Unravelling the genetic control of combined biotic/abiotic stress resistance in *Phaseolus vulgaris* L.
The fusarium wilt – drought interaction

Susana Murtinheira da Trindade Leitão

Dissertation presented to obtain the Ph.D degree in Plant Sciences - Plants for Life

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“I am among those who think that science has great beauty.”

Marie Curie

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List of most used abbreviations

ABA	Abscisic acid
A_n	Net photosynthesis or net CO_2 assimilation rate
AUDPC	Area under disease progress curve
BLUE	Best linear unbiased estimator
BPGV	Portuguese plant germplasm bank
C*a*	Chlorophyll *a* content
C*b*	Chlorophyll *b* content
CIAT	International center for tropical agriculture
C*cx*	Carotenoids (carotenes and xanthophylls) content
CO_2	Carbon dioxide
DAI	Days after inoculation
DART	Diversity arrays technology
DEG	Differentially-expressed gene
DNA	Deoxyribonucleic acid
DS	Disease severity
DSr	Disease progress rate
DW	Dry weight
E	Transpiration rate
f. sp.	*Forma specialis*
FC	Field capacity
Fop	*Fusarium oxysporum* f. sp. *phaseoli*
FW	Fresh weight
gs	Stomatal conductance
GWAS	Genome-wide association study

IRGA	Infrared gas analyzer
JA	Jasmonic acid
LRR	Leucine-rich repeat
MACE	Massive analysis of cDNA ends
Mbp	Mega base pair
PCA	Principal component analysis
PCR	Polymerase chain reaction
Q-Q	Quantile-quantile
QTL	Quantitative trait locus
RH	Relative humidity (%)
REML	Restricted maximum likelihood
RNA	Ribonucleic acid
ROS	Reactive-oxygen species
RWC	Relative water content (%)
SA	Salicylic acid
SNP	Single-nucleotide polymorphism
SWC	Soil water content (%)
TW	Turgid weight
WD	Water deficit
WUE	Instantaneous water use efficiency (A_n/E)
WUE_i	Intrinsic water use efficiency (A_n/gs)
WW	Well-watered

Summary

Common bean (*Phaseolus vulgaris* L.) is among the most important grain legumes for human consumption worldwide. Portugal has a promising common bean germplasm that resulted from more than five centuries of natural adaptation and farmers' selection. Nevertheless, limited characterization of this resource hampers its exploitation by breeding programs. To support a more efficient conservation of the national bean germplasm and promote its use in crop improvement, we performed, for the first time, a simultaneous molecular marker and seed and plant morphological characterization of 175 accessions collected from Portuguese mainland and islands traditional bean-growing regions. Most of the Portuguese accessions grouped with the race representatives and wild relatives from the Andean region. One-third of the national germplasm had an admixed genetic origin and might represent putative hybrids among gene pools from the two original centers of domestication in the Andes and Mesoamerica. The molecular marker-based classification was largely congruent with the three most frequent phaseolin haplotype patterns observed in the accessions analyzed. Additionally, we developed a core collection with 37 accessions that, by maximizing the genetic and morphological diversity of the original collection, represents the Portuguese common bean germplasm with minimum repetitiveness.

Fungal diseases, such as fusarium wilt, and water deficit are among the major constraints to common bean production worldwide, limiting yield, farmer's income, and commercialization. To identify new sources of fusarium wilt resistance and detect resistance-associated SNPs, we explored, for the first time, the Portuguese diverse common bean germplasm using genome-wide association analyses. The collection was evaluated for fusarium wilt resistance under growth chamber conditions, using the highly virulent *Fusarium oxysporum* f.

sp. *phaseoli* (*Fop*) strain, FOP-SP1 race 6. Fourteen accessions were considered highly resistant and 71 intermediate. The same collection was genotyped with single nucleotide polymorphism (SNP)-based arrays and SNP-resistance associations were tested using a linear mixed model accounting for the genetic relatedness among accessions. The results from the genome-wide association study (GWAS) revealed nine SNPs associated with resistance on common bean chromosomes Pv04, Pv05, Pv07, and Pv08, indicating that fusarium wilt resistance is under oligogenic control. Putative candidate genes related to phytoalexins biosynthesis, hypersensitive response, and plant primary metabolism were identified.

Moreover, photosynthesis-related traits, namely leaf gas exchange parameters and photosynthetic pigments, were evaluated in the same Portuguese common bean collection, under both well-watered (WW) and water-deficit (WD) conditions. Once again, the natural variation of the collection was explored and a GWAS performed to identify SNP alleles and candidate genes associated with the studied photosynthesis-related traits. A total of 133 SNP-trait associations were identified for net CO_2 assimilation rate, transpiration rate, and stomatal conductance, and for chlorophylls *a* and *b*, carotenes and xanthophylls contents. Ninety of these associations were detected under WD and 43 under WW conditions. The identified candidate genes revealed that stomatal regulation, membrane translocation of proteins, mechanisms of redox, hormone and osmotic stress signaling were the most relevant processes related to the studied common bean response to water deficit.

Finally, to understand the unique and shared responses of common bean to *Fop* and WD stresses, we characterized the differentially expressed genes in a double resistant and a double susceptible accession when these stresses were applied singly or combined. The susceptible accession revealed a clearly higher level of transcriptional changes in relation to the control condition, in all the three treatments – *Fop* and WD single stresses and in the combined *Fop*WD stress - compared with the resistant accession (944 versus 228 differentially expressed genes (DEGs)). The most represented functional categories within the DEGs were “RNA”, “secondary metabolism”, “stress”, “signaling”, “hormone metabolism”, “transport”, “protein” and “cell wall”. The resistance-related genes found might be targets for functional characterization and development of molecular tools, to expedite the screening and improvement of common bean multiple resistance to biotic and abiotic stresses.

In conclusion, the work developed under this Ph.D. thesis unlocked the door of future common bean breeding in Portugal, improved the knowledge on the molecular and morphological characterization of this national resource, identified new sources of resistance to a highly virulent *Fop* isolate and accessions more tolerant to WD conditions, and unveil the genetic control of disease resistance and photosynthesis-related traits in common bean.

Sumário

O feijão comum (*Phaseolus vulgaris* L.), ou simplesmente feijão, está entre as leguminosas de grão mais importantes para consumo humano em todo o mundo. Portugal possui um germoplasma de feijão com grande potencial, resultante da adaptação natural e da seleção efetuada pelos agricultores ocorridas nos últimos cinco séculos. No entanto, a caracterização desse recurso é limitada, o que dificulta a sua utilização em programas de melhoramento. Para promover uma conservação mais eficiente do germoplasma nacional de feijão e fomentar o seu uso em programas de melhoramento, foi realizado, pela primeira vez neste trabalho de doutoramento, uma caracterização molecular e morfológica de sementes e plantas de 175 acessos de feijão colhidos em diferentes regiões de Portugal continental e ilhas. A classificação baseada em marcadores moleculares foi amplamente congruente com os três haplótipos de faseolina mais frequentemente observados nos acessos analisados. A maioria dos acessos portugueses agrupou-se com os acessos representantes da região andina. Um terço do germoplasma nacional tem uma origem genética mista, sendo híbridos putativos entre os grupos genéticos dos dois centros originais de domesticação desta espécie, Andes e Mesoamérica. Adicionalmente, foi desenvolvida uma coleção nuclear com 37 acessos, representativa da diversidade genética e morfológica da coleção original de feijão português.

As doenças fúngicas, como a fusariose, e a seca estão entre os principais problemas que o cultivo de feijão enfrenta em todo o mundo, limitando a sua produção, o lucro do agricultor e a comercialização. Para identificar novas fontes de resistência à fusariose e detectar alelos de marcadores molecular associados a genes de resistência, foi explorada, pela primeira vez, a diversidade

natural presente no germoplasma de feijão português, utilizando o mapeamento por associação ao longo de todo o genoma de feijão (GWAS, *genome-wide asssociation study*, em inglês). A coleção de acessos de feijão foi avaliada quanto à resistência à fusariose, numa câmara de crescimento de plantas com condições controladas, utilizando um isolado muito virulento de *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*), denominado FOP-SP1 raça 6. Catorze acessos foram considerados altamente resistentes e 71 intermédios. A mesma coleção foi genotipada com *arrays* baseados em polimorfismo de nucleotídeo único (SNP, *single-nucleotide polymorphism*, em inglês) e as associações entre os níveis de resistência e os alelos dos SNPs foram testadas usando um modelo linear misto, tendo em conta a relação genética entre os acessos. Os resultados do estudo de associação (GWAS) revelaram alelos de nove SNPs associados à resistência à fusariose nos cromossomas de feijão Pv04, Pv05, Pv07 e Pv08, indicando que esta resistência está sob controlo oligogénico. Foram identificados genes candidatos envolvidos na biossíntese de fitoalexinas, resposta hipersensitiva e metabolismo primário da planta.

Adicionalmente foram avaliadas, na mesma coleção de feijoeiros, características relacionadas com a fotossíntese, nomeadamente parâmetros relacionados com as troca gasosas foliares e com os pigmentos fotossintéticos, em condições bem irrigadas (WW, *well-watered* em inglês) e em déficit hídrico (WD, *water deficit* em inglês). Uma vez mais, a variação natural da coleção de acessos de feijão foi explorada e um GWAS realizado para identificar alelos de SNPs e genes candidatos associados a características relacionadas com a fotossíntese. Foram identificadas 133 associações entre os alelos dos SNPs e as taxas de assimilação líquida de CO_2 e de transpiração, a condutância estomática, e o conteúdo em clorofilas *a* e *b*, e em carotenos e xantofilas. Noventa destas associações foram detetadas em condições bem irrigadas e 43 em déficit hídrico. Os genes

candidatos identificados revelaram que a regulação estomática, a translocação de proteínas pelas membranas celulares, os mecanismos de redox, a sinalização de stresses hormonal e osmótico foram os processos mais relevantes relacionados com a resposta do feijoeiro ao déficit hídrico estudado.

Finalmente, com o objetivo de estudar as respostas comuns e únicas do feijoeiro à fusariose e ao déficit hídrico, foram identificados os genes diferencialmente expressos em dois acessos – um resistente e um suscetível a ambos os stresses – quando os stresses foram aplicados isoladamente ou em combinação. O acesso suscetível revelou um nível notoriamente mais elevado de alterações transcricionais em comparação com o acesso resistente, nos três tratamentos aplicados – stresses individuais de *Fop* e WD e no stress combinado *Fop*WD. As categorias funcionais mais representadas nos genes identificados foram "ARN", "metabolismo secundário", "stress", "sinalização", "metabolismo hormonal", "transporte", "proteína" e "parede celular". Os genes identificados envolvidos na resistência das plantas serão alvos preferenciais para a caracterização funcional e para o desenvolvimento de ferramentas moleculares que auxiliem o desenvolvimento de acessos de feijão com resistência múltipla a stresses bióticos e abióticos.

Em conclusão, o trabalho descrito nesta tese de doutoramento (Ph.D.) abriu caminho para que se desenvolva um programa de melhoramento de feijão em Portugal com vista ao desenvolvimento de variedades mais adaptadas a múltiplos stresses. Foi caracterizada a diversidade molecular e morfológica deste recurso genético nacional, identificadas novas fontes de resistência a *Fusarium* e de tolerância a condições de déficit hídrico, e ampliou-se o conhecimento existente do controlo genético da resistência a doenças fúngicas e de características relacionadas com a fotossíntese no feijoeiro.

Chapter I

General Introduction

Parts of this chapter are based on:

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Common bean – a worldwide valuable and environmentally friendly crop underused in Europe

Common bean (*Phaseolus vulgaris* L.) belongs to the *Fabaceae* family, genus *Phaseolus* and tropical tribe *Phaseoleae*, which also includes cowpea (*Vigna unguiculata* L.) and soybean (*Glycine max* L.). *Phaseolus* is a diploid genus with most species having 2n = 2x = 22 chromosomes (Gepts et al. 2008). A reference genome of *P. vulgaris* was released in 2014, where 473 Mbp of the 587-Mbp genome were assembled and 98% of this sequence was genetically anchored in 11 chromosome-scale pseudomolecules (Schmutz et al. 2014). This information allows presently not only a deeper understanding of the common bean genetics, by means of genome-wide association studies (GWAS) for instance, but also comparative genomics analysis between *Phaseolus vulgaris* and related model and crop legume species.

Common bean was originated at the Middle American region (Mesoamerica) and it is estimated that 111,000 years ago the two current gene pools and centers of origin - the Mesoamerican and the Andean - diverged from a common ancestor (Mamidi et al. 2013). These two geographically isolated and genetically differentiated wild gene pools were independently domesticated and gave rise to accessions with different seed and leaf sizes, growth habits and variations in seed coat color and patterns. Based on morphological traits and breeding behavior, different races were defined by Singh et al. (1991) within the Mesoamerican and Andean gene pools, namely *Mesoamerica*, *Jalisco* and *Durango* (Mesoamerica), and *Nueva Granada*, *Peru* and *Chile* (Andes).

This legume crop was brought to Europe in the 15th and 16th centuries during the voyages of trade and discovery made by the Portuguese and the Spanish. Since then, it has been conserved and cultivated by generations of farmers, mainly using low-input systems, giving rise to introgression between the two gene pools, which made the Iberian Peninsula considered as a secondary center of origin of common bean (Santalla et al. 2002).

Common bean is a predominantly self-pollinated herbaceous annual plant grown worldwide for its edible green pods and dry seeds. This legume has recognized benefits to human health and nutrition, due to its high content in proteins, dietary fibre, vitamins (C, B6, riboflavin, thiamin, and folic acid) and minerals (magnesium, iron, calcium, and potassium) (Câmara et al. 2013). Common bean is the most important directly consumed legume in the world, especially in countries of Africa and Latin America, where it is part of the staple diet. Indeed it is an important dietary component in some developing African countries, such as Burundi, Rwanda, and Uganda, where it provides 40%, 31%, and 15% of the daily intake of total protein, respectively (Gepts et al. 2008). In Brazil and Mexico, it is also a major source of protein. In Portugal, beans represent 75% of grain legumes consumption (www.ine.pt), and although there are diverse common bean accessions still cultivated in smallholder farms, the national production is insufficient to cover the demand.

Worldwide agricultural statistics on common beans are sometimes difficult to analyze, as the various *Phaseolus* and *Vigna* species are often taken together since in the past some *Vigna* spp. were classified as *Phaseolus*. However, with the necessary precaution, one can say that bean production worldwide is approximately twice of chickpeas and dry peas, the following most consumed grain legumes (average worldwide production 2010-2017: 26, 13 and 12 million tonnes, respectively) (FAOstat 2017). Africa and Latin America are still the regions of greatest production of common beans, with Latin America representing about 50% of world volume, followed by Africa with 25% (Beebe et al. 2013). Common bean is less known in Asia where other grain legumes (*Vigna* sp.) are preferred (Xuzhen and Jing 2011). However, production in China is important and has increased in the last years reaching 0.8 million hectares (ha) in 2017. Myanmar, India, Brazil, China, the United States of America (USA), Mexico and Tanzania represented 2/3 of the world production of dry beans, while China was the main producer of fresh beans (*Phaseolus* and *Vigna* species: 17 million tonnes in 2011, 77% of the world production) (Heuzé et al. 2015). On the other hand, major importing countries of common beans are India, Brazil, USA, Japan, and the United Kingdom, while the main exporters are Myanmar, China, USA, Argentina, and Canada. In the last decade, the annual world exports of common bean totalize 2.6 to 3.5 million tonnes (FAOstat 2017). In the case of Europe, the paradigm of grain legume production has changed dramatically in the last five decades. As an example, in 1961, 9.5 million ha (equivalent to 5.67 tonnes) were cropped with various species of grain legumes, namely common bean (4 million ha, 1.1 million tonnes), cowpea (*Vigna unguiculata* L.), faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.), lupins (*Lupinus* sp.), and soybean (*Glycine max*) (FAOstat 2017). More than half of these crops were for direct human consumption. Diet change, with an increase consumption of meat, and a lack of breeding investment in Europe, rendered legumes less economical attractive to farmers, leading to a different situation. In the period 2010-2014, the total area harvested per year of all the referred grain legumes in Europe was on average less than 5 million ha, with common bean harvested area being 0.25 million ha/year and production 0.5 million tonnes/year (FAOstat 2017).

As other legume species, common bean establishes a symbiotic relationship with rhizobia soil bacteria, which drives the development of root nodules that fix atmospheric nitrogen into ammonia compounds directly available for plant assimilation and growth. The biological nitrogen fixation has a crucial role in environmentally sustainable agricultural systems, since it allows a decrease in the fertilizers inputs, with a consequent reduction of production costs for the farmer. Accordingly, in the case of common beans, it is estimated that 40% of the plant total nitrogen is derived from nitrogen fixation (Herridge et al. 2008).

The diversity of growth habits (from determinate bush types to vigorous climbers), and the range of growth cycles (from 2 to 10 months in length) make beans a crop that fits many production systems (Beebe et al. 2013). Beans are traditionally a small farmer crop, often grown in complex farming systems in association or rotation with maize, sorghum, bananas, or other crops (Broughton et al. 2003). Also in more intensive sole crop common bean cultivation systems, rotations are recommended and commonly used such as a 3 to 4 years rotation with cereals (like maize and winter wheat), potato or sugar beet (www.pulsecanada.com). It is known that crop rotation has several economical, agronomic and environmental benefits when compared to “continuous” cropping. Crop rotation can improve yield and profitability over time, provide an alternative source of nitrogen if the rotation is made with legumes, such as common bean (Dalias, 2015), reduce soil erosion, increase soil organic matter, improve soil tilth, and reduce runoff of nutrients and chemicals (Kirkegaard et al. 2008). The incorporation of resistant cultivars or non-hosts cultures in the rotation will minimize the damage caused by plant diseases, break disease cycles and limit insect and other pathogens infestations. As example, the introduction of host resistance in chickpeas or tomato was reported to be efficient as a control measure for management of fusarium wilt, a fungal disease that inhibits the flow of water and nutrients in plants (Jiménez-Díaz et al. 2015; McGovern 2015). Intensive common bean production systems may be found in the United States of America with 704,000 ha planted in 2015 (major producers in North Dakota and Michigan, USDA agricultural stats) and Canada with 683,000 ha (major producers in Ontario, Quebec, and in Alberta and Manitoba, Saskatchewan, Canadian agricultural stats 2014).

1.1 The unexplored Portuguese common bean germplasm potential

Yield instability, due to diseases associated or not to drought susceptibility, renders beans less attractive to farmers, particularly under Mediterranean conditions where heat waves and drought periods are becoming more frequent. Subsequently, all over the Mediterranean or even in the European region, there is no high-quality raw material production in large amounts for the national grain legumes processing industries, such as the Portuguese, that rely almost exclusively on foreign materials.

Portuguese bean traditional accessions well adapted to local environmental conditions, due to several centuries of natural and human selection, represent important sources of genetic diversity for biotic and abiotic stress resistance. In fact, sources of disease resistance were identified when analyzing 90 accessions of the Portuguese collection for response to rust (*Uromyces appendiculatus*) and powdery mildew (*Erysiphe diffusa*) inoculation (Leitão et al. 2013). In that study, 24 accessions revealed partial resistance against rust and 13 accessions against powdery mildew, with low disease severity despite high infection types. As partial resistance is assumed to be more durable than the complete hypersensitive resistance, due to the reduced selection pressure imposed on the pathogen (Niks and Rubiales 2002), those accessions are promising to be incorporated in common bean resistance pre-breeding programs.

Other preliminary studies in Portuguese common bean accessions revealed high diversity, both at the morphological (Veloso et al., 2015) and genetic levels (Leitão et al., 2012; Dinis et al., 2013). This reinforces the idea that the Portuguese germplasm can be of great value to discover new sources of interesting traits related to human and natural selection such as organoleptic and technological quality of the seeds, and also resistance to biotic and abiotic stresses. These traits will be fundamental to develop high quality multiple resistant varieties attractive to farmers, food processing industry and consumers, which would eventually promote a more common bean sustainable production and consumption. As an example, since the Portuguese common bean germplasm seems to be more genetically related to the Andean gene pool (Dinis et al. 2013), it might be an interesting source of broader durable resistance in cultivars of Mesoamerican origin. This is due to the coevolution of pathogen virulence with common bean gene pools, being the development of lines with resistance genes from both Andean and Mesoamerican origin a recognized strategy for developing broad-based durable resistance (Miklas et al. 2006). However, further characterization of the Portuguese common bean germplasm resistance responses, and of the implicated resistance genetic mechanisms, is needed. Hopefully, the Andean background of this national germplasm can complement the few resistance sources already found and that largely belong to the Mesoamerican gene pool.

1.2 The “unlinked“ common bean major biotic/abiotic constraints

Diseases and pests are frequent in temperate and tropic regions, being important factors compromising common bean production (Assefa et al. 2019; De Ron 2015). Drought is currently the most widespread and yield-limiting abiotic stress in many crops, in particular, in legumes (Vadez et al. 2012). The sensitivity of grain legumes to drought is associated with leaf senescence, oxidative damage of photosynthetic apparatus, reduced carbon fixation, inhibited flowering and reproductive development, sterility of pollen grains and reduced grain development (reviewed by Farooq et al. 2017). During the last decade, some molecular players and physiological pathways underlying water-deficit responses of a broad range of legume species have been elucidated (for a comprehensive review, see Araújo et al. 2015). As an example, water channel aquaporins have been described to be involved in drought tolerance in faba bean and common bean (Cui et al. 2008; Montalvo-Hernández et al. 2008). In chickpea, several transcription factors, such as dehydration responsive element-binding protein 2 A (DREB2A), APETALA2/ethylene-responsive element binding factor (AP2/ERF), zinc finger family, MYB domain, WRKY, and ARF (auxin response factor) were implicated in water-deficit response with a role in major metabolic pathways such as absicic acid (ABA), proline and flavonoid biosynthesis (Hiremath et al. 2012; Mahdavi Mashaki et al. 2018; Nayak et al. 2009). In common bean, DREB2 and late embryogenesis abundant 3 (LEA3) were also identified as candidate targets responsive to drought (Barrera-Figueroa et al. 2007; Cortés et al. 2012). In cowpea (*Vigna unguiculata* (L.) Walp.), several drought stress-inducible genes involved in ABA biosynthesis were identified, namely cowpea responsive to dehydration (CPRD) genes like dehydrin (CPRD22), alcohol dehydrogenase (CPRD12), NADPH-dependent aldehyde reductase (CPRD14), and lipoxygenase (CPRD46), phospholipase D (VuPLD1) or 9-cis-epoxycarotenoid dioxygenase (Muchero et al. 2009).

Besides drought, fusarium wilts and root rots are major constraints to the production of legume crops worldwide (De Ron 2015; Rubiales et al. 2015). The most important pathogens causing wilt in legumes are various host-specific forms of *Fusarium oxysporum*, whereas causal agents of root rots are mainly *F. solani*, *Rhizoctonia* spp., *Verticillium* spp. and *Aphanomyces euteiches* (Bodah 2017; Kraft et al. 1988). Naturally found in fields, all of these soilborne pathogens directly penetrate roots, invade the cortex and colonize the vascular tissue of the host plant. *F. oxysporum* and *F. solani* are responsible for vascular disease in chickpea, common bean, cowpea, pea, and lentil in most growing regions (Afouda et al. 2009; Chaudhary et al. 2006; Chen et al. 2016). Their development is favored by warm and dry conditions and visual symptoms include rotting of the root system, wilting, stunting, chlorosis, and lesions on the stem and/or leaves of infected plants. *Fusarium* spp. can survive in the soil for extended periods in the absence of the host, mainly in the form of thick-walled chlamydospores (Coleman 2016; Di Pietro et al. 2003). On the other hand, *Rhizoctonia* spp., *Verticillium* spp. and *Aphanomyces euteiches* are favored by humid and cooler climate as found in northern Europe and North America and are major constraints in the production of cool-season legumes such as pea, chickpea, lentil or faba bean (Kraft and Pfleger 2001; Wu et al. 2018). The symptoms are similar to those of fusarium wilt, with generalized wilting, dark brown lesions above the collar region on the main stem and lower branches, and drooping of petioles and leaflets (Nene et al. 2012).

To understand the molecular mechanisms and genetic control of legume resistance to some of these pathogens, quantitative trait loci (QTL) mapping studies have been described. Regarding root rot, genomic regions associated with *F. solani* f. sp. *phaseoli*, *R. solani*, and *A. euteiches* partial resistance were detected in common bean (Hagerty et al. 2015; Kamfwa et al. 2013; Oladzad et al. 2019; Schneider et al. 2001). In pea, several QTLs associated with resistance to *F. solani* f. sp. *pisi* and to *A. euteiches* were reported (Coyne et al. 2019; Desgroux et al. 2016; Hamon et al. 2011; Hamon et al. 2013; Pilet-Nayel et al. 2005). Moreover, a major QTL conferring partial resistance to *Verticillium* wilt was identified in the model legume *Medicago truncatula* (Ben et al. 2013). In the case of fusarium wilt, QTLs were identified in chickpea associated with *F. oxysporum* f. sp. *ciceris*, in cowpea associated with *F. oxysporum* f. sp. *tracheiphilum*, and in lentil associated with *F. oxysporum* f. sp. *lentis* partial resistance (Hamwieh et al. 2005; Pottorff et al. 2012; Sabbavarapu et al. 2013; Sharma et al. 2004).

Despite drought and soilborne diseases have in nature interactive effects on plants, research has mainly focused so far in the individually study of these stresses. Next, a review of what is known on fusarium wilt resistance and drought tolerance in common bean, as single “unlinked” stresses, is presented.

a) Fusarium wilt

Fusarium wilt is a vascular disease with great economic importance and in common bean is caused by the soilborne ascomycete *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*). This pathogen has been detected in most of the bean-growing regions of the world and causes a significant yield loss in Africa, Latin America, in the western area of the USA (Buruchara and Camacho 2000), and also in European Mediterranean countries like Spain, Italy and Greece (Alves-Santos et al. 1999; Alves-Santos et al. 2002). The optimum temperature for the development of *Fop* is 28°C, but the most severe symptoms of fusarium wilt may occur at 20°C (Ribeiro and Hagedorn 1979). Depending on the environmental conditions, common bean cultivars, crop developmental stage, prevailing fungi races, production systems, management practices used, occurrence and severity of other stresses in the same field, the yield losses can reach 100% (Singh and Schwartz 2010). Soil moisture, irrigation, field drainage, crop rotation, tillage, administration of fertilizers, planting depth and standing microbiota are all factors that may be manipulated to lower the density of root rot pathogens in the soil (Naseri and Marefat 2011; Naseri and Mousavi 2015). *Fusarium oxysporum* can survive in the soil for extended periods in the absence of the host, mainly in the form of thick-walled chlamydospores. After germination, infection hyphae adhere to the host roots and penetrate them directly (Di Pietro et al. 2001; Rodríguez-Gálvez and Mendgen 1995). The mycelium then advances intracellularly through the root cortex and colonizes the xylem vessels. There, the fungus remains exclusively within the vascular tissue, using it as avenues to rapidly colonize the host (Bishop and Cooper 1983). This is mainly accomplished by the production of microconidia, which are detached and carried upward in the sap stream, penetrating the neighboring vessels. Wilting is most likely caused by the accumulation of fungal mycelium and/or toxin production and host defense responses, including the production of gels, gums and tyloses, and vessel crushing, hampering water and nutrient transport. Wilting is followed by extensive chlorosis and necrosis on the lower leaves. The chlorotic symptoms then propagate to the next higher leaf and continue progressively up the plant, accompanied by the browning of the hypocotyls and stems and vascular discoloration, leading to premature leaf drop and eventual plant collapse and death. After killing the plant, the fungus sporulates profusely on the plant surface (Di Pietro et al. 2003). If young plants are infected they remain stunted. It is widely known that *Fusarium* species are important mycotoxin-producing fungi. Fusaric acid, diacetoxyscripenol, deoxynivalenol, T-2 toxin, and fumonisin B1 were detected in bean seeds where *Fusarium* spp. were isolated (Bacon et al. 1996; Tseng et al. 1995).

As direct field selection is laborious and destructive sampling is needed to identify resistance, indirect selection for fusarium wilt resistance based on markers linked to resistance quantitative trait loci (QTLs) would facilitate the improvement of common bean resistance to this disease. Resistance to *F. oxysporum* f. sp. *phaseoli* seems to be race specific with different associated models of inheritance in common bean. While for some races, resistance is determined by a single, completely dominant gene, for other races it is determined by a single incompletely dominant gene, by a recessive gene or by several genes in a quantitative model (Alves-Santos et al. 2002). Since only a few sources of resistance have been described (Pereira et al. 2013; Román-Avilés and Kelly 2005; Salgado 1995), the risk of resistance breakdown by interactions between host and environment or new pathogen genotypes is always present, especially when resistance is monogenic, and easier to break. A continuous search for novel resistance sources is thus essential, with an emphasis on resistance sources based on heritable quantitative and polygenic mechanisms, potentially more durable. Several differential-expressed genes in contrasting (resistant and susceptible) common bean seedling roots were identified in response to this pathogen, useful for exploring QTL candidate genes for fusarium wilt resistance in common beans (Xue et al. 2015). Those expressed genes were either involved in the mechanism of plant hormone regulation or associated with different defense-related and growth-regulatory genes, namely protein kinases that can be involved in signaling during pathogen recognition and protein receptors with domains of the leucine-rich repeat (LRR) class.

b) Water deficit

Globally, drought can be referred to soil and atmospheric water deficits which, along with high radiation and temperature, can lead to constraints to plant survival and crop productivity due to a decreased water potential in plant tissues (Boyer 1982). Water deficit in plants may occur as a consequence of a seasonal decline in soil water availability, developing in the long term, or may result from dry spells. The timing, intensity, and duration of stress episodes will determine the effects produced by drought (Chaves and Oliveira 2004). In agriculture, the term drought refers to a condition in which the amount of water available through rainfall and/or irrigation is insufficient to meet the transpiration needs of the crop (Tuberosa 2012). Among the physiological processes more affected by water deficit, photosynthesis, growth, and grain filling are typically referred (Barnabás et al. 2008; Farooq et al. 2009).

With the perspective of a global increase in temperature and more frequent periods of heat waves, it is expected that plants will experience recurring periods of water deficit or drought. Based on this climate change scenario, global and regional climate projections and models were developed suggesting that the Mediterranean might be an especially vulnerable and most responsive region (Giorgi and Lionello 2008). This region will face the big challenge of meeting higher water demands with less available water resources. Future warming in the Mediterranean region is expected to exceed worldwide rates by 25%, with summer warming surpassing the global rates mean by 40% (Lionello and Scarascia 2018). A global atmospheric temperature increase of 2ºC will probably be accompanied by a reduction in summer precipitation of around 10-15% in Southern France, Northwestern Spain and the Balkans, and up to 30% in Turkey and Portugal (Vautard et al. 2014). This reflects the importance of understanding the mechanisms that control plant adaptation to drought and use that knowledge to develop varieties with improved water-deficit tolerance.

In common bean, water deficit is considered one of the largest responsible for yield reduction, together with fungal diseases (Rao 2001; Urrea et al. 2009). Water stress affects greatly common bean production worldwide reducing both grain yield and quality (Beebe et al. 2008; Shenkut and Brick 2003; Urrea et al. 2009). It was described that pod and flower abscission were the main causes of reduced common bean yield under water stress (Lizana et al. 2006). More specifically, seed-filling, overall biomass and harvest index, pods per plant, number of seeds per plant, and 100-seed weight were reported of being significantly reduced due to drought stress (reviewed by Asfaw and Blair 2012; Assefa et al. 2019; Assefa et al. 2013).

Plants use multiple strategies to respond to drought stress and have evolved to adapt to water stress via morphological and physiological changes through diverse signaling cascades and osmotic adjustment. Since drought stress response involves different biological processes, multifactorial phenotypes can be originated, which are many times confounded with other plant pathologies. Morphological and biochemical changes to avoid or minimize drought effects include leaf rolling, stomatal density and stomatal aperture control, cell membrane stability, increased thickness of the leaf cuticle, alteration in chlorophyll concentration and root architecture (root depth, distribution and length density), and accumulation of osmoprotectants, antioxidants, and reactive oxygen species (ROS) scavengers (reviewed by Hu and Xiong 2014). The magnitude/intensity of the water stress and the developmental stage of the plant may also influence the stress response. Additionally, plants that escape drought exhibit a high degree of developmental plasticity, being able, for example, to accelerate flowering to complete their life cycle and reproduce before physiological water deficit occurs (Chaves et al. 2003). The morpho-physiological traits referred also influence water use efficiency (WUE), an important determinant of yield under stress especially in a context of a changing climate. A better WUE implies maximal soil moisture capture for transpiration which also involves reduced non-stomatal transpiration and minimal water loss by soil evaporation. It has been used to imply that rainfed plant production can be increased per unit water used, resulting in “more crop per drop”, meaning an increased amount of biomass (or grain yield) per unit of water used by a plant (Kijne et al. 2003). The genetic screening of plants for characteristics directly related to photosynthetic efficiency or reduced respiration was suggested to lead to insights in the potential impacts of climate change in WUE (Hatfield and Dold 2019).

In the last 25 years, efforts have been made to improve common bean resistance to drought. It is notable that more progress has been made in small-seeded Mesoamerican types of Central America and Brazil, than in the large-seeded Andean types that are more popular in Africa and parts of South America (Beebe et al. 2013). In 1995, superior drought tolerance in crosses that combined the genetic diversity found in the bean races *Mesoamerica* (from lowland Central America and Mexico) and *Durango* (from highland Mexico) has resulted in the release of lines with higher yield in drought environments (Singh 1995; Singh et al. 2001; Terán and Singh 2002). Since then, much of the subsequent work on drought resistance has been built around crosses using common bean lines from *Durango* race as the major source of resistance (Beebe et al. 2013; Beebe et al. 2008; Rao et al. 2006; Rao et al. 2013).

A few QTL studies on drought resistance have been conducted in common bean. Asfaw and Blair (2012) were able to identify a QTL associated with drought resistance in common bean, namely related with root traits, in a recombinant inbred line (RIL) population derived from BAT 477 (a drought tolerant line) and DOR 364 (a commercial variety), both of Mesoamerican origin. Mukeshimana et al. (2014) used a RIL derived from a cross between an improved drought resistant line of Mesoamerican origin and a high yield potential and marketability Andean line (large-seeded dark-red bean type) to map QTL for drought tolerance traits associated with yield-components and phenology (e.g.

number of days to flower, maturity, and seed fill). Later on, Trapp et al. (2015) tested similar yield and phenological traits and identified two major QTL for yield under intermittent drought, in a RIL population derived from a cross between a line with moderate tolerance to drought and virus resistance and a higher yielded pinto line from a private seed company.

The lack of sources of drought resistance in common bean led researchers to study sister species of *P. vulgaris* such as tepary bean, *P. acutifolius*, which is well adapted to water-deficit conditions (Rao et al. 2013). Tepary bean, originated from dry subtropical slopes in the deserts of Mexico and the south-western USA exhibits several interesting drought resistance traits: early maturity, excellent remobilization capacity under stress, deep rooting to avoid dehydration, small leaves for reduced water use, and stomatal control but not with osmotic adjustment (Mohamed et al. 2005). Despite the difficulties in crossing these two species, the introgression of cold tolerance from the tepary bean genome into common bean was successfully achieved, augmenting the methods available to increase abiotic stress tolerance in common bean (Souter et al. 2017).

The unexplored Portuguese common bean germplasm has a history of more than five centuries of cultivation in small fields for farmers’ personal consumption and to sell in local markets. However, limited studies exist characterizing its genetic variability and the identification of abiotic and biotic resistance sources. It will be very interesting to verify if, among the Portuguese germplasm, more related to the Andean gene pool (Dinis et al. 2013), there are sources of drought tolerance and disease resistance.

1.3 Understanding abiotic/biotic stress interactions

In nature, plants are exposed to various and complex types of stresses involving numerous environmental factors, both biotic and abiotic. Plant responses to these stress combinations are complex and involve numerous physiological, molecular, and cellular adaptations. An example is water-deficit stress which is often accompanied by other abiotic stresses like heat, and that can promote the severity of diseases caused by pathogenic fungi or bacteria, through the leakage of cellular nutrients into apoplast which facilitates successful pathogen infection (Ramegowda and Senthil-Kumar 2015).

Substantial information is available on the physiological, molecular, and metabolic modifications in plants exposed to individual stresses. These studies have characterized the effect of each stress at cellular and plant level by exposing plants to different stress intensities at different growth stages, under controlled conditions, and the results have been translated to field situations (Atkinson and Urwin 2012; Mittler and Blumwald 2010; Suzuki et al. 2014).

Nevertheless, to achieve high levels of durable resistance, introgression and pyramiding of different stress resistances from different origins like distant market classes, common bean races, and gene pools may be essential. Terán et al. (2009) verified that, for pinto beans and in the USA, despite having available resistance sources, each cultivar was not resistant to more than two of the major diseases affecting beans. Thus, multiple resistances to a wide range of important diseases that are major constraints for the production of high yield and quality cultivars are lacking. However, some breeding efforts had already started. Simultaneous improvement of resistance against angular leaf spot, anthracnose, common bacterial blight, bean common mosaic, and bean rust was performed in three Andean x Middle American inter-gene pool double-cross populations and intermediate to high levels of resistance to the five diseases were accomplished in F5 lines when compared with the parental lines (Terán et al. 2013).

Biotic and abiotic stresses induce specific and overlapping resistance responses (Fujita et al. 2006), which share signaling pathways and genes in many plants species. Abiotic stress resistance is typically governed by polygenic inheritance and may be conditioned by multiple, interacting mechanisms (Pathania et al. 2014). Compatible and incompatible interaction between plant and pathogens has often a quantitative nature also. Recent evidences suggest that plants have the ability to cope with simultaneous biotic and abiotic stresses and to fully understand this capacity one cannot simply extrapolate the results from individual stresses studies where each stress is applied independently (Atkinson et al. 2013; Fujita et al. 2006; Kissoudis et al. 2015; Kissoudis et al. 2014; Suzuki et al. 2014).

Plants response to a combination of drought and pathogen infection strongly depends on the crop involved, developmental stage and intensity and duration of each stress (Rejeb et al. 2014). At field conditions, during the course of drought stress development, pathogens can better infect plants by taking advantage of their weakened status, or drought stress can occur on already infected plants, resulting on plants having to deal with a combined pathogen and drought stress (Ramegowda and Senthil-Kumar 2015). Moreover, pathogen infection may reduce photosynthesis and water use efficiency (Bilgin et al. 2010) and induce abnormal stomata opening patterns (Grimmer et al. 2012) with consequences in plant tolerance to abiotic stress, such as drought. Therefore, precise stress imposition at specific plant growth stages is needed for a better understanding of the combined stress effects.

Little is known concerning the interaction between drought and biotic stresses on common bean. Simultaneous exposure of common bean plants to drought stress and to the fungal pathogen *Macrophomina phaseolina* (causal agent of charcoal rot and seedling blight) resulted in higher transpiration rate and leaf temperature as compared to plants subjected only to drought stress (Mayek-Pérez et al. 2002). Interestingly, while some pathogens attack more plants growing under stressful conditions by taking advantage of their weakened basal defense mechanisms causing further damage to plants, other pathogens require continuous humid conditions to develop and proliferate, and drought-stressed plants seem to be more resistant (Ramegowda and Senthil-Kumar 2015). Fusarium wilt and drought have a known agronomic interaction, and disease progression is favored by dry weather and even more by severe drought (Di Pietro et al. 2001, Sinha et al. 2017). Furthermore, Summerell et al. (2011) reported that plant drought tolerance is a key factor associated with *Fusarium* species distribution. Fungal species that do not produce chlamydospores are frequently found in wetter areas, while the ones that form these structures – and *F. oxysporum* is an example – can survive in more unfavorable dry conditions. However, little is known on the genetic base controlling *F. oxysporum* f. sp. *phaseoli* x drought interaction in common bean.

ABA, salicylic and jasmonic acids (SA, and JA, respectively), together with ethylene signaling, mediates plant adaptation to both biotic and abiotic stresses. The mechanism of *F. oxysporum* perception by plants is not clear, however, it was described that, in *Arabidopsis*, the resistance to this fungus is mediated by ERF1 (Berrocal-Lobo and Molina 2004). It was also reported that this fungus has the capacity of taking control of the plant JA pathway, enhancing the disease symptoms leading to plant death (Thatcher et al. 2009). A complex balance exists between ABA, a major regulator of drought stress response, and JA-ethylene signaling pathways, with antagonistic interactions that modulate defense and stress-responsive gene expression in reaction to combined stresses (Anderson et al. 2004). Ramegowda and Senthil-Kumar (2015) hypothesized that under combined stresses the first signaling effects operate through JA and ABA, in a non-specific manner. However, the modulation of stress signal, crosstalk, and subsequent downstream events are specific and unique to the combination of stresses faced by the plant. Deployment of genes that have a protective function on proteins and cellular components under abiotic stress, such as dehydrins, LEA proteins or RNA chaperones were described to minimize the antagonist interaction with biotic stress signaling (Kang et al. 2013; Kissoudis et al. 2014).

The more efficient way of dealing with abiotic and biotic interactions is through a combined approach with the aim of finding multiple stress resistance sources and identifying genes involved in multiple resistances.

1.4 Closing the gap: understanding the genetic and molecular basis of abiotic/biotic stresses as a tool to the development of multi-stresses common bean varieties

Little is known concerning the complex details underlying the abiotic-biotic interactions in legumes. Among the few reports available there is one on the model legume *M. truncatula* (ecotype Jemalong A17), where simultaneous drought and spider mite attack (*Tetranychus urticae*) stresses were applied and biochemistry assays and gene expression analysis were performed to understand the chemical defense responses involved. An increased in the production of peroxide (representative of ROS) was observed, which enhanced signaling processes and accumulation of antioxidants and osmoprotective compounds, such as proline (Antoniou et al. 2018).

In common bean, Ambachew et al. (2015) described the negative effect of combined drought stress and *Ophiomyia* spp. (bean fly) infestation on the mean seed yield and leaf chlorophyll content. This study examined trait interactions among two diverse recombinant inbred line populations resulting from crosses of parental lines of different gene pools of origin, for the combined effect of drought and bean fly infestation. The authors concluded that vertical root pulling resistance, stem diameter, and pod harvest index should be included as selection criteria for improving seed yield in common beans under drought and bean fly infestation conditions.

Enhanced resistance to powdery mildew (*Erysiphe pisi*) induced by ozone exposure has been demonstrated using two pea cultivars (Rush and Laurence 1993). Although plant defense mechanisms induced by the ozone exposure were not unveiled, the authors concluded it was not correlated with pisatin content, a pea phytoalexin.

Plant-water relations influence plant defense, with stomatal regulation having a critical role (Beattie 2011). Under drought environments, plant-pathogen interaction can inhibit the normal ABA-mediated stomatal closure response of plants to drought, with a clear impact on crop performance (Grimmer et al. 2012). Both pathogen infection and plant host resistance mechanisms can result in alterations in guard cell regulation of stomatal aperture. A wide range of foliar fungi has been described to disrupt the cuticular or stomatal regulation of transpiration, thus affecting plant-water relations. For example*, Erysiphe pisi* in pea, *Uromyces appendiculatus* and *Colletotrichum lindemuthianum* in common bean, and *Botrytis fabae* in faba bean were described to cause a decrease in the stomatal aperture during the day or impairment of stomatal closure during the night (reviewed by Grimmer et al. 2012). The negative influence of foliar pathogens decreasing net photosynthesis and water use efficiency, defined as the ratio of CO_2 assimilation rate to transpiration rate at the leaf level, was described in different plant legume-pathogen systems: *Bean yellow mosaic virus* in faba bean (Radwan et al. 2008), *Phaeoisariopsis griseola* and *U. appendiculatus* in common bean (Jesus-Junior et al. 2001; Meyer et al. 2001) or *E. pisi* in pea (Ayres 1976).

Drought stress has a known agronomic interaction with soilborne pathogens that infect roots causing vascular diseases in grain legumes. For instance, drought stress was reported to increase the incidence of *R. bataticola* and *F. solani* in chickpea (Sinha et al. 2019). In this work, the combination of drought and two fungal diseases reduced the primary root length, crop growth and ultimately yield, as compared to the situation in which drought was combined with a single pathogen stress. Furthermore, QTLs have been reported in pea governing both drought resistance and resistance to the root parasitic weed *Orobanche crenata*, suggesting that they might be controlling water and solute losses due to drought or *O. crenata* presence (Iglesias-García et al. 2015). In this context is worth to mention that often drought stress results in root exudation of amino acids and carbohydrates, which become available for priming plant and soil microbial activity after rewetting (Karlowsky et al. 2018). Actually, these exudates have been reported as a source of nutrients for the growth of soilborne pathogens (Duniway 1977). More recently, Bani et al. (2018) described how pea root exudates can modulate, either stimulating or inhibiting *F. oxysporum* spore germination. In this study, pisatin was identified as having an important role in the constitutive antifungal defense of pea, and its amount in the root exudates was negatively correlated with the extent of *F. oxysporum* germination. On the other hand, pathogen infection also modulates the root exudates profile, leading to the recruiting of beneficial rhizosphere communities, with an effect on the resistance of subsequent plant populations growing in the same soil (Yuan et al. 2018).

