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Project Report

Proposed Research for Innovative Solutions for Chickpeas and Beans in a Climate Change Scenario: The Mediterranean Basin

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Abstract: In order to gain insight into the complex molecular networks driving legume adaptation caused by climate change, it is necessary to deeply characterize the existing germplasm in response to the environmental constraint predicted to worsen in the near future: drought. In this study, we propose to perform a three-year deep agronomic characterization of local genotypes of selected legumes in abiotic stressing conditions through controlled and field experiments conducted in several countries of the Mediterranean basin (Italy, Spain, Algeria, Tunisia, Turkey, Lebanon, and Croatia). These phenotypic analyses will be integrated with a multi-omic approach aiming at identifying the key players involved in the modulation of the analyzed traits that includes the analysis of the plant methylome, transcriptome, and proteome. Following this approach, we propose to deliver epigenomic markers linked with rapid adaptation mechanisms in response to drought. Besides, new genetic variability by breeding could be created in stressing conditions and produce the basis for the obtainment of more productive cultivars in worsening environments. The epigenetic marks identified in "omic" activities will be validated in molecular marker-assisted selection in F2–F4 populations. Finally, specific rhizobia strains for the best evaluated genotypes will be identified in order to enhance symbiotic nitrogen fixation in drought stress conditions with selected cultivars.

Keywords: adaptation; climate change; drought; epigenomic; epigenetic markers; legumes; Mediterranean basin

1. Introduction

According to the Food and Agriculture Organization (FAO) (2015) [1], dried legumes (beans, peas, chickpeas, and lentils) represent an important component of a healthy diet, as they offer a cheaper alternative source of protein compared to meat. In fact, recent investigations recommend doubling the consumption of nuts, fruits, vegetables, and legumes at the expense of reducing meat and sugar intakes as the perfect diet not only for humanity but also for planet health in the current climate change scenario [2]. Enhancement of their consumption will also have important benefits at the environmental level due to the advantages of legume cultivation for the increase of soil fertility, N content, and organic matter balance. When they are used in a biodiversity-based agronomic approach, they significantly increase ecosystem services, such as biotic stress resilience, reduction of pesticides and fertilizers, and maintaining pollinators. Thus, it is critical to generate new knowledge and innovative biotechnological tools to fully exploit, valorize, and expand legume germplasms in response to enhanced drought and salinity caused by climate change, especially in very sensitive areas, such as the Mediterranean basin. In addition to their great nutritional properties, their diversity, and their association with rhizospheric microorganisms, legumes offer an effective alternative for the improvement of plant production, the rehabilitation of degraded sites, and the balance of their ecosystems [3]. Thus, cultivation of important crops such as legumes must be sustained, since the economic impact of climate change on legume cultivation in terms of volatility and income level for farmers is predicted to be worsen in more increasingly severe climate scenarios [4,5], especially in North Africa, where structural agronomic issues are intensified by difficult socio-economic conditions. Here, we focus our attention on two important grain legumes of the Mediterranean basin—chickpea (*Cicer arietinum* L) and bean (*Phaseolus vulgaris* L.). This is because of some well-known evidence. First, the genomes of these crops are recently sequenced and well-annotated [6,7]. Second, there is a gap of knowledge regarding these crops dealing with (epigenetic) molecular markers associated with environmental stresses caused by a harsher climate. Third, these crops are a major source of proteins for Mediterranean basin countries and are an essential part of the typical cuisine of their inhabitants [8]. Forth, there is a high import of grain legume cultivars from North America, implying the urgent need to improve yields of local genotypes [9]. Finally, there is a large genotypic variability in relation to drought responses in both of these two legume crops [10–12].

1.1. Epigenomic Adaptation of Legumes to Climate Change

Besides phosphorus (P) and/or nitrogen (N) deficiencies, drought and salinity are the most limiting factors of grain legume yield, and these constraints will be enhanced by climate change, especially in smallholder systems [13]. The reduction of water availability is particularly threatening for legumes, as demonstrated by the 60% reduction of bean production under rainfed conditions worldwide and the 80% reduction of grain yield in some arid regions [14]. Drought stress also reduces water uptake and affects rapid and long-term adaptation mechanisms of plant species to climate change. Interestingly, drought-resistant cultivars, despite belonging to the same species, show a beneficial strategy to regulate carbohydrate partitioning toward seed filling, counteracting drought during the pod-filling stage [14]. Plant stress responses can be characterized by an initial alarm phase, in which mechanisms for coping with stress are activated as growth-related processes slow down, followed by a resistance phase in which the plant tries to withstand stress and repair any related damages. If the plant persists under this stress, the plant will maintain an imprinting of the stress that will affect the response in case of similar subsequent stress even in following generations (Figure 1). Molecular mechanisms of such plant stress memory are not clearly understood, although epigenomic modifications are thought to play a key role in this phenomenon [15,16]. However, this research field is still largely under-investigated, since very few studies have been conducted on legume epigenomics [17,18]. To our knowledge, the chickpea and bean present no published epigenomic studies, and very few studies deal with their transcriptomic responses to abiotic stresses (including drought) (Table 1).

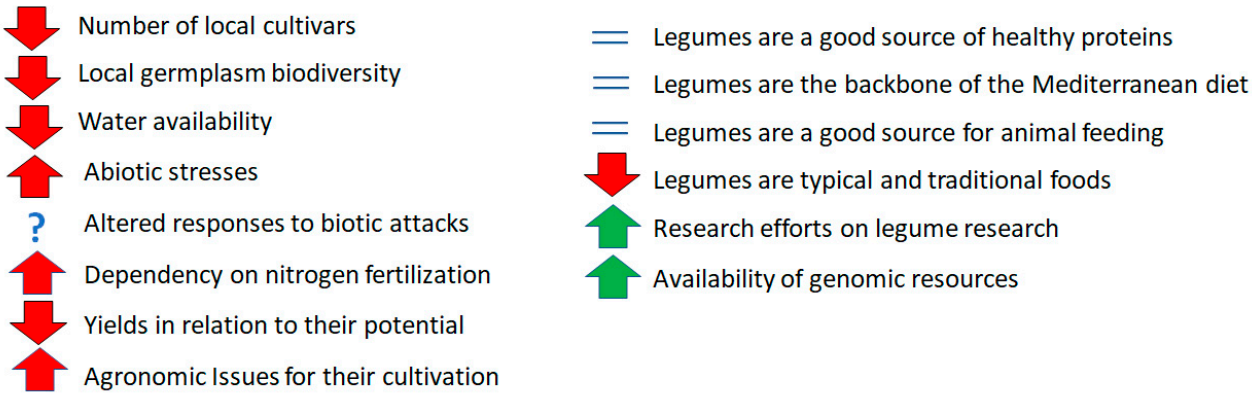

Figure 1. Effects of climate change on legume cultivation in the Mediterranean basin. Red arrows mean detrimental effects, green ones mean beneficial effects, "up arrows" mean increase and "down arrows" mean decrease, "equal sign" means no effects, and "?" means unknown effects.

