„ALEXANDRU IOAN CUZA" UNIVERSITY OF IAȘI
FACULTY OF BIOLOGY
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PHYLOGENETIC AND PHYLOGEOGRAPHIC STUDY OF THE *PHASEOLUS VULGARIS* L. SPECIES FROM THE COLLECTION OF THE PLANT GENETIC RESOURCES BANK "MIHAI CRISTEA" SUCEAVA

PHD THESIS SUMMARY

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CONTENT

LIST OF ABBREVIATIONS

Lista prescurtărilor:

DNA= Deoxyribonucleic Acid

AFLP= Amplified Fragment Length Polymorphism

ARN= Acid Ribonucleic

FAO= Food and Agriculture Organisation

LD= Loading Dye

MW= Molecular Weight

PCR= Polymerase Chain Reaction

RT- qPCR= Reverse-Transcription quantitative Real-Time Polymerase Chain Reaction

SNP= Single-Nucleotide Polymorphism

CBOL= Consortium for the Barcode of Life

ROS= Reactive Oxygen Species

LEA= Late Embryogenesis Abundant

CAM= Crassulacean Acid Metabolism

kDA= Kilodalton

JA= Jasmonic Acid

ABA= Abscisic Acid

UV= Ultraviolet

UV-VIS= Ultraviolet–Visible Spectroscopy

INTRODUCTION

Plant species are considered, in a special way, the lungs of the Earth. Plants are a truly important source of energy for the existence and evolution of species.

Genetic variation plays a key role in the evolutionary process of nature. As Charles Darwin said, evolution is due *to hereditary variation*. Theoretically, the difference between the characteristics of ancestral species and their offspring is caused by genomic variation. This is a genetic transformation, induced by processes such as mutation, recombination, DNA transposition or epigenetic processes, and is the basic material for the evolutionary processes of selection and adaptation (Rasmusson & Phillips, 1997).

Many anthropogenic activities have had devastating effects on the environment, of which extinction is considered a real threat for many plant and animal species. Throughout its existence, man has used approximately 10,000 crop plant species, but according to the latest FAO reports, 90% of food production is currently provided by 120 species. The advent of industrialized agriculture has led to a drastic reduction in specific diversity and at the same time to a marked process of genetic erosion. Modern varieties have been developed at the expense of old genotypes and local varieties (Sæther, 2013), which is not an entirely negative aspect, production yields are much higher with such crops and resistance to biotic and abiotic stress factors may be superior. On the other hand, it is extremely important that the plant germplasm of wild relatives and local populations is conserved ex situ for future studies that may lead to the identification of genetically valuable traditional cultivars.

The world in which we live is developing at breakneck speed, like a *roller coaster*, and everything is affected by technologization and industrialization - humans, animals, plants. Every day, many species of plants and animals are disappearing worldwide, species that man does not know and will never discover. In a report published on May 6, 2019, by IPBES (Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services), it is stated that nature is in decline and the extinction rate of species in the plant and animal kingdoms is at an all-time high (https://www.un.org/sustainabledevelopment/blog/2019/05/nature-decline). Knowing, understanding, discovering nature and trying to live in harmony with it will allow us and future generations to benefit from all biodiversity. The Earth is home to millions of species,

each adapted to different environmental conditions, with species unique to particular areas and with the density of biodiversity varying according to the global area.

MOTIVATION AND OBJETIVES OF THE STUDY

Motivation of the study. The topic revolves around the importance and enormous potential of *Phaseolus vulgaris* L., as well as the need to approach an intelligent management of the valorization and exploitation of this crop in the agricultural system. The FAO states that *Phaseolus vulgaris* L. is the world's most important legume. This importance derives from the nutritional intake provided to the consumer, e.g. the protein content varies between 23-33%, depending on the variety, and the energy intake is provided by the high carbohydrate content (over 50%) (Hayat et al., 2014). Beans are considered a key food in poor regions of the world, contributing to food security. Studies based on genome research are essential for understanding plant physiological processes and the changes that occur at the morphological, cellular or biochemical level when plant organisms are exposed to environmental factors less favorable for growth and development. The information obtained can be essential, in particular, for breeding and can respond to current needs in direct proportion to climate change and, of course, to the expansion of the global population.