The identification and location of genes controlling the interaction of multiple stress resistances in grain legume species is an important challenge in the present context of climate change. This can be accomplished by association mapping studies that explore plant natural variation, validated by functional analysis using transcriptomics, proteomics, and metabolomics of contrasting accessions. The molecular mechanisms of combined multiple-stress have started to be studied using mainly model plants like *Arabidopsis*, tomato, and tobacco (Atkinson et al. 2013; Kissoudis et al. 2015; Prasch and Sonnewald 2015). In *A. thaliana*, the genetic architecture underlying plant responses to a set of different stresses applied single or in combined, including the combination of drought with the necrotrophic fungal pathogen *Botrytis cinerea*, was investigated using a GWAS (Thoen et al. 2017). Many of the candidate genes identified in this particular combination were involved in phytohormone-mediated processes, highlighting the role that phytohormones play on biotic/abiotic stress responses. Another GWAS identified key genes related to the *A. thaliana* adaptive responses to *B. cinerea* under sequential drought stress (Coolen et al. 2019). As a major outcome, this study showed that genes involved in JA biosynthesis and a plant invertase/pectin methylesterase inhibitor were playing a role in resistance against both stresses. The interaction of drought with herbivory by *Pieris rapae* caterpillars in *Arabidopsis* was also analyzed by GWAS (Davila Olivas et al. 2017). This study demonstrated the role played by bHLH (basic helix-loop-helix) transcription factors (e.g. MYC family), as major regulators of JA- and ABA-mediated responses, insect resistance, and drought responses.

Attempts to understand the molecular mechanism underlying the interaction between soilborne pathogens causing vascular diseases and drought in legumes are now giving the first steps. Sinha et al. (2017) investigated the effects of the combination of water deficit and a bacterial wilt disease (caused by *Ralstonia solanacearum*) in chickpea (*Cicer arietinum*) using a transcriptomic approach. Genes implicated in osmoprotectant and phytohormone biosynthesis, signaling, xylem differentiation, and cellulose and lignin deposition were found differentially expressed under this combination. In another work, an increase in osmoprotectants such as sugars, with a concurrent decrease in many organic acids such as citric acid and malic acid was described for *M. truncatula* plants subjected to drought and *F. oxysporum* (Dickinson et al. 2018).

The insufficient understanding of the complex molecular mechanisms of plant response to simultaneous stresses supports the need for an integrated functional genomics approach to accurately identify key genes and biomarkers regulating multi-resistance in legume breeding programs.

1.5 Thesis objectives and layout

The four main objectives of this Ph.D. thesis were:

(1) The identification of fusarium wilt and water-deficit resistance sources within the Portuguese common bean germplasm. These abiotic/biotic stresses were applied individually in the entire collection under controlled growth chamber conditions;

(2) The analysis of the Portuguese common bean collection genetic diversity and position of this germplasm in the common bean worldwide diversity, by comparison with representative and wild accessions of the original Andean and Mesoamerican gene pools;

(3) The assessment of natural variation and detection of SNP-trait associations using genome-wide association studies (GWAS), for

the first time, in the Portuguese common bean germplasm. The identification of SNPs associated with fusarium wilt resistance and photosynthesis-related traits under water deficit enabled the study of the genetic basis of the individual stress resistances;

(4) The transcriptomic analysis of contrasting (susceptible / resistant) accessions to identify core stress-signaling pathways overlapping single and combined water deficit and fusarium wilt stresses, and candidate genes involved in the multiple stress response.

The accomplishment of these objectives, at the same time that will value the national common bean germplasm, will provide targets for functional characterization and novel molecular tools, such as functional markers for selection of multiple traits, to accelerate the screening and increase the efficiency of improvement of common bean multiple resistances to abiotic and biotic stresses.

This thesis is structured as follows:

In Chapter II, we reported a simultaneous molecular marker and seed and plant morphological diversity study of a common bean collection of accessions representative of all Portuguese traditional bean-growing regions. The genetic diversity and structure of 175 Portuguese common bean accessions, characterized using microsatellite markers, was described. In order to position the Portuguese germplasm in the common bean global diversity, the Portuguese accessions were compared with wild relatives and representative accessions from the Andean and Mesoamerican gene pools. The development of a core collection retaining all the Portuguese common bean germplasm diversity with minimum repetitiveness was also reported.

In Chapter III, we described the evaluation of 162 Portuguese common bean accessions for fusarium wilt resistance, using a very virulent *Fusarium oxysporum* f. sp. *phaseoli* strain, FOP-SP1 race 6, under growth chamber conditions. This phenotypic characterization, together with a high-density SNP screening of the same accessions, allowed the study of the genetic basis of fusarium wilt resistance in common bean and the identification of resistance associated molecular markers/candidate genes for precision breeding.

In Chapter IV, we investigated the photosynthetic response of 158 Portuguese common bean accessions, grown under well-watered and water-deficit conditions. We quantified leaf relative water content, gas exchange parameters, and photosynthetic pigments contents. Similarly to chapter III, a GWAS was performed to identify SNP-trait associations and candidate genes implicated in the variation of common bean photosynthetic response under contrasting water treatments.

In Chapter V, we tested if a resistant common bean accession, either to fusarium wilt and water deficit – chosen from the characterization of the Portuguese germplasm described in Chapters III and IV – presented shared transcriptomic responses under single or combined water deficit and fusarium wilt stresses. We compared the different expression profiles using a susceptible and a resistant common bean accessions under (a) water-deficit conditions, (b) after inoculation with FOP-SP1 race 6 isolate, and (c) under the combination of both stresses. The functional characterization of the differentially expressed genes identified was attained using MapMan web tools, providing clues to better understand the regulation of plant response to combined stresses.

Finally, in Chapter VI, the thesis main achievements were discussed, with the identification of key lessons and targets for future action points.

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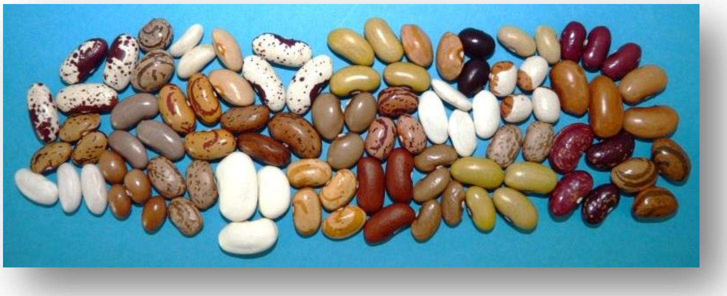
Chapter II

Establishing the bases for introducing the unexplored Portuguese common bean germplasm into the breeding world



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In this research paper, Susana T. Leitão participated in the DNA isolation, SSR genotyping, the seed morphological characterization, data analysis, and drafted the manuscript.

Abstract

Common bean is among the most important grain legumes for human consumption worldwide. Portugal has a potentially promising common bean germplasm, resulting from more than five centuries of natural adaptation and farmers' selection. Nevertheless, limited characterization of this resource hampers its exploitation by breeding programs. To support a more efficient conservation of the national germplasm and promote its use in crop improvement, we performed, for the first time, a simultaneous molecular marker (21 microsatellites and a DNA marker for phaseolin-type) and seed and plant morphological characterization (14 traits) of 175 accessions from Portuguese mainland and autonomous regions traditional bean-growing regions. A total of 188 alleles were identified and an average genetic distance of 0.193 was estimated among accessions. To relate the Portuguese germplasm with the global common bean diversity, 17 wild relatives and representative accessions from the Andean and Mesoamerican gene pools were evaluated at the molecular level. No correlation was detected between the variability found and the geographic origin of accessions. Structure analysis divided the collection into three main clusters. Most of the Portuguese accessions grouped with the race representatives and wild relatives from the Andean region. One third of the national germplasm had admixed genetic origin and might represent putative hybrids among gene pools from the two original centers of domestication. The molecular marker-based classification was largely congruent with the three most frequent phaseolin haplotype patterns observed.

Seed and plant morphological characterization of 150 Portuguese common bean accessions revealed a clear separation among genetic structure and phaseolin haplotype groups of accessions, with seed size and shape and the number of locules per pod the most discriminant traits. Additionally, we used molecular and morphological data to develop a series of smaller core collections that represents the Portuguese common bean germplasm with minimum repetitiveness. A core collection with 37 accessions contained 100% of the genetic variation found in the entire collection. This core collection is appropriate for a more detailed characterization and should be explored, as a priority, in national and international common bean breeding efforts. Furthermore, the identified intermediate accessions may have novel genetic combinations useful in future bean breeding.

Keywords: *Phaseolus vulgaris* L., Portugal, genetic and morphological diversity, admixture, core collection

Introduction

Common bean (*Phaseolus vulgaris* L., 2n = 2x = 22), from now on designated as bean, is a predominantly self-pollinated herbaceous annual plant, grown worldwide for its edible green pods and dry seeds. As a member of the Fabaceae (legume) family, it has an important role in sustainable agriculture due to its capability for fixing atmospheric nitrogen, allowing for the reduction in fertilizer use. Bean has recognized benefits to human health and nutrition, with its high protein content, dietary fiber, and essential vitamins and minerals (Câmara et al., 2013; Petry et al., 2015). This species is the most consumed grain legume in human diets, with 30 million hectares harvested and a global production estimated at 25 million tons in 2014, almost double that of chickpeas and dry peas, the next most consumed grain legumes (FAOSTAT, 2014). As a healthy and inexpensive alternative to animal protein, bean is vital in African (e.g., Burundi and Rwanda) developing countries’ nutrition. In Latin America (e.g., Nicaragua, Brazil and

Mexico), bean is an important part of the staple diet (Petry et al., 2015).

In Portugal, bean represents about 75% of the grain legumes consumed by humans but, although there are many bean landraces still in cultivation, especially in the northern and central regions of Portugal, national production covers only 9.4% of the country’s demand (“Estatísticas Agrícolas 2015”, www.ine.pt).

Wild bean originated in the Mesoamerican region. It is estimated that 111, 000 years ago, a common ancestor diverged into two geographic gene pools, Mesoamerican and the Andean, which were domesticated independently (Bitocchi et al., 2013) giving rise to two gene pools: the Mesoamerican and the Andean. These genetically differentiated pools contain landraces with different seed and leaf sizes, growth habits and seed coat colors and patterns. Based on morphological traits and breeding behavior, different races were defined by Singh et al. (1991), namely *Mesoamerica*, *Jalisco* and *Durango* (within the Mesoamerican pool), and *Nueva Granada*, *Peru* and *Chile* (within the Andean pool). In general, the Mesoamerican germplasm have small (< 25 g 100-seed weight^{-1}) or medium (25 – 40 g 100-seed weight^{-1}) seed sizes and S or B phaseolin types (the major seed-storage proteins of beans). In contrast, in the Andean germplasm, seeds are predominantly large (> 40 g 100-seed weight^{-1}) and have T, C, H or A phaseolin types (Gepts et al., 1986; Singh et al., 1991). Determinacy, characterized by stems ending with a terminal inflorescence, is almost exclusively distributed in the Andean gene pool, probably because in this region bean was domesticated prior to maize and thus grew without a physical support (Koinange et al., 1996; Kwak et al., 2012). In contrast, in the Mesoamerican region, maize and bean have been cultivated together in traditional agriculture and indeterminate types are more common, since bean could use maize plants as supports (Koinange et al., 1996). In addition to these traits, both gene pools present a diversity of seeds colors and shapes, and leaf and bracteole shapes and sizes.

It is known that bean dissemination from the Americas into Europe started during the Columbian Exchange in the 16th and 17th centuries (Gepts and Bliss, 1988). Portuguese and Spanish sailors and traders first introduced bean seeds to Europe from Mesoamerica, given that Columbus arrived in Central America in 1492 and Cortés reached Mexico in 1518 (Rodiño et al. 2003). The introduction of the Andean bean germplasm occurred later, after 1528, with Pizarro's return from Peru (Papa et al., 2006). During the last five centuries, extensive gene flow between these pools, with the development of many intermediate types, has been suggested by several authors, in the Iberian Peninsula as well as in the rest of Europe (Santalla et al., 2002; Logozzo et al., 2007; Gioia et al., 2013). Indeed, about 44% of the European bean germplasm resulted from hybridization between the Andean and Mesoamerican gene pools, a percentage that is almost four to ten times larger than those observed in the Americas (4.3% to 12.3%) (Angioi et al., 2010; Burle et al., 2010). The Iberian Peninsula is considered a secondary center of bean genetic diversity (Santalla et al., 2002), since new forms, better adapted to prevailing conditions in this geographic area, emerged from the initial recombination events between the Mesoamerican and Andean gene pools. These intermediate accessions may contain new combinations of genes of numerous agronomic, technological processing, nutritional and organoleptic bean traits. As an example of these interesting trait combinations, broad-based disease resistance may be achieved in bean by combining resistance genes from both gene pools, since the co-evolution of host and pathogen within gene pools is frequent. As has previously been described, resistance genes from Mesoamerican origin are very effective when transferred to beans of Andean background and vice-versa (Guzman et al., 1995; Geffroy et al., 1999; Miklas et al., 2006).

In Portugal, a great diversity of bean landraces has been cultivated for generations and is still maintained in small fields for farmers' personal consumption and to sell in local markets (Vaz Patto et al., 2007; Leitão et al., 2013). Nevertheless, yield instability, especially due to diseases, pests and drought susceptibility make bean less attractive for extensive farming, particularly under Mediterranean conditions in which heat waves and periods of drought are becoming more frequent. Consequently, in Portugal but also throughout the rest of Europe, there is no high-quality raw bean material production in significant amounts and the processing industries rely completely on foreign materials.

Germplasm collections, hosting traditional landraces, have an important value in biodiversity preservation, development of genetic studies and progress in plant breeding. However, these plant resources must be characterized for morphological, quality and agronomic traits as well as for their genetic diversity, to be effectively conserved and used for breeding. Portugal has considerable bean germplasm resources, which due to their highly diverse and putative intermediate nature, represent important sources of interesting plant traits combinations not yet explored in breeding programs. However, the few studies performed so far to characterize these resources, namely by assessing their genetic variability, were limited to a small number of accessions, collected from a few geographic regions, mainly northern mainland Portugal (Rodiño et al., 2001; Santalla et al., 2002; Martins et al., 2006; Coelho et al., 2009) and Madeira Island (Freitas et al., 2010). Nevertheless, these studies showed that the few Portuguese accessions analyzed were closer to the Andean gene pool and contained significant genetic variability useful for plant improvement in traits such as plant height, seed color and shape, and protein content.

In order to support a more efficient conservation of the national bean germplasm, and to enhance its use in crop improvement, we need to enrich our current understanding of the relationships among accessions and the underlying patterns of diversity through the analysis of a more representative collection. To achieve that, in the present study, we characterized the genetic diversity and structure of an enlarged bean collection, with 175 accessions, originating in all traditional Portuguese bean-growing geographic regions, using microsatellite markers. The genetic diversity of this collection was compared with representative accessions from the Andean and Mesoamerican gene pools, in order to position the Portuguese germplasm in the worldwide diversity of bean. Moreover, we improved the limited available characterization on seed and plant morphological and agronomical traits of the Portuguese bean germplasm. Based on the molecular and morphological data collected, we developed a series of smaller core collections of bean accessions. By capturing, with minimum repetitiveness, the maximum allelic diversity, these collections are more suitable for further detailed characterization of specific traits, and can help to achieve an increased efficiency in the utilization of Portuguese sources in the improvement of this important legume crop.

Material and Methods

Plant Material

The 175 Portuguese bean accessions used in the genetic diversity study were selected based on the national geographical distribution of their collecting sites. One hundred and seven accessions were collected by visiting small farms in a field expedition that took place in 2005 (Vaz Patto et al., 2007), while the remaining accessions were selected from the collection held at the Research Unit of Biotechnology and Genetic Resources, INIAV, Oeiras, Portugal. Based on the available seed, a subset of 150 accessions was selected for morphological evaluation. From the total evaluated accessions, 166 were from mainland Portugal and 9 from the autonomous regions (Azores and Madeira archipelagoes). In more detail, 61 accessions (34.8%) were from the northern interior of mainland Portugal, 11 (6.3%) from the north coast, 68 (38.8%) from the central north, 12 (6.8%) from the central south, 14 (8.0%) from the south, 1 (0.57%) from S. Miguel Island (in the Azores) and 8 (4.6%) from Madeira Island. Maps indicating the collecting sites of the Portuguese accessions, as well as the national climatic data and soil main characteristics are available in Supplementary Figures 1-4. In addition, 17 bean accessions were selected from the CIAT seed bank collection, 11 based on their gene pool and eco-geographic race and 6 wild relatives. From those 17 accessions, 5 were representatives of the Mesoamerican gene pool (2 from race *Mesoamerica*, 2 from race *Guatemala* and 1 from race *Jalisco*), 6 were representatives of the Andean gene pool (2 from race *Peru*, 2 from race *Chile* and 2 from race *Nueva Granada*) and the 6 wild accessions were from Argentina, Bolivia, Colombia, Guatemala, Mexico and Peru. A complete list of the accessions studied, along with their “passport” information, is available in Supplementary Table S1.

DNA isolation

Ten seeds randomly selected from each of the 192 accessions (175 Portuguese plus 17 from the CIAT collection) were sown. Individual young leaves of plant were collected, then immediately frozen in liquid nitrogen and stored at -80ºC until DNA isolation. DNA was isolated using a modified CTAB protocol developed by Torres et al. (1993). DNA quality was evaluated on 0.8% agarose gels (Lonza, Rockland, USA) with SYBRSafe (Invitrogen, Eugene, USA) staining and visualized using a GEL-DOC1000 System (Bio-Rad, Hercules, USA), followed by quantification in a NanoDrop^{TM} 2000c Spectrophotometer (Thermo Scientific, Passau, Germany). DNA was diluted in TE buffer to 10 ng/µL for subsequent experiments. In total, DNA from 1826 individuals was isolated (9.5 individuals per accession on average with a minimum of 6 individuals per accession).

Fluorescent microsatellite analysis

The selection of the 21 microsatellite markers (simple sequence repeats, SSR) used was made based on their uniform distribution and coverage of the 11 chromosomes of the bean genome (Yu et al., 2000; Gaitan-Solis et al., 2002; Blair et al., 2003). Two types of SSRs were included: for coding (gene-derived markers) and non-coding (SSRs derived from non-coding genomic DNA) regions (Supplementary Table S2).

The method for fluorescent labeling of PCR fragments developed by Schuelke (2000) was used. Accordingly, a M13 tail was added to the 5”-end of the forward primers, which allows the annealing of the universal M13(-21) primer labeled with IRDye fluorescence and the visualization of the amplified fragments, resolved in a 6.5% polyacrylamide gel (KBPlus Gel Matrix, LI-COR), using a LI-COR 4300 DNA Analyzer (Lincoln, NE, USA). PCR reactions were conducted in a total volume of 10 µL containing 10 ng of template DNA, 0.04 µM of M13(-21) tagged forward primer, 0.16 µM of IRD700 or IRD 800 M13(-21) and 0.16 µM of reverse primer, 0.2 mM of each dNTP, 1.5 mM of

$MgCl_2$, and 0.2 unit of Taq DNA polymerase (Promega, Madison, USA). The amplification reactions consisted of a denaturing step of 5 minutes at 94 ºC, followed by 30 cycles of 30 s at 94 ºC, 45 s at 56 ºC, 45 s at 72 ºC, and 8 cycles of 30 s at 94 ºC, 45 s at 53 ºC, 45 s at 72 ºC. The reactions were terminated at 72 ºC for 10 minutes. After the amplification, 1 µL from each reaction product was mixed with 25 µL of formamide-loading buffer (98% formamide, 10 mM EDTA pH = 8.0 and 0.1% Bromo Phenol Blue). The total mixture was carefully vortexed, heated for 5 minutes at 95 °C in a denaturation hotblock and then quickly cooled on ice. 0.5 to 0.7 µL of each sample was loaded on the 6.5% denaturing polyacrylamide gel.

Allele sizes (in base pairs) of PCR products were estimated using SAGA^{GT} software from LI-COR and data compiled in a matrix for further analysis.

Phaseolin type analysis

A DNA marker for phaseolin-type diversity analysis was used (Kami et al., 1995), which specifically amplifies a region surrounding the 15-bp tandem direct repeat of the phaseolin gene family. The method for fluorescent labeling of PCR fragments developed by Schuelke (2000) was used and the PCR conditions were the same described above for the SSR markers.

Molecular data analysis

To verify the usefulness of the molecular markers selected to assess the genetic diversity of the accessions analyzed, the Polymorphism Information Content (PIC) of each microsatellite marker was calculated by PowerMarker v3.23 software (Liu and Muse, 2005). GENEPOP v4.0 (Rousset, 2008) was used to calculate the average number of alleles per locus (N_{av}) and the observed (H_O) and expected heterozygosity (H_E, or gene diversity) of each accession. The effective number of alleles (N_e) was estimated using GenAlEx 6.5 (Peakall and Smouse, 2006). The allelic richness (N_{ar}), as the measure of the number of alleles per locus independent of sample size was calculated using FSTAT v2.9.3.2 program package (Goudet, 1995; Goudet, 2002), while the number of private alleles per accession (alleles present in a subgroup of a broader collection of accessions that are indicative of gene pool differentiation) was assessed using MICROSAT software (Minch et al., 1997). Private allelic richness (N_{par}) within each accession was estimated after controlling for differences in sample size using the rarefaction method (Kalinowski, 2004) implemented in the program HP-Rare (Kalinowski, 2005).

To test the significance of the differences in N_{ar}, H_O and H_E between two groups of accessions (Portuguese vs. Andean and Mesoamerican race representatives and wild relatives) as well as among accessions originating from different regions in Portugal, Kruskal-Wallis (among all groups) and Wilcoxon (between all possible pairs of groups) non-parametric tests were performed using SAS/STAT® software (2004). Furthermore, the differences in N_{ar}, H_O and H_E between two groups (Portuguese vs. Andean and Mesoamerican race representatives and wild relatives) were tested on individual level across microsatellite markers by repeated measures analysis of variance using PROC GLM in SAS/STAT® software (2004).

To check for a possible correlation between the geographic distance and the genetic differentiation of the accessions, isolation by distance (IBD) among Portuguese accessions was tested using the method of Rousset (1997). For this analysis, we only considered the 134 accessions from which the GPS coordinates of their local of origin were available and excluded the insular accessions from Madeira and the Azores. A Mantel test (106 permutations of accession locations among all locations) on the matrix of pairwise FST/(1-FST) ratios and that of the natural logarithm of geographical distances (in km) between pairs of accessions was performed using NTSYS-pc v2.02 (Rohlf, 1997).

To test for the existence of genetic structure in this collection, an analysis of molecular variance (AMOVA) was performed, based on SSR markers, using ARLEQUIN v3.0 (Excoffier et al., 2005). We tested four partitions of variance: (A) among and within all bean accessions; (B) among groups of accessions (Portuguese vs. gene pools representatives and wild relatives), among accessions within groups and within accessions, as well as among and within accessions separately for Portuguese (C) accessions and gene pools representatives and wild relatives (D). Variance components were tested statistically by non-parametric randomization tests using 10,000 permutations.

Pairwise Cavalli-Sforza and Edwards' chord distances (Cavalli-Sforza and Edwards, 1967) were calculated and cluster analysis was performed using Neighbor-joining algorithm with 1,000 bootstraps (Felsenstein, 1985) over microsatellite loci as implemented in SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE programs of the PHYLIP v3.6b software package (Felsenstein, 2004). In addition, the genetic distance matrix obtained was used to construct a dendrogram through the Neighbor-joining algorithm.

A model-based clustering method was applied on multilocus microsatellite data to infer genetic structure and define the number of clusters in the dataset using the software STRUCTURE v2.3.3 (Pritchard et al., 2000). Ten runs per each cluster ranging from 1 to 11 were carried out on the Isabella computer cluster at the University of Zagreb, University Computing Centre (SRCE). Each run consisted of a burn-in period of 200,000 steps followed by 10^6 MCMC (Monte Carlo

Markov Chain) replicates assuming admixture model and correlated allele frequencies. No prior information was used to define the clusters. The choice of the most likely number of clusters (K) was carried out by comparing the average estimates of the likelihood of the data, ln[Pr(X|K)], for each value of K, as well as by calculating an *ad hoc* statistic ΔK, based on the rate of change in the log probability of data between successive K values as described by Evanno et al. (2005). The software program STRUCTURE HARVESTER v0.6.92 was used to process the STRUCTURE results files. The runs with the maximum likelihood were chosen and by averaging the estimated membership coefficients of the individuals, the proportion of ancestry of each accession in each of the clusters was calculated. The accessions were assigned to a particular cluster if at least 75% of their genome was estimated to belong to that cluster while those accessions with value < 75% for all the clusters were considered of admixed origin.

Assessment of phaseolin types and relationship with structure memberships

Phaseolin type of each accession was assessed based on the pattern of the fragments amplified with the phaseolin molecular marker (Kami et al., 1995). The phaseolin patterns obtained in the Portuguese accessions were compared with the ones from CIAT accessions (Andean and Mesoamerican race representatives and wild relatives), from which the characteristic phaseolin type is known. The phaseolin pattern of all the accessions was compared to the clustering obtained after structure analysis, in order to verify the correspondence between the phaseolin types and the structure membership obtained. Based on this correspondence, accessions were categorized as true-types or as offtypes according to the following four designations: (1) True-type: unique type of phaseolin within all individuals of each accession, percentage of cluster membership (Q in structure analysis) higher than 75% and phaseolin type matching the cluster membership; (2) Offtype - Composite: Q < 75% at accession level, but with less than 50% of their individuals with Q < 75%; (3) Offtype - Hybrid: Q < 75% at the accession level, but with at least 50% of the individuals with Q < 75%; (4) Offtype - non-corresponding: Q > 75% at accession level, but with more than one phaseolin type and/or no correspondence between clusters and phaseolin types.

Since accessions classified as offtype had an admixture nature and could be considered putative hybrids, the subsequent statistical tests performed, to compare molecular and morphological diversity among groups of accessions, were done only for true-type accessions. To test the significance of the differences in N_{ar}, H_O and H_E among true-type groups of accessions, Kruskal-Wallis (among all groups) and Wilcoxon (between all possible pairs of groups) non-parametric tests were performed using SAS/STAT® software (2004).

Phenotypic data analysis

A total of 150 Portuguese bean accessions were grown during 2014 in a farmer’s field at Cabrela (near Sintra, Portugal) in 3.0 m-rows spaced 50 cm using traditional farmers’ production management. The soil was a haplic luvisol, and during the vegetative cycle (May to August/September) the temperature ranged from 18 ºC to 28 ºC (mean values of minimum and maximum temperatures). Accessions revealing a climbing habit were stacked to permit a full development of the plants.

Data on morphological and agronomic traits were collected from seeds and plants, following the descriptors for *Phaseolus vulgaris* L. (IBPGR, 1982). Ten plants per accession were used to measure the number of seeds per pod, the number of locules per pod (considering only the longest pod) and the plant growth habit (determined or indetermined). Additionally, the weight (g) of 100 seeds of each accession was recorded. For the seed size measurements, an average of 60 seeds per accession, randomly selected, was used. Seed length (L, mm) was measured as the longest distance across the seed parallel to the hilum, seed height (H, mm) as the longest distance perpendicular to length and seed width (W, mm) was measured as the longest distance across the hilum of the seed. In addition, seed flatness (H/W), flatness index ((L+H)/2W) and elongation (L/H) were calculated. Seed shape was also characterized using the qualitative scale: rounded, oval, cuboid or kidney-shaped. Seed coat patterns were classified as absent, spotted bicolored, striped, broad striped, mottled, speckled with marginal color and colored around hilum. Seed coats colors were determined by the naked eye and only for the seeds without pattern.

In total, nine quantitative (seed length, seed width, seed height, 100-seed weight, number of seeds per pod, number of locules per pod, elongation, flatness and flatness index) and five qualitative (growth habit, seed shape, presence or absence of pattern, seed coat color and seed coat pattern) traits were measured.

Univariate analysis of variance (ANOVA) using PROC GLM in SAS software was conducted to test the mean differences between true-type groups for the nine quantitative traits. *Post-hoc* comparisons of the accession means were carried out using Tukey's studentized range test at $P \leq 0.05$. Pearson's correlation coefficients were calculated among the nine quantitative traits using PROC CORR and principal component analysis (PCA) was performed using PROC PRINCOMP from SAS software.

Discriminant analysis (DA) using SAS software was performed to determine which of the nine quantitative traits were the most useful for maximum discrimination among true-type groups of accessions.

With DISCRIM procedure, a discriminant criterion was developed to classify each accession into one of the groups. F test significance level from the analysis of covariance was used as the selection criterion. The STEPDISC procedure allowed the selection of a subset of quantitative traits for use in discriminating among the groups, through a stepwise discriminant analysis. Partial r^2 statistics accounted for the significant discriminative potential of each trait, while Wilks' lambda was used to denote the statistical significance of the discriminatory power of the model function. The discriminant function, with the chosen subset of quantitative traits, was then evaluated for its performance in classifying the accessions correctly into their respective true-type group. Error rates (probabilities of misclassification) were estimated using cross-validation. The discriminant function was finally applied to the total data set (including offtype accessions), once again using the DISCRIM procedure.

For the five qualitative traits, a likelihood-ratio chi-square test in SAS software was used to test for differences in the number of accessions having a particular trait state across true-type groups. The same procedure was used to test for differences across the seven defined Portuguese regions of origin of the accessions evaluated: the north coast, northern interior, central north, central south, south, in Portugal mainland, and the two Autonomous Regions, the Azores and Madeira.

Construction and validation of a core collection

To select a core collection that could represent the whole collection with minimum repetitiveness in future studies, we used molecular marker and morphological data. Based on the allelic diversity profile obtained in the Portuguese bean collection after the screening with 21 SSRs, one representative individual per accession was chosen. This choice relied on an individual with the most frequent genotype among each accession. Following this procedure, we used the molecular data from the 150 representative individuals of the same accessions that were phenotyped in the subsequent analysis.

The qualitative traits used were growth habit, seed shape, seed coat color, seed coat pattern, as already defined, and seed size. Seed size (qualitative) was estimated from the originally measured 100-seed weight trait, by converting it into a categorical variable, based on a classification widely used on bean by several authors for instance, Singh et al. (1991): (1) small seed size (100-seed weight < 25 g), (2) medium seed size (100-seed weight 25 – 40 g) and (3) large seed size (100-seed weight > 40 g).

Two different complementary algorithms were used based on a maximization (M) strategy. On the first approach, we employed the standard M strategy (Schoen and Brown, 1993) as implemented in MSTRAT (Gouesnard et al., 2001) to identify a series of nested core subsets that capture the maximum molecular and morphological diversity given the sample size. This method is considered to be the best in the coverage rate of both molecular and phenotypic data compared with the other conventional methods (Kim et al., 2007). We constructed the optimal core subset of 10 individuals (6.67% of the entire collection) and repeated the analysis for larger subsets (sample sizes: 15, 20, 25 and 30) while constraining the algorithm to include all the individuals already assigned to a core subset of a smaller size. Two hundred independent replicates and 100 iterations were generated for each sampling size and the core subset having the highest Shannon's diversity (Lewontin, 1972) was chosen. The second approach was to use the advanced maximization (M) strategy as implemented in PowerCore v1.0 (Kim et al., 2007) in order to select the accessions representing the total (100%) coverage of alleles and trait states present in the entire collection.

The molecular diversity retained in the core subsets was compared to that of the entire collection based on average number of alleles (N_{avg}) as well as observed (H_O) and expected heterozygosity (H_E), calculated using GENEPOP v4.0. Repeated measures analysis of variance was carried out using PROC GLM in SAS. *Post hoc* Bonferroni's adjustments were used to compare the means of diversity estimates from different core subsets, with the entire collection as control. Morphological diversity of core subsets and of the entire collection was assessed by calculating the average number of traits states and Shannon's diversity index (H_{Sh}). The principal coordinate analysis (PCoA) based on molecular data was performed using NTSYS-pc v2.10 (Rohlf, 2005) to graphically represent genetic relationships among bean accessions. The proportion-of-shared-alleles distances (D_{PSA}) (Bowcock et al., 1994) among representative individuals of each accession were calculated using MICROSAT (Minch et al., 1997).

Results

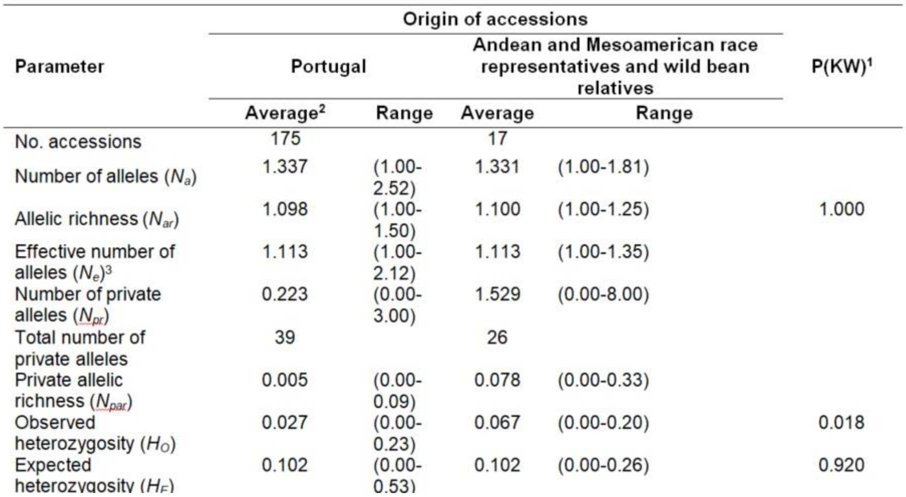
Allelic diversity

The microsatellite markers screening of the 175 Portuguese accessions, and 17 Andean and Mesoamerican race representatives, and wild bean relatives identified 225 alleles (complete dataset available online at Dryad repository). All 21 SSRs analyzed were polymorphic with an average of 10.71 alleles per locus, varying from 2 to 26 alleles each (Supplementary Table S3). Overall, the most informative markers (with the highest PIC) were PVat007, GATS91 and BM143, which were also the ones showing the highest number of alleles per locus. Within the Portuguese accessions, 188 alleles were identified, with an average of 8.95 alleles per locus, and 39 private alleles were found. Within race representatives and wild relatives, 151 alleles were identified, with an average of 7.19 alleles per locus, and 26 private alleles were found (Supplementary Table S3). The effective number of alleles (*Ne*) was 1.113 for both groups (Table 1). Also, there were no significant differences in allelic richness (N_{ar}) among the two groups: 1.098 in the Portuguese accessions and 1.100 in the race representative and wild relative bean accessions (P(*KW*)=1.000). Private allelic richness (N_{par}) was on average 0.005 for the Portuguese accessions and 0.078 for Andean and Mesoamerican race representatives and wild bean relatives. Observed heterozygosity (H_O) was low for both groups, as expected in a mainly self-pollinated species, but significantly lower (P(*KW*)=0.018) for the Portuguese accessions, with an average value of 0.027 in the Portuguese accessions and of 0.067 in the race representatives and wild relatives (Table 1). Sixty-two Portuguese accessions had no heterozygous individuals (H_O = 0.000) and nine of them were genetically uniform (H_E = 0.000). The average value of H_E was equal for the Portuguese bean accessions and for the race representatives and wild relatives, with no significant differences among groups (P(*KW*)=0.920) at the accession level (Table 1).

The diversity parameters N_{ar}, H_O and H_E between the two groups (Portuguese vs. Andean and Mesoamerican race representatives and wild bean relatives), tested on individual level across microsatellite markers, showed that H_O was significantly different (P(*KW*)=0.026) among groups. No significant differences (P(*KW*)=0.479) were found on N_a. In contrast, H_E at the individual level was significantly higher (P(*KW*)=0.000) for the Andean and

Mesoamerican race representatives and wild bean relatives than for the Portuguese accessions (Supplementary Table 4).

Table 1 - Genetic diversity of the common bean accessions as assessed by 21 microsatellite loci.



Parameter	Origin of accessions				P(KW)[1]
	Portugal		Andean and Mesoamerican race representatives and wild bean relatives		
	Average[2]	Range	Average	Range	
No. accessions	175		17		
Number of alleles (N_a)	1.337	(1.00-2.52)	1.331	(1.00-1.81)	
Allelic richness (N_{ar})	1.098	(1.00-1.50)	1.100	(1.00-1.25)	1.000
Effective number of alleles (N_e)[3]	1.113	(1.00-2.12)	1.113	(1.00-1.35)	
Number of private alleles (N_{pr})	0.223	(0.00-3.00)	1.529	(0.00-8.00)	
Total number of private alleles	39		26		
Private allelic richness (N_{par})	0.005	(0.00-0.09)	0.078	(0.00-0.33)	
Observed heterozygosity (H_O)	0.027	(0.00-0.23)	0.067	(0.00-0.20)	0.018
Expected heterozygosity (H_E)	0.102	(0.00-0.53)	0.102	(0.00-0.26)	0.920

[1] Significance of Kruskal-Wallis test
[2] Average (and range) across accessions
[3] Harmonic mean

Genetic diversity across regions

The genetic diversity analysis of the Portuguese common bean accessions grouped by region of origin, as assessed by 21 SSRs, is depicted in Supplementary Table 5. Kruskal-Wallis test indicated that no significant differences were found on genetic diversity parameters such as allelic richness (P(*KW*)=0.506), observed (P(*KW*)=0.147) and expected heterozygosity (P(*KW*)=0.476) among accessions from different regions of origin.

Isolation by distance analysis

The isolation by distance analysis among the 134 Portuguese common bean accessions tested revealed that there was no relationship between the geographic distance and the genetic differentiation of the accessions ($r=0.0054$; $P_{Mantel}= 0.44$, Supplementary Figure 5).

Phaseolin types

Three phaseolin patterns (classified here as haplotypes P1, P2 and P3) were the most common among accessions after screening with the molecular marker for the phaseolin-type analysis. Comparing the haplotypes found in the Portuguese accessions with the ones from the bean gene pool representatives, it was possible to relate the patterns obtained with the phaseolin types already described by Gepts et al. (1986) for the Mesoamerican and Andean gene pools. The accessions representative of Mesoamerican gene pool analyzed were from ecogeographic races *Jalisco*, *Guatemala* and *Mesoamerica* and had phaseolin types S and/or B; while accessions representative of Andean gene pool analyzed were from the ecogeographic races *Peru*, *Chile* and *Nueva Granada* and had phaseolin types H, C or T, according to CIAT gene bank information.

Thus, the pattern obtained for P1 haplotype corresponded to S type phaseolin characteristic of Mesoamerican accessions, while the pattern obtained for P2 haplotype corresponded to H or C types of phaseolin and the pattern obtained for P3 haplotype corresponded to T type of phaseolin, both characteristic of the Andean accessions (Supplementary Table S6).

Analysis of molecular variance

AMOVA was conducted to test for the existence of genetic structure between and within groups of accessions (Portuguese accessions versus gene pool representatives and wild relatives from the CIAT collection). This analysis showed that the differences between the two groups of accessions (Portuguese and CIAT accessions) were significant ($P < 0.0001$) and explained 12.9% of the total genetic variance (Table 2). For the entire set of analyzed accessions, the major source of variance was among accessions and not within them (83.6% versus 16.4%), reflecting again the predominant self-pollinating reproductive system of bean. Similar percentages were observed when the Portuguese accessions were analyzed separately from the race representative and wild relative accessions (Table 2).

Table 2 - Analysis of molecular variance for the partitioning of microsatellite diversity.
(A) among and within all bean accessions, (B) among groups of accessions (Portuguese vs. race representative and wild relatives), among accessions within groups and within accessions, as well as among and within accessions, separately for Portuguese (C) accessions and for race representative and wild relative accessions (D).

Analysis	Source of variation	df	Variance components	% Total variance	ϕ–statistics	$P(\phi)$
(A) All accessions	Among accessions	191	4.311	83.65	0.837	< 0.0001
	Within accessions	3460	0.842	16.35		
(B) Portuguese vs. race representative and wild relatives	Among groups	1	0.746	12.89	0.129	< 0.0001
	Among accessions within groups	190	4.197	72.55	0.833	< 0.0001
	Within accessions	3460	0.842	14.56	0.854	< 0.0001
(C) Portuguese	Among accessions	174	4.093	83.11	0.831	< 0.0001
	Within accessions	3175	0.832	16.89		
(D) Race representative and wild relatives	Among accessions	16	5.416	84.89	0.849	< 0.0001
	Within accessions	285	0.964	15.11		

Genetic analysis and population structure

The average Cavalli-Sforza and Edwards' chord distance, generated from the SSR data, was 0.193 among Portuguese accessions, while it was 0.258 for the race representatives and wild relatives (Table 3 and Figure 1). Although the maximum genetic distance was similar for the two groups of accessions (0.394 for the Portuguese accessions, between accessions 1892 and 5302 as well as between accessions 1938 and 5302, versus 0.389 for the Andean and Mesoamerican race representatives and wild relatives, between accessions g2333 and g9603), the minimum values were substantially lower among Portuguese accessions (0.003, between accessions 1976 and 2126, versus 0.102 for the race representatives and wild accessions, between accessions g51105 and g51294). Three major clusters were visualized in the Neighbor-joining tree (Figure 1). One cluster grouped mainly the race representatives and wild relatives from the Mesoamerican region, with a small part of the Portuguese accessions. The other two clusters grouped most of the Portuguese accessions with the race representatives and the wild relatives from the Andean regions.

Table 3 - Pairwise Cavalli-Sforza and Edwards' chord distances (D_{Chord}) among the analyzed bean accessions.

Origin of accessions	**D_{Chord}**		
	Average	Min	Max
Portugal	0.193	0.003	0.394
Andean and Mesoamerican race representatives and wild accessions	0.258	0.102	0.389
All accessions	0.205	0.003	0.398

Structure analysis of the bean accessions analyzed confirmed the major clusters found using the Neighbor-joining algorithm. The highest ΔK was observed for K = 2 (7028.15) followed by K = 3 (657.21). For K higher than 3, ΔK was always inferior to 2.9. K = 2 showed the major separation between Andean and Mesoamerican gene pools, while K = 3 showed an additional sub-division within the Andean gene pool, separating the representatives of the races *Peru* and *Nueva Granada* from the ones of *Chile*. The Portuguese accessions distributed along with the analyzed representative accessions from both Mesoamerican and Andean gene pools but with a higher prevalence closer to the Andean clusters. Through the combination of the structure analysis with the three major phaseolin haplotypes found (P1, P2, P3), we observed that cluster A (red) contains most of the accessions with P1 phaseolin type, characteristic of the Mesoamerican gene pool, and clusters B1 (blue) and B2 (green) most of the accessions with P3 and P2 phaseolin types, respectively, both characteristic of the Andean gene pool (Figure 1).

Genetic differentiation into subgroups

Based on the classification made, using the results of structure analysis and phaseolin haplotype/cluster membership matching, different groups of accessions were established. From the total of 175 Portuguese accessions, 118 were classified as true-type accessions. Those were distributed in the three true-type groups as follows: 26 accessions in the AP1 group, 36 accessions in the B1P3 group and 56 accession in the B2P2 group. The remaining 57 accessions (33%) were not assigned to any gene pool since they represented putative hybrids among gene pools hereby referred as offtypes (Table 4). Within the offtype accessions, 7 were classified as composite, 20 as hybrid and 30 as non-corresponding.

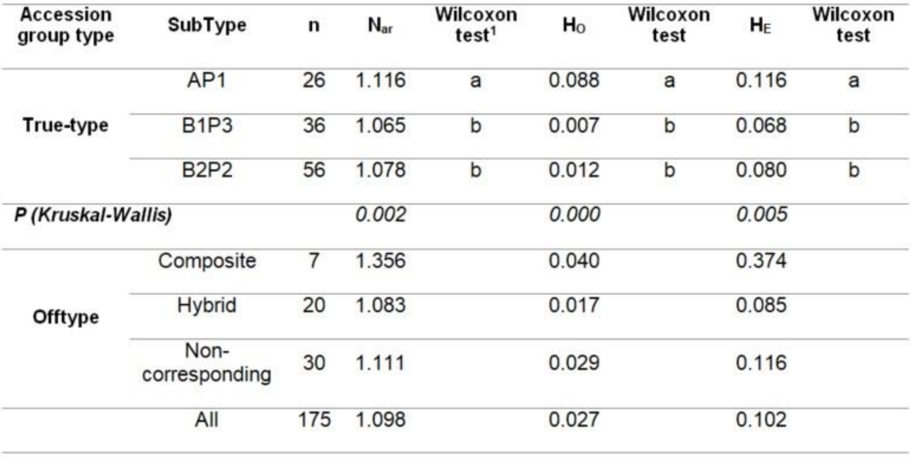
Among the true-type groups of accessions (AP1, B1P3, B2P2), a Kruskal-Wallis test showed that the differences between the average values of $N_{ar,}$ H_E and H_O were statistically significant ($p < 0.05$). A Wilcoxon test (between all possible pairs of groups) revealed that the significant differences found were between the AP1 and both B1P3 and B2P2 groups; that is, between the Mesoamerica and Andean groups (Table 5).