1.2. Previous -omics Studies Performed in Bean and Chickpea under Drought Conditions

As commented above, to our knowledge, no reports dealing with epigenetic modifications in response to drought have been published so far regarding beans or chickpeas. However, the influence of this stressing condition on both legumes has been assessed by means of other -omic approaches. Table 1 shows some examples of high-throughput studies performed on both crops under drought conditions.

Table 1. Examples of reports assessing the influence of drought on beans and chickpeas by means of several -omic approaches, including RNA-sequencing studies (RNA-seq), proteomic analysis (Proteomics), and two types of Genome Wide Association Studies (GWAS)—identification of Quantitative Trait Loci (QTLs) and of Single Nucleotide Polymorphisms (SNPs).

Bean		
Approach	**Results**	**Reference**
RNA-seq	Identification of genes involved in drought stress determining the differentially expressed genes between terminal drought and optimal irrigation treatments and between tolerant and sensitive genotypes, belonging to the cellular pathways plant hormone signal transduction, protein processing in endoplasmic reticulum, ribosome production, starch and sucrose metabolism and purine metabolism.	[19]
RNA-seq	Characterization of genes related to the response to drought stress in leaf and root tissue of drought-susceptible and tolerant genotypes with a predominance of genes involved in oxidative stress, response to stimulus and kinase activity.	[20]
Proteomics	Identification of drought-responsive proteins in leaves of two cultivars differing in their response to drought that could be classified into functional categories that include energy metabolism, photosynthesis, ATP interconversion, protein synthesis and proteolysis, stress and defence related proteins.	[21]
Proteomics	Plants exposed to drought for 17 days and control plants at the same developmental stage were included in quantitative proteomic analysis. Quantified proteins were grouped into several functional groups, mainly into energy, metabolism, photosynthesis, proteolysis, protein synthesis and proteins related to defence and stress.	[22]
QTLs	Identification of QTLs was performed crossing two cultivars (drought sensitive and tolerant), obtaining and genotyping a recombinant inbred population and measuring fourteen traits in both well and water stress conditions. 22 QTLs associated with chlorophyll, leaf and stem fresh biomass, leaf biomass dry weight, leaf temperature, number of pods per plant, number of seeds per plant, seed weight, days to flowering, dry pod weight and total yield were identified under drought stressing conditions.	[10]
QTLs	Identification of QTLs was performed crossing two cultivars (drought sensitive and tolerant), obtaining and genotyping a recombinant inbred population and measuring the inheritance of pod harvest index under well-watered conditions and terminal and intermittent drought stress. 7 QTLs associated with the partitioning of pod biomass to seed biomass were identified with drought.	[23]
SNPs	Two drought-tolerant parental lines were used to generate a recombinant inbred population characterized by 169SNPs. 83 SNPs were significant associated with quantified phenotypes under drought conditions, specially days to flowering and seed biomass. Thirty-seven out of the 83 SNPs were annotated to a gene with a potential function related to drought tolerance, such as starch or proline biosynthesis.	[24]
SNPs	A total of 22,845 SNPs was found across eighty-six American wild bean lines. Allelic associations with a bioclimatic-based drought index were calculated. 115 SNPs were associated with the bioclimatic-based drought index. A gene coding for an ankyrin repeat-containing protein and a phototropic-responsive NPH3 gene were identified as potential candidates involved in drought responses.	[25]

Table 1. *Cont.*

Chickpea		
Approach	**Results**	**Reference**
RNA-seq	RNA-seq of the roots of drought and salinity related genotypes was carried out under control and stress conditions at vegetative and/or reproductive stages. A total of 4954 genes exclusively regulated in drought-tolerant genotypes were identified, including genes coding for enzymes involved in metabolic pathways, photosynthesis, lipid metabolism, generation of precursor metabolites/energy, protein modification, redox homeostasis, and cell wall component biogenesis.	[26]
RNA-seq	RNA-seq of roots and shoots of two contrasting chickpea genotypes at early flowering stage under controlled and drought conditions. A total of 4572 differentially expressed genes were identified. Of these, 261 and 169 drought stress responsive genes were identified in the shoots and the roots, respectively, and 17 genes were common in the shoots and the roots. Several genes coding for proteins involved in response to stress, defense response, and response to stimulus were specific from the tolerant genotype under drought stressing conditions.	[27]
Proteomics	Leaf proteome analysis between drought-tolerant and drought-sensitive chickpeas under controlled and drought conditions. A total of 24 differently expressed proteins were identified in response to drought in both plants. The proteins involved in photosynthesis and energy mechanisms were up-regulated in tolerant chickpea but down-regulated in sensitive chickpea under drought, suggesting that photosynthesis capacity is higher in the tolerant chickpea.	[28]
QTLs	Identification of QTLs was performed crossing four cultivars, obtaining and genotyping two recombinant inbred populations and measuring a total of 20 drought root traits in several seasons and locations under field conditions. Analysis of extensive genotypic and precise phenotypic data revealed 45 QTLs. Some QTLs for several drought tolerance traits appeared clustered. Among these clusters, one cluster harboring most QTLs for 12 traits and explaining most phenotypic variation was identified and referred as "QTL-hotspot."	[29]
QTLs	QTL analysis was used to identify candidate genes in the "QTL-hotspot" region for drought tolerance in chickpeas and was performed crossing two cultivars (drought sensitive and tolerant), obtaining and genotyping a recombinant inbred population and measuring 17 drought tolerance related traits obtained over five seasons and five locations under field conditions. Data split the "QTL-hotspot" region into two subregions namely "QTL-hotspot_a" (harboring 15 genes) and "QTL-hotspot_b" (harboring 11 genes). Functional validation using qRT-PCR indicated four promising candidate genes having functional implications for drought tolerance in chickpea.	[30]
QTLs	Identification of QTLs was performed crossing two cultivars (drought sensitive and tolerant), obtaining and genotyping a recombinant inbred population and measuring traits contributing to plant water use under well-watered conditions. Twenty-one QTLs were identified for plant vigor and canopy conductance traits, some of them located in the "QTL-hotspot" region involved in drought tolerance.	[12]
SNPs	Identification of 828 novel SNPs in a chickpea recombinant inbred line mapping population. Analysis using the genetic map along with phenotyping data for 20 traits collected over seven seasons under field conditions identified 49 SNP markers in the "QTL-hotspot" region, which harbors most drought tolerance QTL in chickpea.	[11]
SNPs	Sequencing of 132 chickpea varieties and advanced breeding lines allowed the identification of more than 144,000 SNPs. Thirteen yield and yield-related traits were correlated with identified SNPs in three drought-prone environments to find putative genes involved in drought tolerance, such as genes coding for auxin production proteins, p-glycoproteins, and nodulin transporters.	[31]