Climate change causes sudden changes in temperature or prolonged periods of drought, which severely affects crops of all types by sharply reducing production yields. In the case of *Phaseolus vulgaris* L., up to 70% of production can be affected by water shortages (Polania et al., 2016). Therefore, research studies based on the identification of variants with increased resistance to various abiotic stress factors, mainly water stress, are of great importance at present, and the findings may be of major importance in the creation of varieties resistant to current climatic conditions.

The aim of the PhD thesis is to contribute to the knowledge of *Phaseolus vulgaris* L. culture, in terms of biogeography of the species, collected in Romania and kept in the collection of the Plant Genetic Resources Bank Suceava. It will also be followed whether the geographical origin of the samples (Mesoamerica/Andean) has an influence on the resistance of *Phaseolus vulgaris* L. to water stress factor in the light of the current climate change situation. In accordance with statements formulated regarding the purpose and objectives of the research, as well as the information currently available in the literature, the following hypotheses were tested:

Hypothesis 1. *Phaseolus vulgaris* L. originating from the Andean geographical basin is currently more prevalent in Europe, including the Balkans, than in populations originating from the Mesoamerican geographical region;

Hypothesis 2. Variants of *Phaseolus vulgaris* L. originating from the Mesoamerica geographic basin show a superior resistance to water stress, due to the adaptation mechanisms they develop, compared to those originating from the Andean region.

Objectives and **activities** related to the 4 years of doctoral studies:

O.1. Analysis of phylogenetic relationships and phylogeography of *Phaseolus vulgaris* L. samples from the collection of the Plant Genetic Resources Bank "Mihai Cristea" Suceava.

1.1. Selection of a representative number of *Phaseolus vulgaris* L. samples, kept in the collection of the Plant Genetic Resources Bank "Mihai Cristea" Suceava and establishment of working methods;

1.2. Germination of *Phaseolus vulgaris* L. samples, under specific conditions of growth and development to obtain leaf tissue;

1.3. Isolation and purification of DNA from leaf tissue;

1.4. Polymerase chain reaction using specific primers (nuclear and chloroplastic);

1.5. Sequencing of the amplified regions and analysis of the obtained data in order to establish phylogenetic relationships between the evaluated samples and to identify the geographical origin.

O.2. Analysis of gene expression, involved in growth, development and resistance to water stress, for varieties originating from the two geographical basins, Mesoamerica and Andean, kept in the collection of the Plant Genetic Resources Bank Suceava.

2.1. Germination of *Phaseolus vulgaris* L. samples under different experimental conditions;

2.2. Analysis of phenotypic (weight, height), biochemical (chlorophyll, carotenoids) and physiological (relative growth rate and relative water content) parameters;

2.3. Expression analysis of genes of interest.

2.3.1. RNA isolation and purification;

2.3.2. Evaluation of gene expression (involved in growth and development processes, but also in plant response to water stress factors) by RT- qPCR method;

2.3.3. Analysis of the data obtained by relative quantification of the expression of the genes of interest.

O.3. Morphoanatomical characterization of *Phaseolus vulgaris L.* samples exposed to a low water environment and those constantly irrigated throughout the experiment.

3.1. Morpho-anatomical analysis of hypocotyl and epicotyl of *Phaseolus vulgaris* L. samples developed under different experimental conditions.

O.4. Dissemination of results - publication in peer-reviewed journals and participation in scientific events.

O.5. Writing and defending your doctoral thesis.

Therefore, according to the data identified in the literature the expected results are the following:

1. The higher frequency of *Phaseolus vulgaris* L samples originating from the Andean geographical basin, compared to those from the Mesoamerica area in Romania;
2. Populations with Mesoamerican origins will show better drought resistance compared to those from the Andean region.

The originality of the study is based on the phylogenetic and phylogeographic analysis of *Phaseolus vulgaris* L samples from all regions of Romania. An essential element is the importance of the biological material, these are local populations that have not been studied so far, and some samples have been in the collection of the Plant Genetic Resources Bank Suceava for over 35 years.

The limitations of the research carried out in this PhD work were largely due to the small number and even absence of similar research at international level, especially in the field of morphoanatomy of *Phaseolus vulgaris* L., as well as in molecular biology, aiming at comparing nuclear and chloroplast genome sequences of populations with different geographical origins, which limited the discussions.