Table 4 - Assignment of the Portuguese bean accessions into subtypes according to the SSR analysis and phaseolin haplotype.

Classification	Membership cluster	Phaseolin haplotype	No accessions	% accessions
True-type	A	P1 – S Mesoamerican type	26	14.86
True-type	B1	P3 – T Andean type	36	20.57
True-type	B2	P2 - H, C Andean type	56	32.00
Offtypes	-	-	57	32.57

Total		175	100

Table 5 - Number of bean accessions (n), mean values of allele richness (N_{ar}), observed (H_o) and expected heterozygosity (H_E) distributed in the different Portuguese bean group types. Kruskal-Wallis test is shown for true-types only. Letters *a* and *b* reflect the result from the Wilcoxon test.



Accession group type	SubType	n	N_{ar}	Wilcoxon test[1]	H_O	Wilcoxon test	H_E	Wilcoxon test
True-type	AP1	26	1.116	a	0.088	a	0.116	a
	B1P3	36	1.065	b	0.007	b	0.068	b
	B2P2	56	1.078	b	0.012	b	0.080	b
P (Kruskal-Wallis)			*0.002*		*0.000*		*0.005*	
Offtype	Composite	7	1.356		0.040		0.374	
	Hybrid	20	1.083		0.017		0.085	
	Non-corresponding	30	1.111		0.029		0.116	
	All	175	1.098		0.027		0.102	

[1] Different letters in the same column indicate significant differences between values at $P < 0.05$ on the basis of the Wilcoxon test.

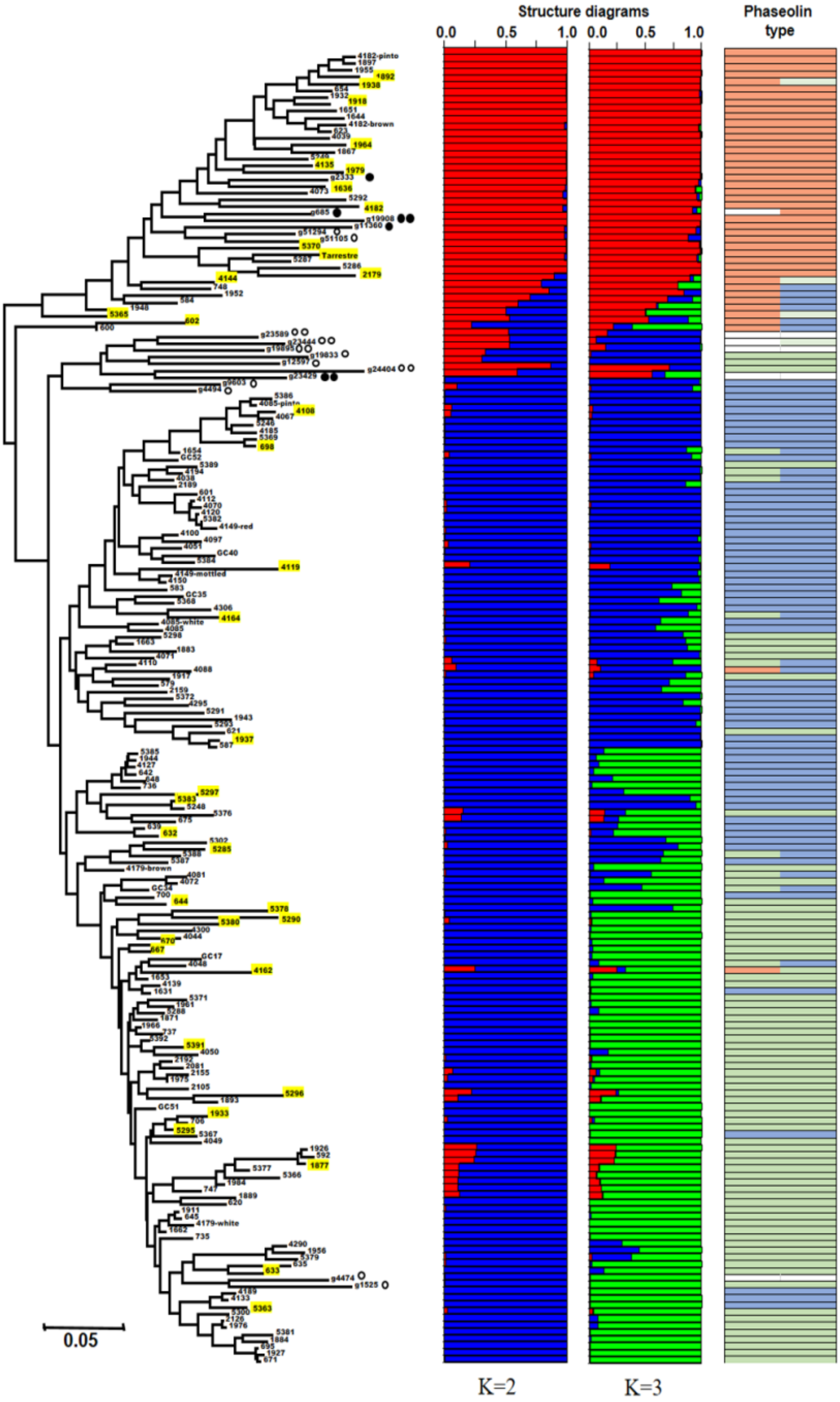

Structure diagrams
Phaseolin type
0.0
0.5
1.0
0.0
0.5
1.0
0.05
K=2
K=3

Figure 1 - Neighbor-joining tree based on chord distance between 192 bean accessions (175 Portuguese and 17 Mesoamerican and Andean race representatives and wild bean relatives). The 37 accessions belonging to the core collection are highlighted in yellow. Structure bar plots of average proportions of membership for K= 2 (in red and blue) and K = 3 (in red, blue and green) clusters are given for all the accessions studied. Bootstrap values above 50 are shown. The most frequent phaseolin haplotypes identified in the accessions are indicated in the last column with a color code (P1 in orange, P2 in light green and P3 in light blue); X stands for other type of phaseolin pattern (colored white). ● accession representative of the Mesoamerican domesticated gene pool;●● wild accession from the Mesoamerican region; ○ accession representative of the Andean domesticated gene pool; ○○ wild accession from the Andean region.

Seed and plant morphological traits

High diversity was found among the 150 bean accessions analyzed for the nine quantitative traits (seed length, seed width, seed height, seed elongation, seed flatness, seed flatness index, 100-seed weight, number of seeds per pod and number of locules per pod) measured. The analysis of variance revealed significant differences among bean true-type groups AP1, B1P3 and B2P2 for the nine quantitative traits evaluated (Supplementary Table S7).

Tukey's studentized test revealed that the three true-type bean groups were significantly different from each other in seed width and flatness. Accessions from the AP1 group were significantly lighter (inferior 100-seed weight) than the accessions from the other groups. Accessions from the B2P2 group had a significantly larger seed height and a lower flatness index, than the rest. Accessions from the B1P3 group had significantly larger seed length, with higher seed elongation, but with a smaller number of seeds per pod as well as a smaller number of locules per pod, than the rest of the accessions from the other true-type groups.

Besides the nine quantitative traits, five qualitative traits were evaluated on the Portuguese bean accessions. True-type groups were tested for significant differences among trait states using the likelihood-ration chi-square test.

Seed shapes were categorized into four different classes: round, oval, cuboid and kidney shape (Table 6).

Half of the 150 Portuguese accessions analyzed possesses a cuboid seed shape, followed by the oval (27.3%) and the kidney-shaped (22%). The cuboid shape was the most frequent seed shape in all three true-type groups. Nonetheless, significant differences were found among the three true-type groups within the number of accessions having a particular seed shape trait state. For instance, accessions from the true-type B2P2 group clearly showed a much higher percentage of oval seeds and a lower percentage of cuboid or kidney-shaped seeds, when compared with the other two true-type group accessions.

Table 6 - Seed shapes found in the collection of 150 bean seeds distributed by accession group types. Significance of the likelihood-ratio chi-square, among true-type groups, is shown.

		Seed shape				
Accession group type	Subtype	Round	Oval	Cuboid	Kidney	Total
True-type	AP1	1	1	15	4	21
	B1P3	0	1	17	7	25
	B2P2	0	22	22	6	50
	P (χ^2)			*0.000139*		
Offtype	Composite	0	3	1	2	6
	Hybrid	0	4	6	9	19
	Non-corresponding	0	10	14	5	29
Total		1	41	75	33	150

From the total of 150 Portuguese bean accessions evaluated, 90 presented plain coat seeds (no seed coat pattern) and 60 presented a patterned seed coat. The chi-square test indicated that there were no significant differences in the number of accessions having or not a seed pattern among the true-type groups ($P\ (\chi^2)$ = 0.563) (Supplementary Table S8).

Within the 60 accessions analyzed that had a seed coat pattern, six different patterns, also described in IBPGR (1982), were found (Table 7).

Table 7 - Pattern types of the 60 Portuguese bean accessions analyzed exhibiting seed coat patterns, distributed according to accession group types. Significance of the likelihood-ratio chi-square is shown among true-type groups.

		Seed coat pattern					
Accession group type	Subtype	spotted bicolor	speckled with marginal colour	striped	mottled	broad striped	colored around hilum
True-type	AP1	0	1	6	0	0	0
	B1P3	0	0	8	0	3	1
	B2P2	2	2	17	2	0	0
	$P\ (\chi^2)$			*0.0581*			
Offtype	Composite	0	0	1	0	0	0
	Hybrid	0	1	4	1	1	0
	Non-corresponding	4	0	5	1	0	0
	Total	6	4	41	4	4	1

The most common seed coat pattern observed among Portuguese accessions was striped. In accordance with the chi-square test performed, no particular true-type group stood out with a significant different number of accessions having a particular seed coat pattern.

Eight different seed coat colors were attributed by the naked eye to the plain coat seeds (with no pattern): white, yellow, pink, light brown, brown, red, purplish red and black (Table 8).

Table 8 - Seed coat colors of the 90 Portuguese bean accessions analyzed with plain seed coat (no patterned seed coat), distributed by accession group types. Significance of the likelihood-ratio chi-square is shown among true-type groups.

		Seed coat color (plain seed coat)							
Accession group type	Subtype	white	yellow	pink	light brown	brown	red	purplish red	black
True-type	AP1	7	0	1	2	3	0	0	1
	B1P3	4	1	2	0	0	5	1	0
	B2P2	1	0	10	3	6	6	1	0
	P (χ^2)				*0.0007*				
Offtype	Composite	3	1	0	0	0	1	0	0
	Hybrid	5	2	2	1	1	1	0	0
	Non-corresponding	12	0	1	1	4	0	1	0
	Total	32	4	16	7	14	13	3	1

The four most frequent colors of plain seed coats among the Portuguese beans were white, pink, brown and red. For this trait, there were significant differences among the true-type groups of accessions (P (χ^2) = 0.0007). Accordingly, the AP1 group of accessions had no red-colored accessions and most of its accessions were white, whereas both the B1P3 and B2P2 groups presented red seed coat accessions. Moreover, the majority of the accessions from the B2P2 group were pink.

Regarding the plant growth habit of the 150 Portuguese bean accessions, 92 accessions had a determinate growth habit, and 58 an indeterminate growth habit (Table 9).

Significant differences among true-type groups were observed. All the accessions from the B1P3 group had a determinate growth habit, whereas the other two true-type groups had accessions with both growth habits. Nevertheless, the AP1 group had a prevalence of determinated plants, while among the B2P2 group the more common growth habit was the indetermined one. Among offtype groups most of the accessions had determinated plants.

Table 9 - Plant growth habit of 150 Portuguese bean accessions. Significance of the likelihood-ratio chi-square is shown among true-type groups.

		Plant growth habit	
Accession group type	Subtype	Determinate	Indeterminate
True-type	AP1	12	9
	B1P3	25	0
	B2P2	20	30
	P (χ^2)	*9.15×10^{-8}*	
Offtype	Composite	4	2
	Hybrid	14	5
	Non-corresponding	17	12
	Total	92	58

There were no significant differences among the true-type groups in the number of accessions collected in each of the seven different Portuguese regions of origin (north coast, northern-interior, central north, central south, south, the Azores, Madeira) (P (χ^2) = 0.316) (Supplementary Table S9). The larger proportion of accessions came from the north central and north interior regions (39% and 35%, respectively).

Moreover, there were not significant differences in the distribution of accessions from each region of origin when comparing true-type and offtype groups (P (χ^2) = 0.211).

Correlation between the quantitative morphological traits

The estimation of the Pearson's correlation coefficients among the nine quantitative morphological traits allowed the measurement of dependence between traits. Accordingly, seed length was highly correlated with 100-seed weight (r = 0.706), elongation (r = 0.841) and flatness index (r = 0.759). Not surprisingly, there were also strong positive correlations between the number of locules per pod and the number of seeds per pod (r = 0.773), between elongation and flatness index (r = 0.771) and between flatness and flatness index (r = 0.740). On the other hand, there was a strong negative correlation between flatness index and seed width (r = -0.704) (Supplementary Table S10).

Principal components analysis

A principal components analysis was performed to assess the relationship among the accessions and to visualize if the different group types, previously defined, cluster separately, based on the results from nine quantitative traits (seed length, seed width, seed height, seed elongation, seed flatness, seed flatness index, 100-seed weight, number of seeds per pod and number of locules per pod).

The biplot projecting the 150 bean accessions (96 true-type and 54 offtype) and the nine quantitative morphological traits was constructed using the two first principal components explaining 70.8% of the variance found among accessions (Figure 2). PC1 differentiates primarily B1P3 (in blue) from B2P2 (in green), with the main contributions of traits like seed length, elongation and flatness index. PC2 differentiates AP1 (in red) from both B1P3 and B2P2, with the main contributions of traits like seed width, seed height and 100-seed weight. The offtype accessions (in yellow) were positioned among the true-types without any clear distribution pattern.

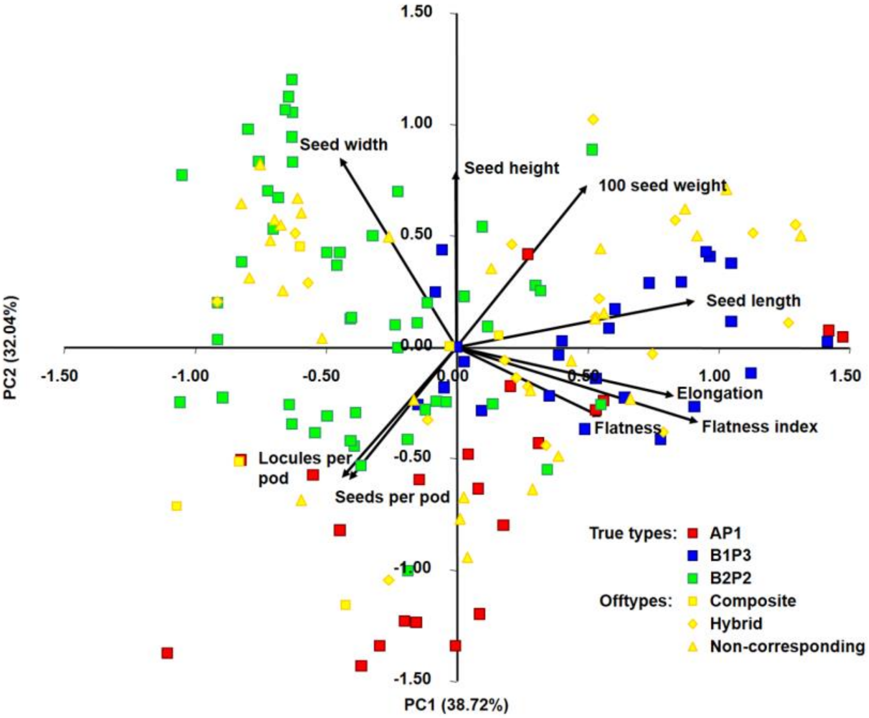


Figure 2 – Principal components analysis of 150 Portuguese bean accessions based on nine quantitative morphological traits. Ninety-six accession assigned as true-types (AP1 in red, B1P3 in blue, B2P2 in green) and 54 accessions assigned as offtypes (composite, hybrid, non-corresponding) in yellow. A, B1 and B2 are the same clusters from structure analysis; P1, P2 and P3 are the phaseolin haplotypes identified.

Discriminant analysis

The stepwise discriminant analysis revealed that seed width, seed elongation, number of locules per pod, seed flatness and seed height were, in this order and in accordance to partial r^2 statistics, the most useful traits in discriminating accessions among the three true-types (Table 10).

Table 10 - Stepwise discriminant analysis summary for the five most informative traits allowing maximum discrimination among bean accessions from true-type groups (AP1, B1P3, B2P2).

Trait	Partial r^2	F-value	P(F)	Wilks' λ	P(λ)
Seed width (mm)	0.521	50.54	***	0.479	***
Elongation (L/H)	0.387	29.05	***	0.294	***
Locules per pod	0.265	16.43	***	0.216	***
Flatness (H/W)	0.119	6.08	**	0.190	***
Seed height (mm)	0.120	6.05	**	0.167	***

P-value significant level: **0.001 <p value <0.01, *** p value < 0.001

These five traits were tested for their performance, and 86.5% of the accessions were correctly classified into their respective true-type groups. Thirteen accessions (out of the 96 with a true-type associated) were misclassified (data not shown).

The discriminant function, resulting from these five traits, was applied to the total data set (including offtype accessions) resulting in a clear separation among the true-type groups, with offtype accessions in intermediate positions (Figure 3).

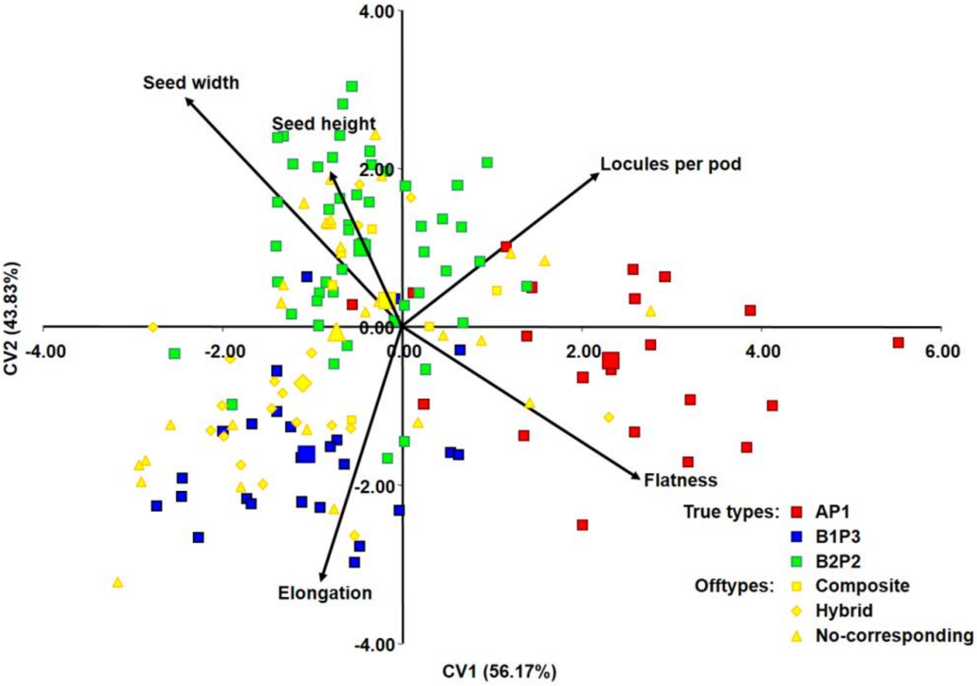


Figure 3 - Discriminant analysis applied to 150 Portuguese bean accessions based on the five quantitative morphological traits that were the most useful for maximum discrimination between true-type groups (AP1, B1P3, B2P2). A, B1 and B2 are the same clusters from structure analysis; P1, P2 and P3 are the phaseolin haplotypes identified. Larger data point marks signal the center of the distribution of different groups**.**

Core collection development

To form core collections based on 150 individual genotypes representing the Portuguese bean germplasm, molecular (21 microsatellites, 187 different alleles) and morphological data (five qualitative traits, with 25 different states) were used. The five qualitative traits used were growth habit, seed shape, seed coat color, seed coat pattern and seed size.

Of the Portuguese accessions analyzed, 73.3% were considered of large seed size (100-seed weight > 40 g), 23.3% of medium size (100-seed weight 25 – 40 g) and 3.3% of small size (100-seed weight < 25 g) (Supplementary Table S11).

With the standard M strategy as implemented in MSTRAT (Gouesnard et al., 2001), five nested core subsets (sample size: 10, 15, 20, 25, 30) were first built. Next, the advanced M strategy as implemented in PowerCore v1.0 (Kim et al., 2007) allowed the selection of a core set of 37 accessions, representing the smallest set of accessions with the total coverage of alleles and trait states present in the entire collection. The composition of each core set is shown in Table 11.

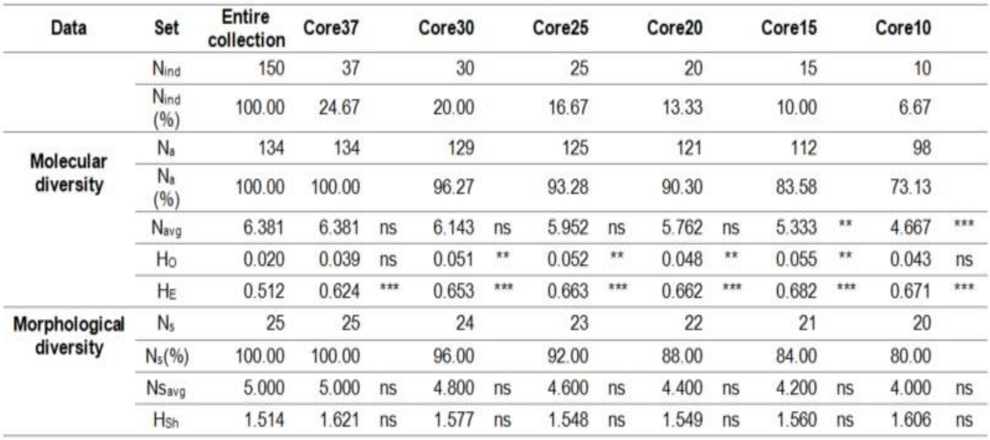
Table 11 - Number of bean accessions from core subsets belonging to each group type.

Accession group type	Subtype	Entire collection	Core 37	Core 30	Core 25	Core 20	Core 15	Core 10
True-type	AP1	21	10	11	10	7	6	3
	B1P3	25	6	6	5	4	2	2
	B2P2	50	10	5	4	4	3	3
Offtype	Composite	6	2	1	1	1	0	0
	Hybrid	19	4	2	2	2	2	1
	Non-corresponding	29	5	5	3	2	2	1
	Total	150	37	30	25	20	15	10

When comparing the composition of each core set with the entire collection, an overrepresentation of accessions from the AP1 group (Mesoamerican) was observed. Accessions from this group represented 14% of the entire collection while, for instance, in core sets with 37 and 20 accessions, this percentage increased to 27% and 35%, respectively (Figure 4).

Molecular and morphological diversity of the entire collection was compared with the core subsets (Table 12). The core collection with 20 accessions is the smallest set, with a non-significantly lower average number of alleles in comparison with the entire collection. Regarding the morphological characterization based on the five qualitative traits, even the core set with only 10 accessions, capturing 80% of these traits states diversity, had an average number of trait states and a Shannon's diversity index not significantly different from the entire collection. The core set with 37 accessions contained 100% of the allelic and morphologic diversity. This core collection was constituted by 10 accessions belonging to the AP1 group (Mesoamerican), 6 accessions from the B1P3 group (Andean) and 10 accessions from the B2P2 group (Andean). The remaining 11 accessions were offtypes: 2 composite accessions, 4 hybrid accessions and 5 non-corresponding accessions (Figure 4).

Table 12 – Comparison between the entire collection and core subsets of molecular and morphological diversity parameters, based on 21 SSRs and 5 qualitative traits.



Data	Set	Entire collection	Core37		Core30		Core25		Core20		Core15		Core10	
	N_{ind}	150	37		30		25		20		15		10	
	N_{ind} (%)	100.00	24.67		20.00		16.67		13.33		10.00		6.67	
Molecular diversity	N_a	134	134		129		125		121		112		98	
	N_a (%)	100.00	100.00		96.27		93.28		90.30		83.58		73.13	
	N_{avg}	6.381	6.381	ns	6.143	ns	5.952	ns	5.762	ns	5.333	**	4.667	***
	H_O	0.020	0.039	ns	0.051	**	0.052	**	0.048	**	0.055	**	0.043	ns
	H_E	0.512	0.624	***	0.653	***	0.663	***	0.662	***	0.682	***	0.671	***
Morphological diversity	N_s	25	25		24		23		22		21		20	
	N_s(%)	100.00	100.00		96.00		92.00		88.00		84.00		80.00	
	Ns_{avg}	5.000	5.000	ns	4.800	ns	4.600	ns	4.400	ns	4.200	ns	4.000	ns
	H_{Sh}	1.514	1.621	ns	1.577	ns	1.548	ns	1.549	ns	1.560	ns	1.606	ns

P-value significant level: * 0.01 < p value < 0.05, **0.001 < p value < 0.01, *** p value < 0.001

ns: not significant (p value > 0.05) N_{ind}: no. of individuals; N_a: no. of alleles; N_{avg}: average no. of alleles per locus; H_o: observed heterozygosity, H_E: expected heterozygosity; N_s: no. of trait states captured in the core set; Ns_{avg}: average no of trait states; H_{Sh}: Shannon's diversity index

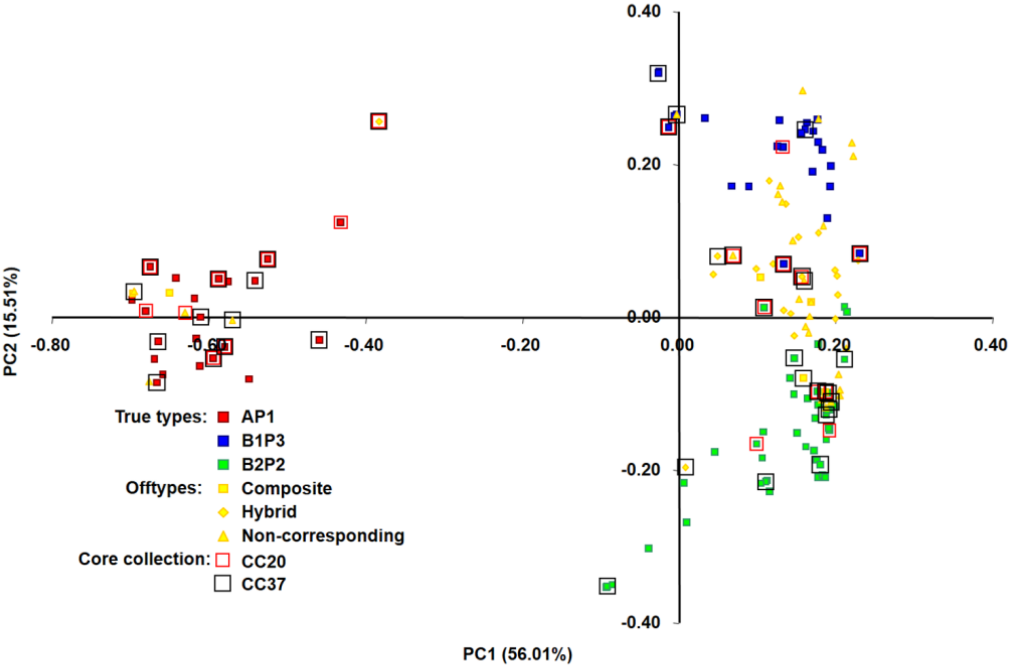


Figure 4 - Principal co-ordinate analysis (PCoA) of 150 bean accessions based on proportion-of-shared-alleles distance matrix. 71.52% of the genetic variation in the Portuguese accessions was explained by the first two axes. A, B1 and B2 are the same clusters from structure analysis; P1, P2 and P3 are the phaseolin haplotypes identified. Accessions included in core collections with 20 and 37 accessions are highlighted by red (CC20) and black (CC37) squares, respectively.

To graphically represent the genetic relationships among the Portuguese bean accessions belonging to the different core sets built, a principal co-ordinate analysis (PCoA), based on proportion-of-shared-alleles distance matrix, was performed. Two axes were generated with a relatively large eigenvalue, capturing 72.03% of the variation in our data (Figure 4). A clear separation of the three true-type groups (AP1 in red, B1P3 in blue and B2P2 in green) was observed. PC1 separated primarily AP1 (Mesoamerican group) from the two Andean types, while PC2 separated the two Andean groups (B1P3 and B2P2). Core sets were scattered along the plot with accessions from all the different group types, but with an overrepresentation of the Mesoamerican group, as was previously noticed.

Discussion

To enrich the current knowledge of molecular and morphological diversity of the Portuguese bean germplasm, we performed, for the first time, a simultaneous molecular marker screen and seed and plant morphological characterization of representative accessions of all national bean-growing traditional regions. Moreover, this bean collection was compared at the molecular level with representatives of the Andean and Mesoamerican gene pools. On the basis of these data, core subsets of the Portuguese germplasm were developed. High genetic and morphological diversity was detected among accessions of this collection. The majority of the Portuguese accessions analyzed were more related to the Andean gene pool. From the entire collection, 37 accessions were selected to form a core collection that retains all the diversity within this germplasm, with minimum repetitiveness. This subset of accessions will allow an increased efficiency of the forthcoming more detailed characterization of this valuable resource. This information is vital to support a more effective conservation of the Portuguese bean germplasm and to promote its use in both national and international breeding programs of this important crop.

The study of the genetic structure and phaseolin type diversity of the collection showed its extent of admixture and the bean gene pool more related to the Portuguese germplasm. One- third of the accessions evaluated were of an intermediate nature. By examining the position of the Portuguese accessions on the Neighbor-joining tree, we conclude that most of the national germplasm is genetically closer to the Andean gene pool, despite the existence of accessions that were distributed with representatives from both gene pools (Mesoamerican and Andean). The analysis of genetic structure and phaseolin types divided the collection into three main clusters: 14.8% of accessions into type AP1 (genetically more related to the Mesoamerican gene pool representatives), 20.6% of accessions into type B1P3 and 32% of accessions into type B2P2 (both genetically more related to the Andean gene pool representatives). The remaining 32.6% of the accessions had an intermediate or admixture nature and were classified as offtypes.

The predominance of Andean bean germplasm found in this Portuguese collection was also reported for other *P. vulgaris* collections from all over Europe, from the Mediterranean countries Portugal, Spain, France and Italy (Rodiño et al., 2003; Sicard et al., 2005; Raggi et al., 2013), to central northern, eastern and southeastern countries (Gepts and Bliss, 1988; Logozzo et al., 2007; Angioi et al., 2010; Angioi et al., 2011). This Andean relationship was further supported by the analysis of phaseolin types, with 77% of the Portuguese accessions having a phaseolin pattern attributable to the Andean gene pool, and by the seed size analysis, in which 73% of the Portuguese accessions depicted large seeds, also characteristic of Andean races (Singh et al., 1991; Blair et al., 2007). When data was graphically represented using principal component analysis (Figure 3), it was clear that higher values of seed width and seed height were found in seeds from the B2P2 group; more elongated seeds belonged to the B1P3 groups, and seeds flatter and with a higher number of locules per pod were predominant in the AP1 group. This evidence is in accordance with previous studies that characterized seeds from the Andean gene pool as larger-sized and accessions from Mesoamerica as higher-yielding but with smaller seeds (Singh et al., 1991; Singh et al., 1993; Kelly et al., 1998; De Ron, 2015). Moreover, the plant growth habit of more than 61% of the Portuguese accessions was determinate, which is mostly typical of the *Nueva Granada* Andean race (Singh et al., 1991). Interestingly, the genetic structure and phaseolin pattern analysis performed was also in accordance with the separation of major known races within the representatives from the Andean gene pool. Thus, cluster B1P3 contained the representatives from races *Nueva Granada* and *Peru,* whereas cluster B2P2 contained accessions from race *Chile*.

The considerable percentage of offtype accessions (32.6%), with novel genetic combinations not typical from the primary centers of domestication, emphasize the potential value of the Portuguese germplasm, providing an additional level of complexity and new gene combinations not yet explored in breeding. In fact, introgression between the Mesoamerican and Andean gene pools had already been detected in the Iberian Peninsula using phaseolin, allozymes and morphological data (Santalla et al., 2002; Rodiño et al., 2006). During this gene flow, new and interesting combinations of traits, such as higher adaptability to environmental stresses, disease resistance or seed quality, may have arisen. In addition, the negative correlation between seed weight and yield potential may have been overtaken (White and González, 1990), with a possible increment of yield among the large-sized Andean germplasm. The existence of a greater proportion of admixed accessions in Europe than in America was already reported by González et al. (2009), who estimated that the percentage of intermediate genotypes in Europe is around 40%, whereas in the Americas it is 12%. Intermediate forms were also previously observed in accessions from the Iberian Peninsula that was considered a secondary center of genetic diversity of bean (Santalla et al., 2002). However, in that study, only accessions from the north of Portugal were analyzed.

The analysis of molecular diversity using 21 microsatellite markers showed that the Portuguese bean germplasm contains a high level of genetic diversity, with 188 alleles found, an average of 9 alleles per locus, and 39 private alleles. Those values were initially considered higher than the ones obtained for the representatives of Mesoamerican and Andean gene pools and wild relatives: 151, 7 and 26, respectively. Nevertheless, when sample size was taken into account, allele richness was identical in both groups (1.1). Similar genetic diversity values for these microsatellites were observed by other authors, in studies with wild and domesticated bean accessions from the Andean and Mesoamerican gene pools (Kwak and Gepts, 2009; Blair et al., 2012), and with accessions from Brazil, also considered a secondary center of diversity (Burle et al., 2010). In our work, the average value of observed heterozygosity was 0.027 for the Portuguese accessions and 0.067 for the Andean and Mesoamerican gene pool representatives and wild relatives. These low values are characteristic of predominantly self-pollinating crops such as bean (Papa et al., 2007). In accordance, the AMOVA results revealed that 83.65% of the overall variance found was among accessions and only 16.35% within accessions.

The presence of many intermediate forms in the collection was also observed at seed level, hampering the seed classification according to the traditional market classes (Voysest and Dessert, 1991). The high morphological diversity of Portuguese bean seeds had already been reported in previous studies with a smaller number of accessions (Gil and Ron, 1992; Rodiño et al., 2001; Santalla et al., 2002; Freitas et al., 2010). Among the accessions studied here, eight different seed coat colors, six seed coat patterns and four seed shapes were detected, once more highlighting the variability within this germplasm. Still, the colors (white, pink, brown and red), shapes (cuboid, oval and kidney) and patterns (absent and striped) more frequent in the Portuguese germplasm were among the ones with higher market value (Jones, 1999).

Five quantitative morphological traits (seed height, seed width, seed flatness, seed elongation and number of locules per pod) proved to be sufficient for discriminating true-type groups of accessions. The discriminant model, built on these five traits, was validated with 86.5% of accessions correctly assigned to the corresponding true-type group, previously classified based on genetic structure and phaseolin type analysis. This procedure was applied only for true-type groups, since accessions classified as offtypes possess intermediate forms between true-type groups that could bias the discriminant function. The discriminant analysis was subsequently applied to all the collection, and the offtype accessions were positioned among the true-type groups, as expected.

Isolation by distance analysis showed that there is no correlation between the geographic distance and the genetic differentiation among accessions. This absence of correlation may be due to an extensive gene flow resulting from traditional seed exchange practices such as trade at local markets or between farmers' family and neighbors. Those practices led to a maintenance of common bean genetic diversity in Portugal, denoted by the presence of almost all identified true-type groups in each region of the country.

On the other hand, we observed that the larger proportion of accessions came from the north central and north interior regions (39% and 35%, respectively). This fact is expected since those are the regions with a more suitable climate to cultivate bean, with higher values of annual precipitation associated with warmer summers, reducing the need for additional costly irrigation.

In order to take full advantage of this valuable germplasm, it is extremely important to complement this initial molecular and morphological characterization with more detailed phenotypic evaluations. This will allow the identification of accessions with increased breeding value; for instance, those more adapted to biotic and abiotic stresses, or possessing particular consumer-oriented quality traits. Yet, this is a time-consuming task in an extended collection such as this. To better exploit this germplasm collection, we developed smaller-size core collections representative of all the diversity from the original collection, with no redundancy. To select the accessions member of the core collection, we combined morphological diversity data (growth habit, seed shape, seed coat color, seed coat pattern and seed size) with molecular marker data. Additionally, Shannon's diversity index was used to evaluate if there were significant differences in the morphological diversity of different core collection sizes. With this combined approach, we had the advantage of using not only simple easy-to-score phenotypic or passport data, normally more prone to missing data and environmental interaction dependence, but also DNA markers that can more accurately represent the genetic relationships and diversity of the entire collection (Díez et al., 2012). By employing the advanced maximization strategy (Schoen and Brown, 1993) we selected 37 accessions (25% of the entire collection analyzed), covering all alleles and morphological trait states present in the entire original collection. This percentage is within the 10%-30% initial number of accessions generally kept in core collections (Wang et al., 2014). The smaller core sets (≤ 20 accessions) developed, although retaining with no significant differences the morphological trait states of the initial collection, had significant different allelic diversity. However, we should take into consideration that if a more detailed phenotypic evaluation (more traits evaluated) had taken place, the differences between the molecular and the morphological diversity of this collection might have been smaller. This highlighted the requirement of phenotyping the collection for other agronomic traits for specific breeding purposes. It is possible that new accessions might be added or removed as new data is accquired. Core collections, along with the germplasm that they represent, should be regarded as a dynamic concept (Díez et al., 2012). We observed a larger proportion of accessions from the Mesoamerican gene pool in the core set of 37 accessions as compared to that of the entire collection, which could be explained by the fact that the accessions of the AP1 type (Mesoamerican) had significantly higher allelic richness than both the B1P3 and B2P2 types (Andean). Additionaly, 11 accessions out of the 37 (30%) were offtype accessions (composite, hybrids and non-corresponding) that, as it was proven, contain novel and relevant allelic information. The intermediate nature of some of these accessions opens new opportunities for using this unexplored material in common bean breeding.

Furthermore, the developed core collections, containing high genetic and morphological diversity, are potentially useful for conducting more detailed phenotypic assessments to deepen the characterization of this germplasm and speed up its use in breeding. This is especially important for traits highly influenced by the environment, such as yield potential. In the present study, since data was collected from one single environment, we limited the characterization mainly to highly heritable morphological traits that generally display low genotype by environment interaction (Debouck and Hidalgo, 1986; Pessarakli, 2001). Low heritable traits will be evaluated in the future using the developed core collection. Its smaller size will be more suitable for a multi-environmental evaluation needed to precisely characterize important agronomic traits such as yield. Also, climatic data and soil characteristics, which differ along the country, will be included in future studies in a more detailed multivariate analysis.

Moreover, the Portuguese core collection developed in this work may now integrate larger international bean collections to deepen the coverage of the worldwide diversity of this important crop. This could be of extreme importance to enrich international breeding or genetic programs and overcome several constraints affecting bean production in different parts of the world. Representative core collections have already proved to be useful tools for conservation purposes and to study resistance to abiotic and biotic stresses or other agronomic traits in several crop collections, such as pea (Grünwald et al., 2003), wheat (Shu et al., 2008), peanut (Chenault Chamberlin et al., 2010) and rice (Wang et al., 2010).

Finally, with this study, the complexity of the Portuguese bean germplasm, reflected by its genetic structure, was revealed. This knowledge is of vital importance for future genetic studies, using this underused but promising germplasm, such as association mapping studies. Those genome-wide studies might contribute to uncovering the genetic basis of several interesting traits and develop molecular tools to assist the selection of bean accessions with improved quality and agronomic performance.

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Supplementary material

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Complete datasets available in FigShare repository:
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Chapter III

Natural variation in Portuguese common bean germplasm reveals new sources of resistance against *Fusarium oxysporum* f. sp. *phaseoli* and resistance-associated candidate genes

This chapter is based on:

Leitão ST, Malosetti M, Song Q, van Eeuwijk F, Rubiales D, Vaz Patto MC (**2020**) Natural variation in Portuguese common bean germplasm reveals new sources of resistance against *Fusarium oxysporum* f. sp. *phaseoli* and resistance-associated candidate genes. **Phytopathology**. DOI: 10.1094/PHYTO-06-19-0207-R

In this research paper, Susana T. Leitão participated in the experimental design, performed the DNA isolation, conducted the fungal inoculations and symptoms evaluation, the genotypic and phenotypic data analysis, participated in the genome-wide association analysis and drafted the manuscript.

Abstract

Common bean (*Phaseolus vulgaris* L.) is one of the most consumed legume crops in the world and fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*), is one of the major diseases affecting its production. Portugal holds a very promising common bean germplasm with an admixed genetic background that may reveal novel genetic resistance combinations between the original Andean and Mesoamerican gene pools. In order to identify new sources of fusarium wilt resistance and detect resistance-associated SNPs, we explored, for the first time, a diverse collection of the underused Portuguese common bean germplasm using genome-wide association analyses. The collection was evaluated for fusarium wilt resistance under growth chamber conditions, using the highly virulent *Fop* strain, FOP-SP1 race 6. Fourteen of the 162 Portuguese accessions evaluated were highly resistant and 71 intermediate. The same collection was genotyped with Illumina BARCBean6K_3BeadChip and DArTseq arrays and SNP-resistance associations were tested using a mixed linear model accounting for the genetic relatedness among accessions. The results from the association mapping revealed nine SNPs associated with resistance on chromosomes Pv04, Pv05, Pv07, and Pv08, indicating that fusarium wilt resistance is under oligogenic control. Putative candidate genes related to phytoalexins biosynthesis, hypersensitive response, and plant primary metabolism were identified. The results reported here highlight the importance of exploring underused germplasm for new sources of resistance and provide new genomic targets for the development of functional markers to support selection in future disease resistance breeding programs.

Keywords: *Phaseolus vulgaris* L., fusarium wilt, association mapping, GWAS, complete and incomplete resistance

Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important food grain legume worldwide, with recognized benefits in health and nutrition (Câmara et al. 2013).

Fusarium wilts have a negative impact on the yield of several legume species and other crops (Okungbowa and Shittu 2012). The causal agent of fusarium wilt disease, *Fusarium oxysporum,* penetrates through root tips or wounds, growing in the plant vascular system. On susceptible plants, it may lead to vessel clogging, internal stem discoloration, and a rapid yellowing of foliage, followed by defoliation and ultimately plant death. Wilting may be caused by a combination of pathogen activity, such as the accumulation of fungal mycelium and/or toxin and host defense responses, including the production of gels, gums, and vessels crushing (Di Pietro et al. 2003).

In common bean, fusarium wilt is caused by *F. oxysporum* (Schlecht.) f. sp. *phaseoli* Kendrick & Snyder (*Fop*) (Agrios 1997) and is among the most important fungal diseases affecting common bean production throughout the world (Alves-Santos et al. 2002; Niño-Sánchez et al. 2015; Schwartz and Pastor-Corrales 1980; Toledo Souza et al. 2012; Xue et al. 2015). At least six different races of *Fop* have been described (Alves-Santos et al. 2002; Salgado 1995) generally associated with a specific geographic area. Race 1 includes isolates found both in the USA and Italy; race 2, isolates found in Brazil; race 3, isolates found in Colombia; race 4, isolates found in the USA; race 5, isolates found in Greece; and race 6, isolates found in Spain. In Portugal, there is no history of the predominant *Fop* race(s) in the fields, but due to the country's geographical proximity to Spain, one may expect that *Fop* race 6 isolates may also be affecting common bean yields in Portugal.

The control of vascular wilt pathogens is not an easy task. Chemical fungicides are ineffective, especially for pathogens like *Fop* that have a soil-borne nature and possess structures that persist for long periods in the soil, even in the absence of host plants (Yadeta and Thomma 2013). Also, biocontrol using antagonistic bacteria or fungi cannot effectively limit these vascular diseases, since abiotic and biotic factors make their performance inconsistent. Consequently, the use of resistant cultivars is the most efficient, environmentally friendly, and economically viable strategy to provide effective fusarium wilt disease control (Dodds and Rathjen 2010).

