp.), have an added value in the near future. The main objective of this study was to evaluate the effect of drought on seed germination and seedling emergence of cowpea genotypes, in order to screen the most tolerant genotypes. Seeds from 58 cowpea genotypes all over the world were submitted to two stress conditions, induced by PEG-6000 (corresponding to osmotic potentials of -0.75 bars and -1.5 bars). Germination and seedling growth parameters, vigor index and proline content were determined to assess drought tolerance. The results revealed significant differences of all parameters among genotypes after treatments and interaction of both. Water stress caused a general decrease in germination and seedling growth, while an increase in proline content was observed. A high variation of drought responses were detected among genotypes, being possible to select seven genotypes (C11, C18, C44, C46, C47, C50 and C54) as tolerant to drought at germination stage. These results will be useful to select the best suitable parents for insertion in future breeding programs.

3.2.2 Introduction

Worldwide agricultural production has been limited by several environmental constrains in the form of abiotic stresses, which affects plants growth, metabolism and development (Muscolo *et al*. 2014; Eftekhari *et al.* 2017). Water scarcity is currently one of the most severe limitations of plant development and production (Jain and Saxena 2016; Eftekhari *et al.* 2017). The predicted temperature increase and rainfall decrease will be responsible for more frequent drought periods, mainly in the Mediterranean region including the Iberian Peninsula (Kröner *et al.* 2017). In this climate change scenario, the selection of drought-tolerant plants gain more importance, particularly the selection during germination. Some studies report several physiological characteristics (including seed germination and seedling growth) as indicators of drought tolerance in specific crop genotypes (Bouslama and Schapaugh 1984; Steiner *et al.* 2017; Yan 2015). Seed germination and seedling emergence are potentially the most critical stages susceptible to water stress (Ahmad *et al.* 2009; Hellal *et al.* 2018; Li *et al*.

2011, 2015) and are pivotal steps for crop propagation (Ravelombola *et al*. 2017). Indeed, water limitation can be responsible for the decline or even complete inhibition of seedling emergence and stand establishment (Kaya *et al*. 2006; Wu *et al*. 2011; Yan 2015). However, tolerance against drought during the germination stage allow an uniform plant stand (Steiner *et al*. 2017).

Cowpea (*Vigna unguiculata* L. Walp.) is a grain legume with high worldwide economic importance, originated in Africa. Seeds of this legume are an important source of protein and other nutritional components for human diet (Ravelombola *et al*. 2017; Timko and Singh 2008) and also an important source to animal fodder (Huang *et al.* 2012). Like many legumes, cowpea has the ability to fix atmospheric nitrogen through rhizobium symbiosis (Ehlers and Hall 1996) and is easily grown in low fertility soils (Eloward and Hall 1987). Some reports referred to the ability of cowpea to grow in regions without irrigation and irregular rainfall, being considered as one of the most tolerant legumes to drought (Agbicodo *et al*. 2009). Taking into consideration the upcoming climate change and increasing protein needs, all these advantages make desirable to increase cowpea production and consumption in European Union. Nowadays, almost all consumed cowpea in Europe is imported from African countries (FAOSTAT 2018). The establishment success of this crop in such semiarid regions depends on the fast and uniform seed germination under low water availability (Muscolo *et al*. 2014).

Several methods and efforts have been employed to identify drought tolerant varieties in different crops (Darkwa *et al*. 2016; Muscolo *et al*. 2014), including in cowpea (Jain and Saxena 2016; Muchero *et al*. 2009). Some studies referred that an *in vitro* screening method based on polyethylene-glycol (PEG) is suitable for selecting tolerant genotypes able to germinate under drought stress conditions (Jain and Saxena 2016; Kocheva and Georgiev 2003; Muscolo *et al*. 2014; Ravelombola *et al*. 2017) being a good alternative method to field experiments (Steiner *et al*. 2017). Indeed, the PEG polymer has been used to mimic drought stress effects in plants with limited metabolic interferences (Murillo-Amador *et al*. 2002). Another important and appropriate methodology for determining drought tolerance levels is proline determination. The accumulation of osmolytes is a plant protection strategy against abiotic stress (Mafakheri *et al*. 2010). Proline accumulation is one of the first plant responses to water-deficit stress, in order to reduce injury to cells (Anjum *et al*. 2011). In general, proline concentration has been considered a good indicator of drought

tolerance, as higher levels are detected in stress-tolerant plants when compared to susceptible ones (Toscano *et al*. 2016).

Recently, germination and growth responses to drought stress have been reported in several crops, including legume crops as chickpea (*Cicer arietinum* L.; Dharanguttikar *et al*. 2015), common bean (*Phaseolus vulgaris* L.; Machado Neto *et al*. 2006), lentil (*Lens culinaris* Medik.; Muscolo *et al.* 2014) and soybean (*Glycine max* L. Merr.; Kpoghomou *et al*. 1990; Vieira *et al*. 1991). In general, these studies indicated a delay in initial germination and a reduction in the different germination parameters due the low water potential. Until now, few studies regarding cowpea seed germination in drought stress conditions have been developed. This is the first report of cowpea germination under drought conditions and makes use of a large set cowpea seeds from Iberian Peninsula and also from worldwide countries. The main objectives of this work are (1) the evaluation of cowpea responses to drought stress during germination, and (2) the screening of drought-tolerant cowpea genotypes from a worldwide collection. Besides the understanding of mechanisms involved in germination under drought stress, the results will be useful for selecting the best genotypes for enhancing the production of this grain legume in Southern Europe (Iberian Peninsula).

3.2.3 Material and Methods

3.2.3.1. Plant material

A total of 58 cowpea (*Vigna unguiculata* L. Walp.) genotypes were used for drought tolerance evaluation at germination stage (Table 3.2.1) being 29 from Iberian Peninsula, 26 originally collected from 17 different worldwide countries and three used as reference. In a previous study, the majority of the cowpea genotypes were already characterized using single nucleotide polymorphism (SNP) through the Illumina Cowpea iSelect Consortium Array (Carvalho *et al*. 2017b). Some of the Iberian Peninsula cowpea genotypes were also characterized through the morphological and agronomical parameters (Carvalho *et al*. 2017a). The references displayed different levels of drought tolerance: Bambey 21 (highly susceptible), CB46 (moderately susceptible) and IT93K-503-1 (highly tolerant), as described by Hamidou *et al*. (2007) and Muchero *et al*. (2008, 2011).

Table 3.2.1 – Cowpea genotypes used in this study with reference to their origin (city and country, when available) and current status.

Code	Origin	Status
C1	Ferreira do Alentejo, Portugal	Landrace
C2	Ansião, Portugal	Landrace
C3	Évora, Portugal	Landrace
C4	Mértola, Portugal	Landrace
C5	Abrantes, Portugal	Landrace
C6	Almeida, Portugal	Landrace
C7	Figueira Castelo Rodrigo, Portugal	Landrace
C8	Pinhel, Portugal	Landrace
C9	Meda, Portugal	Landrace
C10	Trancoso, Portugal	Landrace
C11	Macedo de Cavaleiros, Portugal	Landrace
C12	Penamacor, Portugal	Landrace
C13	Sabugal, Portugal	Landrace
C14	Mogadouro, Portugal	Landrace
C15	Portugal	Variety
C16	Granada, Spain	Landrace
C17	Malaga, Spain	Landrace
C18	Malaga, Spain	Landrace
C19	Orense, Spain	Landrace
C20	Girona, Spain	Landrace
C21	Baleares, Spain	Landrace
C22	Caceres, Spain	Landrace
C23	Pontevedra, Spain	Landrace
C24	Huelva, Spain	Landrace
C25	Jaen, Spain	Landrace
C26	Badajoz, Spain	Landrace
C27	Albacete, Spain	Landrace
C28	Zamora, Spain	Landrace
C29	Cordoba, Spain	Landrace
C30	Sicilia, Italy	Landrace
C31	Puglia, Italy	Landrace
C32	Cuneo, Italy	Landrace
C33	Italy	Landrace
C34	Italy	Landrace
C35	Italy	Landrace
C36	Greece	Landrace
C37	Greece	Landrace
C38	Greece	Landrace
C39	Creta, Greece	Landrace
C40	Nigeria	Cultivar
C41	Angola	Landrace
C42	Benin	Landrace
C43	Egy	Landrace
C44	Ghana	Landrace

C45	Senegal	Landrace
C46	Zambia	Cultivar
C47	Iran	Landrace
C48	Irak	Landrace
C49	Cuba	Landrace
C50	Congo	Landrace
C51	China	Landrace
C52	India	Landrace
C53	Brazil	Cultivar
C54	Bulgaria	Landrace
C55	China	Landrace
	References	
Bambey21	Senegal	Cultivar
CB46	California, USA	Cultivar
IT93K-503-1	Nigeria	Breeding line

3.2.3.2. Determination of optimal PEG concentration

A pilot experiment was performed in order to determine the optimal polyethylene glycol 6000 (PEG-6000) concentration for cowpea seed germination studies. Three cowpea genotypes (C8, C15 and C40) were tested under four PEG-6000 (Merk Millipore, Germany) concentrations, corresponding to final osmotic potentials of -0.75, -1, -1.5, -2 bars. Germination assays (six days) were performed in an incubator (Binder incubator series D, Germany) in the dark. The temperature was set for 26±1ºC, as previous results showed that cowpea genotypes had the highest seed germination rate at this temperature (data not shown), which is also in agreement with the optimal temperature reported by Jain and Saxena (2016). Uniform seeds from each cowpea genotype were selected and sterilized for about 3 min, in a 10% sodium hypochlorite solution, to prevent fungal growth. Seeds were then washed with sterile distilled water for about 3 min, four times. Following the description of Jain and Saxena (2016), ten seeds from each genotype were germinated on a two-folded filter paper, placed in a Petri dish (diameter 11 cm), containing 14 mL of PEG-6000 solutions. Distilled water (without PEG-6000) was used as control. Each Petri dish was sealed with parafilm to avoid evaporation and contaminations. Three replicates of each treatment/genotype combination were performed. Cowpea drought tolerance was evaluated by seed germination rate.