CHAPTER 1

1. *THEORETICAL CONSIDERATIONS*

1.1. *PHASEOLUS VULGARIS* L. - GENERAL ASPECTS AND SYSTEMATIC CLASSIFICATION

According to the FAO, *Phaseolus vulgaris* L. is the world's most important legume. It is an annual, herbaceous and thermophilic plant (Ciofu, 2004). Approximately 230 species are known to belong to the genus *Phaseolus*, of which only 12 are cultivated for human consumption and 8 for fodder, *Phaseolus vulgaris* L. being the best known species. The genus *Phaseolus* is a member of the family *Fabaceae*, a family comprising about 770 genera and more than 19,500 species (Azani et al., 2017; Lewis et al., 2013). At present, leguminous crops are the second most widely cultivated crops in the world, after cereals.

Phaseolus vulgaris L. is a diploid organism *(n=11)* (Zheng et al., 1991), and 60% of this species' genome is made up of unique sequences (Talbot et al., 1984). The first genetic studies using *Phaseolus vulgaris* L. as biological material were carried out by Gregor Mendel in 1866. As regards the morphology of *Phaseolus vulgaris* L. plants, the leaves are trifoliate, the flowers are papilonaceous, zygomorphic (Singh et al., 1991). For *Phaseolus vulgaris* L., the germination process is epigeal (the seed cotyledons are at the soil surface) and takes about 5-7 days. Mature bean seeds are dehiscent (Berglund-Brücher & Brücher, 1976; Singh et al., 1991; Watts, 2012).

Over time, the taxonomic classification of *Phaseolus vulgaris* L. has undergone numerous changes, in particular that of the varieties *vulgaris*:

- **The kingdom:** *Plantae;*
- **Order**: *Fabales;*
- **Family**: *Fabaceae;*
- **Genus:** *Phaseolus;*
- **Species:** *Acutifolius, Coccineus, Longepeduculatus, Maculatus, Polystachios, Vulgaris.*

According to NCBI (Taxonomy Browser) *Phaseolus vulgaris* is classified into two different groups of **varieties:**

- *Phaseolus vulgaris var. aborigineus;*
- *Phaseolus vulgaris var. nanus.*

GRIN Global proposes a slightly different classification for *Phaseolus vulgaris* than previously:

- *Phaseolus vulgaris L var. aborigineus;*
- *Phaseolus vulgaris L var. vulgaris.*

1.2. IMPORTANCE OF *PHASEOLUS VULGARIS* L SPECIES

The world's most important legume for human consumption is *Phaseolus* spp., in particular *Phaseolus vulgaris* L. The importance of bean cultivation is supported by 3 arguments:

- ✓ **chemical composition**: the seeds are rich in protein, fiber, complex carbohydrates, micronutrients and micronutrients (Anonymous, 2001; Reinprecht et al., 2020);
- ✓ **the symbiotic relationship** between *Phaseolus* and *Rhizobium* allows the fixation of atmospheric nitrogen (N2) in the soil (Reinprecht et al., 2020)*;*
- ✓ **the simplicity of producing and maintaining** a crop of *Phaseolus vulgaris*; costs and requirements in relation to soil and climatic conditions are moderate (Myers, 2017).

1.3. SPREAD AND CULTIVATION

Common bean are the main source of food for millions of people worldwide. India and Brazil are the world's biggest bean producers, while China is the world's number one producer of green beans (http://faostat3.fao.org/faostat-gateway/go/to/home/). According to the graphs below, interest in the cultivation of *Phaseolus vulgaris* L. has increased globally, but declined dramatically at national level (Figure 1.1).