A better understanding of the genetic basis of resistance mechanisms deployed by resistance sources is needed for more efficient resistance breeding, taking into consideration the pathogen's evolutionary potential. In common bean, the *Fop* resistances already described are controlled by either single major genes or polygenes according to the common bean geographical and genetic origin (Batista et al. 2017; Cross et al. 2000; Fall et al. 2001; Salgado 1995). Since *F. oxysporum* populations are not very large due to its relatively low potential for gene flow, asexual reproduction, and low mutation rate, the use of major resistance genes in breeding might be a sufficient strategy to achieve durable resistance provided that virulence is monitored and genes effectively deployed spatially (McDonald and Linde 2002). Nevertheless, because the fungus may be seed-transmitted, the risk of gene flow due to human activities is actually high. Under these circumstances, pyramiding of different major genes with the complementary use of quantitative incomplete resistances is advisable. In this way, the continuous search for new sources of resistance is essential for development and deploying more durable resistances on new cultivars.

In Portugal, common bean represents about 70% of grain legumes consumed by humans (“Estatísticas Agrícolas 2017,” www.ine.pt). A very diverse common bean germplasm, resulting from more than 500 years of cultivation and adaptation to the country’s edapho-climatic conditions, is still preserved in farmers’ fields, but underexploited by conventional breeding. An extended representative collection of this national diversity was recently characterized (Leitão et al. 2017). Genetic structure analysis divided this collection into three main clusters, one more related to the Mesoamerican gene pool and two more related to the Andean gene pool. Most of the Portuguese germplasm analyzed grouped with the Andean region race representatives and wild relatives. However, one-third of the national germplasm had an admixed origin and might represent putative hybrids between the original Andean and Mesoamerican gene pools.

As a result of *co-evolutionary* interactions between *pathogens* and *their* host *plants*, virulent isolates for each common bean gene pool have evolved. Accordingly, common bean resistance genes of Mesoamerican origin are more effective when transferred to bean varieties with Andean background, and vice-versa (Geffroy et al. 1999; Guzman et al. 1995; Miklas et al. 2006; Mkandawire et al. 2004). Thus, besides the resistance genes that may be detected in the Portuguese germplasm of Andean origin, the Portuguese gene pool admixed accessions may have novel resistance gene combinations, harder for the pathogen to overcome and, therefore, useful to enhance the durability of resistance. However, little is known about the response of the Portuguese germplasm against *Fop*. To the best of our knowledge, only one short report exists characterizing two Portuguese common bean cultivars (Tarrestre and Oriente) as very susceptible to a *Fop* strain (FA-15) isolated from a greenhouse in Portugal (Santos et al.

2017). Nevertheless, the genetic diversity found by Leitão et al. (2017) on the Portuguese common bean germplasm encouraged the exploitation of the natural variation for fusarium wilt resistance that might exist within a larger number of Portuguese accessions.

Until now, only a few bi-parental linkage mapping-based reports are available on common bean resistance to *Fusarium* sp., namely *Fop* (Fall et al. 2001) and *F. solani* f.sp. *phaseoli* (Hagerty et al. 2015; Nakedde et al. 2016). These studies were, however, limited to the allelic diversity that segregates between the parental lines used in the cross, which eventually also restricts mapping resolution (Korte and Farlow 2013).

Genome-wide association studies (GWAS) are a powerful tool to identify polymorphisms underlying natural variation in genomic regions responsible for the expression of a given trait. This approach can provide higher resolution mapping, greater allelic diversity, and improved efficiency and accuracy in estimating marker effects for quantitative traits than bi-parental linkage mapping (Myles et al. 2009). GWAS has been successfully applied in common bean to analyze the genetic control of resistance to several diseases such as anthracnose, angular leaf spot, or bacterial blight (Choudhary et al. 2018; Perseguini et al. 2016; Wu et al. 2017; Zuiderveen et al. 2016).

The present study was designed to explore the Portuguese common bean natural variation to identify resistance against fusarium wilt and to detect SNP-resistance associations using a GWAS approach. For that, we evaluated a collection of 162 Portuguese common bean accessions for resistance to fusarium wilt under controlled conditions and performed a high-throughput SNP screening of the same collection. SNP-trait associations were tested using a mixed linear model accounting for the genetic relatedness among accessions to identify the genomic regions controlling fusarium wilt resistance. To our knowledge, this is the first time GWAS has been applied to a Portuguese common bean collection and for common bean fusarium wilt resistance.

Materials and Methods

Phenotypic data

Plant material and growing conditions

One hundred and sixty-two Portuguese common bean accessions, belonging to the national *P. vulgaris* germplasm collection, were used in this study. A complete list of the accessions studied along with their passport information is available in Supplementary Table 1. Two Mesoamerican lines – SER16 and Tio Canela-75 – kindly provided by the International Center for Tropical Agriculture (CIAT, Colombia) were also evaluated as international references for comparative purposes. SER16 is an improved bean line for drought resistance (Polania et al. 2016a) and Tio Canela-75 is a commercial variety noted for its resistance to *bean golden mosaic virus* (BGMV), common bacterial blight, rust, and heat tolerance (Rosas et al. 1997). No previous information on the resistance levels of these accessions to fusarium wilt was available.

Common bean seeds were surface-sterilized for 20 min in a 20% solution of sodium hypochlorite, rinsed two times with sterile water for 20 min and sown in 0.5 L pots filled with sterile vermiculite (1–3 mm diameter). One seed was sown per pot and three to five pots per accession were used. Pots were placed in a growth chamber kept at 27 ± 2°C under a photoperiod of 14 h light (~250 $\mu mol.m^{-2}s^{-1}$) and 10 h dark, and with a relative humidity of 60-70%. In total, 1033 plants were evaluated.

Experimental design

Due to growth chamber space constraints, we used an incomplete block design and the 164 accessions (162 Portuguese, SER16, and Tio Canela-75) were assigned to three independent blocks or experiments. Three to five plants (average 4.5) per accession were evaluated in each experiment and averaged. To correct for the block (experiment) effect, 30 accessions were repeatedly evaluated in all experiments. Additionally, in each of the three experiments, three extra plants from eight accessions under evaluation (24 plants in total per experiment) were randomly chosen and used as non-inoculated controls for symptom comparison.

Fungal isolate and cultural conditions

Fusarium oxysporum f. sp. *phaseoli* isolate FOP-SP1 race 6 was kindly provided by Prof. José María Díaz Mínguez (University of Salamanca, Spain) and stored as micro conidial suspensions at -80ºC in 30% glycerol, for use in all the experiments. This fungal strain was identified in common bean cultivars in Avila, Spain, and classified as highly virulent (Alves-Santos, 2002).

For microconidia multiplication, a protocol adapted from Haglund (1989) and Lichtenzveig et al. (2006) was followed. Briefly, the fungal culture was grown in the dark at 28ºC under constant shaking (170 rpm), for four days, filtered using autoclaved cheesecloths to separate both micro and macro conidia from *F. oxysporum* mycelium and centrifuged at 6000 rpm for 10 min at room temperature. The conidial pellets were re-suspended and a suspension of 5.0×10^6 conidia.mL^{-1} was prepared to be used on the same day to inoculate the common bean seedlings.

Inoculation and disease assessment

For inoculation, seven-day-old seedlings were removed from the pots, vermiculite was cleaned from the roots, and the roots were then trimmed by a third and immersed for 5 min in the conidial suspension previously prepared, following a modified version of the dipping technique described by Haglund (1989). The non-inoculated control plants were similarly trimmed by a third but roots were immersed in sterile water instead of conidial suspension. Seedlings were replanted in the pots and maintained in the same growth chamber, under the same photoperiod and temperature conditions. All plants were well watered during the experiment using tap water, and once a week were irrigated with Hoagland nutrient solution (Hoagland and Arnon 1938).

Symptoms were assessed at leaf level every three days, from the 7th to the 30th day after inoculation (DAI), a total of eight time points, using a disease severity (DS) visual scale ranging from 1 (healthy leaf) to 5 (dead leaf) (adapted from Bani et al. (2012) and Rispail and Rubiales (2014)). In more detail: 1 – no symptoms; 2 – light to moderate chlorotic symptoms; 3 – leaves completely chlorotic and bright yellow; 4 – chlorotic leaves with wilt and necrosis symptoms; 5 – leaves and branches exhibiting wilt, chlorosis, necrosis, and defoliation, eventually with plant death (Figure 1). Intermediate scale values were given when appropriate. At 30 DAI, accessions were considered resistant if they had a mean DS score (DS30) of 1, intermediate if they had a mean DS score of 2 or 3, and susceptible if they had a mean DS score of 4 or 5.

Figure 1: Progression of fusarium wilt disease in susceptible bean accessions inoculated with FOP-SP1 race 6. Numbers indicate the disease score based on a severity scale ranging from 1 (healthy leaf) to 5 (dead leaf).

The progression of fusarium wilt disease was monitored, and DS scores taken per plant at the eight time points were averaged per accession and plotted. Then, a linear regression was fitted to obtain the disease progress rate (DSr) given by the slope of the regression line. The accessions were grouped according to the disease progression profile obtained by the eight time points, and four trends were observed: a) accessions that maintained a low DS (1) along 30 DAI; b) accessions that reached and maintained an intermediate DS score (2-3); c) accessions that reached a high DS score (4-5) in the first two weeks of evaluation; and d) accessions that reached a high DS score (4-5) only 16 DAI or later.

To combine the multiple observations of fusarium wilt disease progress taken over time for each accession into a single value, the area under the disease progress curve (AUDPC) was calculated per plant, and then averaged per accession, using the formula:

$$\text{AUDPC} = \Sigma[(x_i+x_{i+1})/2] * (t_{i+1}-t_\text{i})$$

where x_i = score of disease severity at time i, x_{i+1}= score of disease severity at time i+1, and $t_{i+1}-t_\text{i}$ = number of days between scoring times i and i+1. AUDPC scores provided a quantitative summary of fusarium wilt disease severity over 30 DAI for each accession, joining the progression with the extent of disease severity data assessed at multiple observations. Therefore, *the higher* the *AUDPC value, the more susceptible* the accession.

Phenotypic data analysis

The results from the three traits DS30 (disease severity score at the last time point 30 DAI), AUDPC, and DSr (disease progress rate) - were compared using Pearson's linear correlation.

To increase the accuracy and repeatability of the association study, the accessions showing DS30 and AUDPC standard deviations higher than 1.5 and 25, respectively, were excluded from the GWAS analysis. This resulted in the removal of 14 accessions.

The linear mixed model applied was *trait (Fusarium wilt DS30, AUDPC, DSr) = accession* + *block* + *error*. The assumptions of normal errors and homogeneous error variance were checked. *Accession* is the genotypic term and *block* is the term that identifies the three experiments needed to have all the 150 accessions evaluated. With the 30 accessions that were repeatedly evaluated in the three experiments, the experimental effect was estimated. In a first step, the model was fitted with all terms as random to obtain the best linear unbiased predictors (BLUPs). A restricted maximum likelihood (REML) procedure was conducted to estimate the variance components of the linear mixed model and the broad-sense heritability. In a second step, accessions were fitted as a fixed term and the best linear unbiased estimates (BLUEs) for each accession and trait were produced and used as input phenotypic data in the association mapping analysis. All analyses were performed in Genstat® software, 19th edition (VSN, 2017).

Genotypic data

DNA from one representative individual per accession was isolated from young leaves using DNeasy Plant Mini Kit from Qiagen®. The criteria to select the representative individuals were described in a previous work from our team (Leitão et al. 2017). DNA quantification was performed at 260 nm using a NanoDrop™ 2000c Spectrophotometer (Thermo Scientific, USA) and the concentration of all samples was set to values between 50 and 100 ng.μL^{-1}, in a volume of 30 µL. Wavelength ratios at 260/230 and 260/280 nm were examined to assess DNA purity. The DNA quality was also checked in 0.8% SeaKem® LE agarose gels (Cambrex Bio Science Rockland, Inc., USA) stained with SYBR® Safe (Invitrogen, USA).

DNA samples were genotyped using the Illumina Infinium BARCBean6K_3 BeadChip™ assay containing 5,398 SNPs (USDA-ARS, Maryland, USA), designed based on the sequence of *P. vulgaris* 14x and v0.9 *de novo* assemblies (Song et al. 2015), and DArTseq™ analysis (Diversity Arrays Technology sequencing, Canberra, Australia) (Kilian et al. 2012).

Genotypic data analysis

Quality control

Genotypic data quality control was performed by removing SNP markers and accessions with more than 25% of missing data. SNPs called as heterozygous were set as missing data. Moreover, markers with a minor allele frequency (MAF) smaller than 0.01 were removed.

Genetic structure

A subset of 454 SNP markers evenly distributed throughout the common bean genome (average distance between markers of 1.1 Mega base pairs, Mbp) was used to build a similarity matrix to estimate pairwise genetic relatedness among the accessions, as implemented in Genstat software, to calculate principal coordinate scores to study the population structure. The obtained SNP-based structure was compared with the three clusters (Mesoamerican related, Andean related and admixture nature) identified previously using SSR in the same collection (Leitão et al. 2017).

Association mapping

Genome-wide association studies (GWAS) to reveal fusarium wilt DS30, AUDPC, and DSr associated SNPs were conducted using the quantitative trait loci (QTL) library procedures available in Genstat software. The adjusted means (BLUEs) of the three traits were tested for association with 9,825 SNP markers scored in 133 common bean accessions that passed the genotypic and phenotypic quality filters applied.

The association mapping was performed in the mixed-model framework of Genstat software, using the model *Phenotype = SNP + genotype + error*, fitting SNP as fixed and genotype as random terms using REML (Malosetti et al. 2007), with genotype random effects structured following a kinship matrix (*K* matrix). For each chromosome, a different kinship matrix was calculated using only the SNPs located on the remaining 10 chromosomes, as proposed by Cheng et al. (2013). The procedure was performed using the kin function of R package synbreed (Wimmer et al. 2012) and the Van Raden measure (Van Raden 2008). The genome-wide marker-trait association scan was conducted by testing the significance of the marker effect using a marginal Wald test at each SNP location. The observed $-\log_{10}$ (*P*-value) of each SNP was plotted against their chromosomal positions to produce a Manhattan plot. Using a threshold level of $-\log_{10}$ (*P*-value) = 3, the significant marker-trait associations were depicted. This threshold was set to discard the background noise obtained in the

Manhattan plot without compromising the identification of potentially interesting regions, which would be missed by the overly stringent and conservative Bonferroni-corrected threshold of significance. However, as a "conservative" guidance, two additional approaches were followed. On one hand, a LD adjusted Bonferroni-corrected threshold (α/k), considering an $\alpha = 0.05$ and setting the effective number of independent tests as the number of LD blocks per chromosome ($k = 520$) was calculated (Dugal et al. 2008). Additionally, adjusted *P*-values following the Benjamini and Yekutieli (B-Y) false discovery rate (FDR) method (Benjamini and Yekutieli 2001) were also calculated, in this case with $\alpha = 0.2$ and $k = 520$, to control type I errors due to multiple testing.

For every SNP significantly associated with fusarium wilt DS30, AUDPC, and DSr progress rate, the effect of the allele variant in relation to the most frequent allele was calculated. The proportion of variance explained by each SNP-trait association was estimated using the formula V_{QTL}/V_{pheno}, where $V_{QTL} = 2freq(1\text{-}freq)effect^2$ and V_{pheno} is the phenotypic variance of the adjusted means of each trait (Resende et al. 2017). The relation between the frequency of each trait-associated SNP allele, the resistance level and the gene pool of origin of the accessions (Leitão et al. 2017) was also investigated.

Local linkage disequilibrium and candidate gene identification

A gene was considered a putative candidate gene for fusarium wilt DS30, AUDPC, or for DSr progress rate if it contained a significant associated SNP (threshold for significance $-\log_{10}(P\text{-value}) \geq 3$) or if it was in linkage disequilibrium (LD) with a SNP significantly associated with the trait. LD was calculated for each chromosome as a measure of the recombination history, using the *squared coefficient of correlation between marker pairs,* r^2, after correcting for population structure with the principal component scores from Eigenanalysis, as implemented in Genstat software. For this calculation, the entire set of SNPs was used. Average intra-chromosomal LD decay per chromosome was visualized by plotting r^2 against the physical mapping distance in Mb. To consider the existence of adjacent SNP markers in LD with the ones identified as significantly associated with the trait, the r^2 of the neighboring SNPs was investigated, bearing in mind a strict threshold of LD decay ($r^2 > 0.2$). The location of these adjacent SNPs in LD with the significantly associated ones was used to define an LD block and to browse for putative candidate genes mapped within those genomic regions.

The common bean genome sequence, from the Andean common bean accession G19833 (Schmutz et al. 2014), was investigated using the *JBrowse* tool in the *Phaseolus vulgaris* v2.1, available at the Phytozome v12 portal (DOE-JGI and USDA-NIFA, http://phytozome.jgi.doe.gov/). The functional annotation of the genes under the identified genomic regions was given by *KEGG*/*KOG*/*PFAM*/*PANTHER*/*Gene Ontology (GO)* databases identifiers, which were used to make inferences about the pathways involved and the possible role of the common bean candidate genes in the control of Fusarium infection.

Results

Fusarium wilt disease severity variation

To determine the progression of fusarium wilt symptoms among the common bean accessions, disease severity (DS) was scored eight different times during 30 DAI. By 10 DAI, 29 accessions presented leaves already completely chlorotic with visual symptoms of necrosis and were classified as DS 4 (susceptible), whereas 64 accessions still displayed DS 1 (resistant), and 71 DS 2-3 (intermediate). At 20 DAI all five DS scoring values were found among the collection showing the variability of responses among the Portuguese common bean germplasm. By 30 DAI 78 accessions displayed DS 4-5, with 55 of them dead. The Mesoamerican line Tio Canela-75 was included in this group (DS 4). On the other hand, at this final time point, 14 accessions (9%) were considered completely resistant (DS ≤ 1.5) and 72 accessions (44%) were considered intermediate, with leaves showing different levels of chlorosis but no necrosis (DS 2-3) (Figure 2). This last group included the Mesoamerican line SER16 (DS 3).

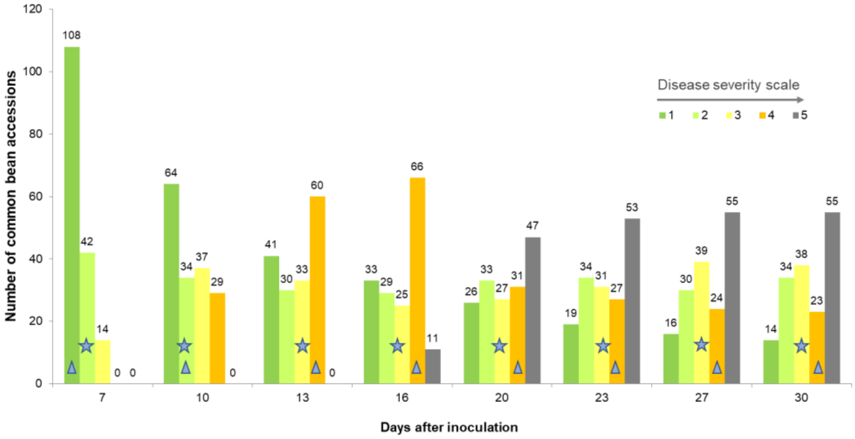


Figure 2: Frequency distribution of disease severity (DS, scale 1-5) in 162 Portuguese common bean accessions caused by *Fusarium oxysporum* f. sp. *phaseoli* (FOP-SP1 race 6). Disease progression was monitored by assessing DS eight different times from the 7th until the 30th day after inoculation. The categorical bins in which SER16 and Tio Canela-75 lines fall are represented by a star and triangle, respectively.

Additionally, the eight DS scores per accession were plotted (Supplementary Figure 1). Out of the 78 susceptible accessions, 60 reached a high DS score (4-5) within the first two weeks after inoculation, while 18 only showed high DS scores 15 DAI or later. On the other hand, the 14 accessions considered resistant (DS ≤ 1.5) maintained the low DS score during the entire experimental time frame. Finally, the 70 intermediate accessions reached and maintained

DS values between 1.6 and 3.5. Fusarium wilt progress rate (DSr), given by the slope of the DS scores regression, ranged from a minimum of 0.000 (resistant accession) to a maximum of 0.571 (susceptible accession) (Supplementary Table 2).

With the fusarium wilt DS values scored every three days during 30 DAI, the area under disease progress curve (AUDPC) was calculated for each accession and their frequency distribution plotted (Figure 3).

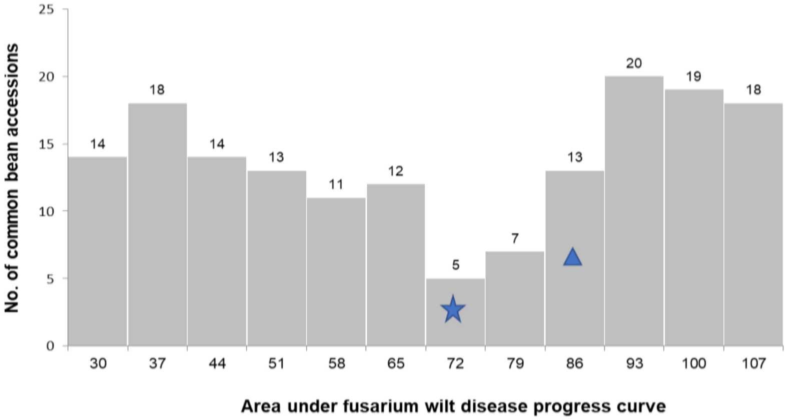


Figure 3: Frequency distribution of the area under disease progress curve (AUDPC) for 162 Portuguese common bean accessions, 7 to 30 days after inoculation, discriminating resistant (low AUDPC values) and susceptible (high AUDPC values) accessions. The categorial bins in which SER16 (AUDPC = 67.2) and Tio Canela-75 (AUDPC = 80.4) lines fall are represented by a star and triangle, respectively.

Fusarium wilt AUDPC mean values per accession ranged from 27.8 to 105.1. The frequencies of AUDPC classes followed a bimodal distribution, with two AUDPC peaks, indicating a clear discrimination between resistant (low AUDPC values) and susceptible (high AUDPC values) accessions. Accessions having AUDPC values below 30 were regarded as resistant, those with AUDPC between 31 and 69 intermediate, and those with AUDPC above 70 susceptible. A complete list of AUDPC values per accession is available in Supplementary Table 3.

The 14 accessions considered resistant – simultaneously with DS ≤ 1.5, AUDPC < 30, and DSr < 0.100 – were either of Andean or admixed origin. Nevertheless, within the intermediate and susceptible accessions, it was possible to identify both Andean and Mesoamerican gene pools in addition to the admixed origin (Figure 4 and Table 1).

Table 1: Response of Portuguese common bean accessions to fusarium wilt according to the gene pool of origin. The minimum and maximum AUDPC values and disease severity scored at 30 DAI are shown.

Gene pool of origin[a]	**AUDPC range**	**Number of common bean accessions**		
		DS30 = 1 Resistant	DS30 = 2-3 Intermediate	DS30 = 4-5 Susceptible
Andean	27.8 – 104.8	11	40	50
Mesoamerican	32.1 – 102.4	0	14	13
Admixed	27.8 – 105.1	3	12	8

[a] Gene pool of origin resulting from the structure analysis performed together with gene pool representatives (Leitão et al. 2017)

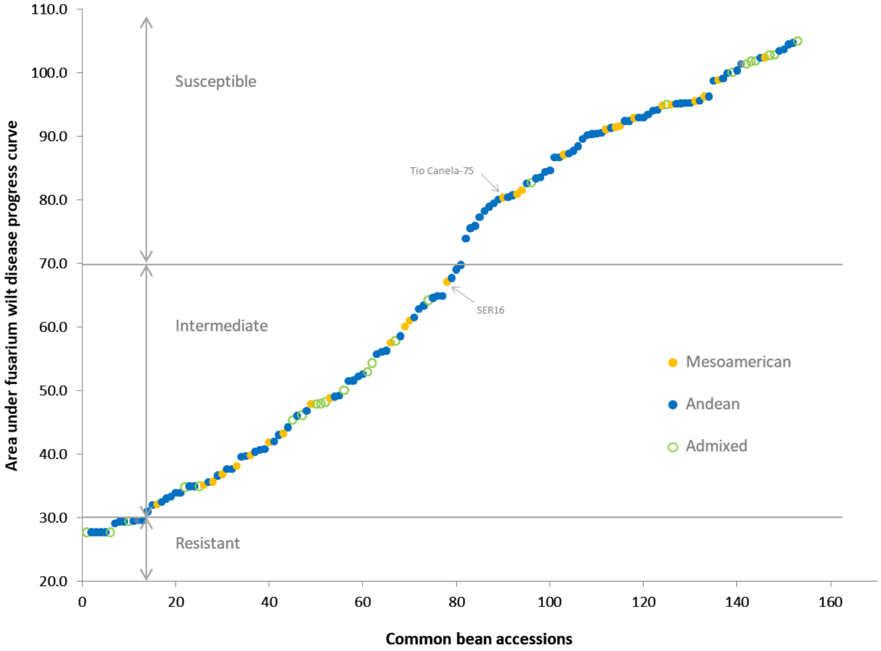


Figure 4: Fusarium wilt AUDPC values of the Portuguese common bean accessions. The accessions are colored according to the clustering resulting from the structure analysis performed together with gene pool representatives (Leitão et al. 2017). Two groups of accessions were depicted (closed circles): one with Mesoamerican origin (in orange) and the other with Andean origin (in blue). Open circles (in green) refer to the accessions of admixed origin between the original gene pools

Pearson’s coefficients revealed strong pairwise correlations between DS measured at 30 DAI (DS30), AUDPC, and disease progress rate values (DSr) (Table 2).

Table 2: Pearson’s linear correlations between disease severity scored at 30 DAI (DS30), AUDPC, and disease progress rate (DSr), measured in 162 Portuguese common bean accessions.

	DS30	**AUDPC**	**DSr**
DS30	-		
AUDPC	0.9683	-	
DSr	0.8152	0.7019	-

Phenotypic data variance components and broad sense heritability

The examination of the histogram of residuals, residuals versus fitted values of the model and the expected versus normal quantiles (Q-Q) plot revealed a random pattern of residuals for the three traits (DS30, AUDPC, and DSr) further used in GWAS (Supplementary Figure 2).

The REML estimators of the variance components of the linear model were obtained with accession and block as random terms, and broad-sense heritability, calculated as the ratio of the genotypic variance to the total phenotypic variance (genetic plus error) (Table 3). With accession term fixed, Wald statistics indicated very strong evidence for differences between accessions (*P*-value < 0.001) for DS30 and AUDPC, and less strong but still significant differences between accessions for DSr (*P*-value = 0.003).

Table 3: Variance components and broad-sense heritability for the three traits measured in 148 Portuguese common bean accessions.

	Variance components			**h^2 heritability (%)**
Trait	$\sigma^2_{genotype}$	σ^2_{block}	$\sigma^2_{residual}$	
DS30	1.0517	0.3812	0.475	71.5
AUDPC	406.9	143.1	201.5	69.6
DSr	0.00877	0.00000	0.0185	40.8

Association panel genetic structure

Based on the 454 selected SNP markers, two main groups of accessions were visualized using principal coordinate analysis (Figure 5). The variance explained by the first two principal coordinates was 65.71%. The observed clustering on the 133 Portuguese accessions was in accordance to their genetic proximity to the two original common bean gene pools in Mesoamerica and in the Andes. Intermediate resistant and susceptible accessions were identified within the accessions more related to the Andean and Mesoamerican gene pools and also among the accessions of admixture origin, whereas the resistant accessions were all of Andean or admixed origin.

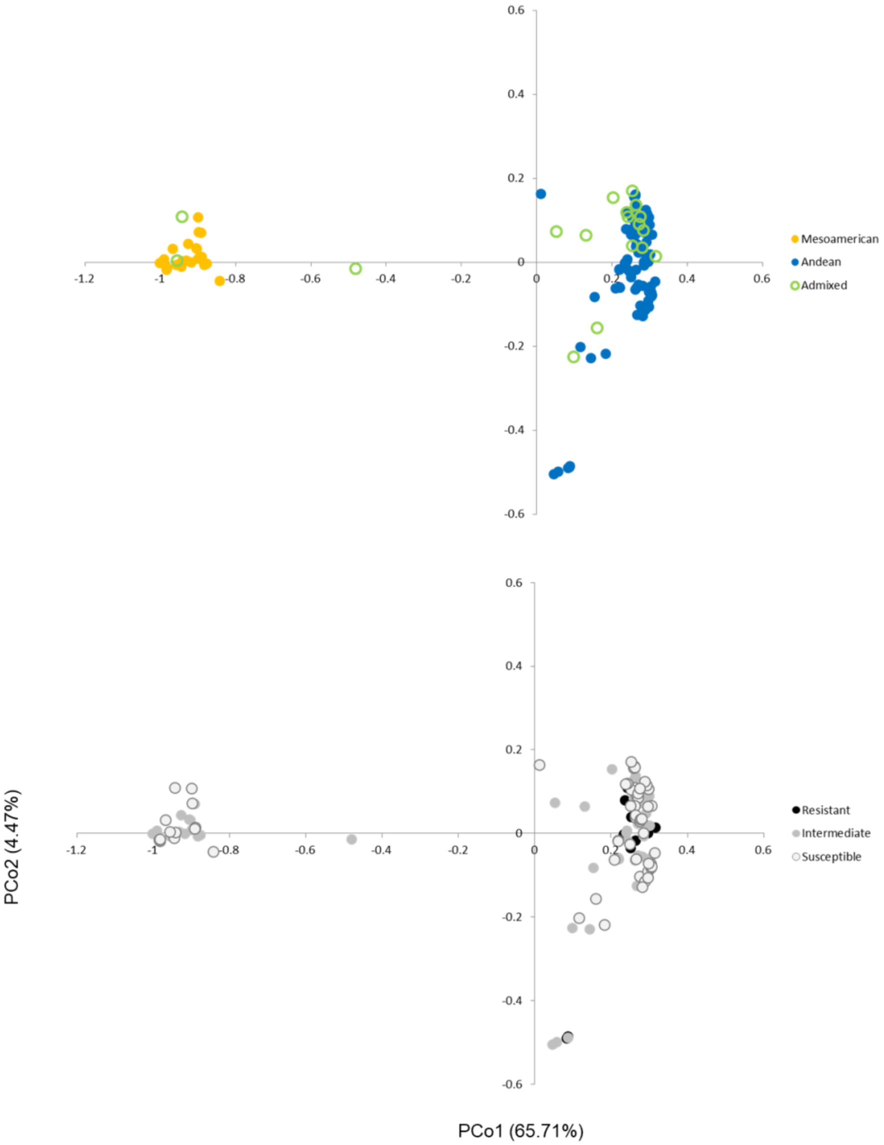


Figure 5: Principal coordinate analysis (PCoA) of the Portuguese common bean collection based on the genotypic profile of 133 accessions using 454 SNP markers evenly distributed along the genome. In the top plot, the accessions are colored according to the structure analysis performed together

with gene pool representatives (Leitão et al. 2017). Two groups of accessions were depicted (closed circles): one with the Mesoamerican origin (in orange) and another with the Andean origin (in blue). Open circles (in green) refer to the accessions of admixed origin between the original gene pools. In the bottom plot, the same PCoA is displayed but with the accessions colored according to their response to fusarium wilt infection (FOP-SP1, race 6): resistant in black, intermediate in dark grey and susceptible in light grey.

Marker-trait associations

Illumina Infinium BARCBean6K_3 BeadChip^{TM} assay and DArTseq^{TM} analysis genotyped together 16,689 SNPs. After quality control, a total of 9,825 SNPs and 133 accessions were used in the association mapping study.

For the three traits under analysis - Fusarium wilt disease severity at 30 DAI (DS30), AUDPC, and disease progress rate (DSr) - the distribution of the -log$_{10}$(*P*-values) from marginal Wald tests was investigated by Q-Q plots (Supplementary Figure 3). Some deflation of the test statistic was observed (*P*-values are slightly under the expected y=x line), but the points corresponding to the significant associations clearly stand out at the high end of the plots (Supplementary Figure 3).

The results from the association mapping revealed nine SNPs significantly associated (using -log$_{10}$(*P*-value) $\geq$ 3) with resistance on chromosomes Pv04, Pv05, Pv07 and Pv08 (Figure 6 and Table 4). The LD adjusted Bonferroni corrected *P*-value set the threshold as -log$_{10}$(*P*-value) = 4.0. The use of this threshold would render the previously detected associations as suggestive. The Benjamini-Yekutiely *P*-values adjustment was found to be highly stringent for all the associations.

By inspecting the allelic variant effect on fusarium wilt resistance of the associated SNPs it was possible to identify the most promising SNPs for marker-assisted selection (SNPs strongly associated and with higher effect on the trait variation). Nevertheless, each of the SNP-trait associations identified for DS30, AUDPC, and

DSr only explained a small portion of the observed phenotypic variance (Table 4).

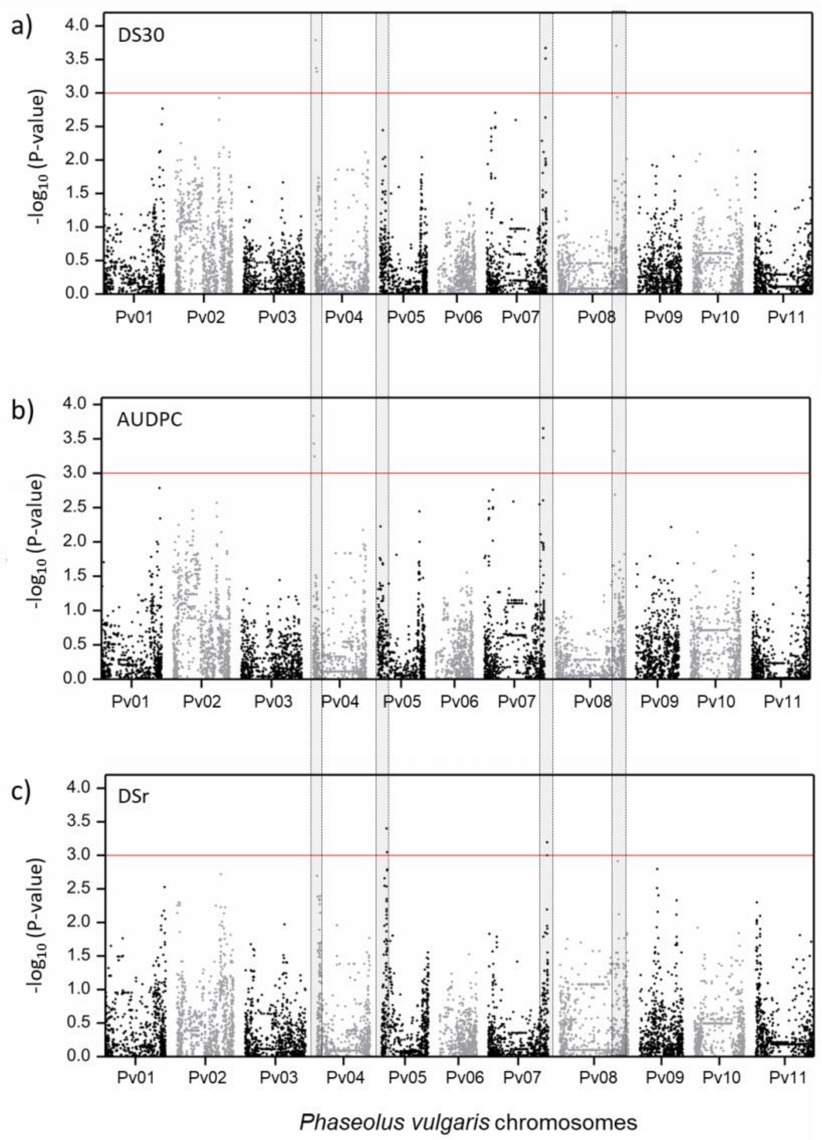
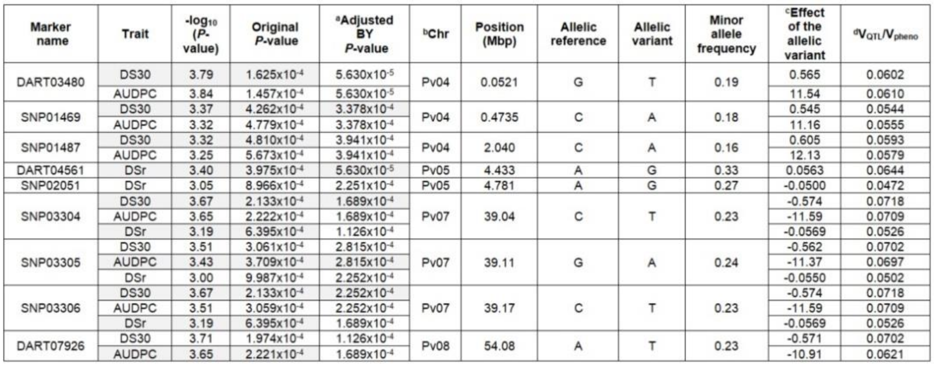


Figure 6: Manhattan plot depicting the genome-wide association results for fusarium wilt DS30, AUDPC and DSr using a panel of 133 Portuguese common bean accessions. The y-axis represents the −log10 (*P*-value) of 9,825 SNPs, and the x-axis shows their chromosomal positions. The horizontal red line indicates a threshold of significance of −log10 (*P*-value) = 3. The four highlighted vertical columns correspond to genomic regions with significantly associated SNPs.

Table 4: SNP associations (-$\log_{10}$(*P*-value) ≥ 3) with fusarium wilt DS30, AUDPC, and DSr, marker position within chromosomes, allelic reference and allelic variant for the associated SNP, minor allele frequency, the effect of the allelic variant, and the proportion of phenotypic variance explained by each associated SNP detected using a panel of 133 Portuguese common bean accessions.



Marker name	Trait	-$\log_{10}$ (*P*-value)	Original *P*-value	[a]Adjusted BY *P*-value	[b]Chr	Position (Mbp)	Allelic reference	Allelic variant	Minor allele frequency	[c]Effect of the allelic variant	[d]V_{QTL}/V_{pheno}
DART03480	DS30	3.79	1.625×10^{-4}	5.630×10^{-5}	Pv04	0.0521	G	T	0.19	0.565	0.0602
	AUDPC	3.84	1.457×10^{-4}	5.630×10^{-5}						11.54	0.0610
SNP01469	DS30	3.37	4.262×10^{-4}	3.378×10^{-4}	Pv04	0.4735	C	A	0.18	0.545	0.0544
	AUDPC	3.32	4.779×10^{-4}	3.378×10^{-4}						11.16	0.0555
SNP01487	DS30	3.32	4.810×10^{-4}	3.941×10^{-4}	Pv04	2.040	C	A	0.16	0.605	0.0593
	AUDPC	3.25	5.673×10^{-4}	3.941×10^{-4}						12.13	0.0579
DART04561	DSr	3.40	3.975×10^{-4}	5.630×10^{-5}	Pv05	4.433	A	G	0.33	0.0563	0.0644
SNP02051	DSr	3.05	8.966×10^{-4}	2.251×10^{-4}	Pv05	4.781	A	G	0.27	-0.0500	0.0472
SNP03304	DS30	3.67	2.133×10^{-4}	1.689×10^{-4}	Pv07	39.04	C	T	0.23	-0.574	0.0718
	AUDPC	3.65	2.222×10^{-4}	1.689×10^{-4}						-11.59	0.0709
	DSr	3.19	6.395×10^{-4}	1.126×10^{-4}						-0.0569	0.0526
SNP03305	DS30	3.51	3.061×10^{-4}	2.815×10^{-4}	Pv07	39.11	G	A	0.24	-0.562	0.0702
	AUDPC	3.43	3.709×10^{-4}	2.815×10^{-4}						-11.37	0.0697
	DSr	3.00	9.987×10^{-4}	2.252×10^{-4}						-0.0550	0.0502
SNP03306	DS30	3.67	2.133×10^{-4}	2.252×10^{-4}	Pv07	39.17	C	T	0.23	-0.574	0.0718
	AUDPC	3.51	3.059×10^{-4}	2.252×10^{-4}						-11.59	0.0709
	DSr	3.19	6.395×10^{-4}	1.689×10^{-4}						-0.0569	0.0526
DART07926	DS30	3.71	1.974×10^{-4}	1.126×10^{-4}	Pv08	54.08	A	T	0.23	-0.571	0.0702
	AUDPC	3.65	2.221×10^{-4}	1.689×10^{-4}						-10.91	0.0621

[a]Adjusted *P*-value for multiple comparisons according to Benjamini-Yekutieli approach.[b]*P. vulgaris* chromosome. [c]A positive effect of the allelic variant represents an increase in susceptibility, while a negative effect represents an increase in resistance to fusarium wilt. [d]Proportion of the variance explained by each SNP-trait association, V_{QTL}= 2freq(1-freq)effect^2 and V_{pheno} = phenotypic variance of the adjusted means of each trait

From the seven significant associations detected for both fusarium wilt DS30 and AUDPC on chromosomes Pv04, Pv07 and Pv08 (Figure 6), DART03480 on chromosome Pv04 had the highest –$\log_{10}$(*P*-value) = 3.79 and 3.84, respectively. The associated SNPs that explained the biggest proportion of variance (7.18% in DS30 and 7.02% in AUDPC) were SNP03304 and SNP03306 on chromosome Pv07 (Table 4).

The allelic variant of four of these seven associated SNPs caused a negative effect in fusarium wilt DS30, in relation to the most frequent allele, meaning that they contributed to an increase in disease resistance. The absolute value of the allelic variant effect was for all the DS30 associated SNPs close to 0.5 in the DS score scale. This corresponded to an increase (or decrease for the SNPs whose allelic variant had a negative effect) in 15% to the DS30 mean value (3.2) of the collection.

Similarly to DS30, the allelic variant of the associated SNPs located in chromosomes 7 and 8 caused a negative effect in fusarium wilt AUDPC, contributing to an increase in fusarium wilt disease resistance. The absolute value of the allelic variant effect was for all the AUDPC associated SNPs close to 11 AUDPC units. This corresponded to an increase (or decrease for the SNPs whose allelic variant has a negative effect) in 17% to the AUDPC mean value (63) of the collection.

The SNPs associated with fusarium wilt progression rate (DSr) on chromosomes Pv07 were the same associated with DS30 and AUDPC. However, the two associations on chromosome Pv05 were unique for DSr. From the five associations detected for DSr (Figure 6), DART04561 on chromosome Pv05 had the highest $–\log_{10}(P\text{-value}) = 3.40$. This SNP also explained the biggest proportion of variance (6.44%) in this trait (Table 4).

All the allelic variants of four out of five associated SNPs caused a negative effect in fusarium wilt DSr, in relation to the most frequent allele, meaning that they contributed to a decrease in the disease progress rate. The exception was DART04561 with a positive effect in DSr. The absolute value of the allelic variant effect for all the DSr associated SNPs was close to 0.05. This value corresponds to a decrease in 19% to the DSr mean value (0.264) of the collection.

SNP allelic variant frequency among gene pool of origin of accessions

The frequency of the favorable allele (providing an increase in resistance) in the nine associated SNPs was different within the gene pool of origin of the Portuguese accessions (Figure 7). The accessions of Mesoamerican origin had, on average, a higher frequency of the favorable alleles than the ones of Andean origin for the SNPs associated in chromosomes Pv04, and Pv07. The most contrasting frequency values were observed for SNP02051, located in chromosome Pv05, for which the frequency of the favorable allele was much higher (0.72) within the accessions of Mesoamerican origin, than within the accessions of Andean or admixed origin (freq. = 0.20). On the other hand, the favorable alleles of DART07926 associated in chromosome Pv08 and DART04561 in chromosome Pv05 were more frequent in the accessions of Andean origin. The average frequency of the favorable allele in the accessions of admixture origin was in most cases intermediate between the accessions of Andean and Mesoamerican origin. Additionally, the frequency of the favorable allele was always above 0.75 for the associated SNPs located on chromosome Pv04, regardless of the gene pool of origin of the accessions.