3.2.3.3. Germination conditions and experimental design

Germination assays with all cowpea genotypes were performed as previously described from December 2017 to February 2018. Drought stress was induced by two different PEG-6000 concentrations, corresponding to final osmotic potentials of -0.75 and -1.5 bars (hereinafter referred to as stress 1 and 2, respectively). Three replicates of each treatment/genotype combination were performed and separately placed on three different incubator shelves (each shelf was considered as a block). The experiment was run multiple times due to space limitations. After each run, the incubator was sprayed with 75% ethanol solution to limit any microbial growth and contamination.

3.2.3.4. Measurements and data collection

A seed was considered germinated if the radicle had one third of seed length, as described by Ravelombola *et al*. 2017. The number of germinated seeds was daily recorded, during six days. At the end, the seed germination percentage (% *G*) was calculated and the roots and shoots length of five plants was measured (*RL* and *SL*, respectively). Seed germination rate (*GR*) was calculated using the formula $GR = \sum \frac{ni}{ti}$ proposed by Silva and Matos (2016), where *ni* is the number of seeds germinated on each observation day and *ti* is the observation day. The vigor index (*VI*) was also calculated following the formula presented by Abdul-Baki and Anderson (1973), $VI = (MRL + MSL) \times \%G$, where *MRL* is the mean of root length and *MSL* is the mean of shoot length.

3.2.3.5. Proline determination

For free proline content determination, the roots of five seedlings were frozen in liquid nitrogen and ground to a fine powder. Root tissue (40 mg) was homogenized in 1 mL of 3% (w/v) sulfosalicylic acid and centrifuged at 12,000 *g* for 20 min, according to Bates (1973) with some modifications. After centrifugation, the supernatant (0.1 mL) was mixed with 0.4 mL of acid-ninhydrin and 0.4 mL of glacial acetic acid. The resulting mixture was heated for 1 h at 100ºC in a water bath. After reaction interruption by placing the tubes on ice, toluene (0.8 mL) was added and vigorously mixed. The toluene phase (upper phase) absorbance was read at 520 nm, using a spectrophotometer (PowerWave XS2, BioTek Instruments, Inc., Winooski, USA). Free proline content was

estimated by referring to a standard curve using L-proline and expressed as µg proline/mg of fresh tissue. Each sample of each combination (treatment/genotype) was used for three technical repetitions.

3.2.3.6. Data analysis

Data from germination (*% G, GR and VI*) and free proline content are presented as the mean of three independent assays with the respective SE bars ($n = 3$). Growth measurements (root and shoot length) and free content were performed from five plants per each plate ($n = 15$) and are presented as the mean of 15 repetitions. Before performing the ANOVA, all measurement data were tested for normality, according to the Kolmogorov-Smirnov and Kruskal-Wallis tests, and homogeneity with the Levene test. Non homogeneity data were observed in germination percentage, being the data transformed with the formula $arcsin\sqrt{(\%\ G/100}$) to obtain homogeneity. Differences between means were analysed with one-way and two-way ANOVA followed by Tukey's test ($p < 0.05$ was considered significant), using *IBM SPSS Statistics version 20* software (IBM SPSS, Inc., Chicago, USA). The statistical significance in mean values among genotypes was examined with Tukey's multiple comparisons tests after two-way ANOVA using the *GraphPad Prism version 7.01* software (GraphPad, Inc., California, USA). Principal component analysis (PCA) was performed using *Past version 3.19* statistical software (Hammer *et al.* 2001). The used values were normalized into percentage, taking into account the maximum value obtained from each assay, and was calculated by the ratio of stress 2 and control.

3.2.4. Results

3.2.4.1. Determination of optimal PEG conditions

A preliminary experiment was performed with the aim to select those PEG-6000 concentrations more suitable for screening cowpea (*Vigna unguiculata* L. Walp.) tolerance to drought at a germination stage. Four PEG-6000 concentrations were chosen based on their osmotic potential (-0.75, -1, -1.5 and -2 bars). In the three tested cowpea genotypes, a germination rate decrease with increasing water stress imposition through PEG-6000 was detected (Fig. 3.2.1). For the most severe stress condition (-2 bars), a

low seed germination rate was detected, not allowing to discriminate the most susceptible genotypes. For this reason, the use of such PEG-6000 concentration could make difficult to screen the most susceptible genotypes. When imposing osmotic potentials of -0.75 and -1.5 bars, a better genotype discrimination was obtained. As one-way ANOVA revealed significant differences among cowpea genotypes (F = 27.219 and 9.296, p = 0.001 and 0.015, respectively; Additional file 3.2.1), these osmotic potentials were further used for determining the drought tolerance level of a set of 55 cowpea genotypes and three references.

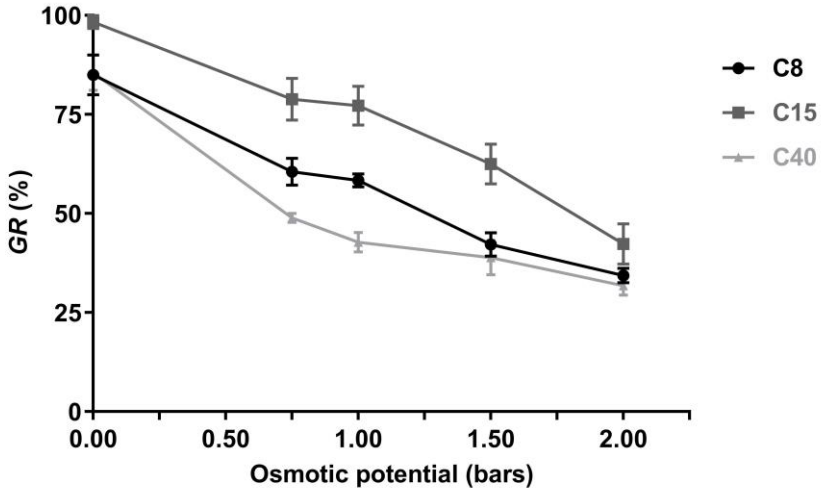


Figure 3.2.1 – Seed germination rates (*GR*) of three cowpea genotypes under four drought conditions induced by PEG-6000 (corresponding to osmotic potentials of -0.75, -1, -1.5 and -2 bars) and control (0 bars) with water (n = 3).

3.2.4.2. Drought effect on seed germination and growth parameters

The drought tolerance level of cowpea genotypes was firstly assessed by determination of seed germination percentage (% *G*), seed germination rate (*GR*), roots and shoots length (*RL* and *SL*, respectively) and vigor index (*VI*) (Table 3.2.2; Additional file 3.2.2). For all evaluated parameters, no significant differences (p > 0.05) between replicas were observed.

Table 3.2.2 - Germination and seedling emergence parameters in the 58 studied cowpea genotypes under drought stress conditions. For reference, maximum, minimum, mean, F-value and Tukey's test (significance level of 0.05) values are indicated. Means were analysed with one-way ANOVA followed by Tukey's test (significance level of 0.05) (*n* = 3 to *%G*, *GR* and *VI* and *n* = 15 to *RL* and *SL*).

Code	*% G*			*GR*			*RL*			*SL*			*VI*		
	Control	Stress 1	Stress 2	Control	Stress 1	Stress 2	Control	Stress 1	Stress 2	Control	Stress 1	Stress 2	Control	Stress 1	Stress 2
C1	100	100	93.33	0.63	0.34	0.29	11.5	8.89	7.11	5.25	1.79	1.13	1675.00	1067.78	764.00
C2	100	100	86.67	0.76	0.56	0.36	8.57	6.58	5.11	4.15	1.94	1.39	1271.94	852.22	568.89
C3	100	100	93.33	0.64	0.4	0.34	11.51	7.24	5.93	4.02	2.28	1.53	1553.33	951.83	699.78
C4	100	100	100	0.8	0.43	0.47	15.59	8.2	7.24	7.74	2.98	1.98	2332.78	1118	921.67
C5	100	100	100	0.56	0.39	0.33	12.89	8.83	6.38	5.78	2.36	1.43	1866.67	1119.44	780.83
C6	100	73.33	66.67	0.67	0.29	0.23	13.86	5.81	5.75	7.87	1.03	1.75	2172.61	502.83	504
C7	100	93.33	100	0.73	0.43	0.33	13.52	8.84	7.99	7.09	3.17	2.03	2060.67	1093.33	1002.22
C8	100	93.33	80	0.7	0.41	0.28	13.64	9.28	8.06	5.83	2.34	1.89	1947.78	1096.67	811.11
C9	100	93.33	86.67	0.8	0.41	0.33	16.6	11.24	10.51	6.67	3.47	2.01	2327.17	1349.44	1074.67
C10	80	93.33	53.33	0.33	0.31	0.16	11.16	7.91	7.22	4.49	3.24	1.44	1252.44	1057.5	458.44
C11	100	100	100	0.87	0.56	0.44	3.89	13.13	10.22	3.8	4.18	3.06	769.17	1731.67	1328.61
C12	100	100	100	0.93	0.54	0.43	12.41	7.97	9.83	7.44	4.87	3.03	1985.78	1283.89	1286.11
C13	100	100	100	0.83	0.5	0.51	13.83	9.88	9.08	8.71	3.84	2.83	2254.17	1372.5	1191.67
C14	100	93.33	100	1	0.52	0.46	8.86	9.36	9.15	3.15	2.4l	2.52	1200.67	1103.73	1166.5
C15	100	100	100	0.93	0.56	0.47	10.35	6.12	6.79	4.13	1.97	1.29	1447.83	809.33	807.44
C16	93.33	86.67	80	0.56	0.47	0.31	10.08	8.78	6.25	3.56	3.69	1.15	1270.00	1118.44	591.33
C17	100	100	100	0.49	0.47	0.38	9.23	8.43	6.2	4.28	3.67	2.42	1351.33	1209.33	861.83
C18	100	93.33	100	0.48	0.41	0.41	7.38	6.73	7.26	3.69	2.62	3.07	1107.33	885.23	1033.5
C19	100	100	93.33	0.9	0.51	0.37	16.28	9.97	7.32	8.67	2.92	2.17	2494.50	1289.00	900.84
C20	100	100	100	0.97	0.51	0.51	11.73	9.06	5.94	6.75	4	2.04	1848.61	1305.83	798.33
C21	100	93.33	100	0.8	0.62	0.58	9.93	6.29	8.36	6.15	2.58	3.18	1607.83	837.72	1153.67