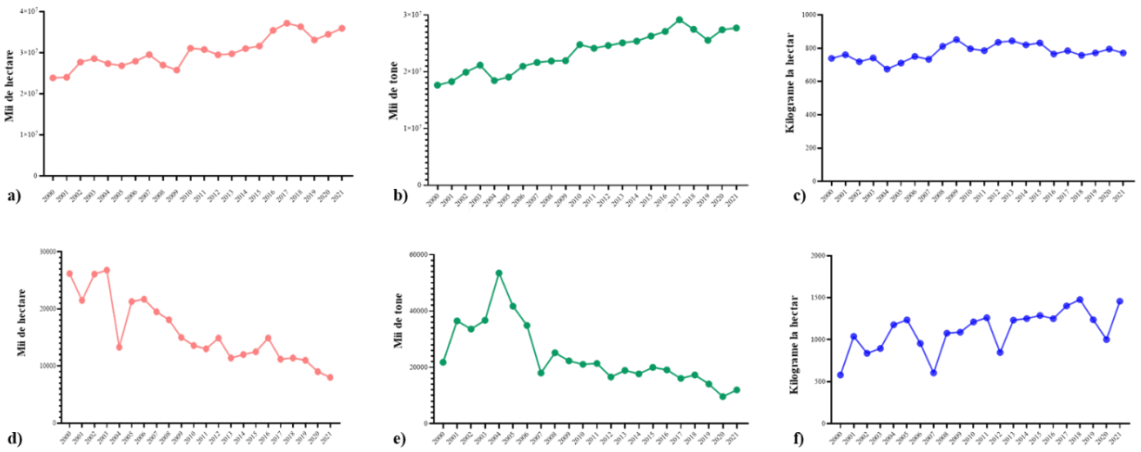


Figure 1.1. Graphical representation of global and national bean bean production, cultivated area and production yield: a) area cultivated at world level (area under cultivation (broad bean), between 2000-2021; b) production at world level (broad bean), between 2000-2021; c) yield of production at world level (broad bean), between 2000-2021; d) area cultivated in Romania (broad bean), between 2000-2021; e) production in Romania (broad bean), between 2000-2021; f) yield of broad bean production in Romania, between 2000-2021

1.4. PHYLOGEOGRAPHY

Knowledge of the origin and domestication areas for species in the plant kingdom is of interest to evolutionary researchers, but also of practical importance for agriculture and *in situ* and *ex situ* conservation, even though the process itself is dynamic and constantly evolving (Zohary, 1999). Phylogeography makes it possible to identify plant populations that have escaped, demographic bottlenecks in plant organisms or the extinction/ extinction of species. On the other hand, phylogeography can also answer questions about the isolation of populations, environmental barriers or geographical distances (Hickerson et al., 2010).

The species *Phaseolus vulgaris* L. was first botanically described in 1542, as evidenced by certain manuscripts called herbals found in Europe. In the Tehucan Valley region of Mexico, fragments of *Phaseolus vulgaris* L., about 7000 years old, have been found in archaeological sites in the Tehucan Valley region, and it has been proven that these plants had undergone domestication by that time (Kaplan, 1999). *Phaseolus* vulgaris L. has its origin in two geographical basins, Central America (the Mesoamerica basin), which is known today as the region stretching from Mexico to Panama, and South America (the Andean basin), the region from northeast Colombia to Argentina (Chacon et al., 2007). Compared to the Andean geographic basin, the Mesoamerican basin is more genetically diverse. *Phaseolus vulgaris* L. from Mesoamerica was introduced to Europe around 1506 from Spain and Portugal (Watts, 2012), and the Andean, followed the same route to the European continent, but a few years later, in 1528, after Pizzaro's exploration of Peru

(Berglund-Brücher & Brücher, 1976). The geographical origin of populations of *Phaseolus vulgaris* L. can be determined by evaluating specific sequences in the nuclear or chloroplast genome (Bitocchi et al., 2012; Konzen et al., 2019; Vidak et al., 2021).

1.5. PHYLOGENY AND GENETIC DIVERISTY

Modern civilization depends on a few plant species for food. Today's crops have been bred over thousands of years, during which time man has intervened through artificial selection and the environment through natural selection, transforming wild ancestors into domesticated descendants (Rendón-Anaya et al., 2017). Genetic diversity is considered the main driver of evolution, both genotypically and phenotypically, and these changes are influenced by environmental conditions (Henry, 2005b). Phylogeny, on the other hand, is a field of biology which aims to study the history or evolution of an organism, but also to establish the relationships between individuals of a group or of different groups (Yang et al. 2012).

1.6. DROUGHT STRESS

Phaseolus vulgaris L. is a globally important crop, and biotic and abiotic stresses can decrease bean yields by up to<600 kg/ha^{-1} (Porch et al., 2013). Accelerated population increase is in direct proportion to the growing demand for food, and global warming is leading to a decrease in agricultural production due to less rainfall and higher temperatures, which ultimately leads to food insecurity, especially in underdeveloped countries.

1.6.1. Response mechanisms of plant organisms subjected to water stress and effects

Drought is considered the most important abiotic stress factor limiting plant growth and development by altering metabolic and biological activities (Hrmova & Hussain, 2021; Konzen et al., 2019; Mahmood et al., 2020; X. Yang et al., 2021). At present, about 45% of the world's agricultural land, where around 38% of the human population is estimated, is subject to frequent or continuous drought. Every stage of plant growth is dependent on water,

from germination to maturity, lack of water leads to reduced yields and the socio-economic consequences can be severe (Hussain et al., 2012). Plant organisms can respond to water stress by two mechanisms, categorized according to response time:

- **long-term response**;
- **short-term response** (Tardieu et al., 2018).