2. Discussion

2.1. Proposed Approach

The main aim of this proposed research work is to improve sustainability in producing grain legumes and their multipurpose use in a climate change scenario through four different objectives / activities performed in a three-year research proposal. Thus, this major objective should be obtained—valorizing the bean and chickpea germplasm as more adapted to drought in the Mediterranean basin, identifying genes and genetic regulatory networks modulating epigenetic adaptation of legumes to environmental abiotic stresses linked to climate change (i.e. drought), and developing molecular tools, methods, and approaches for the genetic improvement of the common bean and chickpea (Figure 2).

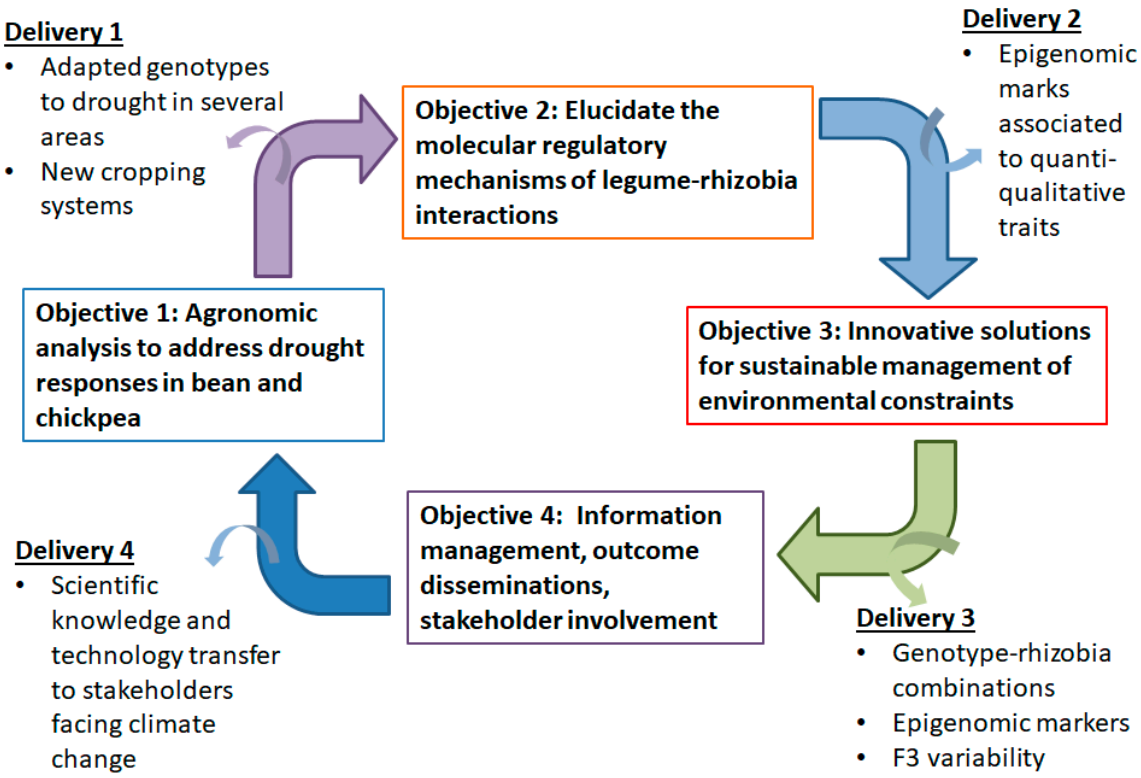

Figure 2. Objectives and deliverables of the research proposal.

2.1.1. First Objective: Agronomic Characterization of Local Germplasm in the Mediterranean Basin

The first objective deals with a deep agronomic characterization of the Mediterranean germplasm of grain legumes in response to the environmental constraints predicted to be worsen in the next future—drought. Thus, the valorization and exploitation of the large spontaneous and domesticated biodiversity of local and neglected bean and chickpea genotypes from different countries of the Mediterranean basin (Italy, Spain, Algeria, Tunisia, Turkey, Lebanon, and Croatia) should be performed (Figure 3).

Figure 3. Countries involved in the proposed research project. Countries of the Mediterranean basin in which agronomic characterization will be performed are tagged with a blue star.

The agronomic and morphological traits of these under-investigated legumes should be evaluated under the occurrence of drought (Figure 4). For this purpose, at least 30 local ecotypes for each country (210 in total) (Italy, Spain, Algeria, Tunisia, Turkey, Lebanon, and Croatia) must be evaluated at agronomic and morphological levels for both chickpea and bean legumes. From these, the five best genotypes per country (35 in total) should be selected to be evaluated in each of the selected Mediterranean regions. Thus, the first evaluation must be conducted in pots under a controlled environment in order to save time and money. For this purpose, each institution of every country will evaluate approximately 30 local genotypes in controlled conditions for both chickpea and bean. This plant material should be represented by local ecotypes, populations, current cultivars, and spontaneous and domesticated genotypes available in their germplasm collections. The experimental conditions should be the same for each partner in order to reduce as much as possible confounding environmental effects. The analyzed parameters should include biomass, root morphology, and other plant growth measurements in order to determine the most resistant genotypes to drought stress conditions, such as plant height; days to 50% flowering; days to 50% seed formation; pods/plant, seeds/pod, and 100-seed weight nutrient uptake (N, P, K, micronutrients); osmotic potential; membrane stability; aquaporin activity; proline content, etc. Each genotype must be sown in five replicated pots using crop soil as a substrate with 10–12 plants, evaluated under two conditions—irrigated (control) and non-irrigated (drought stress). Weights of the pots placed in the greenhouse should be determined every three days, and the amount of water missing in the pots must be determined to obtain evapotranspiration rates. The drought stress would be applied 30 days after emergence. Finally, soil-borne rhizobial species would be isolated from nodules of each genotype grown under drought conditions. From this experimental work, we could identify the five genotypes from each country with the highest resistance to drought (Figure 4).