C22	100	100	93.33	0.93	0.53	0.43	18.19	15.96	8.44	9.62	5.67	2.12	2780.67	2162.67	968.00
C23	100	93.33	100	0.8	0.48	0.57	14.34	8.02	7.61	7.78	3.15	2.78	2211.94	1032.22	1039.17
C24	100	100	93.33	1	0.67	0.44	16.58	8.51	10.75	8.32	5.14	2.86	2489.83	1365	1247.61
C25	100	93.33	80	0.8	0.49	0.42	15.5	6.05	5.58	8.1	4.77	2.22	2360.00	989.28	627.56
C26	100	100	100	1	0.71	0.63	11.05	5.35	4.01	7.02	2.83	2.16	1807.67	817.5	617.22
C27	60	73.33	40	0.24	0.27	0.13	8.57	9.21	5	5.65	4.23	1.75	842.67	986.00	267.67
C28	93.33	93.33	100	0.9	0.48	0.57	11.45	12.75	6.12	6.12	4.61	1.51	1625.33	1634.67	763.33
C29	100	80	100	0.9	0.41	0.47	14.33	12.95	9.59	7.79	3.00	2.83	2212.00	1304.33	1242.22
C30	100	93.33	93.33	0.83	0.57	0.47	12.66	9.42	8.48	5.73	4.77	3.11	1838.89	1313.78	1083.44
C31	73.33	53.33	86.67	0.34	0.19	0.32	12.99	8.64	8.03	6.43	2.88	2.32	1370.83	634.22	871.33
C32	100	100	100	0.48	0.52	0.42	12.66	10.02	9.37	3.84	3.79	2	1650.17	1381.00	1136.67
C33	100	100	100	0.93	0.73	0.5	8.66	7.39	8.47	4.34	3.04	3.19	1300.22	1042.50	1165.17
C34	86.67	80	86.67	0.43	0.3	0.34	10.99	10.3	8.15	3.66	2.31	1.68	1271.33	1015.33	859.33
C35	100	100	100	0.76	0.48	0.46	9.22	7.1	7.13	3.47	1.71	1.69	1269.33	880.56	881.50
C36	86.67	93.33	86.67	0.34	0.4	0.28	12.68	9.78	7.3	6.71	3.81	1.53	1650.67	1298.67	762.00
C37	100	100	100	0.83	0.56	0.54	11.81	10.7	9.43	6.6	4.22	3.12	1840.83	1491.83	1254.44
C38	93.33	100	86.67	0.42	0.46	0.34	12.24	10.12	11.23	5.68	4.75	2.57	1659.06	1486.83	1205.5
C39	93.33	80	93.33	0.51	0.31	0.38	13.78	9.52	10.99	7.17	4.45	2.24	1976.33	1090.17	1238.67
C40	100	93.33	86.67	1	0.49	0.4	8.69	8.34	5.67	5.26	3.43	1.81	1394.67	1094.83	653.61
C41	100	100	100	0.97	0.63	0.49	8.91	6.41	8.89	7.9	5.1	3.9	1681.33	1151.33	1278.67
C42	100	100	100	0.57	0.51	0.47	6.71	4.83	5.55	4.94	3.33	2.51	1165.00	816.00	806.00
C43	93.33	93.33	100	0.73	0.41	0.41	6.43	3.41	3.86	2.41	0.69	1.15	838.89	380.78	500.56
C44	66.67	100	100	0.6	0.53	0.49	8.07	9.47	9.25	4.21	4.58	3.99	880.83	1405.33	1324
C45	80	60	53.33	0.28	0.4	0.25	8.5	6.17	8.56	7.64	3.92	4.33	1357.33	636.53	663.33
C46	100	100	100	0.6	0.5	0.5	5.75	5.53	6.05	4.87	4.12	3.22	1061.5	965.72	927.17
C47	100	100	100	0.56	0.48	0.43	9.49	10.93	10.98	3.54	3.00	2.08	1303.33	1393.33	1306.33

C48	100	100	100	0.9	0.47	0.49	9.24	7.31	6.11	2.71	1.75	1.55	1195.33	905.67	766.00
C49	93.33	86.67	80	0.87	0.46	0.39	7.19	7.22	6.93	2.38	2.05	1.22	884.17	810.22	652.09
C50	100	86.67	86.67	0.42	0.43	0.42	6.54	4.59	5.76	3.04	2.6	2.26	958.67	649.33	719.5
C51	100	86.67	93.33	0.8	0.44	0.41	7.35	8.06	7.5	3.04	2.09	1.53	1039	876.22	850.13
C52	100	93.33	93.33	0.5	0.47	0.47	5.57	5.03	4.35	4.91	2.61	2.4	1048.17	722.89	636.00
C53	100	80	100	0.47	0.42	0.41	9.89	7.31	6.95	3.46	3.93	2.02	1334.67	873.17	896.83
C54	93.33	100	100	0.59	0.5	0.44	6.98	6.13	8.73	3.24	2.09	4.00	959.33	822.00	1272.5
C55	100	93.33	93.33	0.63	0.4	0.33	7.43	7.24	6.24	3.31	2.79	1.88	1074	950.67	746.67
Bambey 21	100	46.67	40	0.17	0.21	0.16	9.8	5.24	3.85	3.32	1.24	1.18	1311.67	295.11	198.67
CB46	80	93.33	93.33	0.67	0.66	0.42	8.24	7.51	7.76	4.44	2.74	1.88	1014.67	949.56	902.06
IT93K-503-1	60	93.33	93.33	0.47	0.77	0.49	3.72	4.38	5.64	1.77	1.36	1.42	329.33	530.67	651.78
Maximum	100	100	100	1	0.77	0.63	18.19	15.96	11.23	9.62	5.67	4.33	2780.67	2162.67	1328.61
Minimum	60	46.67	40	0.17	0.19	0.13	3.72	3.41	3.85	1.77	0.69	1.13	329.33	295.11	198.67
Mean	95.29	92.3	91.15	0.68	0.47	0.41	10.6	8.2	7.45	5.34	3.17	2.23	1530.82	1057.1	891.18
SD	9.91	11.63	14.31	0.22	0.12	0.1	3.29	2.37	1.87	1.95	1.14	0.78	521.68	331.16	274.34
F	14.77^{***}	5.36^{***}	7.16^{***}	2.90^{***}	2.28^{***}	4.03^{***}	11.07^{***}	6.88^{***}	4.75^{***}	6.49^{***}	3.64^{***}	4.96^{***}	8.84^{***}	4.80^{***}	5.64^{***}
Tukey$_{0.05}$	0.19	0.17	0.14	0.35	0.44	0.39	3.32	3.03	2.88	2.57	2.00	1.18	589.69	507.85	388.28

(% *G* - seed germination percentage, *GR* - seed germination rate, *RL* - root length, *SL* - shoot length and *VI* - vigor index. Control with water; stress 1 and 2 correspond to the use of PEG-6000 osmotic for obtaining a potential of -0.75 bars and -1.5 bars, respectively. **Dark gray or** *** - significant differences at level $p < 0.001$; **gray** - significant differences at level $p < 0.01$; ***light gray*** - significant differences at level $p < 0.05$; **white/clear** - no significant differences)

The seed germination percentage decreased with increasing severity of drought in 50% of the evaluated cowpea genotypes, although only three genotypes (C10, IT93K-503-1 and Bambey 21) presented significant differences ($p < 0.05$; Table 3.2.2; Additional file 3.2.2). The germination (%) of the remaining 50% genotypes was not affected even under severe drought treatment (Table 3.2.2). The differences between cowpea genotypes were indeed significant ($p < 0.001$; Table 3.2.3), which could be partially related with variations on germination capacity of each genotype (even under control conditions; Table 3.2.2). Considering all genotypes together, the differences between stress treatments revealed to be significant ($p < 0.01$; Table 3.2.3), suggesting that drought stress imposition affects seeds germination of cowpea. Besides the percentage of germinated seeds, the germination rate is considered as one of the most informative parameters in this type of studies. A drop in germination rate was observed when seeds were exposed to drought stress (Table 3.2.2), revealing that seeds take more time to germinate when subjected to drought. This result is also in accordance with the detected reduced germination percentage. Significant differences ($p < 0.001$) were also detected between treatments (control and drought stresses) and among genotypes (Table 3.2.3). While some genotypes presented a dramatically decrease on germination rate ($p < 0.001$) with increase of drought stress, others did not reveal significant differences ($p > 0.05$) between treatments (Table 3.2.2).