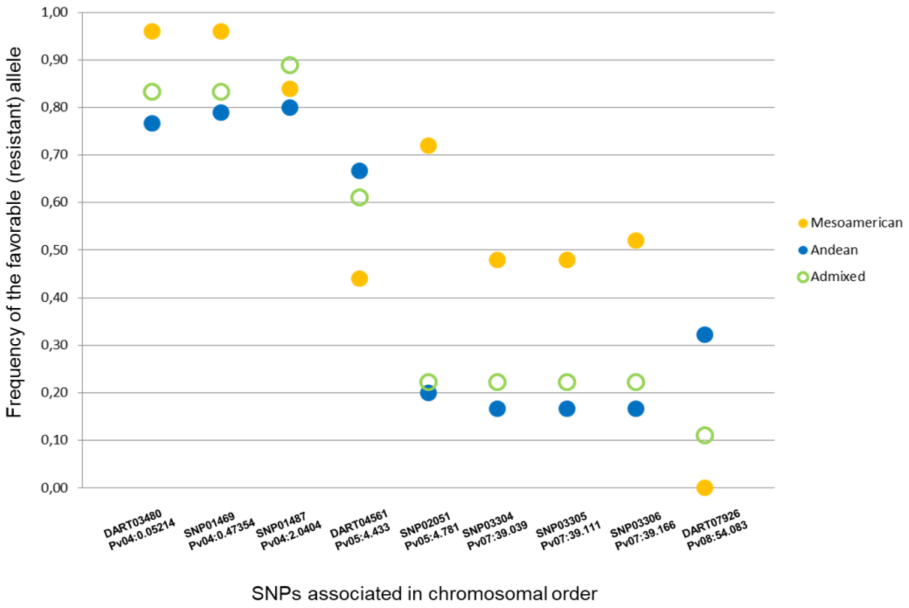


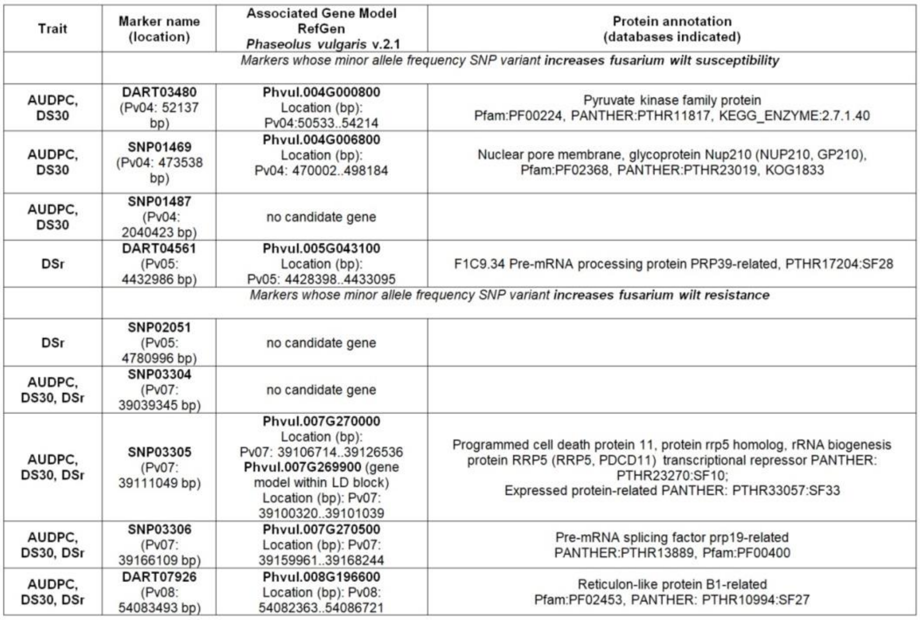
Figure 7: Frequency of the favorable (conferring resistance) allele of the nine SNPs associated with fusarium wilt AUDPC, disease severity at 30 DAI (DS30) and disease progress rate (DSr) according to the main gene pool of origin of the Portuguese common bean accessions (previously determined in Leitão et al. 2017). Each SNP marker is identified in the x-axis by its name and position in the chromosome (in Mbp).

Candidate genes identification

The LD decay to $r^2 = 0.1$ per chromosome varied from 0.5 (on chromosome Pv07) to 1.8 Mb (on chromosome Pv09), with an average graphically estimated of 1.1 Mbp. Supplementary Figure 4 shows the LD decay, measured as r^2 values versus marker distance, and shade plots per chromosome with the correlation between markers highlighted using a color range code.

After identifying the SNPs significantly associated with fusarium wilt response – using the traits AUDPC, disease severity at 30 DAI (DS30), and disease progress rate (DSr) – and the neighboring SNPs in LD, their locations were used to search for putative candidate genes in the *P. vulgaris* genome v2.1 (Table 5). Candidate genes were identified for six of the nine SNP-trait associations.

Table 5: Putative candidate genes based on the gene annotation for the *P. vulgaris* genome v2.1, and on the reference resources for gene and protein annotation, grouped according to the positive or negative effect of the variant allele (SNP allele with minor frequency) in fusarium wilt disease response. The traits evaluated were fusarium wilt disease severity at 30 DAI (DS30), AUDPC and disease progress rate (DSr).



Trait	Marker name (location)	Associated Gene Model RefGen *Phaseolus vulgaris* v.2.1	Protein annotation (databases indicated)
	Markers whose minor allele frequency SNP variant ***increases fusarium wilt susceptibility***		
AUDPC, DS30	**DART03480** (Pv04: 52137 bp)	**Phvul.004G000800** Location (bp): Pv04:50533..54214	Pyruvate kinase family protein Pfam:PF00224, PANTHER:PTHR11817, KEGG_ENZYME:2.7.1.40
AUDPC, DS30	**SNP01469** (Pv04: 473538 bp)	**Phvul.004G006800** Location (bp): Pv04: 470002..498184	Nuclear pore membrane, glycoprotein Nup210 (NUP210, GP210), Pfam:PF02368, PANTHER:PTHR23019, KOG1833
AUDPC, DS30	**SNP01487** (Pv04: 2040423 bp)	no candidate gene	
DSr	**DART04561** (Pv05: 4432986 bp)	**Phvul.005G043100** Location (bp): Pv05: 4428398..4433095	F1C9.34 Pre-mRNA processing protein PRP39-related, PTHR17204:SF28
	Markers whose minor allele frequency SNP variant ***increases fusarium wilt resistance***		
DSr	**SNP02051** (Pv05: 4780996 bp)	no candidate gene	
AUDPC, DS30, DSr	**SNP03304** (Pv07: 39039345 bp)	no candidate gene	
AUDPC, DS30, DSr	**SNP03305** (Pv07: 39111049 bp)	**Phvul.007G270000** Location (bp): Pv07: 39106714..39126536 **Phvul.007G269900** (gene model within LD block) Location (bp): Pv07: 39100320..39101039	Programmed cell death protein 11, protein rrp5 homolog, rRNA biogenesis protein RRP5 (RRP5, PDCD11) transcriptional repressor PANTHER: PTHR23270:SF10; Expressed protein-related PANTHER: PTHR33057:SF33
AUDPC, DS30, DSr	**SNP03306** (Pv07: 39166109 bp)	**Phvul.007G270500** Location (bp): Pv07: 39159961..39168244	Pre-mRNA splicing factor prp19-related PANTHER:PTHR13889, Pfam:PF00400
AUDPC, DS30, DSr	**DART07926** (Pv08: 54083493 bp)	**Phvul.008G196600** Location (bp): Pv08: 54082363..54086721	Reticulon-like protein B1-related Pfam:PF02453, PANTHER: PTHR10994:SF27

Out of those six candidate genes identified, two (Phvul.004G006800 and Phvul.007G270000) encoded proteins involved in the inducible plant response to pathogens, such as phytoalexins biosynthesis and hypersensitive reaction. The others were related to amino acids and secondary metabolite biosynthesis (Phvul.004G000800), pre-mRNA splicing (Phvul.007G270500), signaling of plant immune receptors (Phvul.008G196600) and plant translational regulation and stress adaptation (Phvul.008G203200).

Discussion

The continuous search for new sources of resistance in underexplored plant germplasm collections and the study of their genetic basis is essential for the development of tools to support the breeding of new common bean cultivars with durable resistance to fusarium wilt. By exploring the natural variation of 162 accessions representative of the Portuguese common bean germplasm, we found 14 new sources of complete resistance and 71 new sources of incomplete resistance against the highly virulent *Fusarium oxysporum* f. sp. *phaseoli* isolate FOP-SP1 race 6. Complete and incomplete resistant sources were identified among accessions of Andean, Mesoamerican and genetic admixed origin that constitute the Portuguese germplasm.

Additionally, we identified nine SNPs with small effects associated with this natural variation and six candidate genes, suggesting an oligogenic control of the detected resistances. The identified favorable SNP alleles controlling fusarium wilt resistance will facilitate the resistance transfer into more productive elite cultivars using marker-assisted breeding schemes.

As far as we know, this was the first time that sources of resistance to this particular *Fop* isolate, classified as one of the most virulent in a pathogenicity screening of 16 isolates from Spain and Greece (Alves-Santos et al. 2002), were identified in European common bean germplasm. This was also the first GWAS dedicated to common bean fusarium wilt response and the first report of a GWAS using a panel of Portuguese common bean germplasm.

Different responses were detected among the Portuguese common bean accessions when inoculated with FOP-SP1 race 6, revealing the high variation present within the collection. Thirty days after inoculation, the accessions were categorized from completely resistant (9%) to susceptible (48%), with many intermediate cases (43%) that showed leaves with chlorosis that did not progress to necrosis. Two patterns of disease progression were observed among the susceptible accessions – a fast disease progress rate with accessions reaching high disease severity scores (4-5) within the first two weeks after inoculation, and a slower disease progression with accessions reaching the same high scores but only 16 DAI or later. There is a lack of information on how and where this delay takes place in the host-pathogen interaction (Garcés-Fiallos et al. 2017). In the case of the resistant accessions, no external disease symptoms were observed and they kept their green leaves and a typical development throughout the experiment. Resistant and intermediate accessions have been described as either chemically inhibiting the hyphae growth or physically blocking the conidia spreading up the sap stream (Abawi and Pastor Corrales 1990; Garcés-Fiallos et al. 2017; Niño-Sánchez et al. 2015; Xue et al. 2015). This impairment may occur through the formation of papilla structure, cell wall strengthening, and vessels crushing, as it was described for other legume species (Bani et al. 2018; Cachinero et al. 2002; Castillejo et al. 2015; Grayer and Kokubun 2001). A histological analysis will be needed in the identified common bean resistant accessions to elucidate the underlying physiological mechanisms.

The identification of new sources of resistance to this common bean disease is of extreme importance since the existing ones provide only moderate or incomplete levels of protection to specific *Fop* races isolates (Buruchara and Camacho 2000; Pereira et al. 2013; Salgado 1995; Schwartz and Otto 2005). Moreover, previous studies on Spanish widely cultivated common bean cultivars (of Andean origin), and on other cultivars from CIAT that have been used for race determination in *Fop*, revealed the high virulence of FOP-SP1 race 6 isolate (Alves-Santos et al. 2002). All the screened cultivars in that study were susceptible to this isolate, even the ones that had been described as resistant against other *Fop* isolates.

The Mesoamerican lines from CIAT, SER16 and Tio Canela-75, used in our study for international comparison, were found intermediate and susceptible, respectively. This suggests that SER16, a recognized drought-tolerant elite line (Polania et al. 2016b), may also contain genes (common or not to drought tolerance) that confer resistance to this *Fop* race. Indeed, a transcriptomic analysis revealed that drought stress and vascular pathogen infection induced in chickpea shared differentially expressed genes associated to the cell wall and alkaloids biosynthesis, defense related-proteins and osmoprotectants (Sinha et al. 2017). This might indicate that some of the mechanisms induced by common bean in response to both stresses are coincident, but requires further investigation.

In the present study, we observed a range of plant responses to fusarium wilt inoculation from highly resistant to highly susceptible. Such continuity supports the existence of quantitative resistance mechanisms in common bean against *Fop* race 6. This quantitative nature was already suggested for *Fop* race 4 (Cross et al. 2000), with the involvement of several *genes,* each contributing a small to moderate effect in the resistance level. Both dominant monogenic and oligogenic resistance to *F. oxysporum* have been observed in various legume species (Infantino et al. 2006; Rispail and Rubiales 2014; Sharma et al. 2005). In common bean, studies of the inheritance of resistance to fusarium wilt have been performed using segregating populations derived from contrasting cultivar crosses and *Fop* races isolated from particular geographical regions (Batista et al. 2017; Fall et al. 2001; Xue et al. 2015). Some major resistance genes and quantitative trait loci (QTLs) were identified against *Fop* races 1 and 3, while against race 4 recessive and polygenic resistance were also reported (Fall et al. 2001; Schwartz and Otto 2005). More recently, Batista and colleagues (2017) classified common bean resistance to a putative new *Fop* race as dominant and governed by a few major genes and polygenes.

In our study, we identified a total of nine different associated genomic regions using a $-\log_{10}(P\text{-value}) \geq 3$ (marginal Wald test). Three of the nine SNPs were associated with the three traits DS30, AUDPC and DSr; other four with both DS30 and AUDPC, and two only with DSr, totalizing 19 SNP-trait associations. Considering the more stringent LD-adjusted Bonferroni correction, these detected associations are to be considered only as suggestive associations. Nevertheless, looking at the Manhtattan plots, the threshold of $-\log_{10}(P\text{-value}) = 3$ was clearly above the associations background noise, and, on the other hand, the QQ plots didn’t show much inflation, reassuring the interest of the nine detected associated genomic regions. Probably, the complexity of the measured traits, potentially controlled by multiple genes with small effects on the fusarium wilt resistance, together with the relatively small association panel has hampered the power to detect SNP-trait associations (Korte and Farlow 2013, Pasam et al 2012). Still, the associations detected in the present study were useful for identifying candidate loci related to disease resistance. These candidates need now to be validated by gene expression functional studies in contrasting accessions, and in follow-up studies using different genetic backgrounds or different environments, or through the development/use of segregating bi-parental populations (Ioannidis and Daly 2009).

Most of the associations were coincident for the three traits analyzed (AUDPC, DS30 and DSr), reflecting the high correlation between the traits. Two associations were unique for DSr both on chromosome Pv05. Although the proportion of the observed phenotypic variance explained by each significant SNP-trait associations ranged from 4.7% to 7.2%, the favorable allele of the associations with the highest effect corresponded to an increase in fusarium wilt resistance of 16% and a reduction in the disease progress rate of 19%. This suggests that, even with moderate traits heritabilities (0.72 for DS30, 0.70 for AUDPC and 0.41 for DSr) due to the high influence of the environmental variability, improvements can be attained through selection within this Portuguese germplasm.

The average frequency of the favorable allele of the nine SNPs associated with fusarium wilt resistance varied according to the gene pool of origin of the common bean accessions. For most of the resistance associated SNPs, the accessions of Mesoamerican origin had higher frequencies of the allele conferring resistance, with the exception of the two associated SNPs identified in chromosome Pv08. This indicates that there is room within the accessions of Andean origin to improve their resistance, by introgression of interesting resistance alleles from Mesoamerican lines into Andean breeding germplasm. However, the smaller number of Portuguese accessions of Mesoamerican origin in the association panel in relation to the accessions of Andean origin (25 versus 97) could have biased these results. Although the Portuguese common bean germplasm is predominantly constituted by accessions of Andean origin, one-third of the accessions have admixed genetic origin and might represent putative hybrids among gene pools from the two original centers of domestication (Leitão et al. 2017). Thus, not only the accessions of Andean or Mesoamerican origin identified as resistant to *Fop* infection may be useful for common bean resistance breeding within each particular gene pool, but also the resistant accessions with admixture nature may contain novel and advantageous genetic combinations for both gene pool breeding. We identified among the accessions of admixed genetic origin favorable SNP alleles for fusarium wilt resistance that can reflect a positive selection contributing to adaptation to the local environment. It is known that co-evolution of host and pathogens has led to the development of isolates that infect mainly the common beans from one particular gene pool (Geffroy et al. 1999; Kelly et al. 2003). The development of common bean cultivars with pyramided genes for *Fop* resistance identified in common bean accessions from different origins is accordingly an effective strategy for durable resistance because the pathogen cannot easily overcome the resistance conferred by several genes (Batista et al. 2017; Miklas et al. 2006).

Six of the nine resistance-associated SNPs were located within putative candidate genes, according to the common bean reference genome (v2.1). DART03480 and SNP01469, both on chromosome Pv04, were located within genes that code for a pyruvate kinase protein and for a nuclear pore membrane glycoprotein (Nup210, gp210-related), respectively. Pyruvate kinase is an enzyme that catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP in glycolysis and plays a role in amino acids and secondary metabolites (such as terpenes) biosynthesis (Ambasht and Kayastha 2002). Several studies reported the accumulation of amino acids such as valine, leucine, and tyrosine during plant defense responses; however, knowledge on the mechanisms behind the reconfiguration of the plant metabolism when facing a pathogen is still scarce (Rojas et al. 2014). On the other hand, the role of nuclear pore complex (NPC) in nucleo-cytoplasmic trafficking has been described not only in growth and developmental processes but also in plant response to biotic stresses (Cheng et al. 2009; Yang et al. 2017). For example, in *Nicotiana benthamiana* a nuclear pore protein (NbNup75) is involved in ethylene signaling and induction of defense responses such as the production of phytoalexins or programmed cell death that limits the pathogen spread (Ohtsu et al. 2014). Of note, the release of phytoalexins to inhibit fusarium wilt progression was already described in chickpea and pea (Bani et al. 2018; Cachinero et al. 2002). Furthermore, in Arabidopsis, the transmembrane nucleoporin CRP5 (Constitutive Expresser of Pathogenesis-Related Genes 5) associates with NPC and regulates an essential inhibitory mechanism of ETI/PCD (ethylene-triggered immunity/programmed cell death) (Gu et al. 2016), vital for host recognition of pathogen virulence effectors to induce defense. SNP03305, on chromosome Pv07, was also located within a candidate gene that codes for a protein involved in programmed cell death (pre-rRNA processing protein Rrp5). Programmed cell death is a well-described mechanism in plant-pathogen interactions (Huysmans et al. 2017) with an important role in resistance response. In fact, the hypersensitive response (HR), eliciting localized cell death at the site of the pathogen attack, is often triggered to restrict biotrophic and hemibiotrophic fungi growth and had already been observed in different *F. oxysporum*-plant interactions (Cachinero et al. 2002; Chen et al. 2014; Swarupa et al. 2014; Xue et al. 2015).

Interestingly, some of the loci associated with fusarium wilt detected in this study were located in genomic regions that have been previously associated with resistance to other diseases in common bean. For instance, the already referred SNP01469 and SNP01487 (with no candidate gene associated), co-localized, on chromosome

Pv04, with a major QTL for bacterial resistance. This QTL (HB4.2) confers resistance to multiple races of *Pseudomonas syringae* pv. *phaseolicola*, the bacterium that causes halo blight (Tock et al. 2017). Within the mapping interval of this QTL, some genes were identified and predicted to encode proteins with nucleotide-binding site and leucine-rich domains (NBS-LRR), known to enable pathogen detection and defense signaling and typically associated with hypersensitive cell death (Tock et al. 2017). In the same genomic region, the *Co-34/Phg-3* locus, which confers resistance to leaf angular spot caused by *Pseudocercospora griseola* (Sacc.), was also identified (Valentini et al. 2017). Among the candidate genes for the *Co-34/Phg-3* locus, one contains the serine/threonine kinase domain whose function has been correlated to HR and H_2O_2 accumulation (Cao et al. 2011). In our study, we did not microscopically analyze the roots after *Fop* inoculation. That could be a required follow-up histological task to monitor and confirm the presence of hypersensitive cell death in the resistant accessions of the Portuguese collection.

DART04561 on chromosome Pv05, and SNP03306 on chromosome Pv07 are located within candidate genes that code for pre-mRNA related proteins. While the first coded for a prp39-related protein of unknown function, the second is located within a candidate gene that codes for pre-mRNA splicing factor prp19-related. Alternative splicing has been described as an important mechanism in DNA damage response, plant immunity and defense (Lenzken et al. 2013; Shang et al. 2017; Yang et al. 2014). Moreover, in *Arabidopsis thaliana*, the role of the spliceosomal component prp19 was linked to pathogen defense (Meyer et al. 2015).

DART07926 (on chromosome Pv08) is located within a candidate gene that coded for a reticulon-like protein b1 (RTNLB1)-related, whose absence was described to increase susceptibility to pathogens in *Arabidopsis* by regulating the intracellular trafficking and activity of bacterial flagellin immune receptor (FLS2) (Lee et al. 2011). Downstream to FLS2, essential signal transduction events by mitogen-activated protein kinase (MAPK) cascades are well known to confer resistance to both bacterial and fungal pathogens (Asai et al. 2002), including *F. oxysporum* (Wang et al. 2015).

In addition to the already mentioned SNP01487, SNP2051 (on chromosome Pv05) and SNP03304 (on chromosome Pv07) had no associated candidate gene. Nevertheless, these SNPs might still be useful to select for resistance to *Fop* in common bean breeding. The absence of annotated candidate causal genes at these loci might be due to genetic variability between the Portuguese common bean accessions and the Andean accession whose genome was used as reference (accession G19833).

The nine identified SNP-trait associations provided valuable insights into the genetic basis of fusarium wilt resistance but only explained a fraction of the total phenotypic variance. The success of association mapping in identifying markers effectively associated with the trait under study relies on how well the population structure is corrected in the association model and on the existing levels of linkage disequilibrium (LD). Linear mixed models can successfully correct for genetic relatedness between individuals in a population by incorporating a kinship matrix into the model and have been widely used in genome-wide association studies (Kang et al. 2010; Korte et al. 2012; Zhang et al. 2010). In common bean, LD levels were found to be stronger within the Mesoamerican gene pool and decay more rapidly within the Andean gene pool (Blair et al. 2018). In the Portuguese common bean collection analyzed here, the average intra-chromosomal LD decayed to 0.1 r^2 within 1.13 Mbp. A similar LD decay to 0.1 r^2 within 1 Mbp was reported recently for a common bean panel constituted by 27 Andean and 153 Mesoamerican accessions and using 10,326 SNPs (Diniz et al. 2018). Using 9,825 SNP markers, a significant part of the genome was covered (1 SNP/55.3 kbp), although increasing the number and distribution of markers would increase the probability of identifying additional markers in high LD with any QTL linked to the trait. Additionally, since we are likely dealing with a polygenic trait with trait-associated variants each with a small effect, increasing the sample size, and thus maximizing the phenotypic diversity amongst accessions, would improve the power to recover meaningful associations. In spite of that, most of the associated SNPs detected in our study were located inside or near *candidate genes* related to resistance, which reinforce the usefulness of the association panel used.

The associated SNPs and putative candidate genes identified in the current study increase the number of functional markers available to facilitate resistance breeding in this major crop. Next steps will include the validation of the usefulness of the SNPs associated with fusarium wilt resistance identified here, in controlled conditions, in the field using multi-locations and different years. It will be also interesting to evaluate the level of resistance of this germplasm against other strains of *Fop* than FOP-SP1 race 6 to enhance the insights on the resistance mechanisms and genetic control against fusarium wilt on this underused germplasm. That information is needed to understand if the putative candidate genes found here are only involved in the resistance to this specific *Fop* isolate or if, on the other hand, they present broader resistance to different isolates. We chose FOP-SP1 race 6 since this isolate was already well described and characterized in our neighbor country Spain with a proven high virulence. However, it would be of interest to monitor the presence of fusarium wilt in Portuguese fields and perform characterization and pathogenicity tests using the local isolates.

With the present study, we unveil the potential of the natural variation of the Portuguese common bean germplasm for fusarium wilt resistance. New sources of resistance and incomplete resistance to a highly virulent *Fop* strain were identified on this germplasm under an oligogenic control. The associated functional molecular markers detected will support an effective marker-assisted common bean breeding for more durable resistance against fusarium wilt.

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Supplementary material

Supplementary information is available online in FigShare repository:
https://figshare.com/s/d166f4ca0b78553ba978

Chapter IV

Identification of SNP alleles and candidate genes associated with photosynthesis-related traits in a Portuguese collection of common beans under contrasting water regimes

This chapter is based on:

Leitão ST, Bicho MC, Pereira P, Paulo MJ, Malosetti M, Araújo SS, van Eeuwijk F, Vaz Patto MC. Identification of SNP alleles and candidate genes associated with photosynthesis-related traits in a Portuguese collection of common beans under contrasting water regimes. (**to be submitted shortly to Plant Cell and Environment)**

In this research paper, Susana T. Leitão participated in the experimental design and in the plants phenotyping, performed the genotypic and phenotypic data analysis, participated in the genome-wide association analysis, and drafted the manuscript.

Abstract

Water deficit is a major worldwide constraint to common bean (*Phaseolus vulgaris* L.) production, and photosynthesis is one of the most affected physiologic processes under insufficient water conditions. This is a significant challenge in common bean since the genetic architecture underlying the photosynthetic response in water deficit conditions is still not comprehensively understood in this species.

To gain insights into the genetic basis of the photosynthetic response of common bean under water-limited conditions, a collection of 158 Portuguese common bean accessions was grown under both well-watered and mild water-deficit conditions. To assess the effect of the contrasting water treatments on the photosynthetic performance, leaf gas exchange parameters were measured and photosynthetic pigments quantified, as this information is linked to plants' water status. Additionally, leaf traits such as the specific leaf area and thickness were also calculated. The same collection was genotyped with Illumina BARCBean6K_3BeadChip and diversity array technology (DArTseq) and SNP-trait associations tested using a mixed linear model accounting for the genetic relatedness of accessions.

A total of 133 SNP-trait associations were identified for net CO_2 assimilation rate, transpiration rate, and stomatal conductance, and for chlorophylls *a* and *b*, carotenes and xanthophylls contents. Ninety of these associations were detected under water-deficit and 43 under well-watered conditions. The identified candidate genes revealed that stomatal regulation, membrane translocation of proteins, mechanisms of redox, hormone and osmotic stress signaling were the most relevant processes related to the studied common bean response to water deficit. The genomic regions associated with these physiological adjustments and adaptive mechanisms are now preferential targets for future common bean water-deficit-tolerance breeding. Also valuable for these breeding objectives, and not yet explored: new sources of water-deficit tolerance of Andean, Mesoamerican, and admixed origin were detected in the analyzed Portuguese germplasm collection.

Keywords: *Phaseolus vulgaris* L., water deficit, photosynthesis, association mapping, GWAS

Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes for human consumption worldwide, with recognized benefits for health and nutrition (Câmara et al. 2013). Predominantly self-pollinated annual plant, it is a member of the *Fabaceae* family, with an important role in sustainable agriculture due to its capability for fixing atmospheric nitrogen, improving soil fertility, and enabling reduction in fertilizer usage. Water availability is the major abiotic factor affecting this crop productivity (Urrea et al. 2009) and drought stress can result in yield losses of up to 70% (Smith et al. 2019). Since water-deficit effects are determined by the timing, intensity, and duration of stress episodes (Chaves and Oliveira 2004), the consequences of global climate change have made improving tolerance to water deficit a major breeding priority (Giorgi and Lionello 2008).

Under water-deficit conditions, many physiologic processes, including photosynthesis, are negatively affected. Most plants respond to a mild to moderate water deficit by closing stomata and reducing carbon assimilation that might limit photosynthesis (Chaves 1991; Darkwa et al. 2016; Bolat et al. 2014, Wang et al. 2018). Stomata closure also leads to the exposure of plants to excessive energy that, if not dissipated as heat, may be harmful to photosystem II through the production of reactive oxygen species (ROS) in the chloroplasts (Smirnoff 1993). The cellular antioxidative and photoprotective defense machinery conferred by pigments such as carotenoids may scavenge these ROS (Fu and Huang 2001). Leaf photosynthetic pigments (chlorophyll *a* and chlorophyll *b*) content and the Chl *a*/*b* ratio may also be affected due to water deficit, with reports describing an increase, decrease or maintenance of their values depending on the species and genotype studied (Ashraf and Karim 1991; Jain et al. 2010; Loggini et al. 1999; Moran et al. 1994; Nikolaeva et al. 2010).

The reduction in photosynthesis as a consequence of water deficit can be monitored by the measurement of gas exchange parameters such as net CO_2 assimilation rate (A_n), transpiration rate (E), and stomatal conductance (gs). It is common to relate A_n either to gs, defined as intrinsic water use efficiency (WUE_i), or to E, defined as instantaneous WUE. WUE is a measure of the trade-off between carbon fixation and water loss that occurs due to water evaporation from the interstitial tissues of leaves whenever stomata open for CO_2 acquisition (Bramley et al. 2013). Since E and gs tend to decrease faster than A_n, WUE usually increases when water is scarce. Moreover, plants that have adapted to environments with limited water available frequently present higher values of WUE than plants grown in well-watered conditions (Chaves 2004). It is known that the two common bean gene pools of origin – Andean and Mesoamerican – differ in their molecular, agronomic, morphological, and physiological characteristics including the mechanisms by which common bean tolerates water deficit (Asfaw et al. 2017). As an example, advances in the identification of common bean drought-tolerant sources have been achieved within the Mesoamerican gene pool, using race Durango from the semi-arid highlands of Mexico (Beebe et al. 2008; Rao et al.

2006; Rao et al. 2017; Beebe et al. 2013), whereas only a few sources of tolerance were identified in the Andean gene pool (Polania et al. 2016).

Understanding the mechanisms underlying water-deficit tolerance is of primary importance for devising water-deficit-tolerance precision breeding approaches. In common bean, quantitative trait loci (QTL) studies using recombinant inbred lines (RIL) have been used to identify loci associated with water-deficit tolerance and yield-component traits, especially within the Mesoamerican gene pool (Asfaw and Blair 2012; Blair et al. 2010; Briñez et al. 2017; Diaz et al. 2018; Hoyos-Villegas et al. 2016; Mukeshimana et al. 2014; Trapp et al. 2015; Villordo-Pineda et al. 2015). More recently, and with the release of the first *Phaseolus vulgaris* L. genome (Schmutz et al. 2014), genome-wide association studies (GWAS) have become a powerful approach to identifying candidate regions associated with different traits in this species. As a result, drought-tolerant Mesoamerican accessions have been successfully used in an association study for shoot biomass, lodging and 100-seed weight, performed under irrigated versus rainfed field trials (Hoyos-Villegas et al. 2017). Asfaw et al. (2012) identified QTL associated with SPAD (soil plant analysis development) chlorophyll meter readings (SCMR, which estimate chlorophyll content based on leaf transmittance measured at 650 and 940 nm), using a recombinant inbred line (RIL) from a cross of common bean parental lines DOR364 x BAT477 (both are of Mesoamerican origin; DOR364 is susceptible and BAT477 is tolerant to drought) under drought conditions.

More recently, production traits such as days to flower, days to maturity, *Macrophomina phaseolina* infection, and SPAD measurements were evaluated under drought and heat stress environments, using panels of common bean accessions of both Mesoamerican and Andean origin from Central America and Africa

(Oladzad et al. 2019). In this study, several genetic factors were discovered associated with the four production traits in both heat and drought stress environments. Nevertheless, the genetic basis of photosynthetic response to water deficit is still not comprehensively understood in common bean.

The Portuguese common bean germplasm is the result of more than 500 years of farmers' selection and adaptation to the diverse edapho-climatic conditions of the country. Although preferentially cultivated by small landholders in northern and central regions where the warm spring and summer are accompanied by more frequent rainfalls, it spread to other Portuguese regions with much less water available. This germplasm is genetically characterized by closer proximity to the Andean gene pool, but also by the presence of accessions of admixture nature between the Andean and the Mesoamerican gene pools (Leitão et al. 2017). Because of this, this germplasm may contain unique genetic combinations not yet explored in water-deficit-tolerance breeding that may circumvent the challenge of finding resistance sources useful for breeding within both gene pools. Still, this germplasm was never characterized for water-deficit tolerance to be considered as an effective option for common bean breeding.

To identify mild water-deficit-tolerant sources, and genomic regions or candidate genes associated with the natural variation of common bean photosynthetic response under contrasting water treatments, we phenotyped a diverse Portuguese common bean collection of 158 accessions under well-watered and water-deficit conditions using selected photosynthesis-related parameters. A genome-wide association study (GWAS) combining these phenotypes with previously collected genotypic data was subsequently performed. The knowledge generated will enable the development of molecular tools to increase the efficiency of development of common bean varieties with improved performance under water-deficit conditions. Simultaneously, it will promote better utilization of underused Portuguese common bean resources.

Materials and Methods

Plant material and growing conditions

One hundred and fifty-eight Portuguese common bean accessions, belonging to a germplasm collection previously characterized at molecular and morphological levels (Leitão et al. 2017), were used in this study. This collection includes accessions of Andean, Mesoamerican, and admixed (between the two original common bean gene pools) origin. A complete list of these accessions, along with their passport information, is available in Supplementary Table 1. Two Mesoamerican lines from CIAT (International Centre for Tropical Agriculture, Cali, Colombia) gene bank, SER16 and Tio Canela-75, were also included as international references. SER16 is an elite line with recognized superior drought tolerance and Tio Canela-75 is a commercial cultivar considered drought susceptible (Rao et al. 2006; Rosas et al. 1997).

Ten seeds per accession were sown, one seed per pot, and pots placed in trays in a growth chamber at 26 ± 2°C during the day and 18 ± 2ºC at night, under a photoperiod of 16 h light (~295 $\mu mol.m^{-2}s^{-1}$) and 8 h dark, with a relative humidity of 50% and a CO_2 concentration of 370 ppm, approximately. Sowing was done in 8x8x9 cm plastic pots (0.5L), filled with Montemor soil/peat/vermiculite [2:1:1 (v/v)], watered to full water capacity (100 %) and weighted. Three extra pots were filled with the same mixture and put in an oven at 80ºC for one week to estimate the average dry weight value of the soil mixture.

This dry weight value was used in the calculation of the soil water content of each pot during the experiment.

Experimental design

Due to growth chamber space constraints, the trial consisted of an incomplete block design with 4 blocks. In each block, 32 to 48 accessions were evaluated (10 plants per accession, 5 well-watered, 5 under water deficit growing in the same growth chamber). Eight accessions were occasionally and randomly repeated within blocks. In total, 160 accessions were evaluated: the 158 Portuguese accessions and the two Mesoamerican reference lines - SER16 and Tio Canela 75 - included as drought resistant and susceptible check, respectively.

Water-deficit imposition

All the 10 plants per accession were watered every other day with tap water to maintain the well-watered conditions until the second trifoliate leaf was fully expanded (about 3 weeks). At this point, water deficit (WD) was imposed on 5 plants per accession by withholding watering until the field capacity (FC) of the soil in the pots reached 40%, with the FC monitored daily by weighing the pots. The remaining 5 plants per accession were kept under well-watered (WW) conditions.

Photosynthetic performance

When the soil water content of the pots under water-deficit conditions reached 40% of field capacity, gas exchange photosynthetic parameters – stomatal CO_2 conductance (gs), net CO_2 assimilation (A_n), transpiration rate (E), and sub-stomatal CO_2 concentration (Ci) – were measured in the youngest non-detached and fully-expanded trifoliate leaf. Well-watered plants from the same accessions and at an identical developmental stage were evaluated for the same parameters on the same day. The measurements were carried out using a portable Infra-Red Gas Analyzer system (IRGA, LCpro+ ADC BioScientific Ltd., Hertfordshire, UK), with controlled atmosphere (approximately 370 μmol mol^{-1} CO_2 concentration, 23 ± 2ºC and relative humidity of 50-60 %) and a saturating external light source of 1044 μmol m^{-2} s^{-1}. A_n, E, and gs values were used to calculate instantaneous and intrinsic water use efficiencies (WUE= A_n/E and WUE_i= A_n/gs, respectively).

Leaf photosynthetic pigments

Leaf photosynthetic pigments – chlorophylls *a* (C*a*) and *b* (C*b*), and carotenes and xanthophylls (C*cx*) – were quantified according to Wintermans and De Mots (1965). Briefly, two leaf discs, with 0.636 cm^2 each, were sampled from the same trifoliate leaf previously used for the gas exchange measurements, immediately submerged in 95% ethanol, and kept in the dark at 4ºC until full extraction of the pigments. Then, the absorbance of the extract was measured at 470, 648.6, and 664.1 nm in an Ultrospec 4000 UV-Visible spectrophotometer (Pharmacia Biotech, Cambridge, UK), and the concentrations of C*a*, C*b*, and C*cx* estimated. The sum of C*a* and C*b*, their ratio, and the ratio between the sum of chlorophylls and carotenes and xanthophylls [(C*a* + C*b*) /C*cx*] were subsequently calculated. Photosynthetic pigments concentrations were expressed per dry mass unit.

Leaf water status and related morphological parameters

Leaf relative water content (RWC) was calculated to assess the water status of the leaves at the time of the gas exchange measurements, following a protocol adapted from Catsky (1960).

Three discs per plant, with 0.636 cm^2 each, were punched out of leaves and weighed immediately to obtain the fresh weight (FW). Then, leaf discs were floated in distilled water, in the dark, at 24ºC overnight to obtain the turgid weight (TW). Finally, the discs were oven-dried at 80ºC until reaching a constant weight to obtain the dry weight (DW). Leaf RWC was calculated using the formula RWC (%) = [(FW - DW) / (TW - DW)] x 100. The fresh:dry weight (FW/DW) ratio, as an index of cell water content, was calculated.

Other leaf traits as the specific leaf area (SLA, the ratio of leaf discs area to leaf discs dry mass) and leaf thickness (LT, the ratio of leaf disc fresh weight to leaf discs area) were also calculated.

Phenotypic data analysis

Quality control, variance components, and traits heritability

The phenotypic data acquired (traits related to photosynthetic performance and pigments, leaf morphology, and leaf water status) were subjected to quality control of residuals. Normal distribution was assessed as well as the existence of outliers and homogeneity of variance. A squared-root transformation was applied when needed so that the residuals more closely meet the assumptions of normality.

To test how the two imposed water regimes would affect the different common bean accessions under study, a linear mixed model in the restricted maximum likelihood (REML) framework of Genstat (VSN 2017) was conducted. In this model, accession, water treatment (well-watered and water deficit) and their interaction were included as fixed effects, and block as random. A Wald test for fixed effects was performed, and the significance of the effects evaluated. Additionally, the gene pool of origin of the Portuguese accessions (Andean, Mesoamerican, or admixed) was added to the model and tested as a fixed effect. Tukey's post-hoc tests at 95% confidence level were also performed to compare the means of the 16 photosynthesis-related traits measured among gene pools of origin for each water treatment.

The phenotypic data obtained under well-watered and water-deficit conditions were analyzed separately. REML was used to fit a mixed model per trait as *trait* = *block* + *accession* + *error*. This model, with accession and block fixed, was applied to estimate the best linear unbiased estimates (BLUEs) for each accession, needed as input for the subsequent phenotypic-genotypic association mapping analysis. To assess the distribution of each trait at the two water regimes, histograms were plotted using the BLUEs. A similar model with all terms random was fitted to obtain variance components and the best linear unbiased predictors (BLUPs) of accession values.

Phenotypic correlations of BLUEs were calculated between traits and a principal components analysis (PCA) was performed to inspect the existence of clusters of accessions behaving similarly and verify if those clusters were the same between water treatments.

With our original experimental setup, we could not correctly estimate the variance components for accession and error. Therefore a complementary experiment was performed in the same conditions, using 10 accessions and 5 replications, following an alfa-lattice design. This new experiment was combined with the original experiment in the estimation of variance components for accession and error. Broad-sense heritability was calculated for each trait using procedure *vheritability* in Genstat, based on the same model above, with block and accession as random terms, and using the combined experiments.

Genotypic data

DNA extraction and SNP screening

DNA from one representative individual per Portuguese common bean accession was previously isolated and genotyped using the Illumina Infinium BARCBean6K_3 BeadChip^{TM} assay and DArTseq^{TM} analysis (Leitão et al. 2020). The SNP genotyping data set from this previous study was used, after quality control, for the present association mapping analysis.

Association mapping analysis

Genome-wide association studies (GWAS) to reveal the genetic basis of the common bean photosynthetic response to water deficit were conducted using the QTL library procedures available in Genstat software (VSN 2017). Only the photosynthesis-related traits with comparable error variances (A_n, E, gs, C*a*, C*b*, and C*cx*) and not derived (e.g., sum of or ratios between absolute trait values) were used for the analysis. The adjusted means (BLUEs) of those traits were tested for association with 9,825 SNP markers and for 144 common bean accessions that had passed the genotypic quality filters previously applied (Leitão et al. 2020).

The association mapping was performed separately for well-watered and water-deficit conditions in the mixed-model framework of GenStat software, using the kinship matrix with coefficients of co-ancestry between accessions, and fitting markers as fixed and accessions as random terms on REML (Malosetti et al. 2007). The genome-wide marker-trait association scan was conducted by testing the significance of the marker effect using a marginal Wald test at each SNP location. The observed $-\log_{10}$ (*P*-value) of each SNP was plotted against their chromosomal positions to produce Manhattan plots. Using a threshold level of $-\log_{10}$ (*P*-value) = 3 the significant marker-trait associations were highlighted. This threshold was set to discard the background noise obtained in the Manhattan plot without compromising the identification of potentially interesting regions, which would be missed, for instance, by the too stringent and conservative Bonferroni-corrected threshold of significance. For every SNP significantly associated with a trait, the effect of the minor frequency SNP variant in relation to the most frequent allele was calculated.

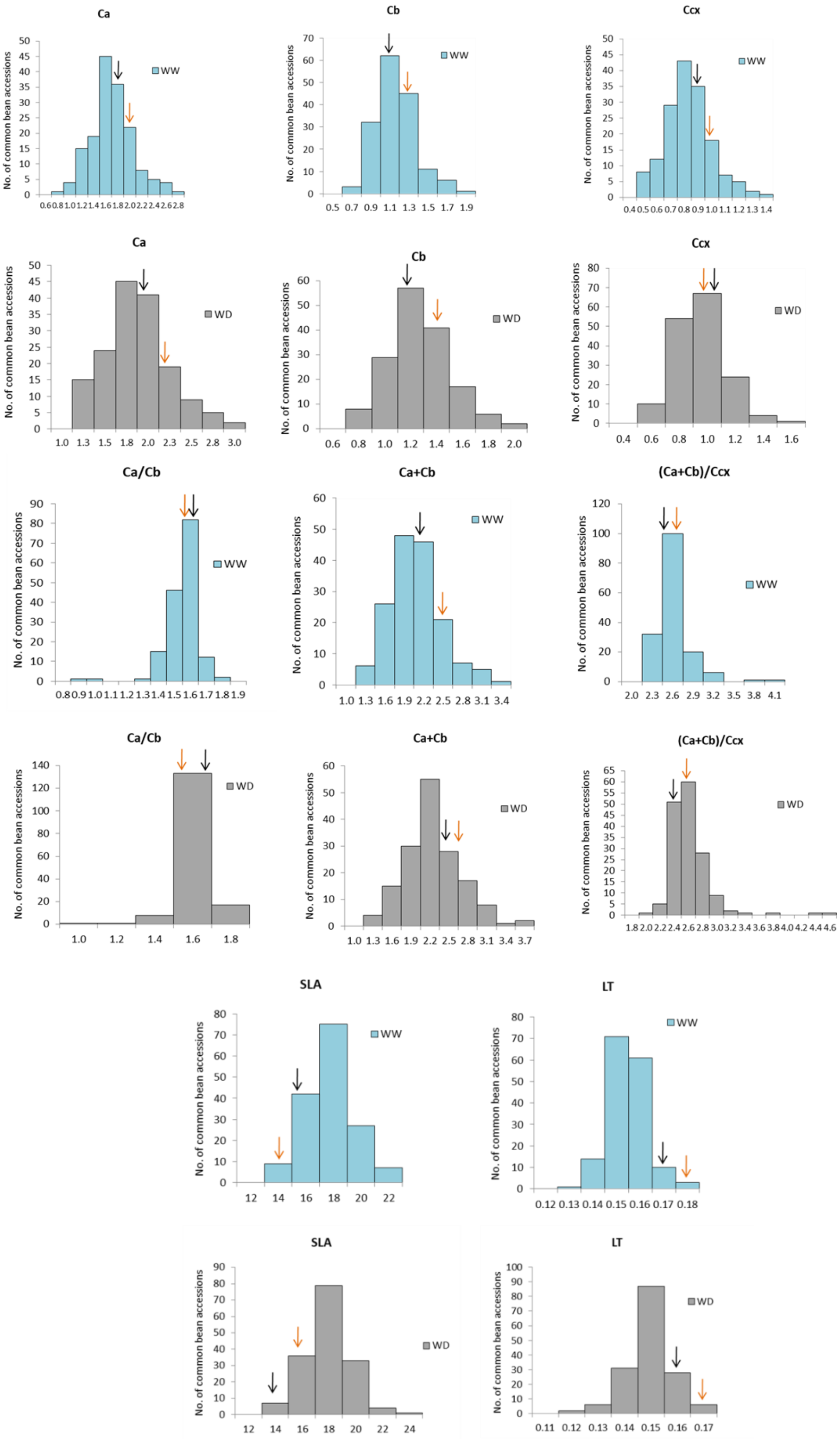
Candidate genes identification

After GWAS, a gene was considered a putative candidate gene for the phenotypic trait under analysis if it contained a significant associated SNP (threshold for significance $-\log_{10}$ (P-value) > 3) or if it was in Linkage Disequilibrium (LD) with a SNP significantly associated with the trait. LD was previously calculated for each common bean chromosome using the squared coefficient of correlation between marker pairs, r^2 (Leitão et al. 2020) and retrieved for the present post-GWAS procedures. To consider the existence of adjacent SNP markers in LD with the ones identified as significantly associated with the traits, the r^2 of the neighboring SNPs was investigated bearing in mind a strict threshold of LD decay ($r^2 > 0.2$). The location of these adjacent SNPs in LD with the significantly associated ones was used to define an LD block or genomic region to look for putative candidate genes. For that, the common bean genome sequence, from the Andean common bean accession G19833 (Schmutz et al., 2014), was investigated using the *JBrowse* tool in the *Phaseolus vulgaris* v2.1, available at the Phytozome v12 portal (these sequence data were produced by the US Department of Energy Joint Genome Institute, DOE-JGI and USDA-NIFA, http://phytozome.jgi.doe.gov/). The annotation information of the genes under the identified genomic regions was obtained from the file "Pvulgaris_442_v2.1.annotation_info.txt", available in the same portal, and *KEGG*/*KOG*/*PFAM*/*PANTHER*/*Gene Ontology (GO)* databases identifiers were used to make inferences about the pathways involved and the possible role of the candidate genes in the control of the traits under analysis.