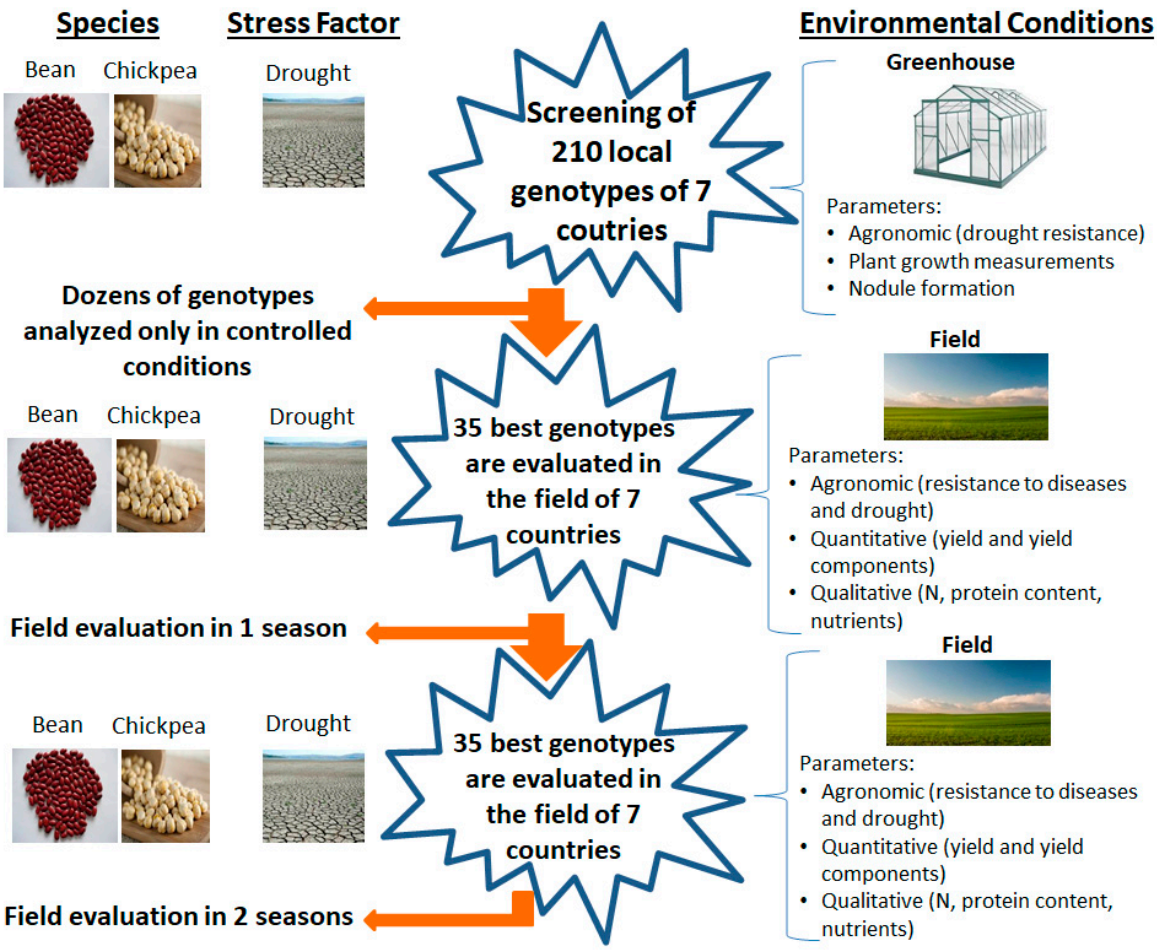

Figure 4. Experimental design for agronomic evaluation of legume germplasm of bean and chickpea.

These best genotypes will be evaluated also in the field of each country of the Mediterranean basin participating in the proposal. For this purpose, the 35 best genotypes (five genotypes each from seven Mediterranean regions) must be evaluated in each field condition in the second and third year. Research should be conducted in four replications in accordance with the design of randomized blocks. Each parcel must be made up of four rows of 4 m length, keeping 45 cm between rows, and around 60 seeds should be placed in each row. Thus, each parcel will consist of 0.45 m × 4 rows × 4 m = 7.2 m^2. During the experiment, the following vegetative parameters should be investigated: flowering time, poding time, maturity time, plant height, height of first pod, seed yield, 100 seed weight, drought tolerance index, measurements of energy (kcal), dietary fiber, proteins-nitrogen content, amino acid composition, sugars, lipids, polyphenols, dry matter, micronutrient compositions, viscosity, and water absorption capacity. The total number of analyzed samples for each legume plant in both trials should be 42 genotypes × 2 conditions (control, drought) × 4 biological replicates = 252. Agronomic analysis should be conducted in two stress stages identified by early and late symptoms.

Finally, a link between the presence of specific rhizobial strains and drought tolerance could also be determined in order to identify those strains that contribute significantly to the drought tolerance of chickpea and bean genotypes. It is well known that the nitrogen nutritional level of plants exerts a strong effect on the sensitivity to following water deprivation [32,33]. In legumes, there is a significant delay in drought-induced leaf senescence in nodulated relative to non-nodulated plants, independent of rhizobial strain. The major mechanisms consist of increased potassium concentrations, balancing the carbon partitioning between starch and sugars and enhance the reserves of osmolytes during drought. Consequently, in general, nodulated plants recovered more effectively from drought than non-nodulated plants [34].