Table 3.2.3 – Statistical analysis of seed germination, seedling emergence and proline content evaluated in the 58 studied cowpea genotypes under drought stress conditions. Means were analysed with one-way and two-way ANOVA followed by Tukey's test (n = 3 to *%G*, *GR*, *VI* and n = 15 to *RL* and *SL* and proline content).

Treatment	Source	*DF*	*F* ratio	*Prob* > *F*
	Genotype	57	5.968	<0.001
% *G*	Treatment	2	6.330	0.002
	Genotype * Treatment	114	1.523	0.002
	Genotype	57	20.427	<0.001
GR	Treatment	2	495.982	<0.001
	Genotype * Treatment	114	4.754	<0.001
	Genotype	57	14.680	<0.001
RL	Treatment	2	187.136	<0.001
	Genotype * Treatment	114	4.472	<0.001
	Genotype	57	9.367	<0.001
SL	Treatment	2	415.854	<0.001
	Genotype * Treatment	114	3.352	<0.001
	Genotype	57	11.893	<0.001
VI	Treatment	2	26.131	<0.001
	Genotype * Treatment	114	4.287	<0.001
	Genotype	57	10.865	<0.001
Proline content	Treatment	2	78.039	<0.001
	Genotype * Treatment	114	2.928	<0.001

(% *G* - seed germination percentage, *GR* - seed germination rate, *RL* - root length, *SL* - shoot length and *VI* - vigor index)

The results also showed the commitment of seedling emergence with drought stress imposition, since root and shoot growth were generally inhibited under drought stress treatments (Table 3.2.2). Indeed, both parameters were significantly ($p < 0.001$) affected by drought stress treatments and genotypes presented significant differences for both parameters ($p < 0.001$; Table 3.2.3). A decrease in the root length was generally detected (by the means of all genotypes) when compared to control (23% and 30%, for stress 1 and 2, respectively). Many cowpea genotypes did not show variations in the root length with drought stress, but four genotypes in particular (C6, C20, C25 and Bambey 21) were significantly affected. Similarly, a reduction in shoot length was registered in many cowpea genotypes, corresponding to general decreases (means of all genotypes) of 41% and 59% (for stress 1 and 2, respectively) in relation to control. Interestingly, many genotypes that were significantly

affected in their root length, were not significantly affected in their shoot length. This result suggests that root length is more sensitive to drought conditions than shoot length.

As germination and seedling emergence may interfere with plant vigor and ultimately with crop yields, the vigor index was also determined (Table 3.2.2). The vigor index decreased significantly ($p < 0.001$) with drought severity and among genotypes (Table 3.2.3). However, while many genotypes were significantly affected ($p < 0.001$) by drought stress, some were not affected ($p > 0.05$). The genotypes C11, C54 and IT93K-503-1 (highly tolerant reference) increased their vigor under drought stress conditions (Table 3.2.2).

3.2.4.3. Drought effect on proline accumulation

Free proline content, in general (means of all genotypes), increased 1.4-fold (stress 1) and 1.7-fold (stress 2) in relation to control condition (Table 3.2.4). Differences between stress treatments were significant ($p < 0.01$; Table 3), suggesting that drought stress imposition induces the production of proline in roots. The highest increase of proline was detected in several genotypes, including the tolerant IT93K-503-1 genotype, while others did not reveal significant differences in proline content with drought stress imposition (Table 3.2.4; Additional file 3.2.2). The highly susceptible reference (Bambey 21) genotype had, in all treatments, the lowest proline content (Table 3.2.4).

Table 3.2.4 – Proline content evaluated in the 58 studied cowpea genotypes under drought stress induced by PEG-6000 and control. For reference, maximum, minimum, mean, *F*-value and Tukey's test (significance level of 0.05) values are indicated. Means (n = 15) were analysed with one-way ANOVA followed by Tukey's test (significance level of 0.05).

	Proline content		
Code	Control	Stress 1	Stress 2
C1	0.49	0.33	0.41
C2	0.21	0.33	0.35
C3	0.73	0.30	0.27
C4	0.15	0.26	0.47
C5	0.21	0.31	0.38
C6	0.15	0.54	0.91
C7	0.14	0.28	0.43
C8	0.29	0.40	0.30
C9	0.17	0.06	0.03
C10	0.23	0.56	0.35
C11	0.65	0.38	0.37
C12	0.14	0.26	0.28
C13	0.17	0.42	0.23
C14	0.17	0.38	0.8
C15	0.58	1.09	0.99
C16	0.19	0.26	0.35
C17	0.26	0.28	0.24
C18	0.11	0.24	0.32
C19	0.16	0.35	0.77
C20	0.18	0.41	0.49
C21	0.19	0.21	0.24
C22	0.13	0.13	0.22
C23	0.12	0.14	0.14
C24	0.14	0.12	0.21
C25	0.20	0.40	0.74
C26	0.16	0.32	0.40
C27	0.13	0.25	0.31
C28	0.15	0.15	0.30
C29	0.15	0.18	0.20
C30	0.11	0.17	0.32
C31	0.29	0.24	0.23
C32	0.54	0.43	0.52
C33	0.37	0.41	0.42
C34	0.17	0.31	0.55
C35	0.42	0.55	0.68
C36	0.19	0.24	0.46
C37	0.26	0.26	0.39

C38	0.19	0.20	0.34
C39	0.16	0.92	0.4
C40	0.31	0.42	0.50
C41	0.30	0.53	0.45
C42	0.34	0.42	0.35
C43	0.59	0.65	1.10
C44	0.38	0.56	0.78
C45	0.36	0.34	0.25
C46	0.25	0.36	0.41
C47	0.42	0.41	0.64
C48	0.30	0.53	0.84
C49	0.16	0.34	0.8
C50	0.32	0.46	0.51
C51	0.13	0.33	0.55
C52	0.20	0.57	0.54
C53	0.15	0.47	0.57
C54	0.08	0.46	0.76
C55	0.23	0.44	0.67
Bambey 21	0.52	0.59	0.42
CB46	0.32	0.27	0.32
IT93K-503-1	0.62	0.89	1.38
Maximum	0.73	1.09	1.38
Minimum	0.08	0.06	0.03
Mean	0.27	0.38	0.48
SD	0.15	0.19	0.25
F	14.77^{***}	5.36^{***}	7.16^{***}
$Tukey_{0.05}$	0.3	0.26	0.31

Control with water; stress 1 and 2 correspond to the use of PEG-6000 osmotic for obtaining a potential of -0.75 bars and -1.5 bars, respectively. **Dark gray or** *** - significant differences at level $p < 0.001$; **gray** - significant differences at level $p < 0.01$; ***light gray*** - significant differences at level $p < 0.05$; **white/clear** - no significant differences.

3.2.4.4. Screen of genotypes to drought tolerance

Different development measures and proline accumulation, evaluated in the most severe stress condition (stress 2), were normalized in relation with control conditions and used for discriminating cowpea genotypes tolerance/susceptibility with a principal component analysis (PCA; Fig. 3.2.2). First two principal components of PCA explained 97.52% of total variation (PC1 = 88.75% and PC2 = 8.77%), being proline (PC1, 0.99) and vigor index and root length (PC2, 0.64) the three most contributive parameters. PCA clustered the genotypes C18, C46, C47, C50, and in particular C44, close to the tolerant reference IT93K-503-1. On

the other hand, the C3 and C9 genotypes were grouped close to the susceptible reference genotype Bambey 21. C11 and C54 genotypes, which presented enhanced responses to drought stress, are distant from the remaining genotypes, suggesting a different performance than other genotypes.