Results

Phenotypic traits variation under contrasting water treatments

The distribution of the data for each trait under well-water and water-deficit conditions showed the variability of responses within the Portuguese common bean collection (Figure 1).


Ca
No. of common bean accessions
0 5 10 15 20 25 30 35 40 45 50
0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8
WW
Cb
No. of common bean accessions
0 10 20 30 40 50 60 70
0.5 0.7 0.9 1.1 1.3 1.5 1.7 1.9
WW
Ccx
No. of common bean accessions
0 5 10 15 20 25 30 35 40 45 50
0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4
WW
Ca
No. of common bean accessions
0 5 10 15 20 25 30 35 40 45 50
1.0 1.3 1.5 1.8 2.0 2.3 2.5 2.8 3.0
WD
Cb
No. of common bean accessions
0 10 20 30 40 50 60
0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
WD
Ccx
No. of common bean accessions
0 10 20 30 40 50 60 70 80
0.4 0.6 0.8 1.0 1.2 1.4 1.6
WD
Ca/Cb
No. of common bean accessions
0 10 20 30 40 50 60 70 80 90
0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9
WW
Ca+Cb
No. of common bean accessions
0 10 20 30 40 50 60
1.0 1.3 1.6 1.9 2.2 2.5 2.8 3.1 3.4
WW
(Ca+Cb)/Ccx
No. of common bean accessions
0 20 40 60 80 100 120
2.0 2.3 2.6 2.9 3.2 3.5 3.8 4.1
WW
Ca/Cb
No. of common bean accessions
0 20 40 60 80 100 120 140
1.0 1.2 1.4 1.6 1.8
WD
Ca+Cb
No. of common bean accessions
0 10 20 30 40 50 60
1.0 1.3 1.6 1.9 2.2 2.5 2.8 3.1 3.4 3.7
WD
(Ca+Cb)/Ccx
No. of common bean accessions
0 5 10 15 20 25 30 35 40 45 50 55 60 65
1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6
WD
SLA
No. of common bean accessions
0 10 20 30 40 50 60 70 80
12 14 16 18 20 22
WW
LT
No. of common bean accessions
0 10 20 30 40 50 60 70 80
0.12 0.13 0.14 0.15 0.16 0.17 0.18
WW
SLA
No. of common bean accessions
0 10 20 30 40 50 60 70 80 90
12 14 16 18 20 22 24
WD
LT
No. of common bean accessions
0 10 20 30 40 50 60 70 80 90 100
0.11 0.12 0.13 0.14 0.15 0.16 0.17
WD

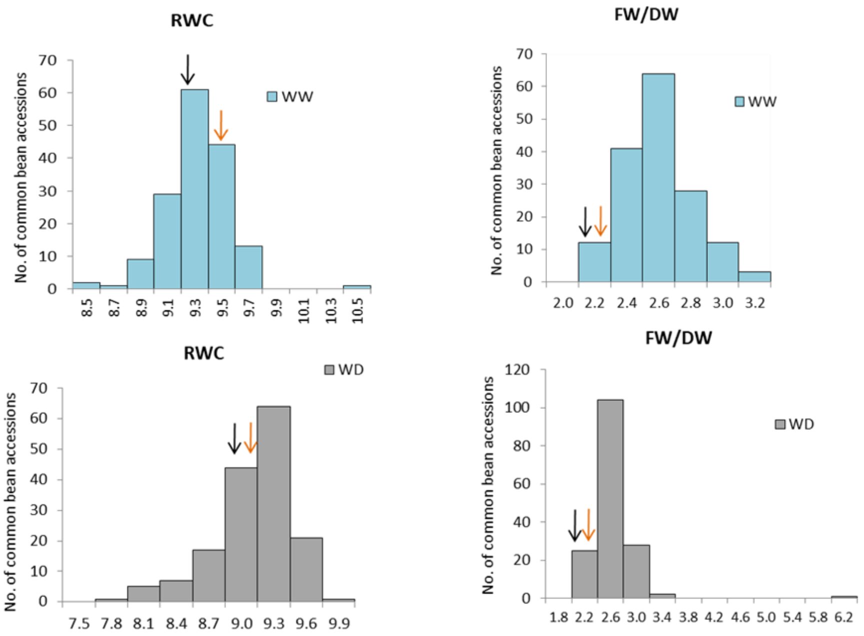


Figure 1: Histograms of the BLUEs for the 16 traits evaluated in 160 common bean accessions and measured under well-watered (WW, in blue) and water-deficit (WD, in grey) conditions. The Mesoamerican reference lines SER16 and Tio Canela-75 are represented by a black and an orange arrow, respectively.

For the majority of the traits measured, the CIAT references SER16 (WD resistant) and Tio Canela-75 (WD sensitive) had an intermediate position in relation to the Portuguese accessions variation, with few exceptions. Both these Mesoamerican lines had lower FW/DW and SLA, and higher LT values than the Portuguese accessions, under both water treatments. Tio Canela-75 had A_n values close to the ones observed for SER16 under well-watered conditions. Nevertheless, Tio Canela-75 showed a stronger decrease in A_n upon WD, when compared with the values observed for SER16 under the same treatment.

The effect of common bean accession and water treatment (well-watered and water deficit) in the observed trait variation was estimated to assess the source of variation using a linear model

(Supplementary Table S2). Significant differences were detected between water treatments (*P*-value ≤ 0.005) for all the parameters evaluated with the exception of specific leaf area (SLA, *P*-value = 0.110), chlorophyll *a*:*b* ratio (C*a*/C*b, P*-value = 0.229) and chlorophylls:total carotenoids ratio ((C*a*+C*b*)/C*cx*, *P*-value = 0.387) (Supplementary Table S2). Nevertheless, SLA and C*a*/C*b* were among the traits that varied significantly among the common bean accessions, together with FW/DW, A_n, Ci, LT, and C*a*.

Overall, gas exchange photosynthetic parameters (A_n, E, gs, Ci) and the ratio FW/DW decreased with the reduction in the soil water content to 40% of field capacity. In contrast, the photosynthetic pigment contents and the water use efficiencies increased under WD. Contrary to what was observed between water treatments, no significant differences were detected among accessions for RWC, E, gs, C*b*, C*a*+C*b*. The interaction between accession and water treatment (Acc x Treat) was only significant for WUE (ratio A_n/E, *P*-value = 0.002) (Supplementary Table S2).

Furthermore, significant differences among the gene pool of origin of the Portuguese accessions (Andean, Mesoamerican, and admixed) were detected or all the traits, with the exception of FW/DW, C*i*, LT, A_n/E, A_n/gs, C*a*/C*b*, and (C*a*+C*b*)/C*cx* (Supplementary Table S3). These differences were mainly observed in the well-water treatment. Under WW conditions, the Portuguese accessions of Mesoamerican origin had significantly (*P*-value < 0.05) higher mean values of A_n, E, and gs than the accessions more related to the Andean gene pool. For these traits, accessions with admixed origin responded similarly to the ones of Andean origin. On the other hand, under WD conditions, only the RWC mean values were significantly higher (*P*-value < 0.05) for the accessions of Andean origin than for the Mesoamerican origin accessions. The accessions of admixed origin presented intermediate RWC mean values.

For the gas exchange parameters, there were no significant differences among gene pools of origin, under WD conditions (Supplementary Table S3).

Correlation between traits

Two groups of highly correlated traits (Pearson's correlation > 0.75) under both treatments (WW and WD) were detected: 1) traits related to gas exchange measurements, namely A_n, E, and gs; and 2) traits related to leaf pigments levels, namely, C*a*, C*b*, and C*cx* (Figure 2). Additionally, traits related to leaf morphology and water content (namely, SLA and FW/DW) were also highly correlated (r = 0.80 under both water treatments). Leaf pigments and the traits related to leaf morphology were the ones more autocorrelated when comparing both water treatments. As example, the correlation of C*a* under WW and WD was 0.76. The correlation matrix with all the pairwise correlations between traits is available in Supplementary Table S4.

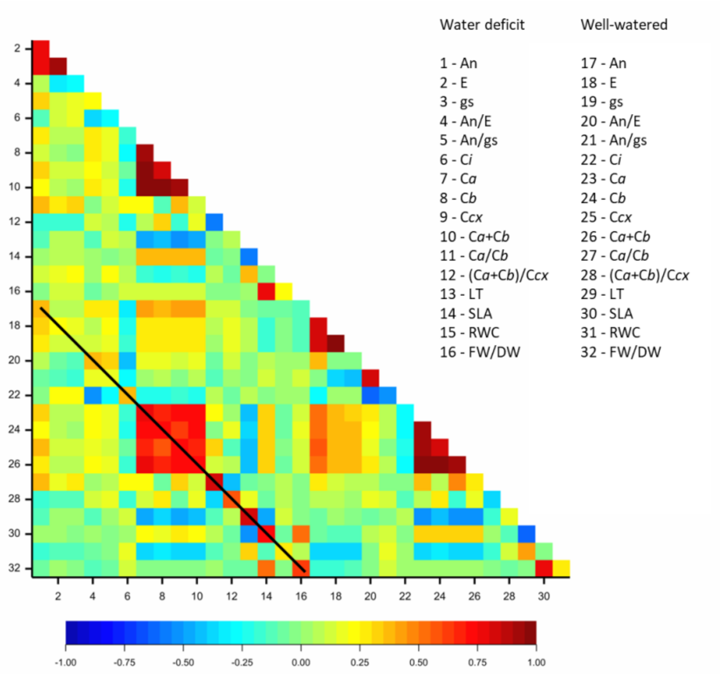
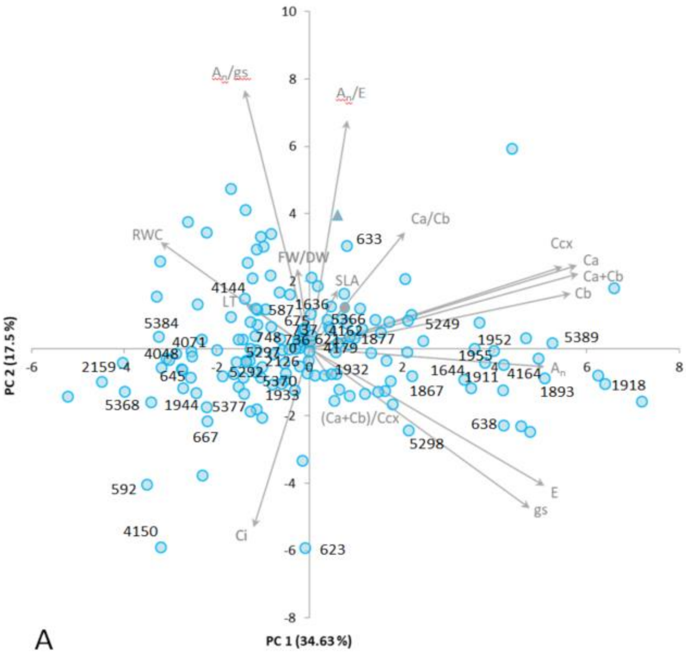
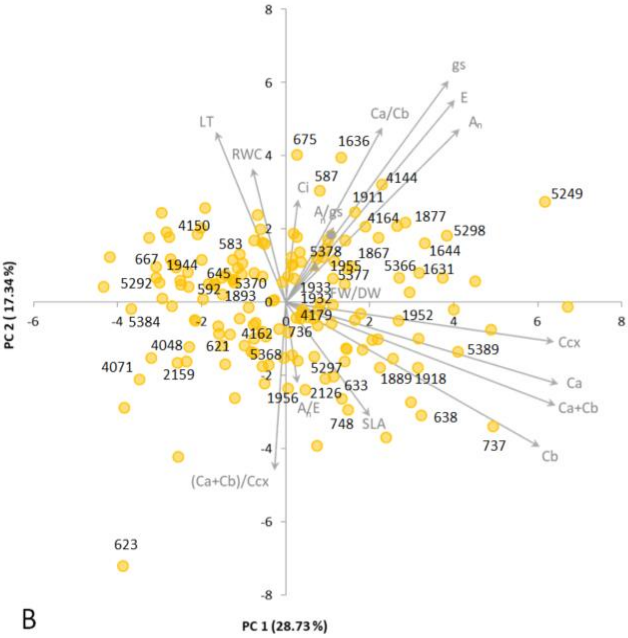


Figure 2: Heat map of the correlation matrix with Pearson coefficients based on BLUEs for 160 common bean accessions, colored by spectrum from blue (correlation = -1.0) to red (correlation = 1.0). The 16 traits under well-watered and water-deficit conditions are shown. A black line was crossed along the diagonal containing the pairwise correlations of the same traits under both water treatments (autocorrelations).

Accessions phenotypic relatedness

A principal component analysis (PCA) was conducted with the phenotypic data obtained from the 16 traits evaluated under WW and WD conditions. The biplots generated simplified the multidimensional data set and allowed us to visualize the interrelationships of the data (Figure 3). Two approaches were followed, plotting phenotypic data for each water treatment separately or combined. Figures 3A (WW) and 3B (WD) display the 160 common bean accessions (158 Portuguese, SER16 and Tio Canela-75) scattered in the space defined by the first two components from the *Eigenanalysis*. The first two principal components explained 52.1% and 46.2% of the variance observed under WW and WD conditions, respectively. In both water treatments, the loading vectors of C*a*, C*b*, C*cx*, and C*a*+C*b* grouped together indicating that these variables contributed similar information irrespective of the water treatment applied. The same occurred for the gas exchange traits A_n, E, and gs. Under well-watered conditions (Figure 3A), the RWC vector pointed out in the opposite direction of E and gs vectors. The smaller magnitude of FW/DW, SLA, LT, and (C*a*+C*b*)/C*cx* vectors indicated that these variables had less influence in explaining the phenotypic variation observed under WW conditions. Under water-deficit conditions (Figure 3B), RWC was negatively correlated with WUE (A_n/E) and SLA, and the vectors with smaller magnitude were FW/DW, C*i*, A_n/E, and A_n/gs. Figure 3C displays the 160 accessions and 32 traits in total since the 16 traits measured under both treatments were differentiated. The first two principal components explained almost 39% of the variance observed. This biplot evidenced that the loading vectors of A_n, E, and gs pointed out to different directions according to the water treatment. On the other hand, the direction and magnitude of the vectors representing C*cx*, C*a*/C*b*, (C*a*+C*b*)/C*cx*, and the traits related to leaf morphology (SLA and LT) were similar under WW and WD conditions. Accessions with high A_n values, regardless of the water treatment, were identified (e.g. 587, 1877, 1911, 4164, 5298, 5366, and 5389 of Andean origin; 1636, 1644, 1867, 5249, and SER16 of Mesoamerican origin; and 675 of admixed origin). From those, the ones with higher water-use efficiencies (both A_n/gs and A_n/E) under WD were 587, 5366, and 5389.




gs
E
Ca/Cb
A_n
LT
RWC
Ci
A_n/gs
FW/DW
Ccx
Ca
Ca+Cb
Cb
SLA
A_n/E
(Ca+Cb)/Ccx
675
1636
587
4144
5249
1911
4164
1877
5298
4150
583
5378
1867
1644
667
1944
1955
5366
1631
5377
645
5292
5370
592
1893
1933
1932
4179
1952
5384
4162
736
4048
621
5389
5368
4071
2159
5297
1889
1918
633
2126
1956
638
748
737
623
8
6
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2
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2
4
6
8
PC 2 (17.34 %)
PC 1 (28.73 %)
B

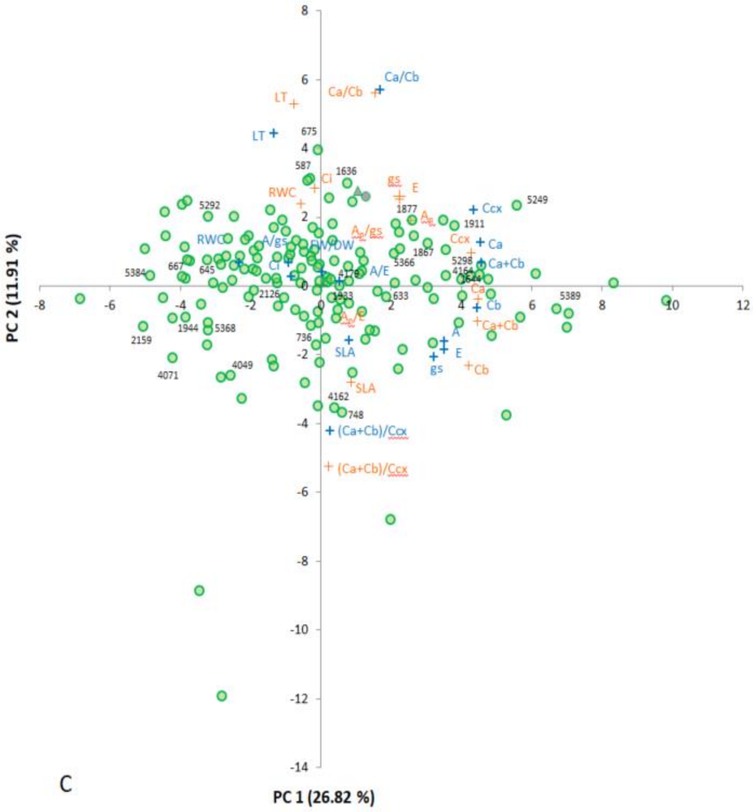

Figure 3: Principal components analysis based on the BLUEs values for 16 phenotypic traits, using 160 common bean accessions (158 Portuguese, SER16 and Tio Canela-75) under well-watered (A) and water-deficit (B) conditions. In C, the loadings of the 16 traits under both water treatments are represented together (well-watered in blue and water deficit in orange). The Mesoamerican lines SER16 and Tio Canela-75 are represented in grey by a circle or triangle, respectively. Accession numbers depicted in the plots are the ones mentioned throughout the text.

To identify water-deficit-resilient accessions another PCA was performed (Figure 4) using the differences between the BLUEs values obtained for each trait under well-watered and water deficit conditions. This allowed us to detect the accessions with little variation under both water treatments, which are thus considered resilient. Since some trait vectors were reductant, only 9 out the 16 traits are shown to simplify the readability of the plot. We selected C*a*+C*b*, (C*a*+C*b*)/C*cx*, A_n, E, gs, A_n/E, A_n/gs, LT, and RWC. The accessions with similar values under WD and WW for the traits measured were highlighted in a central circle (Figure 4) and were 633, 645, 667, 736, 1933, 1944, 4179, 5298, and 5384 of Andean origin, 1644, 5292, 5370, and SER16 of Mesoamerican origin, and 4162 of admixed origin. Moreover, among the accessions with a resilient high A_n (smaller A_n variation), it was still possible to distinguish the ones for which the RWC variation was also minimal (e.g. 587, 675, and 1636) from the ones with a larger decrease in RWC values under WD (e.g. 1867, 5249, 5298, and 5389). On the other hand, the following accessions showed small WUE (A_n/E) variation even with a reduction of RWC under WD: 1932 and 1955, both of Mesoamerican origin, and 4048 of Andean origin.

Additionally, we identified accessions that increased WUE under WD despite decreasing the leaf RWC, e.g., 621, 633, and 700 of Andean origin. We also found accessions in which C*a*+C*b* increased more than 1/3 with WD, such as 748 and 5249 of Mesoamerican origin, 737 of Andean origin, and 5368 of admixed origin.

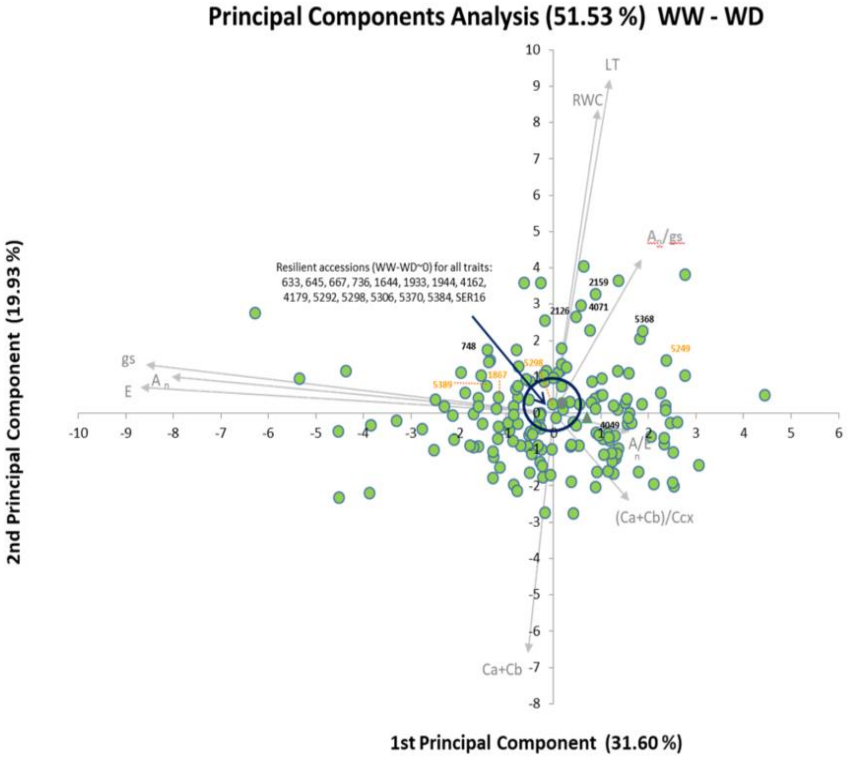

Figure 4: Principal components analysis based on the differences observed between the BLUEs values under well-watered (WW) and under water-deficit (WD) conditions of 9 phenotypic traits, for 158 Portuguese common bean accessions, and the Mesoamerican lines SER16 (represented by a grey circle) and Tio Canela-75 (represented by a grey triangle). The first two components explained 51.53 % of the observed variation. The accessions inside the circle are the ones for which all the traits varied less between water treatments. The accessions highlighted had high A_n values (in orange) and high WUE (A_n/E, in black) under both conditions but also a relatively high leaf RWC variation between water treatments.

Traits heritabilities and variance components

Broad-sense heritability estimates of the traits (h^2) were in general below 0.5 and higher under well-watered conditions than under water deficit (Supplementary Table 5). Moreover, the variance component analysis showed that the highest percentage of variance was for some traits due to the differences between accessions, while for others the residual variance component has a higher percentage (Supplementary Table 5).

Genomic regions associated with photosynthesis-related traits

A total of 133 significant marker-trait associations were identified, 43 under well-watered (WW) and 90 under water-deficit (WD) conditions (threshold set as $-\log_{10}$ (*P*-value) = 3), for the six traits more directly related to photosynthesis: A_n, E, gs, C*a*, C*b*, and C*cx* (Supplementary File S6). Of those, 112 significant marker-trait associations were related to the gas exchange parameters (A_n, E, and gs), while the remaining 21 were related to the leaf pigment contents (C*a*, C*b*, and C*cx*). The traits with the strongest positive correlation presented identical marker-trait associations. The marker-trait associations were scattered throughout the common bean chromosomes, except for chromosomes 4 and 7 where no significant associations were identified (Figure 5). Chromosome 10 was the one with more SNP-trait associations detected: apart from C*b*, the variation for all traits was associated with this chromosome. Under WW conditions, gs was the trait with more associated genomic regions, located in chromosomes 2, 5, 6, 8, 9, 10, and 11. This trait, under WD, was only associated with SNPs located in chromosomes 1, 2, and 10 (different SNPs from the ones associated under WW condition). Only two SNPs were associated with a trait under both water treatments. This was the case of SNP00315, at 37.69 Mbp in chromosome 1, associated with C*b,* and of DART09339, at 3.84 Mbp in chromosome 10, associated with E. In both cases, the association was slightly stronger ($-\log_{10}$ (*P*-value) increment of 0.08 and 0.14, respectively) under WD conditions (Supplementary File S6 and Figure 5).

Regions with the strongest marker-trait associations

Under WD conditions, the strongest significant marker-trait associations ($4.03 < -\log_{10}$ (P-value) < 4.25) were identified for A_n, E, and gs in chromosome 10, between 3.31 and 4.76 Mbp (Supplementary File S6 and Supplementary Figures 1-3). Under WW conditions, SNP01123 located in chromosome 3 at 5.30 Mbp was strongly associated with C*a* and C*b* ($-\log_{10}$ (P-value) = 4.82, the strongest association identified), while SNP04526 in chromosome 10 at 27.9 Mbp was strongly associated with E and gs ($-\log_{10}$ (P-value) = 4.05 and 4.56, respectively).

Regions with multiple traits associations

Interestingly, 75 SNPs were responsible for the 133 marker-trait associations detected. This indicates that, frequently, the same SNP was associated with different traits. For instance, SNP00315, located in chromosome 1 at 37.69 Mbp, was associated with C*a*, C*b*, and C*cx,* under WW and with C*b* under WD conditions.

Under WW conditions, SNP04526, SNP04627, and SNP04633 were associated with both A_n and E, in chromosome 10 at 27.88, 36.89 and 37.28 Mbp, respectively. Under WD conditions, the region between 3.16 and 5.96 Mbp of the same chromosome contained 29 SNPs associated with A_n, and 22 SNPs with E (Figure 5).

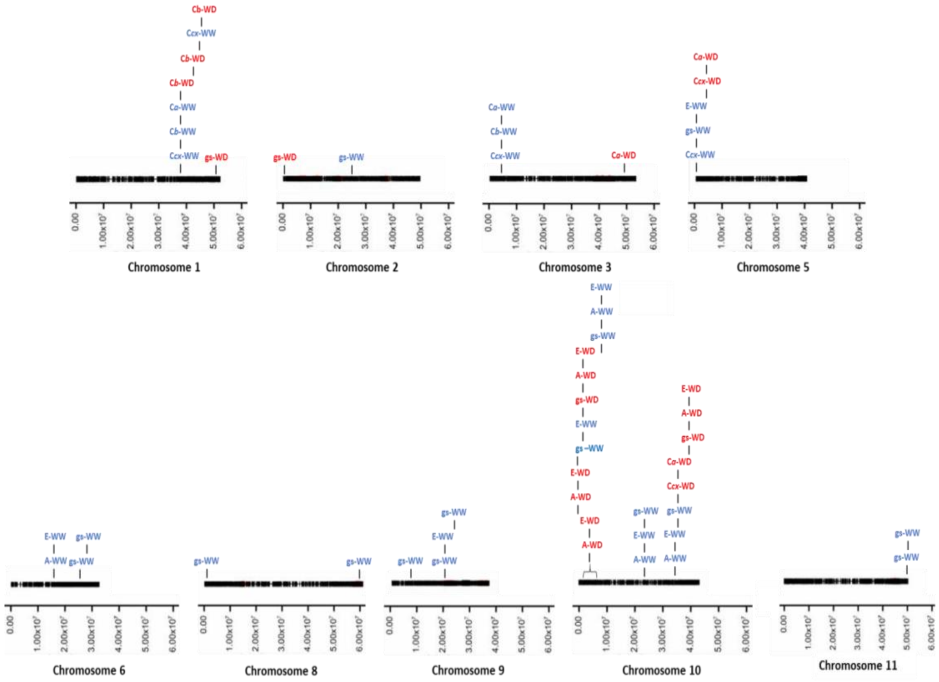

Figure 5: Schematic representation of the common bean chromosomal regions significantly associated with the traits A_n, E, gs, C*a*, C*b*, and C*cx*, under well-watered (in blue) and water-deficit (in red) conditions, using 144 Portuguese accessions (the accessions that fulfilled genotypic quality filtering). Traits in the same column were associated with the same SNP markers. Horizontal bars represent common bean chromosomes and the position of the SNP is indicated in base pairs, based on the *Phaseolus vulgaris* genome v2.1. Only chromosomes with significant trait-marker associations are depicted.

The complete list of the marker-trait associations with markers names, positions in the genome, effect and frequency of the variant allele, and $-\log_{10}$ (*P*-value) is available in Supplementary File S6. Manhattan plots per trait and water treatment, showing the *P*-values (on the $-\log_{10}$ scale) from marginal Wald tests of the marker effects along the chromosomes, are available in Supplementary Figures 1-6. As an example, the Manhattan plot depicting the genome-wide association results for A_n under WW and WD conditions is shown (Figure 6).

Under WW conditions, one SNP was significantly associated with A_n in chromosomes 6 and four in chromosome 10. Under WD conditions, 36 SNPs were significantly associated with A_n in chromosome 10, but none were in common (or in LD) with the ones significantly associated under WW conditions.

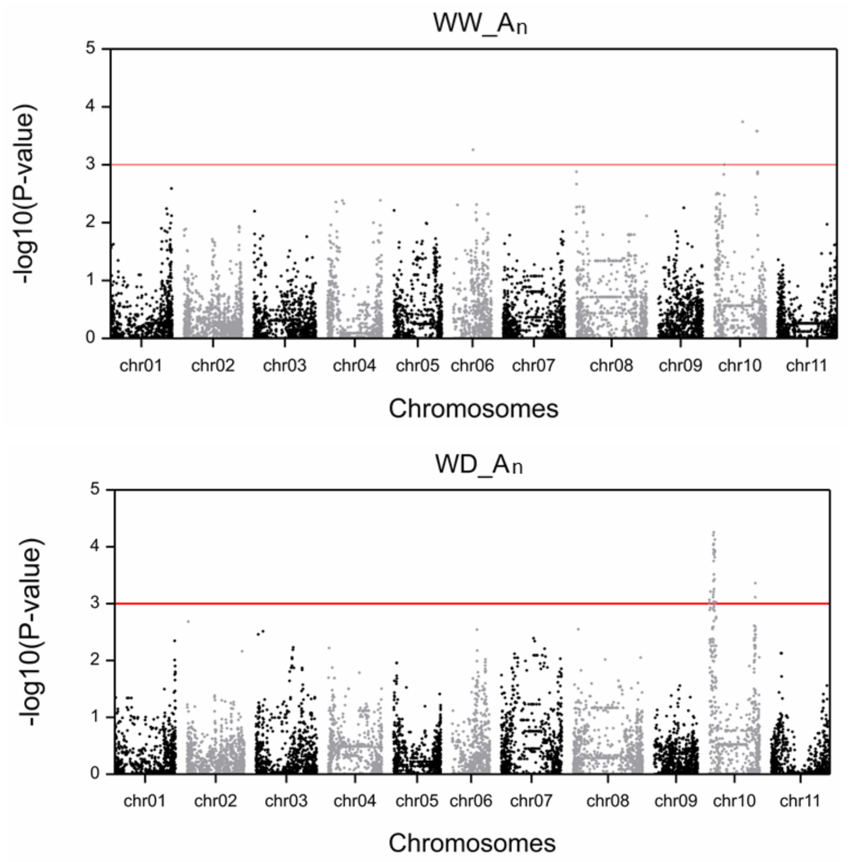

Figure 6: Manhattan plot depicting the genome-wide association results for net CO_2 assimilation rate (A_n) in 144 Portuguese common bean accessions, under well-watered (WW, on the left) and water deficit (WD, on the right) conditions. The y-axis represents the $-\log_{10}$ (P-value) of 9,826 SNPs, and the x-axis shows their chromosomal positions across the common bean genome. The horizontal red line indicates the significance threshold (P-value = 1×10^{-3}).

Effect of variant allele and proportion of variance explained by each SNP-trait association

The effect of the variant allele was larger for A_n, E and C*a* and was positive in almost all the SNPs significantly associated with the traits under WD conditions (Supplementary Table S7). Under this water treatment, the variant allele had a negative effect only in three SNPs: DART01093 associated with gs (variant allele effect = -0.0363), DART03370 associated with C*a* (variant allele effect = -0.2721), and SNP00315 associated with C*b* (variant allele effect = -0.0888). Under WW conditions, the effect of the variant allele was positive for 29 out of the 35 (82.9%) SNPs significantly associated with A_n, E, and gs, and negative for 7 out of 8 (87.5%) SNPs significantly associated with C*a*, C*b* and C*cx*.

For all the traits, each significant SNP-trait association only explained a small portion of the observed phenotypic variance (3.92% to 14.2%). The trait with the largest proportion of variance explained by a significantly associated SNP was C*b* under WD conditions (14.2%), followed by C*a* under WD conditions (12.4%), and gs under WW conditions (12.0%) (Supplementary Table S7).

SNP allelic variant frequency among gene pool of origin of accessions

The frequency of the variant allele in the 75 associated SNPs was different between the gene pool of origin of the Portuguese accessions (Figure 7). On average, the accessions of Mesoamerican origin had a higher frequency of the variant allele than the accessions of Andean and admixed origin. The average frequency of the variant allele in the accessions of admixture origin was in most cases intermediate between the accessions of Andean and Mesoamerican origin.

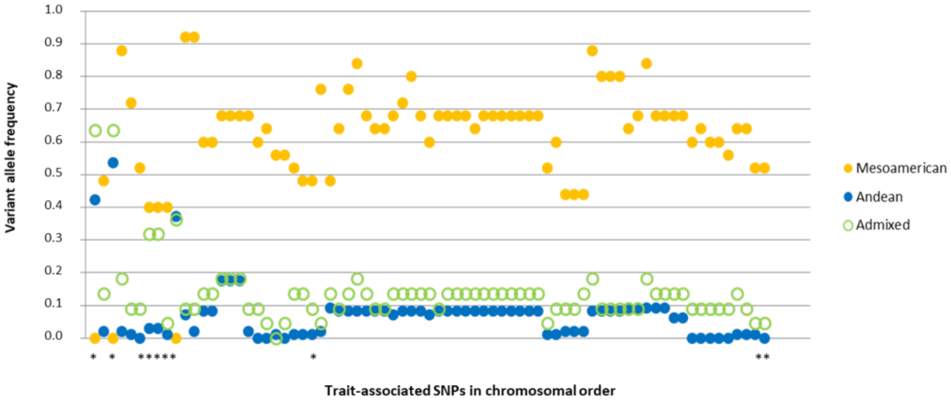

Figure 7: Frequency of the variant allele of the seventy-five SNPs associated with six photosynthesis-related traits (A_n, E, gs, C*a*, C*b*, C*cx*) according to the gene pool of origin of the Portuguese common bean accessions. The ten SNPs highlighted with an asterisk (*) are those having a variant allele with a negative effect on the trait.

Candidate genes identification

The genomic locations of the SNPs significantly associated with the traits were inspected using the *JBrowse* tool in *P. vulgaris* v2.1 genome and positional or in LD candidate genes identified. The average LD decay for the regions significantly associated with the traits is for our association panel 35.24 kbp for LD $r^2 > 0.2$ (Leitão et al. 2020). This value varied across the genome with a maximum LD decay block observed in chromosome 10, with a 1.15 Mbp region spanning from 3.52 to 4.67 Mbp (between SNP04354 and DART09493) identified as associated with A_n and E under WD conditions. In brief, from the 90 SNPs found significantly associated with the traits under WD conditions, 66.7% mapped within genes or were in LD with SNPs located within candidate genes. On the other side, from the 43 SNPs significantly associated with the traits under WW conditions, 60.5% mapped within genes or were in LD with SNPs located within candidate genes. A mapping resolution to the gene level was achieved in 55% of the cases, in which a single gene was identified within the LD block around the associated SNP. A complete list of the identified candidate genes, their functional annotation, and putative role in controlling the traits under scrutiny can be found in Supplementary File S6.

In the frame of this work, it was not possible to describe all putative candidate genes located within the associated genomic regions in detail. Therefore, we restricted ourselves to describing the candidate genes that were located in regions where the strongest significant associations were detected, or in regions associated with multiple traits. This description will be made, separately, for WW and WD conditions.

Candidate genes under well-watered conditions

In chromosomes 5, 9, and 10 several candidate genes were located on a genomic region identified as being associated with different traits.

SNP04396 located at 8.18 Mbp in chromosome 10 was associated with A_n, E, and gs (-$\log_{10}$ (*P*-value) = 3.003, 3.208 and 3.705, respectively) and mapped within the gene Phvul.010G053300, which encodes for a phosphoglycerate mutase family protein (KOG3734).

SNP01992, DART08825, and DART09339 mapped within genes and were associated with E (-$\log_{10}$ (*P*-value) = 3.312, 3.225 and 3.497, respectively) and gs (-$\log_{10}$ (*P*-value) = 3.047, 3.817, and 3.126, respectively). SNP01992, in chromosome 5, was mapped within the gene Phvul.005G004500 encoding for nuclear matrix protein-related (THOC1). In chromosome 9, DART08825 was mapped within the gene Phvul.009G147200 encoding for nodulin MtN21/EamA-like transporter family protein (PF00892). Finally, DART09339, in chromosome 10, was mapped within the gene Phvul.010G026100 encoding for a disease resistance protein (TIR-NBS-LRR class) family.

The SNP with the strongest association with gs ($-\log_{10}$ (*P*-value) = 3.996) was DART08325, mapped within a candidate gene (Phvul.008G291300) in chromosome 8. This gene encodes for O-fucosyltransferase family protein. SNP01983 was the only marker significantly associated with a photosynthetic pigment content (C*cx*) that mapped within a candidate gene under WW conditions. This SNP, located in chromosome 5, was mapped within the gene Phvul.005G002100 annotated as mitochondrial import inner membrane translocase, subunit TIM44.

Candidate genes under water deficit conditions

Under WD, among the strongest marker-trait associations detected for A_n (chromosome 10, between 3.31 and 3.72 Mbp), two were mapped within putative candidate genes. These were the already described DART09339 ($-\log_{10}$ (*P*-value) = 4.254) and SNP04353 ($-\log_{10}$ (*P*-value) = 4.032). SNP04353 was located within Phvul.010G024250, which encodes for a disease resistance protein (TIR-NBS-LRR class) family). SNP04354, also located in the same genomic region interval of the previous SNPs, was also associated with A_n, but with a lower $-\log_{10}$ (*P*-value) = 3.398. This SNP was located within the gene Phvul.010G024800 annotated as a sterol regulatory element-binding protein belonging to basic helix-loop-helix (bHLH) DNA-binding superfamily protein.

Focusing now on the genomic regions associated with multiple traits under WD conditions, three SNPs associated with the gas exchange parameters (A_n, E, gs), DART09337, SNP04694, and DART09907 in chromosome 10 (at 3.75, 39.43, and 39.52 Mbp) were located within candidate genes. DART09337 was located within the gene Phvul.010G025200 encoding for a leucine-rich repeat-containing protein, SNP04694 within the gene Phvul.010G124400 encoding for a subtilase family protein, and DART09907 within the gene Phvul.010G125000 encoding for a sac3-related phosphoinositide phosphatase.

Also, in chromosome 10, the region between 4.65-4.70 Mbp constituted a LD block containing six SNPs (DART09397, DART09398, DART09399, DART09400, DART09401, and DART09403) associated with A_n and E. Within this genomic region, five candidate genes were identified: Phvul.010G031700 encoding for a gamma-tubulin complex component 2 (TUBGCP2, GCP2), Phvul.010G031800 encoding for an ATP/DNA binding protein, Phvul.010G031900 and Phvul.010G032000 encoding for receptor-like serine/threonine-protein kinase 1, and Phvul.010G032100 encoding for a small subunit ribosomal protein S6e. Moreover, at 4.79 Mbp, next to the LD block previously described, DART09405 was also significantly associated with A_n and E. This SNP mapped within the gene Phvul.010G032700 annotated as Sec23/Sec24 zinc finger, a transport family protein.

Regarding the SNPs associated with leaf pigments under WD conditions, for which candidate genes were identified, two markers were listed: SNP02054 and SNP02055 in chromosome 5 (at 4.84 and 4.92 Mbp, respectively) associated with C*a* ($-\log_{10}$ (*P*-value) = 3.269 for both SNPs) and C*cx* ($-\log_{10}$ (*P*-value) = 3.098 for both SNPs). SNP02054 was mapped within the gene Phvul.005G045500 annotated as an aspartyl protease family protein, and SNP02055 within the gene Phvul.005G045700 annotated as nuclear pore anchor (TPR, MLP1, MLP2). Considering the LD block between the two associated SNPs, the gene Phvul.005G045600 was additionally identified as a candidate, annotated as YTH domain family protein from evolutionarily conserved C-terminal region 8 (YT521-B-like).

Discussion

Drought is a major concern in agricultural production affecting a wide range of crops including common bean. It is estimated that 60% of bean production occurs in agricultural land prone to water deficit at some time during the cropping season (Beebe et al. 2013). Drought periods result in losses that may reach up to 70% of common bean yield reduction (Smith et al. 2019). The capacity of plants to adapt to water deficit and prevent its negative impact on plant growth and reproduction is related to the plasticity and resilience of the photosynthetic process (Chaves et al. 2011). The genetic basis of photosynthesis-related traits controlling plant response mechanisms under well-watered and water-deficit conditions is still not totally understood in common bean. In this context, this study characterized for the first time the natural variation in leaf morphology, pigment contents, and photosynthesis-related traits found in a collection of 158 Portuguese common bean accessions. This Portuguese collection is known for its genetic admixture between the original Mesoamerican and Andean gene pools (Leitão et al. 2017). Additionally, and using a genome-wide association approach, we identified a total of 133 common bean genomic regions controlling the variation of parameters related to photosynthetic performance (gas exchange and pigment contents) and leaf traits under well-watered and moderate water-deficit conditions.

Accessions with higher CO_2 assimilation rate, water-use efficiency, and pigment contents under water deficit were highlighted within each gene pool and SNP markers and candidate genes associated with this resilient photosynthetic performance identified. Moreover, the evaluation of photosynthesis-related traits under well-watered conditions allowed the identification of accessions with higher CO_2 assimilation rates and water-use efficiencies more suited to be grown in regions where water deprivation is not a constraint. This new knowledge provides an opportunity to develop novel molecular tools to achieve a more effective selection of water deficit-tolerant germplasm adapted to different environments.

A diversity of physiological responses to water deficit

The leaf RWC content was found to decrease less than 10% under WD, indicating that leaf water status did not change severely with the diminished water availability on the soil. Nevertheless, this was enough to trigger relevant physiological responses. As expected, a general reduction in the photosynthesis-related parameters (A_n, E, and gs) was observed when soil water content decreased to 40% of field capacity (WD). The decline of photosynthetic and transpiration rates and the closure of the stomata are among the most frequent responses of plants facing WD (Fahad et al. 2017). Indeed, stomatal responses are frequently more strictly linked to soil moisture content than to leaf water content (Chaves et al. 2002) and our results are in agreement with this. Considering the entire collection, A_n, E and gs decreased on average 46%, 56% and 71% under WD. Stomatal conductance response was the photosynthetic parameter most affected by the imposed water deprivation. This proves that common bean plants subjected to WD gave dominance to the mechanisms that allow better water retention, as the first line of defense against water scarcity. Indeed, this behavior has been reported in many studies describing response of C3 plants under mild to moderate drought (Flexas and Medrano 2002), including common bean (Arruda et al. 2019; Mathobo et al. 2017; Montero-Tavera et al. 2018; Polania et al. 2016; Rosale et al. 2012).

The Portuguese collection presented a large variability of photosynthetic performance in response to the studied conditions. For instance, we identified accessions with high A_n values that were stable in WW and WD. These accessions can be considered resilient to WD, maintaining a good photosynthetic performance even under stress. Among those accessions, 587, 675, 1636, 5249, and 5298 had a better A_n under WD conditions than, for example, SER16, the elite accession from CIAT with recognized drought tolerance used for comparison in this study. We also identified accessions, such as 623, 638, 1893, and 1918, that were among the ones with the highest A_n values under WW but were very susceptible under WD. These accessions are more adequate for irrigated farming systems. Interestingly, we also found accessions in which the WD treatment enhanced their photosynthetic performance (A_n values) such as 592, 4144, 4150, and 5377.