The experimental work, including the number of microbial species and genotypes to be tested, should be divided into several research units. This activity aims at developing new, improved microbial inoculants for genotypes resistant to drought previously identified for both legume plants (Figure 5). In Year 1, we must characterize the microbial strains involved in symbiotic nitrogen fixation from the root nodules of the best five plant genotypes that are resistant to drought from the seven soils used in pot experiments under controlled conditions. Thus, at least one bacterial strain would be selected from the seven different regions for each plant and taxonomically characterized by 16S rRNA and by *nod*/*nif* genes sequencing. All strains must be assayed by "in vitro" short time tests (about one month) for their ability to nodulate plant seedlings in pot-controlled conditions under low nitrogen availability with the 35 best genotypes of bean and chickpea identified in the previous activity. A restricted water regime should be used to simulate soil aridity (the number of nodules per plant will be checked). The "in vitro" assay will be conducted three times consecutively. Through this "in vitro" experiment, we can select the four best strains (two for each plant species) in combination with the 35 genotypes for a total of 70 combinations to be tested in field conditions (Year 2). We should use three randomized replications for a total of 240 randomized blocks. This experiment must be repeated in Year 3. The field experiments are to be conducted in drought conditions (not irrigated), plant responses to the inoculation (plant health and yield) must be evaluated, and the best combinations kept for further commercial applications.

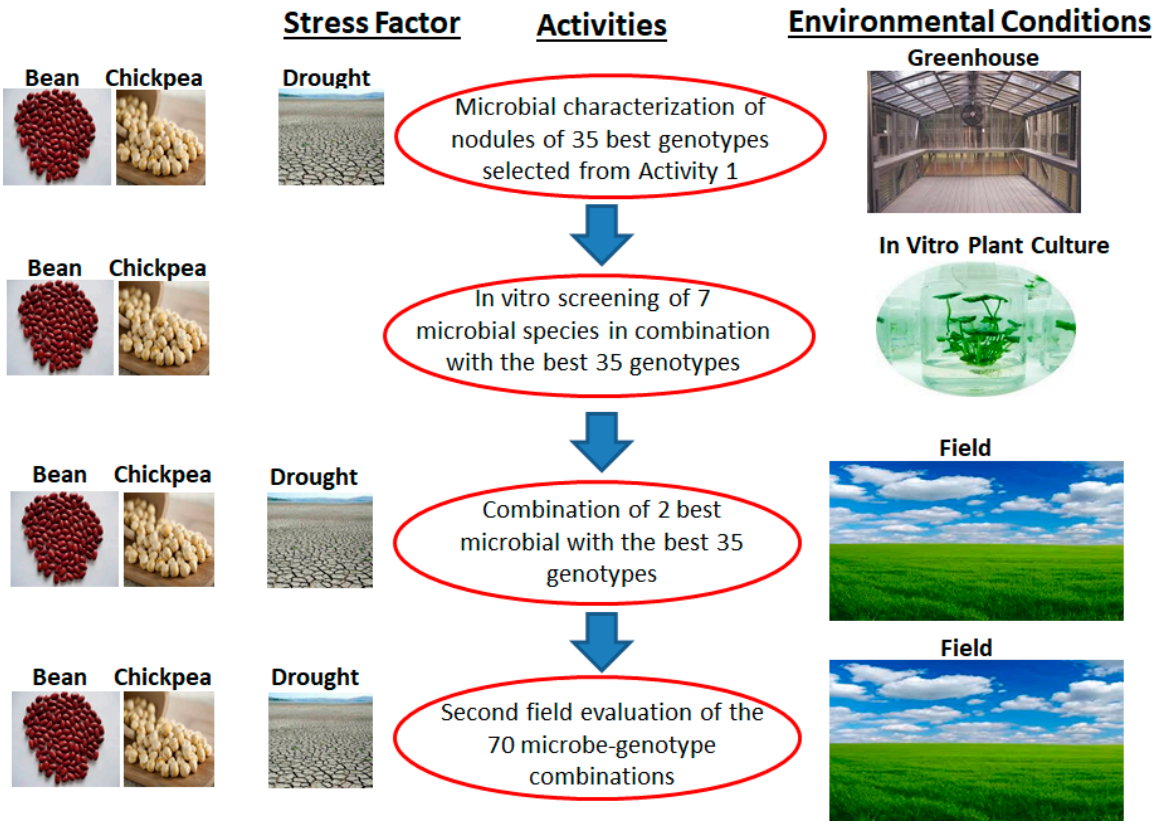

Figure 5. Experimental workflow for the development of genotype-specific microbial inoculants for bean and chickpea.

2.1.2. Second Objective: Epigenomic Adaptation to Climate Change

A second objective may deal with the elucidation of the complex molecular regulatory networks underlying legume responses to drought stress. This should be performed through an integrated -omic approach analyzing the plant methylome, transcriptome, and proteome comparing epigenetic, transcriptomic, and proteomic profiles among susceptible and tolerant genotypes in well water

or drought conditions (Figure 6). This part of the work should shed light into the transient and transgenerational epigenomic modifications modulating key legume traits such as stress resistance and resilience, symbiotic nitrogen fixation and qualitative nutritional aspects. Expected outcomes of this objective will be (1) the identification of thousands of methylation sites and single nucleotide polymorphisms (SNPs) linked with tolerance/susceptibility to these environmental constraints, (2) the establishment of the link between epigenetic information and previous identified molecular clusters in model legumes such as bean or chickpea (Table 1). Similar approaches have been performed to identify key players in molecular regulatory networks in plant responses to environmental stresses [35–37].