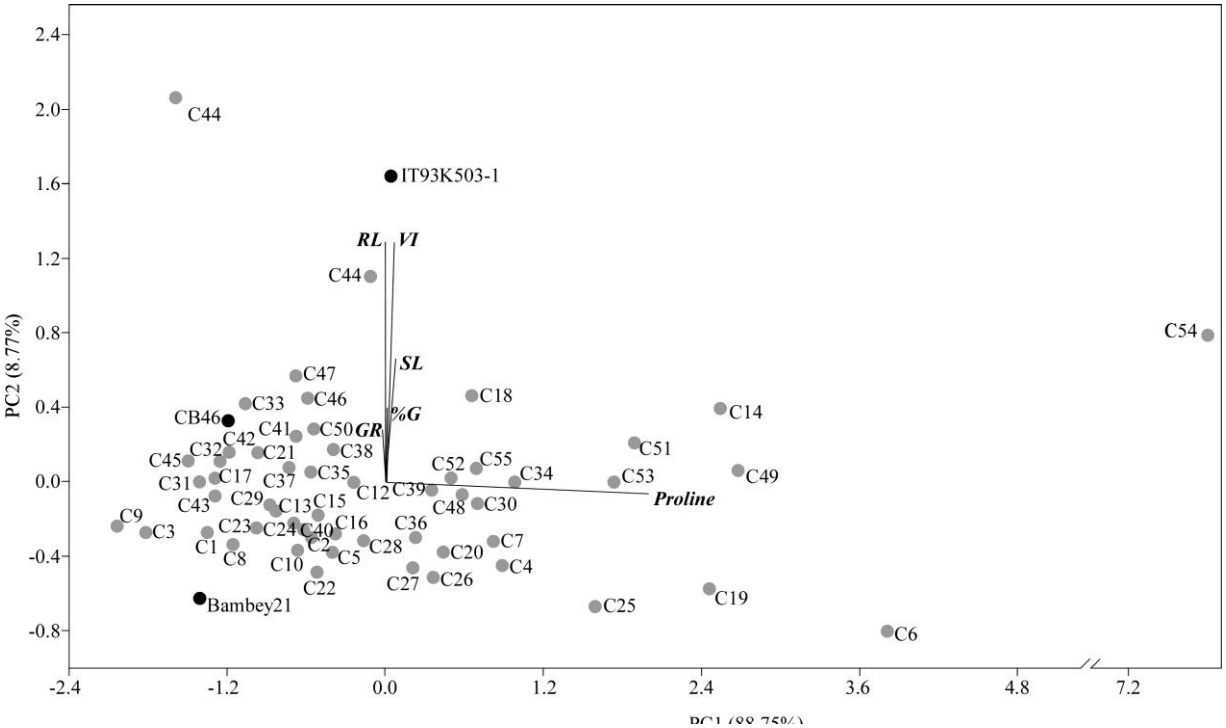


Figure 3.2.2. Principal component analysis with all the cowpea genotypes data, obtained from stress 2 versus control. PCA was performed using the results of seed germination percentage (% *G*) and rate (*GR*), root (*RL*) and shoot (SL) length, vigor index (*VI*) and proline content.

3.2.5. Discussion

One of the most serious limitations to crops yield is drought. This multifaceted stress condition is differently sensed by plants depending on their growth stage, stress duration and severity (Ahmad *et al*. 2009). Drought stress during germination can impose a critical limitation to plant development, mainly because seed germination is the most sensitive stage in plant life cycle (Ahmad *et al*. 2009; Muscolo *et al*. 2014; Hellal *et al*. 2018). When seeds are exposed to water stress (or to other unfavorable environmental condition), plants establishment can be compromised (Ahmad *et al*. 2009; Muscolo *et al*. 2014). The selection of cowpea (*Vigna unguiculata* L. Walp.) genotypes with increased seed tolerance to drought is a reasonable strategy for the selection of accessions for enhancing cowpea production in a

climate change scenario. The use of an *in vitro* screening method on seeds, where drought imposition was artificially imposed by PEG-6000, allowed the assay of 58 cowpea genotypes, which were compared with three susceptible/tolerant genotypes. A wide range of PEG-6000 osmotic potentials was initially tested and presented a dose-dependent detrimental effect on seed germination. This polymer adversely affected the germination and seedling growth of cowpea genotypes, as observed in previous studies (Khodarahmpour 2011). PEG-6000 concentrations corresponding to osmotic potentials of -0.75 and -1.5 bars were considered adequate to induce stress for the cowpea, while displaying discriminatory resolution among cowpea genotypes. For this reason, both PEG-6000 concentrations were used for imposing drought stress conditions and evaluate the most tolerant drought genotypes.

Considering that tolerant genotypes have higher capacity to germinate and emerge from seeds than susceptible ones, the obtained results could indicate which are the most susceptible and tolerant cowpea genotypes. Previous results obtained under different drought stress conditions, but using mature plants, revealed Bambey 21 as highly susceptible, CB46 as moderately susceptible and IT93K-503-1 as highly tolerant genotypes (Hamidou *et al*. 2007; Muchero *et al*. 2010, 2008). This classification was previously confirmed by us, using different physiological and biochemical approaches on drought-stressed mature plants (unpublished data). The results here presented revealed that a similar trend was observed at germination/seedling stages, suggesting that the mentioned genotypes (Bambey 21, CB46 and IT93K-503-1) could be used as susceptible/tolerant reference genotypes. On the other hand, several studies in other crop species (e.g. Beshir *et al*. 2016; Dodig *et al*. 2015), including in cowpea (Singh *et al.* 1999), have revealed a close correspondence of drought tolerance observed in seedlings and reproductive stage plants. Different seed germination and seedling emergence capacities were displayed by distinct cowpea genotypes under stress conditions. As a large proportion of cowpea genotypes did not present any difference on seed germination percentage between treatments, PEG-6000 treatments could not have a strong influence during this stage. According to Mickky and Aldesuquy (2017), the use of PEG-6000 causes a delay in seed germination, as it happens naturally in the drought, but the seed germination percentage is not affected. In the present work, the decrease of seed germination rate was indeed more evident that the decline in germination (%, six days after sowing) and one of the most pronounced parameters under study. This result is in accordance with other cowpea studies, where the seed germination rate (*GR*) also decreased with drought stress induced by PEG-6000 (Araújo *et al*. 2018; Ferreira *et al.* 2017; Murillo-Amador *et al*. 2002). Several

cowpea genotypes revealed significant alterations in their germination with PEG-6000 treatments, while others were not so affected, suggesting that distinct genotypes could be differently disturbed by drought. This variability can be considered as a valuable tool for screening cowpea genotypes more tolerant and adapted to climate change. From the assayed cowpea genotypes, 16 cowpea genotypes, including the moderately susceptible CB46, revealed non-significant changes on both germination parameters. These results can be a valuable information about the possible drought tolerant genotypes.

Root length is pointed as another key trait for the selection and differentiation of drought tolerant genotypes, due to the role of roots in providing water and maintaining an adequate water balance in plants. Roots are thus deeply affected when plants are subjected to water stress and are the first plant organs suffering from water stress during seedlings development (Silva and Matos 2016; Trachsel *et al*. 2013). Water stress causes decrease of cellular division, increase of rigidification of cell wall resulting in a reduction of root elongation and root-hair development during germination (Muscolo *et al*. 2014; Silva and Matos 2016). In general, with increasing of water stress, cowpea seedlings presented a higher decrease of root length than shoot length. This result is in agreement with others studies that verified that the symptoms observed in shoots are normally softer and can be delayed relative to the root (Silva and Matos, 2016). The C6 and Bambey 21 (susceptible reference) genotype (together with C20 and C25 genotypes) were the most affected under drought stress conditions, indicating a higher susceptibility to osmotic stress. In contrast, the drought-tolerant reference (IT93K-503-1) and several other genotypes did not present significant differences between treatments in root length and shoot length, suggesting them as drought tolerant genotypes.

Seedling vigor index is another important parameter that combines seed germination percentage and seedling growth data. The values obtained for this parameter decreased in all genotypes with increasing water stress, except for the tolerant reference (IT93K-503-1) and also C11 and C54 genotypes. In all these cowpea genotypes, the vigor index values increased with water stress conditions, indicating that they display some capacity to tolerate drought. Furthermore, C11 and C54 could be so adapted to water limiting conditions that seem to have a preference for water scarcity during its development. Furthermore, the moderately susceptible reference (CB46) and other four genotypes (C33, C14, C46 and C47) were the least affected by drought stress presenting the lowest decrease of vigor index in the three treatments, being also considered as possible drought tolerant genotypes. On the other hand, a

drastic decrease on vigor was observed in the susceptible reference (Bambey 21), similar to decreases observed for other three cowpea genotypes (C6, C25 and C26), followed by others genotypes such as C19, C20 and C22. These results suggest higher susceptibility of these genotypes to drought. Moraes *et al*. (2005) in common bean (*Phaseolus vulgaris* L.) and Cokkizgin (2013) in pea (*Pisum sativum* L.) also reported a decrease of seedling vigor index with the increasing of PEG-6000 concentrations. The same result was also obtained in cowpea by Jain and Saxena (2016) using PEG-4000.

As proline is one of the compatible solutes that plants accumulate under water stress being the accumulation of this osmolyte correlated with stress tolerance (Anjum *et al.* 2011). Proline accumulation is commonly associated with the increase of cell osmotic potential, facilitating the water absorption (Ashraf and Foolad 2007; Toscano *et al.* 2016), but can also reduce cells injury (Anjum *et al*. 2011). Our data agree with proline protective role, as proline content generally increased in all genotypes under stress conditions, presenting the tolerant reference (IT93K-503-1) the highest contents. A significant increase in proline with drought imposition was also observed for other 13 cowpea genotypes. Other studies revealed similar increases in other cowpea genotypes (Cavalcanti *et al*. 2004; Merwad *et al*. 2018), as well in other species, such as in soybean (*Glycine max* L. Merr.; Mwenye *et al*., 2016) or chickpea (*Cicer arietinum* L.; Mafakheri *et al.* 2010). On the other hand, in the present study some genotypes did not reveal any difference in the proline content under drought conditions, as the moderately susceptible reference CB46 (together with C8, C32 and C42). In others genotypes, the proline content decreased with drought stress, such as in the highly susceptible reference Bambey 21 (and also in C1, C3, C9, C31 and C45).