1.6.2. Effects of water stress in plants - morphological changes

When plant organisms are subjected to water stress, the first changes that occur are at the morphological level, in the external (leaf tissue, stem, root system) and internal structure of the plant. The first visible signs of water shortage are a slowing down of growth and, finally, plant death. Plant organisms adapt over time to soil and climatic conditions, an obvious example being xerophytes[1] (Yang et al., 2021). When plants are deprived of water, their stems become smaller, leaves wither, and the number and area of leaf surfaces are greatly reduced (Anjum et al., 2017; Marais, 2017; Nasir & Toth, 2021; Patmi et al., 2020). Roots have a remarkable ability to sense and eventually respond to physico-chemical parameters in the soil by adjusting their development and thus their uptake of water. Increasing the length and density of the root system in plant organisms are considered as drought adaptation mechanisms (Bengough et al., 2006).

1.6.3. Water stress effects in plants - physiological and biochemical changes

When plants are deprived of their water source, they undergo a number of changes, not only at the morphological but also at the physiological and biochemical level. This includes changes related to photosynthesis, osmotic regulators, drought-induced proteins, antioxidant enzymes and various phytohormones (Yang et al., 2021). Other changes that may occur at the physiological stage are related to relative water content (RWC) and relative growth rate (RGR), and a decrease in RGR has been reported in *Phaseolus vulgaris* L. in plants that were not irrigated compared to those that were adequately hydrated (Costa et al., 2000) and a decrease in leaf tissue water content in water-stressed lots was also indicated.

[1] *Xerophytes* - are plant species that are able to survive in areas with low water levels, such as deserts or snow and ice-covered regions in the Alps or the Arctic. This class of plant organisms includes cacti, pineapples and some species of *Gymnosperms*.

1.6.3.1. Photosynthesis

Photosynthesis is one of the biological processes essential for the survival of plant organisms. Drought can reduce photosynthesis by decreasing leaf area and photosynthetic rate (Arunyanark et al., 2008; Razi & Muneer, 2021; Yang et al., 2021).

1.6.3.2. Osmotic regulation

Osmoprotective substances or osmolytes are defined as small molecules that allow plant organisms to cope much more easily and survive under severe osmotic stress (Hussain et al., 2013). The accumulation of osmoprotectins in plants during water stress is one of the immediate responses and is essential for maintaining turgor pressure (Ozturk et al., 2021).

1.6.3.3. Proteins synthesized in drought periods

During periods of drought, plants can synthesize a variety of protective proteins that improve the drought tolerance of plant organisms. These proteins can be categorized into functional and regulatory proteins. The first category, functional proteins, includes LEA proteins, membrane proteins, OSM proteins, metabolic enzymes, etc., which play an important role in the direct protection of cell structures. In contrast, regulatory proteins are protein kinases, phospholipase C, calmodulin, transcription factors, and signaling factors, all of which are involved in signaling pathways at the molecular level or in regulating gene expression, and their protective role becomes indirect (Yang et al., 2021).

1.6.3.4. Reactive oxygen species (ROS) production and removal systems during droughts

Under normal conditions, plants produce reactive oxygen species (ROS) within tolerable limits, so that the removal systems for these molecules are effective. However, during periods of drought, plants produce much more reactive oxygen species and ROS removal mechanisms may be overwhelmed (Yang et al., 2021). The most susceptible molecules to free radical damage at the cellular level are proteins, lipids and carbohydrates (Blokhina et al., 2003), but ROS can interact with purines and pyrimidines in the DNA molecule and can cause cleavage, degradation and modification of double-stranded

molecules (Marnett, 2000). Indirectly, reactive oxygen species can suppress plant growth and development by inactivating enzymes.

1.6.3.5. Phytohormones involved in water stress

Abscisic acid (ABA) is a plant hormone, and the implications and effects produced, both under normal conditions and under various biotic and abiotic stresses in the plant cell, have been intensively studied over time. ABA is a phytohormone involved in triggering many physiological processes, including meristematic dormancy, seed germination, stomatal closure or regulation of the expression of stress-responsive genes (Ali et al., 2020).