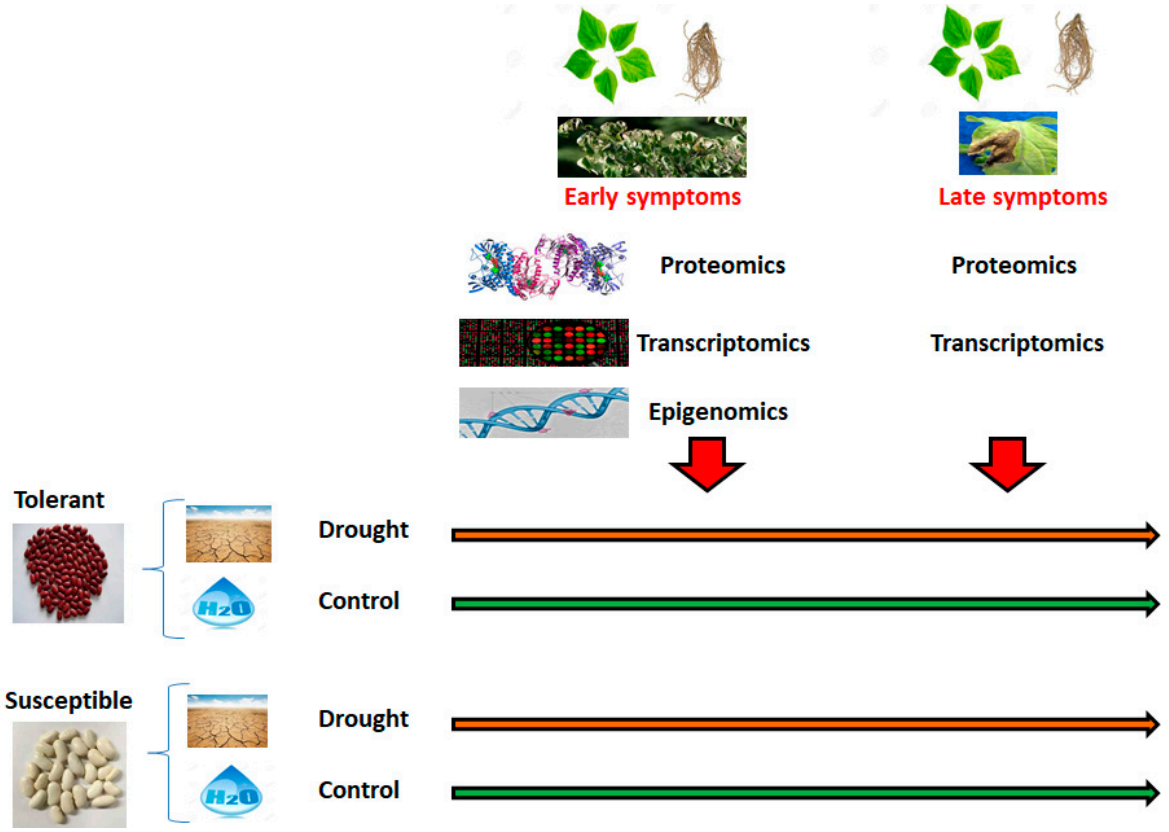

Figure 6. Experimental design of -omic analysis performed in bean and chickpea in response to drought stress.

Thanks to the agronomic evaluation of first objective, we would select one bean genotype with high resistance and one genotype with high susceptibility to drought stress. Thus, we can analyze both DNA methylations and chromatin remodeling using whole genome bisulfite sequencing (WGBS) and ChIP-seq. Nucleic acid samples would be taken from the experiment set up in activities of the objective 1. The following experimental design should be considered for each platform: 2 genotypes × 2 treatments (control, drought) × 2 tissues (roots and leaves) × 1 time point (early stress) × 3 biological replicates = 24 samples. The obtained sequences must be trimmed, quality-controlled, and annotated to the reference genome. We should also filter this huge preliminary list of epigenetic patterns focusing on those genes that are previously known to play a key role in abiotic stress resistance through a deep literature search. The RNA-seq analysis should provide the list of the differentially regulated genes and guide the epigenomic analysis focusing only on key genes affecting drought resistance/susceptibility. The RNA-seq analysis conducted on this activity could allow us to focus on 25–50 candidate sequences that will be analyzed with a targeted epigenetic approach. This validation must be conducted combining PCR and bisulfite sequencing using two other additional genotypes

with variable resistance. This will enhance the power of the validation in determining the effect of genotypic variability on epigenetic mechanisms.

Thus, we could perform RNA-seq using the same samples of the two bean genotypes analysed at epigenomic level (1 resistant and 1 susceptible for each stress) and employing the Illumina 2500 Hiseq platform to produce 50–125 bp reads. Considering the lower costs of RNA-seq compared to epigenomics, we could analyze two time points (early and late stress). Indeed, the experimental design will conform to the following structure: 2 genotypes × 2 conditions (control, drought) × 2 tissues × 2 time points × 3 biological replicates = 48 samples. After read trimming, mapping, annotation, and differential expression analysis, the list of the genes differentially regulated by drought should be determined. This list could be used to perform gene set and pathway enrichment analysis, gene visualization and discovery, and gene interaction network analysis. We plan to use our functional genomic pipeline composed by publicly available software such as Mapman, Pageman, David, NetworkAnalysist, and Cytoscape [38,39]

Finally, proteomics analysis should be conducted using isobaric tags for relative and absolute quantification (iTRAQ) using the same samples analyzed at transcriptomic levels (2 genotypes × 2 conditions (control, drought) × 2 tissues × 2 time points × 3 biological replicates = 48 samples). Proteins must be extracted by powdered tissues from both plants using a phenol extraction procedure described by Schuster and Davies [40]. The digested peptides must be analyzed using a QExactive mass spectrometer coupled with an Easy-LC (Thermo Fisher Scientific, Waltham, MA, USA) and a nanospray ionization source. The peptides should be loaded onto a trap (100 micron, C18 100Å 5U) and data will be acquired using a data dependent ms/ms method. Raw data must be analyzed using X!Tandem and visualized using Scaffold Proteome Software (Version 3.01). Samples would be searched against Uniprot databases (https://www.uniprot.org/) appended with the cRAP database (https://thegpm.org/cRAP/), which contains common laboratory contaminants. The list of differentially abundant proteins between genotypic and control/stress comparisons must be compared with the list of genes identified from RNA-seq analysis. Functional data mining could be performed using the same tools used for RNA-seq data.

Overall, once we obtain the data from epigenomics, transcriptomics and proteomics, we could integrate all these -omic data in order to gain insight into the complex molecular regulatory networks underlying resistance to drought in bean and chickpea. The integration of the -omic data could be performed determining the *Arabidopsis* orthologs, using the most recent interactome data available for this plant model. Additional interactome data obtained from other model species should be also used.