Although a common trend is observed for all cowpea genotypes under drought stress (germination and seedling development alterations), each genotype displays a more specific response, probably due to the processes to which they are more susceptible/tolerant. For example, C21 genotype is greatly affected in shoot development, while C33 is significantly affected in seed germination rate. The most susceptible genotypes will be affected in most of evaluated parameters, as detected for Bambey 21, while the most tolerant will be unaffected, like observed for IT93K-503-1. Taking this into consideration, our data suggest that the most tolerant cowpea genotypes were C16, C18, C44, C46, C47, C50, C53, and in a lesser extent C38, C43, C52. In contrast, the most susceptible genotypes seem to be C6, C22, C24, C25, and in a lesser extent C7, C20, C28, C40. A PCA performed with normalized data (ratio between the highest drought stress imposition and control) showed that the reference

genotypes Bambey 21 and IT93K-503-1 had divergent drought responses, corroborating the previous studies of Hamidou *et al*. (2007) and Muchero *et al.* (2008, 2010). Close to the tolerant reference (IT93K-503-1) was the genotype C44, and also C18, C46, C47 and C50. Regarding, C11 and C54 genotypes, the PCA revealed that they present a different drought response from all the others genotypes under study, presenting a general increase of studied traits, consistent with drought tolerant genotypes. For other hand, the susceptible reference (Bambey 21) was very close to the genotypes C3 and C9.

Most of the evaluated genotypes had been previously included in a genetic diversity study using single nucleotide polymorphisms (SNPs), revealing that they were grouped based on their geographical origin (Carvalho *et al*. 2017b). The suggested tolerant genotypes C46 (Zambia), C47 (Iran) and C50 (Congo) were considered admixed due they have information from several subpopulations. Probably, these genotypes are the result of introgression of genetic material on other lines and subsequent selection by farmers based on their adaptation to specific environmental conditions, in these cases all tropical and sub-tropical weather. The other suggested tolerant genotypes (C11, C18 and C44) were from Portugal, Spain and Spain, respectively, and belong to different subpopulations from genetic analysis (Carvalho *et al*. 2017b). These genotypes could be a source of variability and could be useful for the improvement of new varieties to mitigate the effects of climate change.

3.2.6. Conclusions

The selection of cowpea (*Vigna unguiculata* L. Walp.) genotypes well-adapted to upcoming climate change (including drought) is a key step for improving crop production. Drought can inhibit the germination and subsequent seedling growth, impairing the crops establishment. Therefore, the germplasm screening at an early growth stage is a reasonable approach for selecting tolerant genotypes to drought conditions. PEG induction is a simple, cost effective and fast method of drought induction allowing to screen a large number of genotypes. Various seed germination and seedling emergence features could be evaluated to have a complete picture of drought responses in an early stage, but root length, vigor index and proline contents were the most consistent and informative, enabling to infer about genotypes drought tolerance. A response variation was identified in this collection of cowpea genotypes that can be further explored by plant breeders. Our results suggest that C11

(Portugal), C18 (Spain), C44 (Ghana), C46 (Zambia), C47 (Iran), C50 (Congo) and C54 (Bulgaria) cowpea genotypes showed a high drought tolerance at germination stage. These accessions could be further used as parents for developing segregating populations for cowpea drought tolerance and to get of new varieties.

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3.2.8. Additional files

Additional file 3.2.1. Statistical analysis of seed germination rates of three cowpea genotypes (C8, C15 and C40), under four drought conditions induced by PEG-6000 (corresponding to osmotic potentials of -0.75, -1, -1.5 and -2 bars) and control with water (*n* = 3).

Osmotic potencial (bars)	Source	DF	Sum of square	Mean square	F ratio	Prob > F
Control	Genotype	2	0.034	0.017	3.591	0.094
	Error	6	0.029	0.005		
-0.75	Genotype	2	0.179	0.089	27.219	0.001
	Error	6	0.020	0.003		
-1	Genotype	2	0.137	0.069	18.841	0.003
	Error	6	0.024	0.004		
-1.5	Genotype	2	0.098	0.049	9.296	0.015
	Error	6	0.032	0.005		
-2	Genotype	2	0.018	0.009	2.587	0.155
	Error	6	0.021	0.004		

Additional file 3.2.2. Statistical significance differences of control (upper table) and stress 2 (down table) treatments among genotypes in all parameters evaluated (*% G, GR, VI, RL, SL* and proline) using Tukey's multiple comparisons tests after two-way ANOVA. *** - significant differences at level $p < 0.001$; ** - significant differences at level $p < 0.01$; * - significant differences at level $p < 0.05$; ns - no significant differences.

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CHAPTER 4

Concluding remarks and future perspectives

The detailed discussion about cowpea genetic diversity and drought stress responses was presented in the previous chapters. Accordingly, this chapter provides a summary of thesis main conclusions and future perspectives.

Global climate change is one of the most critical challenges for the near future, enhancing both abiotic and biotic stresses. This will have a huge impact on plant development and yield. Among abiotic constraints, drought and heat stresses are key factors that will restrain the production of grain legumes within the Mediterranean area. This impairment is expected to have a special effect during reproductive stage (Daryanto *et al.* 2015). Cowpea farming can play an important role for a more sustainable agriculture, due to its ability of growing in a wide range of soils and ability to fix nitrogen from atmosphere. This crop also revealed a high capacity to tolerate drought and heat (Agbicodo *et al*. 2009). Furthermore, cowpea is an affordable dietary source of proteins (Sultani *et al*. 2007; Jensen *et al*. 2012; Daryanto *et al*. 2015). Due to all these characteristics, the increase of cowpea production is highly required for meeting the plant protein demands in European countries, where the Southern European countries have the most favorable climatic conditions for cowpea growing. Considering the climate projections pointing to temperatures increasing and limited water resources, new effective approaches are fundamental for obtaining more productive cowpea varieties with increased drought tolerance.

For the identification of those cowpea genotypes more suitable for sustainable and resilient farming systems, the assessment of genetic diversity through morphological, agronomical and molecular traits is fundamental. **Chapter 2** gives an overview about cowpea genetic diversity, suggesting some cowpea genotypes that could be grown in Southern European countries. Landraces are farmer-developed populations that result from centuries of local adaptation and represent a valuable source of genes for plant breeding (Corrado and Rao 2017). For this reason, they were the basis of this study. When using morphological and agronomical characterization tools, a high genetic diversity was found in a set of Iberian Peninsula cowpea landraces (**sub-chapter 2.1**). The number of pods and seeds per plant and the seed weight were the most variable traits. An overall analysis revealed that Portuguese and Spanish genotypes were separately grouped, indicating a possible specific gene pool structure of this genotypes set. Taking this into consideration, the ten most promising genotypes were selected and, in order to evaluate their environmental stability, their

morphological and agronomical features were compared in three distinct environments (Cartagena in Spain; Elvas and Vila Real in Portugal), during two consecutive years (2015 and 2016) (**sub-chapter 2.2**). Significant interactions among genotypes, environments and years were observed. The best environment for the production of these set of cowpea genotypes was Elvas, South of Portugal, being yield the most informative trait. Other important trait for cowpea genotypes selection is the flowering date, once an early flowering allows to avoid periods with higher heat and drought. The obtained results suggest that Vila Real and Cartagena are the most suitable environments for early flowering of the studied set of cowpea genotypes. The genetic diversity of Iberian Peninsula cowpea genotypes, studied in sub-chapters 2.1 and 2.2, revealed somehow contrasting results, probably due to the environmental effect on the morphological and agronomical characterization. Accordingly, the conventional morphological and agronomical characterization has been reported to be difficult for getting clear genotypes evaluation, owing to the effects of environmental conditions. For this reason, the use of molecular markers, such as single nucleotide polymorphisms (SNPs), is crucial for complementing and more accurately determine the genetic diversity among genotypes, allowing an effective use of the germplasm, especially in plant breeding programs. SNPs are environmental independent and give a more precise and objective evaluation, ensuring that a trait can be selected regardless of environmental conditions. A publicly available resource, *Cowpea iSelect Consortium Array*, was recently developed (Muñoz-Amatriaín *et al.* 2017) and allowed the evaluation of cowpea genetic diversity and population structure. This tool allows the screen of 51,128 SNPs and was used in this work in a worldwide cowpea genotypes collection that included the two most cultivated *Vigna unguiculata* subspecies, *unguiculat*a and *sesquipedalis* (**sub-chapter 2.3**). This was the first study that used the high-density SNP genotyping through *Cowpea iSelect Consortium Array* for evaluating the affinity between both sub-species. All the set of cowpea genotypes was grouped in four subpopulations, being one of them only composed by genotypes from *V. unguiculata* ssp. *sesquipedalis*. The other three subpopulations were grouped based on the geographical origin of cowpea genotypes and allowed to suggest possible cowpea dispersion routes. This work is in agreement with other studies that have suggested an important role of Iberian Peninsula countries in the exchange and acclimatization of new and old world crops during the discoveries period (Saúco and Cubero 2011). The Iberian Peninsula cowpea genotypes were mainly grouped in the same sub-population and revealed a low genetic diversity in comparison to other worldwide collected

genotypes. Three Iberian Peninsula cowpea genotypes (one belonging to another subpopulation and two considered as admixtures of different subpopulations) were suggested as a source of genetic diversity. These genotypes could introduce additional diversity into the Iberian Peninsula genepool, which could be an interesting strategy for increasing cowpea yields, under a climate change scenario, without compromising environmental adaptation.