1.6.4. Water stress effects in plants - changes at the molecular level

Plant organisms are able to survive and grow during periods of water stress due to adaptive mechanisms. Angiosperms are able to receive the signals produced by the lack of water. The signals are then transduced through different pathways at the cellular level, resulting in the synthesis of molecules that produce physiological, biochemical and morphological changes, which are reflected in plant resistance to drought. During periods of water stress, numerous genes can be activated in plants to protect plant organisms from stressors. These genes have thus been classified according to their role and direct or indirect action into functional and regulatory genes. The first category, functional genes, includes sequences encoding protective proteins (aquaporins, LEAs, chaperone proteins), osmoregulatory factors (proline, betaine, various carbohydrates) or synthetase genes. Regulatory genes are involved in message transduction and in regulating gene expression, they play an indirect role in plant defense during drought periods (Yang et al., 2021).

CHAPTER 2

2. MATERIALS AND RESEARCH METHODS

2.1. RESEARCH MATERIALS

The biological material used is represented by local populations of the *Phaseolus vulgaris* L species. The analyzed samples were collected from different areas of Romania and are currently kept in the collection of the Plant Genetic Resources Bank "Mihai Cristea" Suceava. Within this study, 27 varieties of *Phaseolus vulgaris* L. were studied. The selection of the biological material was based on the following criteria: the color of the samples should be white; the shape and size of the seeds of *Phaseolus vulgaris* L. should be slightly similar; the plant germplasm should come from different counties of Romania.

The study on drought stress resistance of *Phaseolus vulgaris* L. landraces with different geographical origins (Andean/Mesoamerica), required the selection of a smaller number of samples. Thus, three varieties (SVGB-1988 - Andean origin, SVGB-2087 - Mesoamerican origin and the Lechința variety, with superior resistance to drought stress, the variety is in the collection of the Research and Development Station for Legumiculture of Bacău) were grown under normal irrigation conditions (control lots) and exposed to drought (drought stressed lots).

2.2. METHODS OF RESEARCH

2.2.1. Morphological analysis of *Phaseolus vulgaris* L. germoplasm

Previous to the development of molecular biology, the geographic origin of *Phaseolus vulgaris* L. samples was determined by measuring the weight of 100 grains or by macroscopic assessment of seed shape. Thus, small seeds (M100 < 25 g) were considered to be of Mesoamerican origin, and medium (M100 25-40 g) and large (M100 > 40 g) of Andean

origin (Cichy et al., 2015). Morphological analysis of *Phaseolus vulgaris* L. seeds involved the determination of quantitative and qualitative traits, such as seed length, width, height, mass of 100 seeds, weight, Flatness Index (FI) and Eccentricity Index (EI), color and shape of germplasm. From each population 20 seeds were randomly selected and subsequently analyzed.

2.2.2. Germination of *Phaseolus vulgaris* L.samples

Seeds assessed morphologically in the previous step were germinated in order to provide leaf tissue for the DNA isolation and purification reaction. Initially, seeds were disinfected with a 1% sodium hypochlorite solution. Germination was carried out under controlled temperature and humidity conditions (25°C and 80%).

2.2.3. DNA isolation and purification

DNA isolation and purification was carried out using the *Wizard® Genomic DNA Purification Kit (Promega)* from leaf tissue resulting from the germination of *Phaseolus vulgaris L.* seeds.

2.2.4. Quantification of nucleic acids

In the current study, the concentration and purity of DNA was determined using a *ThermoScientific NanoDrop One* spectrophotometer

2.2.5. Amplification of coding regions and intergenic regions for the phylogeny and phylogeography studies

At first, 13 regions from the nuclear and chloroplast genomes were amplified: 5 coding regions from the nuclear genome (*PvLEA3, PvDREB1, PvDREB2A, PvDREB5A, PvDREB6B*) and 8 genes and intergenic regions from the chloroplast genome (*rpoC1-rpoC2, trnL-trnF, trnT-tnL, accD, rps4, rps14-psbaB, trnL, rpl16*). Finally, only 6 coding regions and intergenic regions, (*PvLEA3, PvDREB6B, PvDREB5A, rpoC1-rpoC2, rps4, trnL-trnT*) were selected for the study of phylogeography and phylogenetic relationships between

populations. Amplification of the sequences was performed using *GoTaq® Green Master Mix (Promega).*