2.1.3. Third Objective: Innovative and Sustainable Biotechnological Solutions to Climate Change

The generated knowledge obtained by these two basic objectives could be transformed into the following translational genomic solutions: (i) new genetic variability (F3–F4 generations) through the crossing of resilient genotypes identified in the first objective; (ii) delivering DNA methylation markers associated to enhanced drought tolerance (key agronomic traits) in Genome-Wide Association Studies (GWAS), and (iii) obtainment of new specific rhizobia–legume combinations for the best evaluated genotypes to enhance symbiotic nitrogen fixation in abiotic stress conditions (described in the first objective and in Figure 5).

The experimental plan for breeding for chickpea and bean genetic improvement is shown in Figure 7. For each plant, we must perform crossings between two parental lines—one should have drought tolerance and the other one should have important agronomic traits such as high yields and/or resistance to biotic stresses (*Ascochyta rabiae* and *Fusarium* wilt) and/or growth habit (production at apical parts) and/or cold resistance (for winter sow) (10 combinations in total). All genotypes must be grown in crossing blocks that would consist of 2 m length and 50 cm intra row spacing. Each parcel would consist of two lines for mother plant and one line for father plant. All genotypes would be sown at 10–20 days interval and, for each combination, we should have 10 crossings whereby we can select one seed per plant (10 F1 seeds). Considering the low number of seeds needed for F1, this

process could be performed in greenhouse. On the third year, these seeds would be sown in high spatial size (50 cm × 50 cm) to produce at least 30 seeds/plant (300 F2 seeds). Using the infrastructure available by the agronomic partners involved in the proposed research, we should arrive to at least the F3 generation using a single seed descent breeding scheme that does not provide any selection procedure at early generation stages to collect the maximum of the variability.

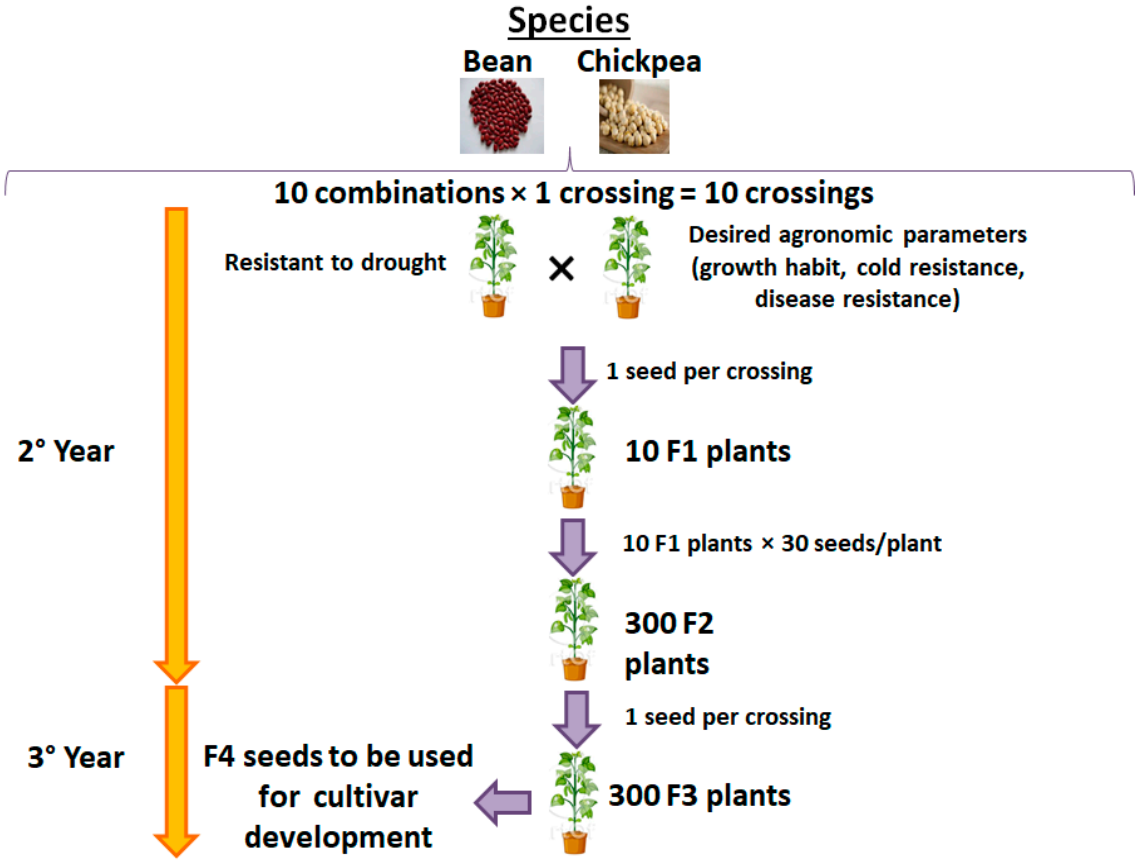

Figure 7. Breeding plan for bean and chickpea in relation to drought stress tolerance. The nine combinations will be obtained by plant breeding of drought tolerant genotypes crossed with better growth habit genotypes and cold or disease resistant genotypes for each legume plant.

The experimental procedure for GWAS mapping is showed in Figure 8. The aim of this activity is to identify epigenomic markers that represent next-generation quantitative trait loci (QTLs) associated with drought tolerance. For this purpose, we should conduct GWAS in chickpea and bean by means of statistical association between the quanti-qualitative traits analyzed in Objective 1 and epigenetic markers obtained in Objective 2 (Figure 8). The analysis should be conducted at the second and third year. First, we would use at least 25 local genotypes for each plant agronomically evaluated in the first objective. At the third year, in case results are not clear, we should extend this analysis up to more than 50 genotypes for each plat. As commented above, the agronomic parameters evaluated in Objective 1 must be linked to the targeted analysis of the epigenetic marks identified from objective 2. Best epigenetic markers should also be analyzed in 100 F2 plants in order to determine their statistical association with the considered agronomic parameters. For each plant, GWAS must be done using analysis of association based on linear models of nine traits (root dry weight, root length, shoot dry weight, plant height, days to 50% flowering, days to 50% seed formation, pods/plant, seeds/pod, and 100-seed weight) and candidate epigenetic markers. The phenotype-associated epigenetic marker localized inside a coding exon or a promoter could be considered associated with drought tolerance. This work should confirm and/or identify new genes related to drought response in chickpea and bean.