Despite the negative effect of WD on gas exchange observed for the majority of accessions, most of them improved the WUE (A_n/E) under WD when compared to WW conditions. Indeed, in our study, a significant accession x treatment interaction for WUE was observed. This feature was already described for other bean cultivars under WD (Rosales et al. 2012). In response to WD, stomatal control and low conductance avoid water losses and increase water use efficiencies (Traub et al. 2017). However, the closure of the stomata also prevents CO_2 from entering the leaf and, consequently, photosynthetic carbon assimilation is decreased in favor of photorespiration. The selection of accessions with higher WUE, with a reduced need of water irrigation, has been considered an essential approach to breeding for drought-tolerant bean cultivars, maximizing crop yield in a more sustainable manner (Beebe et al. 2013; Muñoz-Perea et al. 2007; Polania et al. 2016 and 2017; Ruiz-Nieto et al. 2015). Following this idea of selection for a better WUE under water deficit, common bean cultivars, mainly of Mesoamerican origin, with superior drought tolerance have been identified (Polania et al. 2017). We found several Portuguese accessions with high WUE values under water deficit. Among those, accessions 587, 5366, and 5389 of Andean origin stood out, for having both high A_n and WUE values under WD. Interestingly, accession 587 was among the most resilient, with high A_n and little changes in leaf RWC upon WD, while accession 5389 had high A_n values that were maintained in WD, despite the decrease of its RWC. This is important information since few sources of water-deficit tolerance of Andean origin had been identified until now and they are lacking especially for the improvement of bush-type beans usually cultivated in dry environments (Beebe et al. 2013).

Chlorophylls *a* and *b*, and carotenes and xanthophylls content increased, on average, 20% among the Portuguese accessions under WD conditions. A variability of responses was, however, observed among the Portuguese collection for all photosynthetic pigments and respective ratios established. Although an increase in total contents was detected, few exceptions still occurred, in which a decrease in these contents was observed in response to WD. As an example, accessions 1867, 2126, and 5297 of Mesoamerican, Andean, and admixed origin, respectively, showed a decrease in total chlorophylls (C*a*+C*b*) in response to decreased RWC. Other field studies using common bean also described a reduction in chlorophyll content in response to WD, due to the damage in chloroplasts caused by the formation of ROS species such as O_2^- and H_2O_2 (Karimzadeh Soureshjani et al. 2019; Mathobo et al. 2017). On the other hand, accessions 1952, 4048, and 1956 of Mesoamerican, Andean and admixture origin, respectively, are examples of Portuguese accessions with high values of C*a*+C*b* under WD, despite their decrease in RWC.

Most of the above-mentioned studies on water deficit effect in crops were conducted in plants grown under field conditions and at the reproductive stage. This hampers the comparison with our results, since growing conditions and plant life cycle stage on which the measurements take place greatly influence the response to WD. Reproductive stages are generally more sensitive to stress than vegetative ones (Mouhouche et al. 1998).

Regarding carotenoids, accessions 748, 1889, and 2159 of Mesoamerican, Andean, and admixed origin, respectively, showed an increase in total content in response to a decrease in RWC triggered by WD. Since carotenoids play a role in the antioxidant defense system and scavenging of ROS, their accumulation can be part of the strategy of these common bean accessions to cope with WD. The mobilization of these carotenoids is a well-described strategy to counteract the negative effects of oxidative damage caused by the accumulation of ROS derived from the excess of light excitation energy (Fahad et al. 2017).

Based on our results, we suggest that besides the time-consuming gas exchange measurements, which require costly specialized equipment, C*a* and C*cx* contents could be used to distinguish both Portuguese common bean accessions and water treatments in controlled conditions experiments using young plants, but more studies are needed to validate this approach. To make these discrimination analyses more efficient, another interesting approach would be the use of non-destructive and expeditious methods to assess chlorophyll contents, such as SPAD measurements, instead of the laborious and destructive pigment quantification we have performed in this study. In our controlled conditions experiment, gas exchange measurements were performed in young plants and at a single time point using a photoperiod restrict window, due to the large number of accessions under study. One of the main limitations of our study is that we did not measure biomass and yield parameters in those same experiments. Thus, we could not establish correlations between the leaf and photosynthesis-related traits measured with each accession’s yield, the main driver of any breeding program. However, this preliminary larger evaluation, will allow us to more efficiently select a smaller group of accessions that behaved differently in the present study to expose to different levels of WD, at different developmental stages, including a recovery period until harvest.

Candidate loci and candidate genes associated with photosynthetic performance

The GWAS performed increased the knowledge available on the common bean genetic basis of photosynthesis-related traits controlling plant response mechanisms to different water conditions. To our knowledge, this is the first association mapping report using photosynthetic-related traits in common bean. Several of the SNP-trait associations detected in the present study were located within or near an *a priori* candidate gene involved in water-deficit response, which strengthened and validated the usefulness of the used association panel. The SNPs with greater potential for an effective marker-assisted selection will be the ones strongly associated with a trait and for which the SNP variant allele have a higher effect on the trait variation. Accordingly, we identified strong SNP-trait associations that could result in an improvement of 19% of net CO_2 assimilation rate, 16% of chlorophyll *a* concentration, and 14% of chlorophyll *b* concentration, under water-deficit conditions. Interestingly, we also observed that the traits more correlated had similar marker-trait associations.

For the great majority of the detected associations with A_n, E, gs, C*a*, C*b*, C*cx*, the variant allele had a positive effect on the trait, increasing its value. On the other hand, the average frequency of the variant allele varied according to the gene pool of origin of the common bean accessions. For most of the associated SNP, the accessions of Mesoamerican origin had higher frequencies of the variant allele. This might reflect background selection events during domestication and breeding history, with selection and fixation of alleles involved in water-deficit response due to adaptation to different environmental ecosystems in Mesoamerica (in general more prone to drought scenarios) versus the Andes.

Several markers were simultaneously associated with different gas exchange parameters, under each water treatment, reflecting the high correlation existing between these traits.

Some of the candidate genes identified as associated with A_n, E, and gs, under water-deficit conditions, provided clues on the mechanisms common bean activate to overcome water deficit. For example, the candidate gene Phvul.010G125000 located in chromosome 10 encodes for a polyphosphoinositide phosphatase that in *Arabidopsis thaliana* is associated with an increased water deficit tolerance due to the reduction of water loss (Perera et al. 2008). The water-retaining capacity is essential in water-deficit-avoidance and -tolerance mechanisms. Moreover, four other candidate genes located in chromosome 10 (Phvul.010G025100, Phvul.010G031700, Phvul.010G032000, and Phvul.010G032700) have functional annotations indicating that osmotic adjustments and stomatal closure might be one of the first mechanisms triggered by common bean in response to water deficit. Phvul.010G025100 codes for a NB-ARC leucine-rich repeat (LRR)-containing domain disease resistance protein. The LRR-containing domain is evolutionarily conserved in many proteins associated with innate immunity in plants and has been implicated in diverse signaling events, including the ones involved in the early steps of osmotic stress regulation (Ng and Xavier 2011, Osakabe et al. 2013). Phvul.010G031700 codes for a gamma-tubulin complex component 2, a cytoskeleton protein described as drought-responsive and implicated in cell growth (Wang et al. 2016). Phvul.010G032000 codes for a receptor-like serine/threonine-protein kinase 1, and this class of proteins has known roles in signaling, development regulation, and plant defense (Afzal et al. 2008). For instance, the SnRK2 family members are plant-specific serine/threonine kinases involved in plant response to abiotic stresses and abscisic acid (ABA)-dependent signaling (Kulik et al. 2011). Finally, Phvul.010G032700 codes for a zinc finger protein involved in the transcriptional regulation responsive to abiotic stresses through the induction of ABA (Joshi et al. 2016). ABA is known to accumulate in cells and to be very abundant under water deficit conditions, inducing the expression of many stress-related genes (Bray 2002; Yang et al. 2011). Moreover, ABA controls stomatal aperture through the biochemical regulation of ion and water transport processes (Kim et al. 2010).

The candidate gene Phvul.001G259400 located at chromosome 1 contained a SNP strongly associated with gs under water-deficit conditions. This gene encodes for a 3-oxoacyl-[acyl-carrier-protein] reductase that catalyzes the first reduction step in fatty acid biosynthesis and is repressed by ABA in guard cells of *Arabidopsis thaliana* (Frolov et al. 2017). Additionally, the candidate gene Phvul.010G024800, located at chromosome 10, contained a SNP only significantly associated with A_n, under water deficit conditions. That gene encodes for a sterol regulatory element-binding protein described as having a role in abiotic stress signaling in the endoplasmic reticulum of *A. thaliana* (Liu et al. 2008).

Regarding the SNP associations with leaf pigments under water-deficit conditions, the region between 4.79 and 4.88 Mbp in chromosome 5 was significantly associated with both chlorophyll *a* (C*a*) and total carotenoids concentrations (C*cx*). Within this region, two candidate genes were mapped: Phvul.005G045500 and Phvul.005G045600. The first encodes an aspartyl protease and the second for a YT521-B-like domain. The relation of aspartic protease to drought susceptibility was found in *P. vulgaris* leaves with drought-tolerant and drought-susceptible plants that differed regarding aspartic protease precursor gene expression (Contour-Ansel et al. 2010; Cruz de Carvalho et al. 2001). YT521-B homology (YTH) domain-containing RNA binding proteins in plants were described as having a responsive function to the oxidative stress caused by the generation of reactive oxygen species (ROS) (Wang et al. 2014, 2017).

Under well-watered conditions, gs was the trait with the higher number of associations. From the candidate genes detected for the 14 SNPs significantly associated with gs (or in LD), we discuss four whose function seemed more relevant to the photosynthetic response. In chromosome 2, Phvul.002G127100 encodes a mitochondrial alternative oxidase. This enzyme is involved in a large number of plant physiological processes, such as growth, development and protection of the photosynthetic apparatus against photodamage by regulating cellular redox homeostasis and ROS generation (Vishwakarma et al. 2015). Interestingly, in the same chromosomal region, a second candidate gene Phvul.002G127200 might also be involved in the autophagy pathway of recycling cytoplasmic components through the lysosomes or vacuoles. This gene encodes for a sodium:solute symporter family, reported as mediating water flux to maintain proper water balance within and outside both the plasma and the vacuole membranes (Dong et al. 2014). Additionally, the candidate gene Phvul.002G127400 encodes the transcription initiation factor TFIID. The nuclear factor NF-YA that interacts with TFIID is believed to contribute to the reduction of H_2O_2 production under dehydration conditions and increased plant growth and photosynthetic rate under normal watering conditions and drought stress (Pereira et al. 2018). Finally, in chromosome 8, the candidate gene Phvul.008G017800 encodes a protein from aldo/keto NAD(P)-linked oxidoreductase superfamily protein. It is known that members of the aldo–keto reductase family, including aldose reductases, are involved in antioxidant defense by metabolizing a wide range of lipid peroxidation-derived cytotoxic compounds (Fehér-Juhász et al. 2014).

Fewer associations were mapped for C*a*, C*b* or C*cx*, under well-watered conditions. As an example, only one candidate gene was identified for C*cx*, in chromosome 5, Phvul.005G002100, which encodes for a mitochondrial import inner membrane translocase. However, we could not find in the literature any direct relationship between this gene and carotenes or xanthophylls concentration.

Water deficit impacts many aspects of the physiology of plants and particularly photosynthetic capacity. By identifying SNP-photosynthesis-related traits associations and underlying candidate genes, we presented insights into the genetic basis of those physiological mechanisms, and of the different pathways involved in common bean response to water limitation. The biological function of the candidate genes identified here suggests that stomatal regulation, membrane translocation of proteins, mechanisms of ROS accumulation avoidance, and hormone and osmotic stress signaling were the more relevant processes that characterized the Portuguese common bean photosynthesis-related-traits response to water deficit.

The Portuguese common bean accessions evaluated here under two water treatments presented a large variability in their photosynthesis-related traits. As confirmed by the genetic study the response of this collection to WD conditions was dependent on many different traits underlying specific physiological responses. In this germplasm, we were able to identify accessions that revealed, to a certain extent, a capacity to activate a physiological response to cope with water deficit, namely through stomatal conductance regulation, increased water use efficiency, or avoidance of ROS accumulation.

Additionally, the existence of interesting accessions more related to the Andean or to the Mesoamerican gene pools but also of intermediate accessions with an admixture nature between the two original gene pools offers complementary alleles and novel genetic combinations valuable for improving water deficit tolerance in both gene pools. Indeed, accessions that trigger different but complementary physiological mechanisms in response to stress are expected to perform better under water-deficit conditions and should be the target of future breeding selections.

Next steps will include analyzing the most promising accessions identified here, under field conditions, using a multi-location design to assess the environmental effect on these traits through a genotype-by-environment interaction analysis, validating the usefulness of results obtained in the current controlled study under field conditions.

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Supplementary material

Supplementary information is available online in FigShare repository:
https://figshare.com/s/21be39ac74aa766584ea

Chapter V

Impact of combined biotic and abiotic stresses in common bean: Insights from a fusarium wilt – drought imposition transcriptomics approach

This chapter is based on:

Leitão ST, Santos C, Araújo SS, Vaz Patto MC. Impact of combined biotic and abiotic stresses in common bean: Insights from a fusarium wilt – drought imposition transcriptomics approach (**in preparation)**

In this research paper, Susana T. Leitão participated in the experimental design, performed the experiment and the data analysis, and drafted the manuscript.

Abstract

Common bean (*Phaseolus vulgaris* L.) is one of the most consumed legumes worldwide. In nature, plants are exposed to various and complex types of stresses involving numerous environmental factors, both biotic and abiotic. Water deficit (WD) and fusarium wilt, caused by *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*), are constraints to yield that are found frequently together in nature. To understand the shared and unique responses of common bean to *Fop* and WD stresses, we identified the differentially expressed genes (DEGs) in a double resistant and a double susceptible Portuguese common bean accessions exposed to single and combined stresses. Transcriptomic analysis was complemented with a phenotypic follow-up of photosynthesis-related traits and disease progression 48 and 96 hours as well as 8 days after stress imposition.

The susceptible accession revealed a clearly higher level of transcriptional changes in relation to the control condition, in all the three treatments – *Fop* and WD single stresses and in the combined *Fop*WD stress – compared with the resistant accession (944 versus 228 DEGs. A total of 57 DEGs were common to the two single and combined stresses for the susceptible accession, whereas in the resistant accession 24 DEGs were found. On the other hand, for the susceptible accession, 328 DEGs were unique for WD and 36 for *Fop* single stresses. The resistant accession displayed a constitutive defense to *Fop* with zero DEGs, while 145 DEGs were identified under WD stress. Furthermore, a total of 89 and 35 DEGs were identified only in the combined stresses for the susceptible and resistant accession, respectively.

The most represented functional categories within the DEGs were “RNA”, “secondary metabolism”, “stress”, “signaling”, “hormone metabolism”, “transport”, “protein” and “cell wall”. The resistance-related genes identified are potential targets for functional characterization and the development of molecular tools, to expedite the screening and improvement of common bean multiple resistances against biotic and abiotic stresses.

Keywords

Stress interaction, *Phaseolus vulgaris*, water deficit, *Fusarium oxysporum* f. sp. *phaseoli,* transcriptome, gene expression

Introduction

In nature, plants are simultaneously exposed to a combination of different stresses that influence both crop growth and productivity. Moreover, forecasted global climate change effects increase the chances of plants' multi-stress interactions (Kissoudis et al. 2014), challenging breeding programs to develop crops that thrive and maintain a high yield at constantly varying field conditions. Global warming is increasing the frequency of heatwaves, especially in the Mediterranean area and facilitating pathogen spread, altering the habitat range of pathogens (Giorgi and Lionello 2008; Luck et al. 2011; Madgwick et al. 2011).

Limited data is available on plant responses in general – and on grain legumes in particular – under abiotic and biotic stress combinations hampering breeding progress. Thus, it is essential and urgent to better understand the molecular mechanisms used by plants to balance growth and defense against diverse combinations of environmental constraints. This knowledge is needed to improve grain legumes fitness through plant breeding (Huot et al. 2014). The response of plants to simultaneous stress conditions is of particular interest, as one stress response pathway might interact and antagonize another, a process mainly controlled by phythormones (Atkinson et al. 2013). Furthermore, unique responses occur in plants simultaneously exposed to two or more stresses. These responses – called tailored – are different from the responses that occur in plants exposed to the same individual stresses (Ramegowda and Senthil-Kumar 2015).

The molecular mechanisms behind plant multiple stress interactions started to be studied using mainly model species like Arabidopsis, tomato and tobacco (Kissoudis et al. 2014; Prasch and Sonnewald 2015; Prasch and Sonnewald 2013; Atkinson et al. 2013).

In the particular case of a biotic-abiotic stress combination, several studies demonstrated that a preceding abiotic stress may induce pathogen resistance responses in plants (Sharma and Pande 2013; Carter et al. 2009; Wiese et al. 2004). For instance, the generation of reactive oxygen species (ROS) and the accumulation of abscisic acid (ABA) caused by moderate drought stress was reported to enhance plant defense against pathogens, by inducing expression of defense-related genes (Achuo et al. 2006; Pandey et al. 2015). Besides phytohormone balance, the tradeoff between development and defense under abiotic and biotic stress conditions relies on soluble sugars, transcription factors activation, and interconnected signaling pathways, including Ca^{2+} sensing, production of ROS and secondary metabolites, and activation of protein kinase cascades (Kudla et al. 2018; Liang and Zhou 2018; Prasch and Sonnewald 2015; Rejeb et al. 2014; Zhang et al. 2018; Zhang and Sonnewald 2017). As an example, ROS generation by RBoh (respiratory burst oxidase homolog) proteins, after recognition by pathogen elicitors, leads to ABA-induced stomatal closure and to hypersensitive cell death (Fujita et al. 2006).

Additionally, it has been described that many abiotic stress conditions, such as water deficit and heat, weaken the defense mechanisms of plants and enhanced their susceptibility to both aerial and root pathogen infection (Campo et al. 2012; Mittler and Blumwald 2010; Atkinson and Urwin 2012; Goel et al. 2008). On the other hand, pathogen infection has been shown to reduce photosynthesis and water use efficiency (Bilgin et al. 2010) and induce abnormal stomata opening patterns (Grimmer et al. 2012), with consequences in plant tolerance to abiotic stress. Indeed, and despite some existing overlap, each stress condition induces a unique mechanism of response and each combination of two or more different stresses triggers a more complex plant response than the individual stresses (Suzuki et al. 2014; Fujita et al. 2006).

Common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes for human consumption in the world. This crop is threatened by a series of biotic and abiotic constraints during the life cycle, severely limiting its yield. Diseases and pests are frequent important factors compromising common bean production in temperate and tropic regions (De Ron 2015; Assefa et al. 2019). Drought stress affects over 60% of the common bean production worldwide (Beebe et al. 2013). Few studies exist on the interaction of abiotic and biotic stresses in common bean. For example, Mayek-Pérez et al. (2002) characterized phenotypically the simultaneous exposure of four common bean cultivars to drought stress and a fungal pathogen, *Macrophomina phaseolina* (causal agent of charcoal rot). The combined stresses resulted in higher transpiration rate and leaf temperature as compared to plants only subjected to drought stress. Ambachew et al. (2015) on the other hand, described the negative effect of combined drought stress and *Ophiomyia* spp. (bean fly) infestation on the mean seed yield and leaf chlorophyll content of two diverse common bean recombinant inbred line populations. In particular, the transcriptional profile linked to concurrent stresses adaptation in common bean is still poorly understood as well as the central hub genes and key pathways controlling abiotic and biotic stresses interaction. As a consequence, it is difficult to predict core stress-signature pathways overlapping single and combined stresses that could be relevant for the development of multi-stress resistant plants.

Fusarium wilt caused by the soilborne fungus *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*), and water deficit have a known agronomic interaction, being disease progression favored by dry weather (Di Pietro et al. 2001). The wilt fungi are known to interfere with the water relations of plants by colonizing the xylem vessels where they proliferate and obstruct the transportation of water and nutrients (Yadeta and Thomma 2013). This pathogen has been detected in most of the bean-growing regions of the world, causing significant yield losses (Alves-Santos et al. 2002; Buruchara and Camacho 2000; Pereira et al. 2013), and the species distribution is strongly associated with the ability of pathogenic strains to survive periods of drought (Summerell et al. 2011).

The Portuguese common bean germplasm has a history of more than 500 years of cultivation with high genetic and morphological diversity (Leitão et al. 2017). Its tolerance to water deficit and to fusarium wilt (as single stresses) was recently characterized with the identification of resistance sources (and associated genomic regions) some of which common to both single stresses (Leitão et al. 2020, Leitão et al. submitted chapter IV of this thesis). Nevertheless, it was never subjected to a combined stresses genetic study. The present work was designed to identify transcriptomic changes in common bean in response to single and combined water-deficit and fusarium wilt stresses. The identification of molecular signatures common to different stresses will be relevant for the development of multi-stress resistant varieties.

To attain this objective, we focused on two Portuguese common bean accessions, with contrasting responses to water deficit and fusarium wilt. The two accessions were exposed to single and combined stresses and the transcriptome profiling of roots was compared using massive analysis of cDNA ends (MACE). Roots were chosen since both water deficit and fusarium wilt interact primarily with this tissue. The identification of common bean differentially expressed genes (DEGs) and key pathways activated against *Fop* and water deficit interaction will contribute to a better understanding of the combined common bean responses to two stresses frequently concurrent in nature.

Material and Methods

Plant material and growing conditions

Two Portuguese common bean accessions – 645 of Andean origin, and 1955 of Mesoamerican origin – were chosen based on their contrasting response to water deficit and fusarium wilt (Leitão et al. 2020, Leitão et al. submitted chapter IV). Accession 645 is resistant and accession 1955 is susceptible to both stresses. The disease severity score (DS visual scale 1-5, where 1 represents no symptoms and 5 represents dead plant) 30 days after inoculation with *Fusarium oxysporum* f. sp. *phaseoli* (isolate FOP-SP1 race 6, herein called *Fop*) was 1.8 for accession 645, and 5.0 for accession 1955. Also, accession 645 was more resilient than 1955 to water deprivation, with photosynthesis-related traits showing higher variation in the latter accession when comparing plants from well-watered and water deficit conditions. For example, in accession 645 the net CO_2 assimilation rate was only 10% lower under water deficit than under well-watered conditions, while for accession 1955 the same trait was 3.4 times lower under water deficit.

Thirty-six seeds per accession were sown, 72 seeds in total, one seed per pot, and pots were placed in trays in a growth chamber kept at 26 ± 2°C during day and 18 ± 2ºC during night, under a photoperiod of 16 h light (~295 µmol.m^{-2}s^{-1}) and 8 h dark, with a relative humidity of 50% and a CO_2 concentration of 370 ppm, approximately. Sowing was done in 8x8x9 cm plastic pots (0.5L), filled with vermiculite, which were previously watered to full water capacity and weighted. Three pots were filled with dry vermiculite and weighed to estimate the value of the dry weight of the pots. This dry weight value was used to calculate the soil water content of each pot during the experiment.

Experimental design

The seventy-two pots were divided into three groups for stress response phenotypic evaluation and root sampling for RNA extraction at three different time points - 48 h (T1), 96 h (T2) and 8 days (T3) after stress imposition. At each time point, 12 plants were phenotypically evaluated per accession as follows: three plants were evaluated for response to soil water deficit, defined as 40% of field capacity, other three plants for resistance to fusarium wilt (*Fusarium oxysporum* f. sp. *phaseoli* inoculation), other three for the two stresses combined, and the final three plants were used as controls with no stress applied (Figure 1).

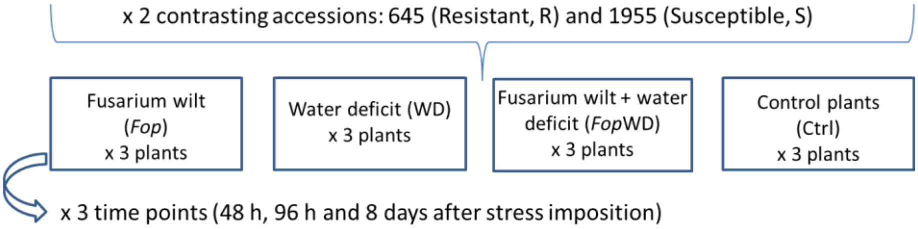


Figure 1: Schematic representation of the biotic-abiotic experimental design. Accessions 645 (resistant) and 1955 (susceptible) were evaluated for fusarium wilt, water deficit and the combined stresses (fusarium wilt plus water deficit). Roots from all the plants were collected at 48 h, 96 h and 8 days after stress imposition (*Fusarium oxysporum* f. sp. *phaseoli* inoculation and/or water deficit) for molecular analysis.

Water deficit imposition

All pots were watered every other day with tap water, to keep the well-watered conditions until the first trifoliate leaf was fully expanded. At the defined growth chamber conditions and for the soil mixture used, three days without watering were needed for the pots to reach the 40% of field capacity. Based on this, the water supply was interrupted 3 days before the *F. oxysporum* inoculation day for the plants under the water deficit treatment (single or combined). These plants were then kept at 40% of field capacity until the end of the experiment. The remaining plants (fusarium wilt single stress and control group) were kept under well-watered conditions until the end of the experiment.

Fungal isolate and inoculation

Fusarium oxysporum f. sp. *phaseoli* isolate FOP-SP1 race 6 was kindly provided by Prof. José María Díaz Mínguez (University of Salamanca, Spain), and stored as micro conidial suspensions at -80ºC in 30% glycerol. This fungal strain was identified in common bean cultivars in Avila, Spain, and classified as highly virulent (Alves-Santos, 2002).

For microconidia multiplication, a protocol adapted from Haglund (1989) and Lichtenzveig et al. (2006) was followed as described in Leitão et al. 2020 (Chapter III). Briefly, the fungal culture was let to grow at 28ºC under constant shaking (170 rpm), for 4 days, and a suspension of 5.0x10^6 conidia.mL^{-1} was prepared and used on the same day to inoculate the common bean seedlings.

For the inoculation, seedlings were removed from the pots, vermiculite was cleaned out of roots that were immersed in the fungal suspension for 30 minutes. Roots from control plants were immersed for 30 min in water, to mimic the stress of immersing the root in the fungal suspension during inoculation. Seedlings were re-planted in the pots and maintained in the same growth chamber, under the same photoperiod and temperature conditions, until sampling.

To verify the full development of the disease, three extra plants of each accession were inoculated together with the trial plants and kept under well-watered conditions. The progression of disease severity in both accessions was assessed every 3 days for 30 days, by visual analysis of the leaves symptoms.

Photosynthetic performance

At the three defined time points, gas exchange photosynthetic parameters - stomatal CO_2 conductance (gs), net CO_2 assimilation (A_n) and transpiration rate (E) - were measured in every plant of each treatment and afterwards roots were sampled. The measurements were carried out using a portable Infra-Red Gas Analyzer system (IRGA, LCpro+ ADC BioScientific Ltd., Herfordshire, UK), with controlled atmosphere (approximately 370 μmol mol^{-1} CO_2 concentration, 23 ± 2ºC and relative humidity of 50-60 %) and a saturating external light source of 1044 μmol $m^{-2}.s^{-1}$. Measurements were made in the non-detached first trifoliate fully-expanded leaf. A_n, E, and gs values were used to calculate instantaneous and intrinsic water use efficiencies (A_n/E and A_n/gs, respectively).

Leaf photosynthetic pigments

Leaf photosynthetic pigments - chlorophylls *a* (C*a*) and *b* (C*b*), and carotenes and xanthophylls (C*cx*) – were quantified for all the plants according to Wintermans and De Mots (1965), at each of the three time points. Briefly, two leaf discs, with 0.636 cm^2 each, were sampled from the same first trifoliate leaf used in the previous gas exchange measurements, immediately submerged in 95% ethanol and kept in the dark at 4 ºC until full extraction of the pigments. The absorbance of the extract was measured at 470, 648.6 and 664.1 nm in an Ultrospec 4000 UV-Visible spectrophotometer (Pharmacia Biotech, Cambridge, UK), and the concentrations of C*a*, C*b* and C*cx* estimated. The sum of C*a* and C*b*, their ratio, and the ratio between the sum of chlorophylls and carotenes and xanthophylls [(C*a* + C*b*) /C*cx*] were subsequently calculated to characterize the physiological state of plants.

Leaf water status

Leaf relative water content (RWC) was calculated to estimate the water status of the leaves at the time of the gas exchange measurements following a protocol adapted from Catsky (1960). Three discs per plant, with 0.636 cm^2 each, were punched out of leaves and weighed immediately to obtain the fresh weight (FW). Then, leaf discs were put in distilled water, in the dark, at 24 ºC overnight to obtain the turgid weight (TW). Finally, the discs were oven-dried at 80 ºC, till constant weight to obtain the dry weight (DW). RWC was calculated using the formula RWC (%) = [(FW - DW) / (TW - DW)] x 100.

Phenotypic data analysis

The restricted maximum likelihood (REML) framework of Genstat® software, 19th edition (VSN 2017) was followed with accession (resistant, susceptible), treatment (control, fusarium wilt, water deficit, combined stresses fusarium wilt + water deficit) and time point (48 h, 96 h and 8 days after stress imposition) as effects to determine the variance components and consequently the contribution of these effects in the variation of each of the 12 traits. A Wald test for fixed effects was performed and their significance as well as of the interaction between effects evaluated. A Tukey's post-hoc multiple comparison test at a significance level of 95% was used for means comparison between accessions, treatments and time points.

Phenotypic correlations (Pearson's r) were calculated between traits, and a principal component analysis (PCA) performed based on the eigenvalue decomposition of the correlation matrix obtained.

Sample collection, RNA isolation, quantification and quality assessment

After trait measurements at each of the three time points, plants from all treatments were removed from the pots and quickly washed under tap water to remove the vermiculite. Roots samples were collected, immediately frozen in liquid nitrogen and stored at -80 ºC for the subsequent molecular analysis. Sampling was performed 48 h, 96 h and 8 days after single and combined stress imposition. Roots from control plants were also sampled at the same time.

For total RNA isolation, frozen roots were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated separately for each of the accession and treatments, using the GeneJET^{TM} Plant RNA Purification Mini Kit (Thermo Scientific^{TM}, Massachusetts, USA) according to the manufacturer's protocol. Trace amounts of DNA contamination were removed from RNA after treatment with TURBO^{TM} DNase (Ambion, Texas, USA), following the manufacturer's instructions. RNA quantification was performed using Qubit RNA BR (Broad-Range) Assay Kit (Life Technologies^{TM}, California, USA) on a Qubit 2.0 Fluorometer (Invitrogen^{TM}, ThermoFisher Scientific^{TM}, California, USA). RNA purity was estimated based on the 260/280 and 260/230 absorbance ratios using NanoDrop^{TM} 2000c Spectrophotometer (Thermo Scientific^{TM}, Passau, Germany), which were 2.1 and 1.8 on average, respectively, after DNAse treatment. RNA integrity was also checked by electrophoresis in a 1% agarose gel stained with SYBR^{TM} Safe (Life Technologies^{TM}, California, USA).

Massive analysis of cDNA ends (3′ mRNA-Seq) and data analysis

Total RNA isolated from roots 96 h after inoculation from three plants per accession and treatment, totalizing 24 samples (2 accessions x 4 treatments x 3 biological replicates), was sent to RNA-seq variant MACE – massive analysis of cDNA ends - provider (GenXPro GmbH, Frankfurt am Main, Germany). This time point (96 h after stresses imposition) was chosen since it showed the largest phenotypic differences between resistant and susceptible accessions (see Result section).

Triplicated MACE libraries for each accession and treatment were prepared and sequenced by the service provider following in-house developed protocols (Zawada et al. 2014; Yakovlev et al. 2014). Poly-adenylated mRNA was isolated from 1 µg of the large fraction of total RNA using Dynabeads® mRNA Purification Kit (ThermoFisher Scientific^{TM}, California, USA). First- and second-strand synthesis of cDNA was performed using SuperScript® III First-Strand Synthesis

System (Invitrogen^{TM}, ThermoFisher Scientific^{TM}, California, USA), with modified bar-coded 5'-end biotinylated poly-T adapters suitable for the Illumina Hiseq2000 flow cell (Illumina, San Diego, USA). Subsequently, the cDNA was fragmented to yield 250 base pair (bp) fragments. The 3'-ends of the fragmented cDNA were captured with streptavidin beads, while PCR bias-proof technology "TrueQuant" was used by ligation of TrueQuant adapters (GenXPro GmbH, Frankfurt am Main, Germany) to distinguish PCR copies from original copies (Eveland et al. 2008; Torres et al. 2008). The barcoded samples were sequenced simultaneously in one lane of an Illumina Hiseq2000 with 1 x 100 bps. Low-quality sequence-bases were removed using "cutadapt" tool (Martin 2011). Poly(A)-tails were clipped by an in-house Python-Script.

The reads were aligned to "Pvulgaris_442_v2.0.fa.gz" (from Phytozome v12.1 *P. vulgaris* v2.1, http://phytozome.jgi.doe.gov/) using Bowtie 2 (Langmead and Salzberg 2012). The annotation information was taken from the files "Pvulgaris_442_v2.1.gene.gff3" and "Pvulgaris_442_v2.1.annotation_info.txt" (these sequence data were produced by the US Department of Energy Joint Genome Institute, DOE-JGI and USDA-NIFA). Normalization and test for differential gene expression between libraries were performed using DEGseq R-package version 1.16.0 (Wang et al. 2009). Genes were considered expressed when they presented a raw read value number ≥ 100 in the three biological replicates of each condition. Differential gene expression was quantified as the log base 2 fold change ($\log_2$FC) of the ratio of normalized values in pair-wised comparisons (Table 1). The *P*-value and correction for multiple testing with the Benjamimi-Hochberg false discovery rate (FDR) were computed to determine the significance of gene expression differences. Genes were considered differentially expressed (DEGs – differentially expressed genes) between conditions when the $|\log_2FC| \geq 1.5$, P-value ≤ 0.05 and FDR ≤ 0.01.

Table 1: List of the transcriptome comparisons performed between MACE libraries. R = resistant accession, S = susceptible accession, Ctrl = control, WD = water deficit single stress, *Fop* = fusarium wilt single stress, *Fop*WD = combined fusarium wilt + water deficit stress.

Comparisons between MACE libraries
R Ctrl x R WD
R Ctrl x R *Fop*
R Ctrl x R *Fop*WD
S Ctrl x S WD
S Ctrl x S *Fop*
S Ctrl x S *Fop*WD
R Ctrl x S Ctrl

Sequencing data validation by quantitative real-time PCR

Gene selection and primer design

Five target genes were selected from the DEGs dataset for MACE data validation: Phvul.001G145600 – Disease resistance-responsive (dirigent-like) family protein, Phvul.001G201300 – RCIA peroxidase superfamily protein, Phvul.G003G096700 Drought-induced 21, Phvul.007G203400 – GolS2 galactinol synthase 2, and Phvul.G231800 – MYB79 myb domain protein 79. These genes were selected based on their level of expression and transcript count, in order to represent a broad range of expression profiles.

Phvul.002G104100, encoding for RNA-binding (RRM/RBD/RNP motifs) family protein, and Phvul.003G082700, encoding for indole-3-butyric acid response 1, were used as reference genes, after testing their expression stability using the geNorm and

NormFinder software package, from GenEx v.5 software (MultiD, Goteborg, Sweden).

Specific primers were designed for reference and target candidate genes using the Primer3Plus online tool (Boston, USA), and checked for specificity using the Primer-BLAST NCBI tool (National Center for Biotechnology Information, USA). Primer design parameters were defined using the default setting for qPCR optimal conditions, on Primer3Plus online tool (Untergasser et al. 2007). Primers were designed in the 3' intra-exonic region and were synthesized by STABVida (Caparica, Portugal) (Supplementary Table S1).

Quantitative real-time PCR

The relative expression of the five selected genes was determined by quantitative real-time PCR (qRT-PCR) to validate RNA-sequencing (MACE). The qRT-PCR reactions were performed using three biological replicates per accession (resistant and susceptible) and treatment (Ctrl, WD, *Fop*, and *Fop*WD) for the time point 96 h (T2) after stress imposition, in a total of 24 samples. cDNA was synthesized from one µg of total RNA from each sample following the manufacturer's instructions of the iScript™ cDNA synthesis kit (Biorad, California, USA). The qRT-PCR reactions were carried out in a PikoReal™ Real-Time PCR System (Thermo ScientificTM) using PerfeCTa® SYBR® Green SuperMix™ (Quantabio, Massachusetts, USA). PCR amplification efficiencies were tested for all primer pairs using a 10-fold dilution series of the cDNA samples. Each reaction of 10 µL was performed twice (technical replicates) containing 2.5 ng of cDNA, 200 nM of each primer, and 5 µL of PerfeCTa® SYBR® Green SuperMix™. Thermal cycling for all genes started with a denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s and 60°C for 30 s. For each reaction, a melting curve (dissociation stage) was performed to verify non-specific PCR products or contaminants. Also, a negative template control (NTC) without cDNA was included in each PCR plate to detect possible contaminations.

The relative expression values (fold change) of the five target genes were normalized to control samples and to the two reference genes using the Pfaffl method (Pfaffl 2001). Finally, fold change data were transformed into a logarithmic scale (base 2) for graph representation and statistical analyses. Student's t-tests were performed to compare the expression levels of each sample in relation to the control.

Bioinformatic data analysis

Venn diagrams were set up using the Bioinformatics & Evolutionary Genomics platform (http://bioinformatics.psb.ugent.be/webtools/Venn/) to compare DEGs identified between each accession/treatment.

PCA with normalized reads counts of DEGs as loading vectors was used to assess the relative distribution of all biological replicates of each experimental condition, using Genstat® software, 19th edition (VSN 2017).

The functional categorization of DEGs was performed using MapMan web tools (Thimm et al. 2004). Unspliced gene sequences retrieved from Phytozome v12.1 were used to create a mapping file for the Mercator pipeline from MapMan (Schwacke et al. 2019).

Results

Phenotypic traits variation

The REML variance components analyses showed that there were significant differences between accessions in C*a*, C*b*, C*cx*, A_n,

A_n/gs, and A_n/E (Supplementary Table S2). For these traits, the resistant accession had a higher mean value compared to the susceptible accession. Significant differences were also detected among treatments for all the photosynthetic pigments contents and ratios and for the gas exchange parameters A_n, E, gs, and A_n/E. Despite the decrease in soil water content and physiological differences observed, no significant variation on RWC was detected between accessions and in response to treatments (Supplementary Table S2).

For the resistant accession, when compared to control conditions, C*b* and C*a*+C*b* values were higher under the combined stress *Fop*WD. On the other hand, A_n, E, and gs values were lower under *Fop*WD. For the susceptible accession, when compared to control conditions, C*b* and (C*a*+C*b*)/C*cx* increased under *Fop* and *Fop*WD, whereas C*a*+C*b* increased under *Fop*. However, C*a*/C*b* decreased under *Fop* and *FopWD*. Additionally, E decreased under *Fop* and *Fop*WD, and gs decreased under *Fop*, WD, and *Fop*WD (Supplementary Table S2). Conversely, A_n, E, and gs were higher under WD in the susceptible accession, but only at the first time point evaluated (Supplementary Tables S3 and S4).

The photosynthetic pigment (C*a*, C*b*, and C*cx*) and gas exchange parameters (A_n, E, gs) values decreased significantly from T2 to T3 (Supplementary Figure 1 and Supplementary Table S2).

The accession x time point interaction was significant for A_n and (C*a*+C*b*)/C*cx*, and treatment x time point interaction was significant for C*a*, C*b*, C*cx*, and C*a*+C*b* (Supplementary Table S2). The gs was the only parameter with significant interactions between accession x treatment, accession x time point, and treatment x time-point.

The largest diversity of responses (for A_n, gs, C*a*, C*b* and C*cx*) between accessions and treatments was observed at T2 (Supplementary Figure S1 and Supplementary Tables S3-S4).

At T2, regarding gas exchange parameters, we observed that the two accessions behaved very differently under single WD, with the A_n value of the resistant accession being 8 times higher than the one of the susceptible accession (Supplementary Table S3). However, for both accessions, *Fop* single stress did not cause a significant change in the A_n values, in relation to control conditions. Also at T2 and in relation to control conditions, gs and E were significantly reduced under all the treatments in the susceptible accession. The same was not observed for the resistant accession (Supplementary Table S3). Finally, *Fop*WD combined condition resulted in a reduction of A_n values to almost half in the resistant accession, and a reduction of 2.5 times in the susceptible accession. A strong effect of the combined stresses was also found for E and gs in the susceptible accession, with these parameters showing a highly significant decrease in comparison with control conditions (Supplementary Table S3).

Still at T2 and for both accessions, C*a*, C*b*, C*cx*, and C*a*+C*b* values under the three treatments were not significantly different from the control samples. Nevertheless, significant differences were found for these traits comparing WD (the lowest values) and *Fop*WD (the highest values), for both accessions (Supplementary Table S4). Under *Fop*WD, C*a* and C*cx* values of the resistant accession duplicated in comparison to WD single stress and increased 51% in comparison to *Fop* single stress. In the susceptible accession, C*a* and C*cx* increased, respectively, 2.4 and 2.2 times, in *Fop*WD in relation to WD single stress (Supplementary Table S4).

At T3, the differences between treatments within accessions decreased, and only C*b* values of the resistant accession under *Fop*WD were significantly different (higher) from the control samples (Supplementary Table S4).

A principal component analysis was performed to assess the relationship among the two contrasting common bean accessions and to visualize if the different treatments and time points cluster separately, based on the results from the 12 traits measured (Figure 2). All traits contributed with a similar weight, with the exception of intrinsic water-use efficiency (A_n/gs) with a smaller vector. The first principal component, explaining 38.42% of the total variation observed, separated the majority of the samples by time point (T1 with an intermediate position between T3 and T2). The exception was the susceptible accession at T2 under WD (single stress) that was displayed together with the accessions at T3. The second principal component, explaining 26.69% of the total variance observed, mostly separated the plants under control and WD single stress from the plants under *Fop* single stress and *Fop*WD combined stress. From the biplot, we inferred that in general samples at T3 had the smallest photosynthetic pigments concentrations and A_n/gs, and samples at T2 presented higher differences among treatments.

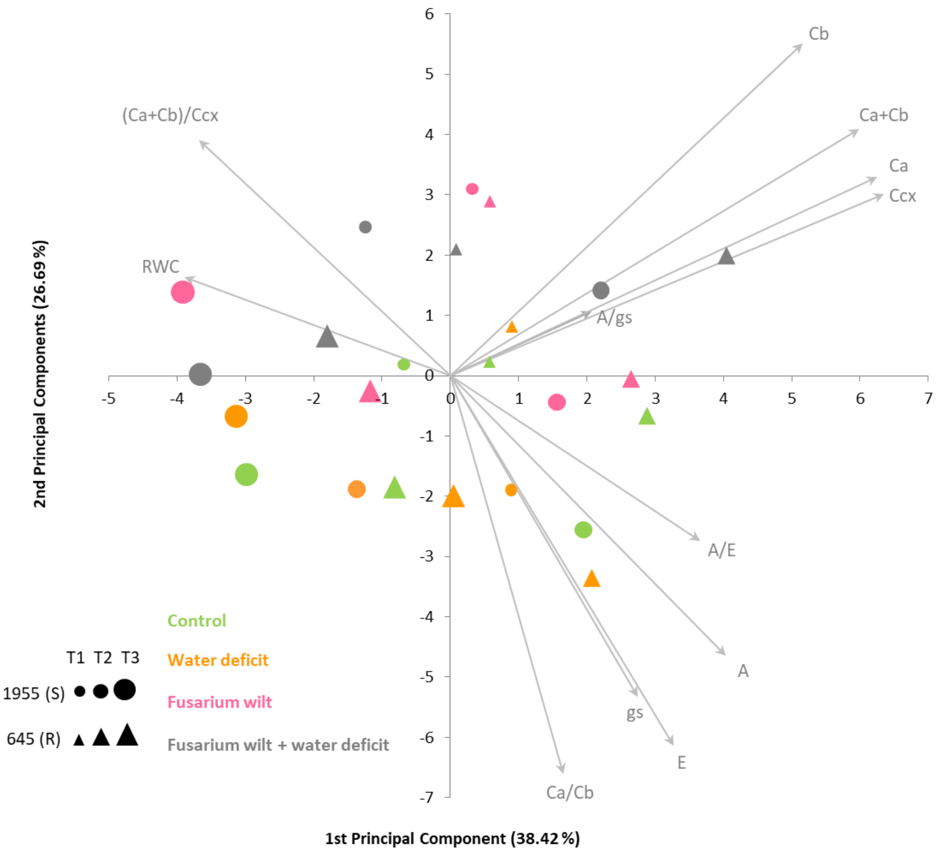


Figure 2: Principal components analysis based on the photosynthesis-related-parameters average values and leaf relative water content, for each condition (control in green, water deficit in orange, fusarium wilt in pink, fusarium wilt + water deficit in grey) and time point (T1 smaller symbols, T2 intermediate symbols, and T3 larger symbols) for the resistant (triangles) and susceptible (circles) Portuguese common bean accessions. The first two principal components explained 65.11% of the variance observed.

During the first 96 hours after inoculation with *Fop*, no visible symptoms were observed in the plants from both susceptible and resistant accessions. Eight days after inoculation, the susceptible accession showed some wilting and chlorotic symptoms in the leaves.