Plants develop different morphological, physiological, biochemical (including metabolic) and molecular stress strategies to overcome drought (Mitra 2001; Chaves *et al*. 2003). Until now, the exact processes underlying drought tolerance in cowpea are not established and the most suitable approaches for evaluating cowpea drought tolerance are still controversial. An overview about the fundamental knowledge on cowpea drought stress responses and methodologies that could be useful to screen cowpea drought tolerance is presented in **chapter 3**. Several physiological (stomatal conductance and chlorophyll *a* fluorescence), biochemical (stress markers and antioxidant activity enzymes) and molecular (gene expression of drought stress-related and oxidative stress-related genes) approaches were evaluated in four cowpea genotypes under drought conditions (**sub-chapter 3.1**). An overall analysis suggested that stomatal conductance, free proline and anthocyanins content were the most discriminating methods for determining cowpea drought tolerance level. Among several described drought stress-related genes, two candidate genes (*VuCPRD14* and *VuHsp17.7*) were associated to cowpea drought responses. Screening and selection of cowpea genotypes for drought tolerance are pivotal for the development of new varieties. Some reports referred that screen of tolerant genotypes during seed germination stage could be a good approach (Muscolo *et al*. 2014; Jain and Saxena 2016). Taking into consideration this information, worldwide cowpea genotypes were submitted to different polyethylene glycol 6000 (PEG-6000) osmotic potential, during seed germination and seedling emergence (**sub chapter 3.2**). This study allowed to identify seven cowpea genotypes as drought tolerant, from a collection of 58 genotypes. All these genotypes can represent an advantage and raw material for future breeding programs for achieving higher cowpea resilience to climate change.

The main focus of this thesis was the evaluation of cowpea genetic diversity and plant responses to drought. This was only a starting point to future research. Based on the work presented in this thesis, there are several issues that would be important to study in a near future.

Currently, cowpea is mostly consumed as grain. However, leaves and immature pods and seeds could be also consumed as green vegetables (Timko and Singh 2008). For example, immature pods have been consumed at a small scale in some parts of Southern Europe. the upscale of consumption to other cowpea products (other than grain) would be an opportunity for producers to increase the incomes taken from this crop. The diversity evaluation of cowpea immature pods and seeds traits, as well as the definition of the most promising landraces regarding yield, antioxidant capacity and phenolic composition, would be an interesting starting point for increasing the market potential and consumption of novel cowpea products.

Data from high-density SNP genotyping arrays, combined with consensus genetic maps of cowpea, provide an excellent opportunity for the management of biodiversity conservation through genome-wide association studies (GWAS) (Muchero *et al*. 2009; Muñoz-Amatriaín *et al*. 2017). GWAS of cowpea germplasm allows to discover promising alleles, either for simple or complex traits, like already performed in cowpea for pod length (Xu *et al*. 2017) and seed coat (Herniter *et al*. 2018). A similar work for flowering days and seed number per pod would be interesting, as these two traits are of great value to legume (including cowpea) producers. The determination of which genes are related to drought would be also a crucial step to breeders for selecting better genotypes for mitigating climate change constrains.

The screening of drought tolerant genotypes could also be performed using other perspectives. Cowpea has the capacity to support drought stress during its vegetative-stage, without a considerable decrease on yield and displaying a rapid recover ability (Muchero *et al*. 2008). Therefore, the screening of drought tolerance levels during the vegetative-stage, by evaluation of drought recovery capacity would be interesting. This approach could be used for screening cowpea genotypes by the identification of several shoot phenotypic responses, such as stem greenness, unifoliate senescence, wilting, trifoliate abscission, visual anthocyanin score and type of recovery. Other aspect that could be evaluated is the root system. The root system is fundamental for plant water acquisition and in legumes has been described to be closely related to drought tolerance mechanisms (Matsui and Singh 2003). The evaluation of root system traits (as root length density, rooting depth and root dry matter) would be useful as a screening methodology for drought-tolerance.

Plant seeds are rich in several nutrients, including proteins. Storage proteins, commonly known as globulins, are the most common proteins in legumes seeds (Sales *et al*.

2000). These proteins have an important role during plant establishment and development, mainly due to their capacity to act as a storage reserve for nitrogen, carbon and sulphur (Wobus *et al*. 1995; Krishnan and Coe 2001). For these reasons, the gene expression pattern of seed storage globulins from cowpea plants under drought stress conditions would be important for understanding the seed germination capacity under a climate change scenario. An evaluation of drought stress effects in seed storage protein gene expression in different seed development stages (immature pods and seeds from growing pods) was already performed in two distinct environments (Vila Real and Cartagena). Currently, a manuscript containing these results is being prepared for publication.

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ANNEX

Published papers and submitted manuscripts

Review

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Cowpea: a legume crop 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 a good protein quality with a high nutritional value, its nitrogen-fixing ability, and an ability to be more drought- and heat-tolerant than most of its legume relatives. In a research perspective, studies of cowpea are relatively scarce, despite its relevance to agriculture in the developing world and its resilience to stress. The present review provides an overview of different aspects of cowpea, with a special emphasis on the molecular markers for assessing genetic diversity, as well as on biochemical and transcriptomic data with respect to evaluating cowpea drought stress tolerance. The integration of both datasets will be useful for the improvement of cowpea because research on drought stress tolerance is of major interest for this crop in a challenging environment.

Keywords: cowpea; genetic diversity; morphological traits; molecular markers; drought stress; gene expression

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 the human diet, as well as in 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, in salads, snacks and cakes, amongst others. Also, young leaves, fresh pods and fresh seeds have been consumed in some world regions.[4,5] Green organs could be used as a vegetable and are often served boiled, as well as being consumed fried or fresh.[5] One of the most important characteristics of cowpea is the high nutritive content value of all plant parts.[3,4,6] The dry grain is rich in proteins (23–32%), as well as essential amino acids such as lysine (427 mg g^{-1} N) and tryptophan (68 mg g^{-1} N), although it is low in sulphur-containing amino acids.[7,8] Accordingly, 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 vitamins (folic acid and vitamin B) has also been reported to be important in preventing birth defects during pregnancy.[3,9,10] Dry grain is also high in fibre and low in fat.[8] Taking into account these advantages, an increase in cowpea production and consumption in the European Union is highly desirable. Currently, the European Union imports almost all of the cowpea consumed from African countries, more specifically from Niger and Nigeria. During the period 2009–2013, the world cowpea planting area was 5 million hectares and the worldwide production was 12 million tonnes. Africa has been responsible for 95.4% of worldwide cowpea production,[11] with the drier savannah and the Sahelian region of West and Central Africa being responsible for producing 72% of the total. Nigeria and Niger are the largest producers, with 3.4 and 1.4 million tonnes, respectively. By contrast, Europe is only responsible for 0.4% of worldwide cowpea production and the European Union has 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 is related to its ability to establish associations with distinct microorganisms, mainly nitrogen-fixing bacteria (e.g. rhizobia) and vesicular-arbuscular mycorrhizal fungi. Cowpea tolerance to low fertility soils[8,12,13] and 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. The present review provides an overview of different issues about genomic and transcriptomic studies in cowpea, with an emphasis on studies related to genetic diversity and cowpea drought stress tolerance that could be useful with respect to integration in cowpea breeding programs.

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Research Article

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European cowpea landraces for a more sustainable agriculture system and novel foods

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Abstract

BACKGROUND: Genetic diversity is fundamental to breeding programs and consequently has an important role in obtaining new varieties. To properly use the genetic diversity present in germplasm collections, a good knowledge of the agro-morphological traits of each accession is needed. The aim of this study was to explore the production capacity of 24 cowpea landraces from southern Europe, through phenotypic characterization and evaluation in three different locations in Greece and Portugal.

RESULTS: Most qualitative parameters tested showed a high stability among the three locations. A wide difference was observed among the three locations with respect to number of days to flowering, ranging from 55 to 99 days. Quantitative traits showed a higher genotype × environment than genetic variance component. In general, an inverse relationship between σ^2_{ge}/σ^2_g ratio (where σ^2_{ge} is genotype × genotype interaction and σ^2_g is genotype impact) and heritability value was observed. Principal component analysis was able to group accessions based on their origin. The first two principal components explained 97.52% of variation, being the number of seeds per plant, plant height and seed protein content, the traits which contributed most to variability.

CONCLUSION: The results show that sufficient variation exists in different traits within landraces in the studied cowpea germplasm to pursue a breeding program. However, the quantitative traits showed a higher genotype × environment component.