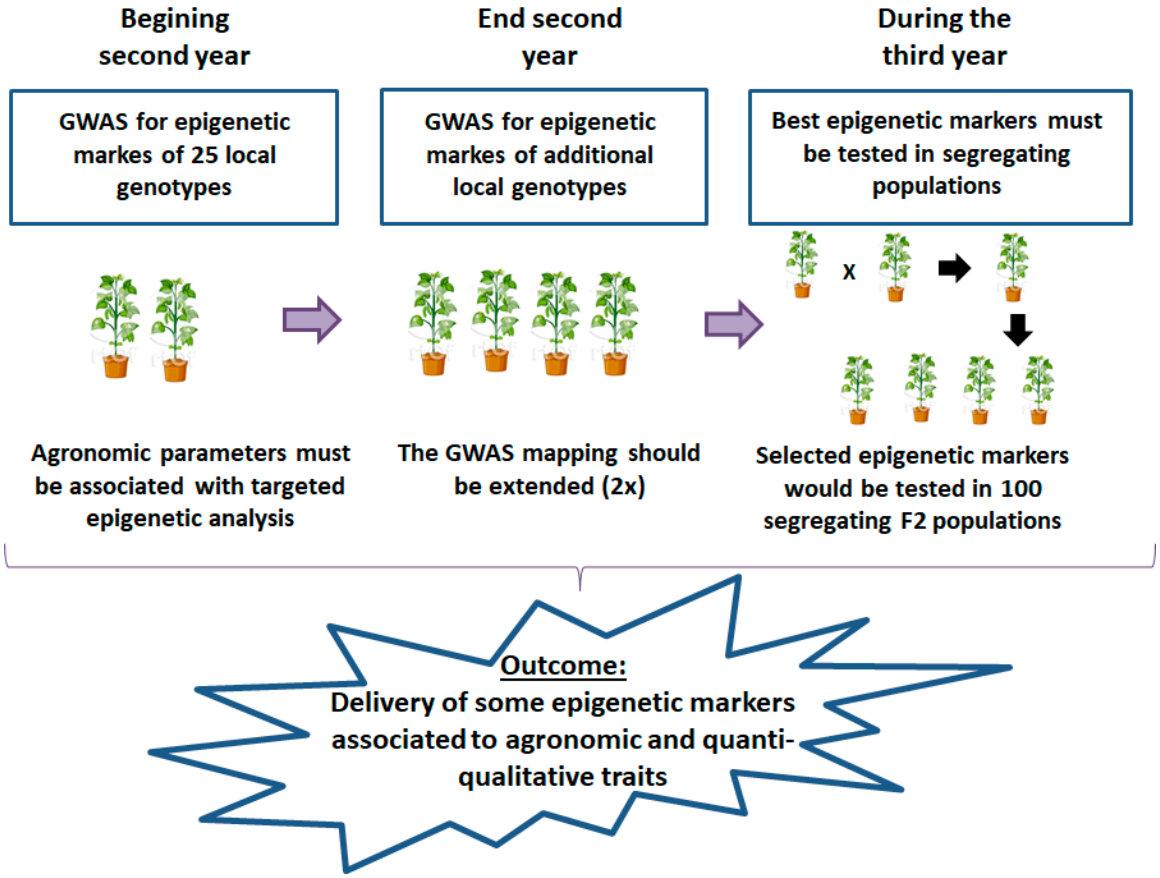

Figure 8. Experimental plan of Genome-Wide Association Studies (GWAS) for epigenetic marker delivery.

2.1.4. Forth Objective: Information Management, Outcome Dissemination, Stakeholder Involvement

Finally, it would be desirable to conduct an intensive series of actions aimed at disseminating, exploiting, transferring, and communicating as much as possible the results obtained during the research proposal. For this purpose, we suggest to detail and define a plan of action to disseminate and exploit, results such as project reports, regular meetings among all partners, organization of scientific congress and stakeholder workshops, trade magazine and ISI peer-reviewed publications, oral presentations at external congresses, creation of a Website as a public repository of all obtained deliverables.

2.2. Deliverables of the Proposed Approach

Briefly, agronomic evaluations of bean and chickpea biodiversity will provide information about the different tolerance/susceptibility responses to drought stress. This knowledge will allow us to select genotypes for (i) functional genomic analysis (discovery of the epigenetic marks), (ii) the testing of genotype-rhizobia combinations, and (iii) the development of epigenetic molecular markers. The analysis of "omics" responses to stresses will allow us to identify candidate (epi)-genetic markers for GWAS. To our knowledge, no epi-genomic markers have been delivered yet in beans and chickpeas linked with drought tolerance and with symbiotic nitrogen fixation under these stressing conditions. Indeed, this aspect of the proposed approach represents a ground-breaking objective, since it will deliver novel concepts and approaches dealing with DNA–environment interactions in plants and will shed lights into the plant genetic plasticity to environmental constraints. Very limited information is known regarding the transgenerational DNA methylations inherited by offspring and due to the environmental stresses at the parental generation. The agronomic knowledgebase will be exploited to gain insight into the molecular regulatory networks of legume adaptation to environmental stresses.

Finally, new products/solutions will be represented by (i) wide agronomic evaluation of legume biodiversity in relation to environmental constraints, (ii) identification of novel genes and epigenetic mechanisms linked plant adaptation to climate changes, (iii) discovery of novel (epi)-genomic markers linked with DNA-environment interactions, (iv) delivery of new genotype-rhizobia combinations adapted to extreme stress events, (v) an improved knowledge of hereditability of quantitative traits, (vi) new genetic variability more resilient to abiotic stresses and finally, and (vii) scientific knowledge and technology transfer to stakeholders facing climate change (Figure 2).

3. Expected Impacts

In sum, the main long-term aim of this proposed approach is to sustain and enhance legume production, the main source of healthy vegetal proteins in an expected more arid and harsher Mediterranean environment. This aim is accomplished through both traditional agronomic evaluations of local germplasm and understanding under-investigated subjects, such as drought stress memory and developing new genotypes and innovating translational genomic tools. At the end, we plan to have good and coherent information on the improvement in a climate change scenario of soil fertility and structure, microbial community, resilience to emerging environmental stresses, and improved management of pest and diseases across the farming systems of the participating countries at both the agronomic level and from a socio-economic perspective. Besides, this proposed approach might not be restricted for legumes of the Mediterranean basin but also for any sensitive crop to climate change worldwide.

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