As expected from our previous work (Leitão et al. 2020, Chapter III), accession 1955 was susceptible to *Fop* and the three extra plants that were used to verify the development of the disease were dead 20 days after inoculation, while the extra plants from accession 645 only presented minor symptoms (slight yellowing of leaves margins) that did not aggravate during 30 days.

MACE libraries characterization

Twenty-four MACE libraries were constructed to overview transcriptomics changes in roots collected 96 h after stress imposition. These corresponded to the two accessions (645 resistant and 1955 susceptible to both stresses), four treatments (Ctrl, WD, *Fop*, and *Fop*WD), and three biological replicates for each condition. The number of raw reads per condition ranged from 11,601,286 (sample R Ctrl) to 20,749,104 (sample R WD) (Table 2).

Table 2: List of raw, cleaned, and read counts superior to 100 obtained for each library after Massive Analysis of cDNA Ends (MACE). R = resistant accession, S = susceptible accession, Ctrl = control, WD = water deficit single stress, *Fop* = fusarium wilt single stress, *Fop*WD = combined fusarium wilt + water deficit stresses. The values are averages of the three biological replicates per condition.

Sample/MACE library	Raw read counts	Cleaned read counts	Read counts ≥ 100
R Ctrl	11,601,286	6,336,407	5,589
R WD	20,749,104	11,150,960	6,197
R *Fop*	13,001,084	7,193,821	5,289
R *Fop*WD	12,847,265	6,899,819	5,870
S Ctrl	16,234,892	8,506,887	6,112
S WD	12,954,307	6,305,977	5,447
S *Fop*	12,533,221	6,419,584	5,785
S *Fop*WD	12,846,063	6,465,866	5,752

Phvul.003G109300 - MLP423 MLP-like protein 423 (144,602 counts in average), Phvul.002G207400 - bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein (71,536 counts in average), and Phvul.007G099700 – RSH extensin 3 (48,212 counts in average) were amongst the genes with highest total raw read counts, suggesting no ribosomal RNA (rRNA) contamination during library preparation.

Differentially expressed genes (DEGs) between accessions and treatments

A total of 1,171 genes were considered DEGs (Supplementary Table S5). For each accession, DEGs were identified for each stress condition (WD, *Fop*, *Fop*WD) compared with control (Ctrl).

In response to the applied treatments, a higher level of transcriptional changes was seen in the susceptible accession. While 944 DEGs were found in the susceptible accession, only 228 DEGs were found in the resistant accession. In the susceptible accession, WD was the treatment with more DEGs (242 up-regulated and 239 down-regulated genes) (Figure 3). In contrast, no DEGs were observed for the resistant accession under fusarium wilt single stress, whereas in the *Fop*WD combined stress 26 up-regulated and 33 down-regulated genes were identified. In general, the number of genes up-regulated and down-regulated in each condition was similar. The exception was *Fop* for the susceptible accession in which the number of up-regulated genes was twice the number of down-regulated genes (109 vs 51) (Figure 3).

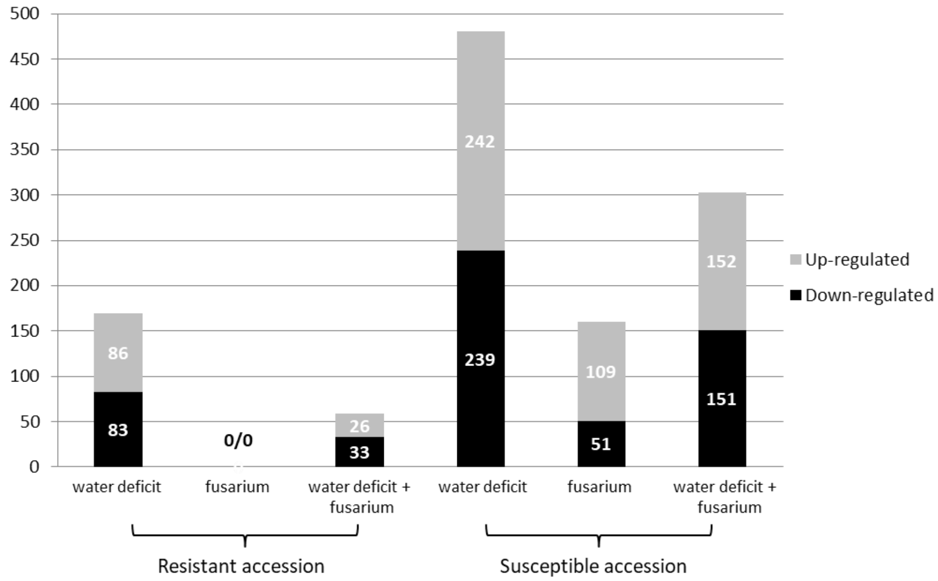


Figure 3: Up and down-regulated genes identified 96 h after stress imposition in comparison with the control condition. The thresholds applied were *P*-value ≤ 0.05, FDR ≤ 0.01, $\log_2$ FC ≥ |1.5|.

A PCA was performed (Figure 4) using a dataset of raw read counts of 294 randomly selected DEGs, instead of the total 1,171 DEGs, due to the statistical software limitation. In general, the three biological replicates per accession/conditions clustered reasonable together, with the exception of three samples of resistant accession, one at each condition. The first two principal components (PCs) explained 35 and 23% of the variance, respectively. PC2 separated the control samples of both accessions and the resistant accession inoculated with *Fop* (single stress) from the remaining samples. The third PC explained 13% of the observed variance and separated clearly the susceptible from the resistant accession under all conditions.

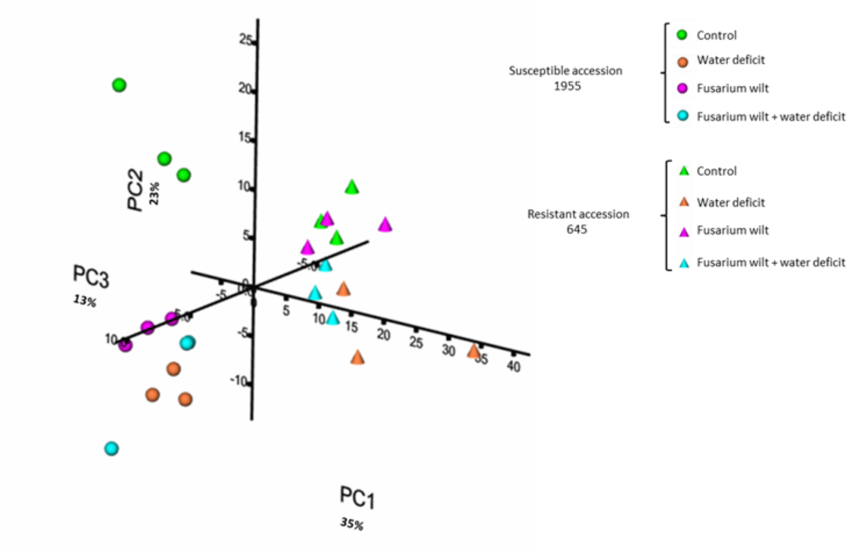


Figure 4: Principal component (PC) analysis of gene expression levels (read counts of 294 shared genes) in two common bean accessions (one susceptible and one resistant) 96 h after exposure to water deficit (orange), fusarium wilt (pink), and to the combination of both stresses (blue). The control situation is in green. The susceptible accession is represented by circles and the resistant accession by triangles. Together the first three PCs explained 71% of the total variance observed.

Functional categorization of DEGs

Functional categories were assigned to the identified DEGs using the MapMan web tools (Supplementary Table S6 and Figure S2). The most represented categories for all the conditions/treatments were “miscellaneous” (bin code (BC) 26), “RNA” (BC 27), “secondary metabolism” (BC 16), “stress” (BC 20), “signaling” (BC 30), “hormone metabolism” (BC 17), “transport” (BC 34), “protein” (BC 29) and “cell wall” (bin code 10) (Figure 5 and Supplementary Table S5).

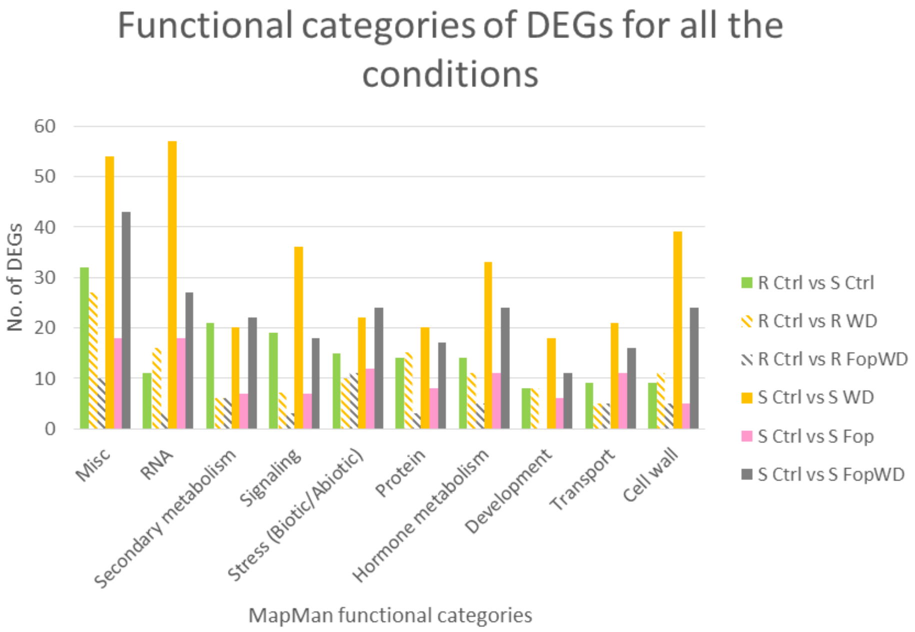


Figure 5: Number of DEGs in the most represented functional categories, assigned by MapMan, *for the resistant and susceptible accessions in the different conditions.* R = resistant accession (diagonal stripes), S = susceptible accession (solid bars), Ctrl = control (green), WD = water deficit single stress (orange), *Fop* = fusarium wilt single stress (pink), *Fop*WD = combined fusarium wilt + water deficit stress (grey).

Some functional categories less represented were only assigned to the susceptible accession, namely, “amino acid metabolism” (BC 13) and “redox” (BC 21), categorizing DEGs under both WD and *Fop*WD, and “cell” under all treatments. Moreover, some functional categories were only assigned to DEGs exclusively up or down-regulated. For instance, “minor CHO metabolism” (BC 3) was only assigned to up-regulated genes in all the treatments for both accessions. On the other side, “OPP.non-reductive PP.transaldolase” (BC 7) was only assigned to down-regulated genes, in *Fop*WD for the resistant accession, and in WD and *Fop*WD for the susceptible accession. The other functional categories assigned to DEGs were present in all the accessions and treatments, categorizing both up and down-regulated genes.

One hundred ninety-eight genes were constitutively expressed by both accessions under control conditions. Among them, we identified a chaperone DnaJ-domain superfamily protein (Phvul.001G226300), a MLP-like protein 43 (Phvul.005G058600), a NB-ARC domain-containing disease resistance protein (Phvul.008G071300), a HEN3 cyclin-dependent kinase E;1 (Phvul.002G275500), and a multidrug resistance-associated protein 12 (Phvul.005G015500).

Shared and unique DEGs between accessions and conditions

In the resistant accession, 24 DEGs were found in common between WD and *Fop*WD (Figure 6A). WD condition had four times more unique DEGs than the combined stressed. On the other hand, when comparing the transcriptome of the susceptible accession, under the three stressed conditions, 57 DEGs were found in common to all treatments (Figure 6B). Sixty (57+3) DEGs were shared between *Fop* and WD conditions (single stresses), 150 (93+57) were shared between WD and *Fop*WD, and 121 (57+64) were shared between *Fop* and the *Fop*WD (Figure 6B).

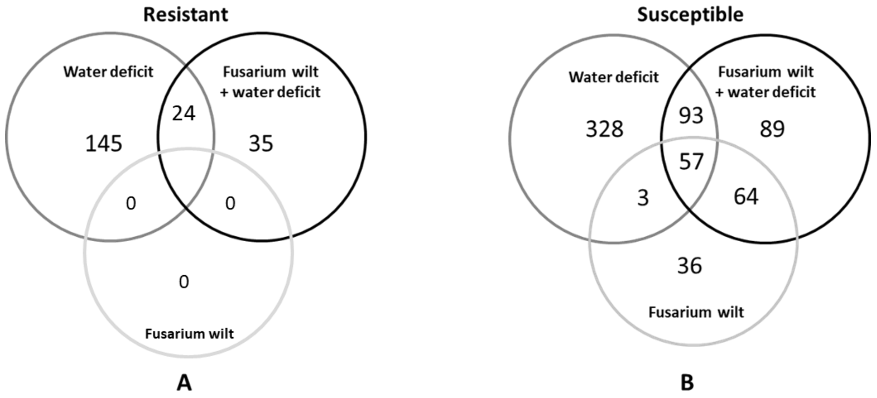


Figure 6: Venn diagrams with the unique and shared differentially-expressed genes identified in the resistant (A) and in the susceptible (B) accessions under the different treatments. All treatments were compared with control conditions. No differentially expressed genes were identified in the resistant accession when comparing fusarium wilt treatment with the control condition.

Sixty DEGs were common between *Fop* and WD single stresses for the susceptible accession (Figure 7B). Among the down-regulated ones, we identified wound-responsive family proteins (Phvul.011G110800 and Phvul.011G110900), a beta-1,3-glucanase 1 (Phvul.001G128500), alcohol dehydrogenase 1 (Phvul.009G149500), a xyloglucan endotransglucosylase/hydrolase 32 (Phvul.001G179900), and a chloroplast-targeted copper chaperone protein (Phvul.002G207200).

On the other hand, a fatty acid desaturase 8 (Phvul.006G068600) was only differentially expressed in the susceptible accession in *Fop* single stress, whereas an integrase-type DNA-binding superfamily protein (Phvul.009G22500), a myb domain protein 79 (Phvul.007G231800), a cellulase 2 (Phvul.001G239900), and a peroxidase superfamily protein (Phvul.008G086800) were only differentially expressed in the susceptible accession under WD.

Seventy-seven DEGs were common to both accessions under WD conditions (Figure 7). From those 77 DEGs, 41 were down-regulated and 34 were up-regulated in both susceptible and resistant accessions, whereas two were up-regulated in the resistant accession and down-regulated in the susceptible one. These two genes, Phvul.009G134300 and Phvul.004G054100, encode for Nodulin MtN3 family protein and an unknown protein, respectively.

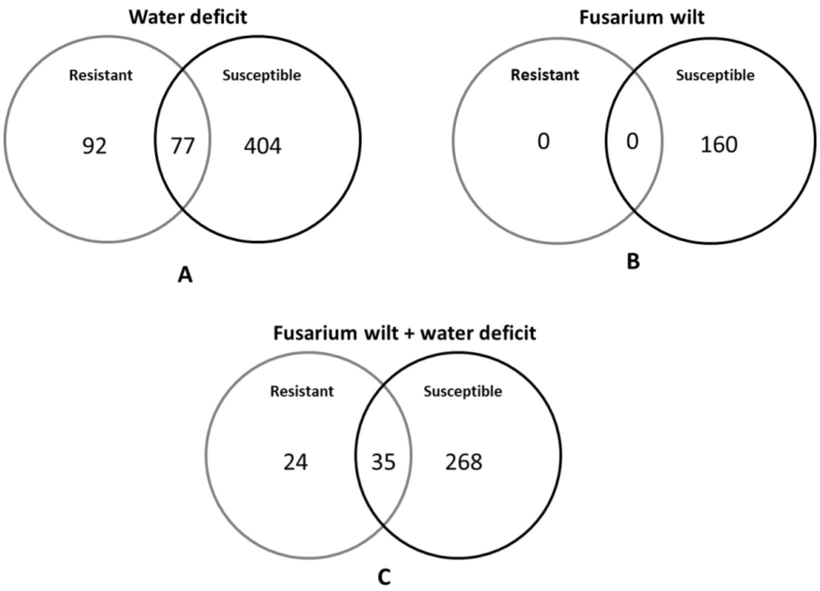


Figure 7: Venn diagrams with the unique and shared differentially-expressed genes in the resistant accession versus the susceptible accession, A) under water-deficit conditions, B) with fusarium wilt, and C) under the combined stresses fusarium wilt + water deficit. Each treatment was compared with control conditions.

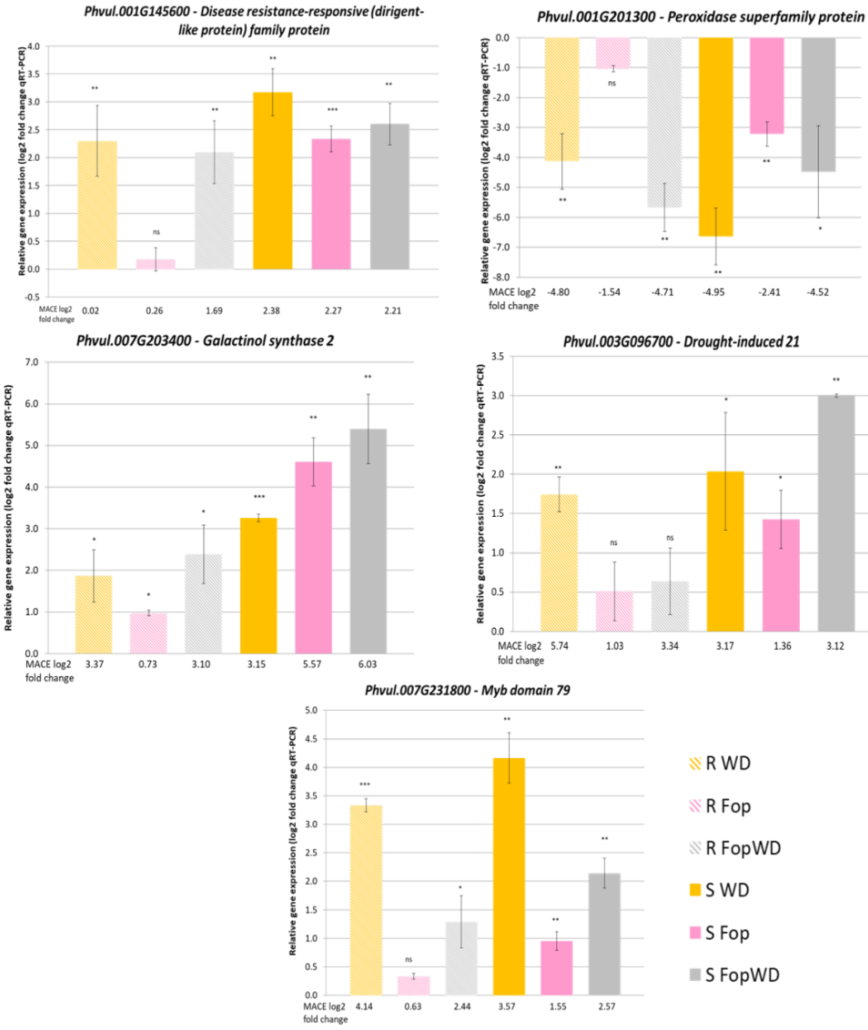
Ninety-two DEGs were unique to the resistant accession under WD (Figure 7A). Among those, four were upregulated and related with hormone metabolism: a lipoxygenase 3 (Phvul.002G228700), an ABA-responsive elements-binding factor 2 (Phvul.009G065500), an highly ABA-induced PP2C gene 2 (Phvul.008G231200) and highly ABA-induced PP2C gene 3 (Phvul.001G075400 and Phvul.001G23600), and a HAB1 homology to ABI1 (Phvul.009G229900). A cold-regulated 413-plasma membrane 2 (Phvul.011G049500), a phytoene synthase (Phvul.008G241500), a sucrose synthase 3 (Phvul.001G209600), and a late embryogenesis abundant protein group 6 (Phvul.003G237400) were found also upregulated. Among the down-regulated ones, we found a SKU5 similar 5 (Phvul.011G025400), a NSHB1 hemoglobin 1 (Phvul.011G025400), a beta-1,3-glucanase 1 (Phvul.001G128500), a peroxidase superfamily protein (Phvul.001G201300), and a matrix metalloproteinase (Phvul.002G076400).

Thirty-five DEGs were common in both accessions under the *Fop*WD, from which 17 down-regulated and 18 up-regulated in both accessions (Figure 7B). Twenty-four DEGs were unique to the resistant accession under *Fop*WD. Among these 24 genes, eight were up-regulated, including a ribulose bisphosphate carboxylase (small chain) family protein (Phvul.004G064800), a CAP (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 protein) superfamily protein (Phvul.006G197200), a UDP-glucosyl transferase 74B1 (Phvul.002G075200), and a pectin lyase-like superfamily protein (Phvul.007G165300) (Figure 7B). Among the down-regulated ones, we found a leucine-rich repeat protein kinase family protein (Phvul.008G244400), an AP2/B3 transcription factor family protein (Phvul.007G102800), a PYL5,RCAR8 Polyketide cyclase/dehydrase and lipid transport superfamily protein (Phvul.008G220600), and a nitrate transporter 2:1 (Phvul.011G083500). Among the 268 DEGs unique to the susceptible accession under *Fop*WD, we found up-regulated an alcohol dehydrogenase 1 (Phvul.001G067300), an integrase-type DNA-binding superfamily protein (Phvul.009G161000), and a bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein (Phvul.009G211200). Among the ones down-regulated in the susceptible accession under *Fop*WD, we found a ferric reduction oxidase 2 (Phvul.010G101500), a wound-responsive family protein (Phvul.011G110800), transcription regulators (Phvul.006G075400) and a protein kinase family protein (Phvul.001G226900).

The above are just a few examples of DEGs that might be involved in the resistance mechanisms deployed by common bean. The comprehensive list with all the DEGs identified is available in Supplementary Table S5.

qRT-PCR validation

To validate the MACE results, the expression of five selected DEGs were analyzed by qRT-PCR using three biological replicates per condition. A high Pearson's correlation (0.93) was observed between the $\log_2$ fold changes measured by MACE and qRT-PCR, for both resistant and susceptible accessions (Figure 8).


Phvul.001G145600 - Disease resistance-responsive (dirigent-like protein) family protein

Phvul.001G201300 - Peroxidase superfamily protein

Phvul.007G203400 - Galactinol synthase 2

Phvul.003G096700 - Drought-induced 21

Phvul.007G231800 - Myb domain 79

Figure 8: Expression patterns of five genes in the resistant and susceptible accession, under three treatments, obtained by quantitative real-time PCR. The $\log_2$ fold change values are

represented in relation to the non-inoculated and well-watered control. A comparison with MACE $\log_2$ fold change is shown. R = resistant accession (diagonal stripes), S = susceptible accession (solid bars), WD = water deficit single stress (orange), *Fop* = fusarium wilt single stress (pink), *Fop*WD = combined fusarium wilt + water deficit stress (grey). Error bars correspond to the standard deviation of the mean of the three biological replicates. Paired Student's t-test indicates the significance between the expression levels of each sample in relation to control samples. ns – not significant $\alpha > 0.05$, * $0.01 \le \alpha < 0.05$, ** $0.001 < \alpha < 0.01$, *** $\alpha \le 0.001$.

Linear regressions were applied to the gene expression correlation between RT-qPCR and MACE sequencing data, separately for each accession. The R^2 values of the regressions were 0.75 and 0.96, for the resistant and susceptible accessions, respectively (Figure 9).

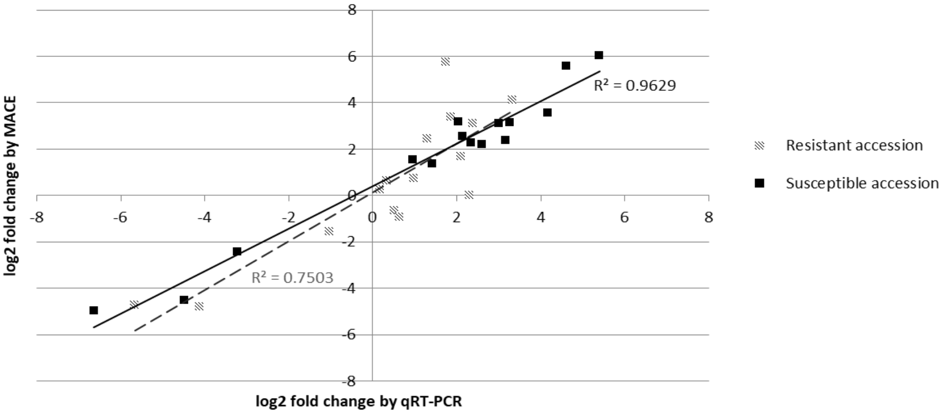


Figure 9: Comparison of relative expression levels of five genes, using $\log_2$ fold change of MACE and qRT-PCR, for the resistant (in striped grey) and susceptible (in solid black) accessions, under water deficit, after inoculation with *Fop* and under both stresses combined. Linear regressions for both accessions are shown.

Discussion

This study identified shared and unique phenotypic and transcriptomic responses of a resistant and a susceptible common bean accessions under a combination of fusarium wilt and water-deficit stresses applied individually or combined. This is important for breeding because plants exhibit tailored physiological and molecular responses when exposed to simultaneous stresses, which are not the addition of the responses observed in individual stresses alone (Ramegowda and Senthil-Kumar 2015). Fusarium wilt (*Fop*) and water deficit (WD) are two stresses frequently concurrent in nature (Sinha et al. 2017). However, few studies exist on the interaction of abiotic and biotic stresses in common bean. Thus, there is a scarce understanding of key genes and signaling pathways triggered by concurrent stresses that could be relevant for the development of multi-stress resistant plants.

The treatment that affected more photosynthetic pigment concentrations was water-deficit single stress, whereas the gas exchange parameters suffered a higher reduction under the combined fusarium wilt and water deficit stress (*Fop*WD). Interestingly, the photosynthetic pigment concentrations decreased under WD single stress, increasing under *Fop* single stress, and had their highest value under the combined *FopWD* stress. This is indicative that the response to the combined stresses is not simply the sum of the individual responses to single stresses.

The time-frame of the experiment with three sampling points – 48 h (T1), 96 h (T2) and 8 days (T3) after stresses imposition – allowed a phenotypic response analysis along time. For RWC, C*a*, C*cx*, C*a*+C*b*, C*a*/C*b*, A_n, E, and gs, the biggest differences in relation to control conditions were observed at the second time point. Consequently, for MACE, we decided to extract RNA from roots – the first tissue sensing both water deficit and the pathogen infection – at T2.

After 96 h of stress imposition, the two contrasting accessions were clearly distinguishable at the transcriptomic level. Indeed, the total number of DEGs, in relation to control conditions, was four times higher for the susceptible accession than for the resistant accession. Similar results were described in common bean response to a cyst nematode infection (caused by *Heterodera glycines* Ichinohe) where 353 DEGs were found in a resistant accession and 990 DEGs in a susceptible accession (Jain et al. 2016).

In our work, water deficit (single stress) was the condition that triggered more DEGs in both accessions, followed by the combined water deficit + fusarium wilt stresses. In the scope of this discussion, it is not possible to discuss every DEGs identified in this work. Thus, we will highlight only the ones exclusive of the resistant accession, in the combined stresses, or in the two single stresses and/or whose functional annotation seemed to be more relevant to unveil the molecular mechanisms behind the studied stress interactions.

DEGs in the combined FopWD stress in the resistant accession

Twenty-four DEGs were exclusive of the resistant accession and only identified in the combined *Fop*WD condition (not present in the single stress conditions), 8 up-regulated and 16 down-regulated. Among the up-regulated, we found Phvul.006G197200 that encoded a CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein (PR-1)) superfamily protein. The implication of PR-1 in sterol binding and the identification of the CAP-derived peptide suggest multiple roles in immune defense, from antimicrobial function and defense signal amplification to potential sterol or effector recognition (Breen et al. 2017). Moreover, Phvul.004G064800 encoding ribulose bisphosphate carboxylase (small chain) family protein was also up-regulated only in the combined treatment for the resistant accession. RuBisCO is the major protein in the stroma of chloroplasts and in higher plants exists as a complex of 8 large and 8 small subunits. The small subunit is coded by several different genes, which are distributed in a tissue-specific manner, but their function is unknown (Inter Pro). The increase in the abundance of its small subunit transcripts may indicate an increased RuBisCO synthesis, and thus an improved photosynthetic rate. Finally, in this particular subset of DEGs, we identified Phvul.010G012700 encoding CYP76C4 cytochrome P450, family 76, subfamily C, polypeptide 4. In *Arabidopsis*, several cytochrome P450 genes were induced by both abiotic and biotic stresses and are known to participate in the regulation of plant defense (Narusaka et al. 2004). CYP76C4 cytochrome P450 seems to be involved in the terpenoid metabolism and has been previously reported as expressed in *Arabidopsis* roots (Höfer et al. 2014). It is known that several terpenoids have a role in plant defense against biotic and abiotic stresses (Singh and Sharma 2015). On the other hand, among the down-regulated genes we found a leucine-rich repeat protein kinase family protein (Phvul.008G244400), the largest group of receptor-like kinases in plants with crucial roles in development and stress responses after pathogen recognition (Liu et al. 2017); and Phvul.008G220600 a PYL5,RCAR8 Polyketide cyclase/dehydrase and lipid transport superfamily protein. This family proteins function as regulatory components of ABA drought stress response (Ng 2016).

Unique DEGs in single stress for the resistant common bean accession

The resistant accession under single WD stress revealed 145 DEGs, 58% of them up-regulated. Interestingly, in our work, the only ABA-induced genes detected were expressed by the resistant accession under water deficit (single stress). In fact, Phvul.008G231200 encodes a HAI2 highly ABA-induced PP2C gene 2, while Phvul.001G075400 and Phvul.001G236000 encode a HAI3 highly ABA-induced PP2C gene 3. These protein phosphatases were described to be involved in the negative regulation of ABA signaling in *Arabidopsis* (Schweighofer et al. 2004).

On the other hand, the following genes were down-regulated exclusively in the resistant accession under WD: Phvul.011G025400 encoding SKU5 similar 5, a glycoprotein that is related structurally to the multiple-copper oxidases, expressed most strongly in expanding tissues, such as cell wall, and is required for normal root growth (Sedbrook et al. 2002); and Phvul.011G048700, encoding hemoglobin 1, a plant hemoglobin that has an extremely high affinity to oxygen and their main function seems to be related to scavenging nitric oxide with influence in biotic and abiotic stress response (Gupta et al. 2011).

For the resistant accession, no DEGs were identified in *Fop* single stress in comparison to the control condition. This result suggests that this accession displayed a constitutive defense with resistance genes always expressed. In agreement, in *Arabidopsis*, constitutive expression of ERF1 was described to mediate and confer enhanced resistance to *F. oxysporum conglutinans* and *F. oxysporum* f. sp. *lycopersici* (Berrocal-Lobo and Molina 2004).

Conclusions

With this study we concluded that the transcriptome profile of common bean, when stresses were combined, was not the sum of the individual ones, and could not be predicted from individual single stress treatments. Several genes were exclusively differentially expressed when *Fop* and WD stresses were combined, such as a CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein; a ribulose bisphosphate carboxylase (small chain) family protein; and a cytochrome P450, family 76, subfamily C, polypeptide 4.

We observed an accumulation of DEGs belonging to the MapMan functional categories of “RNA”, “stress”, “signaling”, and “secondary metabolism”, for both contrasting common bean accessions and stresses applied, single or in combination.

Moreover, we verified that the number of DEGs was much higher under WD conditions than under *Fop* stress. This is in agreement with previous reports that showed that resistance to pathogens seems to be genetically simpler than tolerance to abiotic stresses, such as drought, (Kissoudis et al. 2016). Taking this into account, a first step in the development of multi-stress-tolerant varieties could be the integration of resistance genes against fusarium wilt and test their stability across different environments. The subsequent introgression of quantitative water-deficit resistance governed by several minor genes/QTLs will be more challenging. This might be achieved in a more efficient way through genomic selection than via marker-assisted selection (Annicchiarico et al. 2019). The recent advances in genomic selection research for complex traits in many crops, including legumes; Roorkiwal et al. 2018; Varshney et al. 2019), foresee alternative to increase the efficiency of breeding for multi-stress resistance (Leitão et al. submitted to Springer).

Overall, our results provide clues to further understand the regulation of plant response to simultaneous stresses. The resistance-related DEGs identified in this study implicated in water deficit tolerance and at the first 96 h of *Fop* infection, allow a better understanding of the mechanisms of defense and resistance to these stresses, and will aid in the selection of a set of genes more likely to be major regulatory hubs that can be used as targets for resistance.

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Supplementary material

Supplementary information is available online in FigShare repository: https://figshare.com/s/e78b7e234f15ba7df82a

Chapter VI

General Discussion

Breeding for resistance against multiple stresses is a challenge. Little is known concerning the complex details underlying the genetic architecture of abiotic-biotic interactions in legumes, such as common bean. Few studies exist on the interaction of abiotic and biotic stresses in common bean leading to a poor understanding of key genes and signaling pathways triggered by concurrent stresses adaptation that could be relevant for the development of multi-stress resistant plants. The identification and location of genes controlling these traits may be accomplished by association mapping studies based on the existing natural variation, validated by functional analysis using transcriptomics, proteomics, and/or metabolomics data of contrasting accessions (Davila Olivas et al. 2017; Thoen et al. 2017). The success in developing legume varieties resistant to multiple abiotic/biotic stresses depends on the availability of good sources of resistance and on the inheritance of that resistance.

The Portuguese common bean germplasm is the result of more than 500 years of farmers' selection and adaptation to the diverse edapho-climatic conditions of the country. A great diversity of common bean landraces has been cultivated for generations and is still maintained in small fields for farmers' personal consumption and to sell in local markets (Vaz Patto et al. 2007; Leitão et al. 2013). Nevertheless, the limited characterization of this resource performed up to now hampered its exploitation by breeding programs.

Fusarium oxysporum f. sp. *phaseoli* (*Fop*), the causal agent of fusarium wilt in common bean, and water deficit are two stresses that frequently coexist at field conditions.

Fusarium wilt is among the most important fungal diseases affecting common bean production throughout the world (Alves-Santos et al. 2002; Niño-Sánchez et al. 2015; Schwartz and Pastor-Corrales 1980; Toledo Souza et al. 2012; Xue et al. 2015). The control of vascular wilt pathogens is not an easy task. Chemical fungicides are ineffective, especially for pathogens like *Fop* that have a soil-borne nature and possess structures that persist for long periods in the soil, even in the absence of host plants (Yadeta and Thomma 2013). Also, biocontrol using antagonistic bacteria or fungi cannot effectively limit these vascular diseases, since abiotic and biotic factors make their performance inconsistent. Consequently, the use of resistant cultivars is the most efficient, environmentally friendly, and economically viable strategy to provide effective fusarium wilt disease control (Dodds and Rathjen 2010).

Water availability is the major abiotic factor affecting common bean productivity (Urrea et al. 2009) and drought stress can result in this crop yield losses of 70% (Smith et al. 2019). Water-deficit effects are determined by the timing, intensity, and duration of stress episodes (Chaves and Oliveira 2004), which tend to aggravate under global climate change scenario. Thus, tolerance to water deficit has become a major breeding priority for this crop species (Giorgi and Lionello 2008).

The research described in this Ph.D. thesis constitutes an important contribution in the valorization of the underused Portuguese common bean germplasm, identifying new sources of multiple stress resistance and providing new genomic targets for the development of functional markers to support selection in future disease resistance and drought tolerance breeding programs.

The morphological and genetic diverse Portuguese common bean germplasm brought to the stage:

The assessment of the genetic and morphological diversity of a Portuguese common bean collection with 175 accessions and the selection of a core collection that retains all the diversity within this germplasm, with minimum repetitiveness, was achieved in Chapter II of the present Thesis.

The Portuguese collection was compared at the molecular level with Andean and Mesoamerican representatives and wild relatives. Furthermore, eight different seed coat colors, six seed coat patterns and four seed shapes were detected, once more highlighting the variability within this germplasm. Moreover, the colors (white, pink, brown and red), shapes (cuboid, oval and kidney) and patterns (absent and striped) more frequent in the Portuguese germplasm were among the ones with higher market value.

The information gathered in this collection is vital to support a more effective conservation of the Portuguese bean germplasm and to promote its use in both national and international breeding programs. Despite the predominance of accessions of Andean origin, one-third of the accessions analyzed have admixed genetic origin and might represent putative hybrids between the original Mesoamerican and Andean gene pools, having novel and advantageous genetic combinations not yet explored in breeding.

Interestingly and although the majority of the common bean resistance sources known so far against biotic and abiotic stresses are of Mesoamerican origin, it was possible to identify double-resistant (against fusarium wilt and water deficit) accessions among the Portuguese common bean of Andean origin (used later on Chapter V). Indeed, this thesis was the first to explore the Portuguese common bean germplasm to identify new sources of fusarium wilt (Chapter III) and water-deficit resistance (Chapter IV). The collection was evaluated for fusarium wilt resistance under growth chamber conditions (Chapter III), using the highly virulent *Fop* strain, FOP-SP1 race 6. Fourteen of the 162 Portuguese accessions evaluated were highly resistant and 71 intermediate. Complete and incomplete resistant sources were identified among the accessions of Andean, Mesoamerican, and genetic admixed origin that constitute the Portuguese germplasm. The exploration of water-deficit-tolerant accessions was undertaken by studying the natural variation of common bean photosynthetic response under contrasting water treatments (Chapter IV). Water deficit impacts many aspects of the physiology of plants and, particularly, photosynthetic capacity. We identified accessions with high CO_2 assimilation rates that were stable in well-watered and water-deficit conditions. These accessions were considered resilient to water deficit, maintaining a photosynthetic performance, even under stress, better than SER16, the elite accession from CIAT with recognized drought tolerance.

Settling the basis for common bean stress resistance precision breeding with "a little help" from the Portuguese germplasm natural variation

The detection of fusarium wilt resistance- and photosynthesis-related-traits- associated SNPs was accomplished in the present thesis by exploring, for the first time, the Portuguese common bean germplasm through a genome-wide association study (GWAS) (Chapter III and Chapter IV). For the establishment of the phenotypic-genotypic associations, the collection of Portuguese common bean accessions was, additionally to the phenotypic screening, genotyped with SNP-based arrays.

Several SNP-fusarium wilt resistance (9) and SNP-photosynthesis-related traits associations under contrasting water regimes (133) were detected using a mixed linear model accounting for the genetic relatedness among accessions.

Although the proportion of the observed phenotypic variance explained by each significant SNP-*Fop* resistance associations ranged from 4.7% to 7.2%, the favorable allele of the associations with the highest effect corresponded to an increase in fusarium wilt resistance of 16% and a reduction in the disease progress rate of 19%. This suggests that improvements can be attained through selection within this Portuguese germplasm (Chapter III). Not only the accessions of Andean or Mesoamerican origin identified as resistant to *Fop* infection may be useful for common bean resistance breeding within each particular gene pool, but also the resistant accessions with admixture nature may contain novel and advantageous genetic combinations for both gene pool breeding. We identified among the accessions of admixed genetic origin favorable SNP alleles for fusarium wilt resistance that can reflect a positive selection contributing to adaptation to the local environment.

By identifying SNP- photosynthesis-related traits associations and underlying candidate genes, this thesis provided insights into the genetic basis of those physiological mechanisms, and of the different pathways involved in common bean response to water limitation (Chapter IV). The biological function of the candidate genes identified suggested that stomatal regulation, membrane translocation of proteins, mechanisms of reactive oxygen species accumulation avoidance, hormone and osmotic stress signaling were the more relevant processes that characterized the Portuguese common bean photosynthesis-related traits response to water deficit.

The whole is greater than the sum of its parts: Common bean-Fop-water-deficit stress triangle

The identification of molecular signatures shared by different stresses could be relevant for the development of multi-stress resistant varieties (Chapter V). Based on the single stress screening described in Chapter III (*Fop*) and Chapter IV (water-deficit) of this thesis, two Portuguese common bean accessions, one susceptible (with

Mesoamerican origin) and one resistant (with Andean origin) both to water-deficit and fusarium wilt, were used in Chapter V on the identification of differentially expressed genes (DEGs) in common bean in response to combined water-deficit and fusarium wilt stresses. The two contrasting accessions were exposed to single and combined stresses and the transcriptome profiling of the different treatments and accessions were compared using massive analysis of cDNA ends (MACE). Common bean differentially expressed genes and key pathways activated against *Fop* and water-deficit (WD) interaction were identified allowing a better understanding of the induced combined and single common bean responses to these two stresses frequently concurrent at field conditions. The susceptible accession revealed a clearly higher level of transcriptional changes in relation to the control condition, in all the three treatments – *Fop* and WD single stresses and in the combined *Fop*WD stress – compared with the resistant accession (944 versus 228 DEGs). The most represented functional categories within the DEGs were “RNA”, “secondary metabolism”, “stress”, “signaling”, “hormone metabolism”, “transport”, “protein” and “cell wall”. The genes found increased our understanding of the mechanisms of defense and resistance implicated in water-deficit tolerance and/or during the first 96 h of *Fop* infection. The resistance-related DEGs identified in the present thesis could be used together with the candidate genes or resistance-associated SNP from the GWAS (Chapter III and IV) for the development of breeding programs aiming for the improvement of multiple-stress tolerance in common bean.

The challenge of breeding for resistance against multiple stresses

The success in developing legume varieties resistant to abiotic/biotic stresses depends not only on the availability of good sources of resistance but also on the inheritance of the resistance. The identification and location of genes controlling these traits may be accomplished as in this thesis Chapter III and IV by association mapping studies based on the existing natural variation, or like in Chapter V of this thesis by functional analysis using transcriptomic or other approaches such as proteomics, and metabolomics data of contrasting accessions (Davila Olivas et al. 2017; Thoen et al. 2017). The omics combination will narrow down the number of candidate genes for combined stress tolerance, identifying major regulatory hubs, such as transcription factors (Kissoudis et al. 2014). It would be optimal if these genetic studies are performed under combined stress conditions (Bai et al. 2018). By analyzing several traits simultaneously, these studies would enable a comprehensive and integrative view of the genetic architecture and molecular pathways underlying combined abiotic and biotic stress resistance in legumes (Dickinson et al. 2018; Sinha et al. 2017).

Nevertheless, and in the case of multiple stresses studied individually (such as in the case of GWAS analyses in Chapter III and IV of this thesis), besides the deployment of these individual common regulators in novel varieties, an educated pyramiding of non-(negatively) interacting resistant components appears as an alternative breeding strategy to achieve tolerance to combined stress in crops. These might be disease resistance genes (R-genes) and inducible abiotic stress resistance genes involved in physical barriers against pathogen penetration (Bai et al. 2018; Kissoudis et al. 2014). Resistance to pathogens seems to be genetically simpler than tolerance to abiotic stresses, such as drought, as it is often conferred by single major genes (Kissoudis et al. 2016). In this way, a marker-assisted backcross could be the first step in the development of multi-stress tolerant varieties with the prerequisite of testing the stability of R-genes under different stress scenarios. The subsequent introgression of quantitative abiotic stress resistance governed by several minor genes/QTLs will be more challenging. However, with the advances in genomic selection research for complex traits in many crops, as well as in legumes (Annicchiarico et al. 2019; Roorkiwal et al. 2018; Varshney et al. 2019), this might become an alternative to achieve balanced signaling pathways in novel varieties, contributing to a sustained performance of crops under diverse environmental conditions.

In conclusion, the work developed in this Ph.D. thesis paved the way to a more efficient utilization of the Portuguese common bean germplasm in future breeding programs. The complexity of this germplasm, reflected by its genetic structure, was revealed. Moreover, the core collection developed may now integrate larger international common bean collections to deepen the coverage of the worldwide diversity of this important crop. This could be very important to enrich international breeding and genetic programs and overcome several constraints affecting common bean production in different parts of the world.

Furthermore, several sources of complete and incomplete resistance against a highly virulent isolate of *Fusarium oxysporum* f. sp. *phaseoli* were identified among accessions of Andean, Mesoamerican and genetic admixed origin that constitute the Portuguese common bean collection. In this thesis, the photosynthetic response of the Portuguese common bean collection to water deficit was also characterized. Accessions that showed a better capacity to activate a physiological response to cope with water deficit were identified, namely through stomatal conductance regulation, increased water use efficiency, or avoidance of reactive oxygen species accumulation.

Importantly, favorable SNP alleles in the common bean genome were located associated with fusarium wilt resistance and photosynthesis-related traits under contrasting water regimes. These SNP markers will enable the development of molecular tools that will facilitate the resistance transfer into more productive cultivars using marker-assisted breeding schemes.

Finally, molecular signatures shared by combined abiotic and biotic stresses were identified taking as a case study the water deficit and fusarium wilt interaction, which will be important for the development of multi-stress resistance common bean varieties.

The next steps will include analyzing the most promising accessions, identified here using controlled conditions, under field conditions. A multi-location design will enable us to assess the environmental effect on these traits through a genotype-by-environment interaction analysis, needed to validate the results obtained during this Ph.D. work.

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