Keywords: *Vigna unguiculata* L. Walp; G × E interaction; landraces; qualitative traits; quantitative traits

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is a primarily self-pollinated species of the genus *Vigna*, a member of the Leguminosae family. Different areas have been proposed as cowpea domestication centers,[1,2] although it is unquestionably of African origin.[3] Introduction of cowpea in Europe has been reported to occur throughout the eastern part of the Mediterranean Basin, as it was certainly cultivated by the Romans in the first century AD.[4,5] This grain legume is cultivated in many tropical and subtropical regions of the world.[6]

Nowadays, cowpea is cultivated on a small scale in southern European countries, representing only 0.43% of the total cowpea seed production, amounting to 5.59 million tonnes in 2014.[7] Cowpea is mainly used in the human diet but also as forage for animal feeding. It is mainly cultivated for its dry grain, although in some regions young leaves, fresh pods and fresh seeds are also consumed,[8] constituting a significant source of proteins, essential amino acids, minerals, vitamins and fiber.[9,10]

Agricultural productivity of food legumes, grown in semi-arid areas or drylands, e.g. the Mediterranean Basin, is usually characterized by instability, as it is influenced by several environmental constraints, such as water scarcity and extreme temperatures[11,12] that prevail in these areas.[13] Tolerance to low water regimes and

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Genotype by environment interactions in cowpea (*Vigna unguiculata* L. Walp.) grown in the Iberian Peninsula

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Abstract. The aim of this work was to determine the variance components and genetic and environmental stability of 12 cowpea genotypes at three locations (South-east of Spain: Cartagena, South and North of Portugal: Elvas and Vila Real, respectively) in the Iberian Peninsula in two consecutive years (2015 and 2016). The genotype, the environment and the genotype × environment interaction significantly influenced all the morphological and agronomical parameters evaluated. For both years, the highest yields were observed at Elvas, whereas Cartagena and Vila real were the most suitable places to obtain crop precocity. Cartagena was the place where the filling of the seed was the fastest, probably due to the higher temperatures and radiation. The thermal time model (effective degree-days) could be used to predict the period of cowpea development, therefore predict flowering and pod maturity date. Correlation analysis showed that days to flowering, days to maturity and the seed yield vs protein content exhibited negative correlations. The highest heritability was found for plant height and pod length at Cartagena and for 100-seed weight at Elvas and Vila Real. In conclusion, the variations that exist in the studied accessions could give rise to a breeding program to develop cowpea cultivars with interesting agronomic traits.

Additional keywords: degree-days, heritability, legumes, protein content, yield.

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Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is originated in Southern Africa and belongs to the family *Fabaceae*, tribe *Phaseoleae* and genus *Vigna*, which comprises several species, subspecies and varieties depending on morphology and domestication (Padulosi and Ng 1997). Cultivated cowpea belongs to *V. unguiculata*, spp. *unguiculata*, which contains the cultigroups Unguiculata, Biflora, Sesquipedalis and Textilis (Ng and Marechal 1985). This annual warm-season legume is one of the most widely adapted, versatile, and nutritious grain legumes (Ehlers and Hall 1997). During the 2010–2014 period, the world cowpea planting area was 58.1 million hectares and the production was 33.5 million tonnes. Africa has been responsible for 95.8% of worldwide cowpea production (FAOStat 2017). Nigeria and Niger are the largest producers with 3.4 and 1.6 million tonnes, respectively. In contrast, Europe is only responsible for 0.4% of worldwide cowpea production (FAOStat 2017). Now-a-days, cowpea is mainly grown by subsistence farmers in west and central sub-Saharan Africa, but also is an important food source in the rest of Africa, Central and South America, South-east Asia and in the southern United States (Davis *et al.* 1991; Timko and Singh 2008). In addition, cowpea is being cultivated at a small scale in many parts of Southern Europe and countries around the Mediterranean Basin (Domínguez-Perles *et al.* 2015), providing

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Genetic diversity and structure of Iberian Peninsula cowpeas compared to world-wide cowpea accessions using high density SNP markers

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Abstract

Background: Cowpea (*Vigna unguiculata* L. Walp) is an important legume crop due to its high protein content, adaptation to heat and drought and capacity to fix nitrogen. Europe has a deficit of cowpea production. Knowledge of genetic diversity among cowpea landraces is important for the preservation of local varieties and is the basis to obtain improved varieties. The aims of this study were to explore diversity and the genetic structure of a set of Iberian Peninsula cowpea accessions in comparison to a worldwide collection and to infer possible dispersion routes of cultivated cowpea.

Results: The Illumina Cowpea iSelect Consortium Array containing 51,128 SNPs was used to genotype 96 cowpea accessions including 43 landraces and cultivars from the Iberian Peninsula, and 53 landraces collected worldwide. Four subpopulations were identified. Most Iberian Peninsula accessions clustered together with those from other southern European and northern African countries. Only one accession belonged to another subpopulation, while two accessions were 'admixed'. A lower genetic diversity level was found in the Iberian Peninsula accessions compared to worldwide cowpeas.

Conclusions: The genetic analyses performed in this study brought some insights into worldwide genetic diversity and structure and possible dispersion routes of cultivated cowpea. Also, it provided an in-depth analysis of genetic diversity in Iberian Peninsula cowpeas that will help guide crossing strategies in breeding programs.

Keywords: *Vigna unguiculata*, Single nucleotide polymorphism, Genetic diversity and variation, Population structure

Background

Cowpea (*Vigna unguiculata* L. Walp., 2n = 2× = 22) is a member of the Fabaceae family and one of the most important grain legumes growing in tropical and sub-tropical regions [1]. Grain-type cowpea, also known as common cowpea or African cowpea belongs to subspecies *unguiculata* while vegetable cowpea, commonly known as asparagus bean or 'yardlong' bean, belongs to subspecies *sesquipedalis* [2]. These two subspecies are differentiated mainly by their plant architecture, pod size and thickness, and end use [3, 4], but they both possess a high protein content [3, 5]. Other important characteristics of cowpea are the capacity to fix atmospheric nitrogen through symbiosis with root nodule bacteria [6], the ability to grow in low fertility soils [7], and the high tolerance to high temperatures and drought [8]. These attributes make cowpea a key crop in the context of global climate change and food security. In Southern Europe, namely the Iberian Peninsula, rainfall is projected to decrease while temperature is projected to increase [9].

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Keywords: Biochemical and molecular markers; Genotype discrimination; Physiological parameters; Vigna unguiculata L. Walp.; Drought stress

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Abstract: Drought impact on plants is an increasing concern under the climate change scenario. Cowpea (Vigna unguiculata L. Walp.) is considered as one of the most tolerant legume crops to drought, being the search for the best well-adapted genotypes crucial to face the future challenges. Different approaches have been used for differentiating plant responses to drought stress. Plants of four cowpea genotypes were submitted to three watering regimens (a severe and moderate drought stress, and well-watered control) during 15 days, and several physiological, biochemical and molecular parameters were evaluated. Stressed plants revealed commonly-described drought stress characteristics, but not all assayed parameters were useful for discriminating plants with different drought severities or genotypes. The analyses which have contributed most to genotype discrimination were those related with stomatal function, and biochemical markers such as proline and anthocyanin contents. Antioxidant enzymes activities and related genes expression did not differed among genotypes or upon drought stress treatments, suggesting that scavenging enzymes are not involved in the differential ability of cowpea plants to survive under drought stress. This information will be useful to evaluate and use genetic resources, as well as design strategies for breeding cowpea resistance to drought stress.

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Screening of worldwide cowpea collection to drought tolerant at a germination stage

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ABSTRACT

Global warming has an increasing impact on the availability of water for agriculture. Crops tolerant to high temperatures and drought, such as cowpea (*Vigna unguiculata* L. Walp.), have an added value in the near future. The main objective of this study was to evaluate the effect of drought on seed germination and seedling emergence of cowpea genotypes, in order to screen the most tolerant genotypes. Seeds from 58 cowpea genotypes all over the world were submitted to two stress conditions, induced by PEG-6000 (corresponding to osmotic potentials of −0.75 bars and −1.5 bars). Germination and seedling growth parameters, vigor index and proline content were determined to assess drought tolerance. The results revealed significant differences of all parameters among genotypes after treatments and interaction of both. Water stress caused a general decrease in germination and seedling growth, while an increase in proline content was observed. A high variation of drought responses were detected among genotypes, being possible to select seven genotypes (C11, C18, C44, C46, C47, C50 and C54) as tolerant to drought at germination stage. These results will be useful to select the best suitable parents for insertion in future breeding programs.

1. Introduction

Worldwide agricultural production has been limited by several environmental constraints in the form of abiotic stresses, which affects plants growth, metabolism and development (Eftekhari et al., 2017; Muscolo et al., 2014). Water scarcity is currently one of the most severe limitations of plant development and production (Eftekhari et al., 2017; Jain and Saxena, 2016). The predicted temperature increase and rainfall decrease will be responsible for more frequent drought periods, mainly in the Mediterranean region including the Iberian Peninsula (Kröner et al., 2017). In this climate change scenario, the selection of drought-tolerant plants gain more importance, particularly the selection during germination. Some studies report several physiological characteristics (including seed germination and seedling growth) as indicators of drought tolerance in specific crop genotypes (Bouslama and Schapaugh, 1984; Steiner et al., 2017; Yan, 2015). Seed germination and seedling emergence are potentially the most critical stages susceptible to water stress (Ahmad et al., 2009; Hellal et al., 2018; Li et al., 2011, 2015) and are pivotal steps for crop propagation (Ravelombola et al., 2017). Indeed, water limitation can be responsible for the decline or even complete inhibition of seedling emergence and stand establishment (Kaya et al., 2006; Wu et al., 2011; Yan, 2015). However, tolerance against drought during the germination stage allow an uniform plant stand (Steiner et al., 2017).

Cowpea (*Vigna unguiculata* L. Walp.) is a grain legume with high worldwide economic importance, originated in Africa. Seeds of this legume are an important source of protein and other nutritional components for human diet (Ravelombola et al., 2017; Timko and Singh, 2008) and also an important source to animal fodder (Huang et al., 2012). Like many legumes, cowpea has the ability to fix atmospheric nitrogen through rhizobium symbiosis (Ehlers and Hall, 1996) and is easily grown in low fertility soils (Eloward and Hall, 1987). Some

Abbreviations: ANOVA, analysis of variance; *GR*, germination rate; PC, principal component; PCA, principal component analysis; PEG, polyethylene-glycol; *RL*, root length; SE, standard error; *SL*, shoot length; *VI*, vigor index; *%G*, germination percentage

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