2.2.6. Agarose gel electrophoresis

Agarose gel electrophoresis is a qualitative technique used in the current study to assess genomic DNA and to also validate PCR products. DNA fragments migrated between 80 and 120 and minutes and the voltage value was in the range of 55-75V, depending on the type of DNA migrated (genomic, amplicons). The electrophoresis and visualization systems used in this instance are *Wide Mini-Sub Cell GT Cell, BioRad* and *GelDoc Go Imaging System, BioRad.*

2.2.7. Purification of PCR amplicons

Purification of PCR products was carried out using the *Wizard® SV Gel* and *PCR Clean-Up System (Promega).*

2.2.8. SANGER sequencing

The sequencing reaction was performed for the 6 nuclear and chloroplast regions, in both directions *for PvDREB6B, PvLEA3, trnL-trnT* and *rpoC1-rpoC2* sequences and in reverse for *PvRPS4* and *PvDREB5A*. Three individuals were analyzed each from all 27 local populations sampled. The Sanger sequencing method was outsourced and performed at *CeMIA* laboratory, Greece.

2.2.9. The experimental design

Twelve lots of 5 individuals were established, each cultivar being represented by 2 control and 2 drought stressed lots, which constituted 2 experiments: one for 30 days and the second for 34 days. One control was irrigated daily for 30 days and the second control for 34 days. The drought stressed lots were composed as follows: both lots were irrigated until day 22 (V2 growth phase), and then water stress was applied for 8 days (experiment I - 30 days) and 12 days (experiment II - 34 days), respectively.

2.2.10. Morphological evaluation of *Phaseolus vulgaris* L. seedlings

Measurements of total plant height, root system length, above-ground height, root system biomass, shoot biomass and total weight were carried out. The observations were performed for all individuals in the experimental lots.

2.2.11. Morphoanatomical analysis of *Phaseolus vulgaris* L. samples from control and non-irrigated lots

The photon microscopy method was used to highlight the differences at the morphoanatomical level. Examinations were carried out in cross-section of the hypocotyl and epicotyl parts for individuals from the three populations studied. Double staining of the preparations with iodine green (to highlight lignin) and ruthenium red (to highlight cellulose and pectin) was necessary to visualize cellular structures. Subsequently, the stained sections were examined in water droplet (provisional preparation) using a Novex binocular microscope.

2.2.12. Quantification of biochemical compounds

The content of assimilatory pigments, chlorophyll a, chlorophyll b and carotenoids, was determined spectrophotometrically using the method developed by Nayek et al, (Nayek et al., 2014), using the *PG Instruments T70 UV/VIS* Spectrophotometer, Wibtoft, UK, at wavelengths 665.2 nm, 652.4 nm and 470 nm. Determinations were performed for all individuals in the experimental lots.

2.2.13. Determination of certain physiological parameters

Au fost evaluați parametrii fiziologici precum **rata relativă a creșterii plantelor** (*relative growth rate* – RGR) (Lizana et al., 2006) și **conținutul relativ de apă** (*relative water content* - RWC) (Qayyum et al., 2021).

2.2.14. RNA isolation and purification

RNA was isolated from leaf and root tissues of both control and drought stressed plants using the *SV Total RNA Isolation System (Promeg*a) kit.

2.2.15. Real Time Quantitative PCR (RT-qPCR)

Quantification expression of the genes expression(*PvLEA3, PvDREB1, PvDREB2A, PvDREB6B, PvABA8'H, PvPP2C-12, PvP5CS10, PvWRKY53, PvWRKY57, PvMYB03, PvMYB07, PvMYC, PvERF, PvACT* - housekeeping) was performed by the RT-qPCR method, using the *qRT-PCR- GoTaq® 1-Step RT-qPCR System Promega* and Amplification and Detection System *CFX96 BioRad*. Results were expressed as $\log_2 2^{-\Delta\Delta Ct}$.

CHAPTER 3

3. RESULTS AND DISCUSSION

3.1. ASSESSMENT OF THE GERMINATION RESPONSE

All *Phaseolus vulgaris* L. landraces germinated at 100% (Figure 3.1), under species-specific temperature and humidity conditions, while the germination rate differed by cultivar. Germination evaluation was performed on day 4 and day 8. On day 4 the samples showed a germination percentage between 56-70%, and on day 8, its value ranged between 95-100%. The samples were free of any contaminants and were kept in the germinator under favorable conditions of growth and development until day 20.

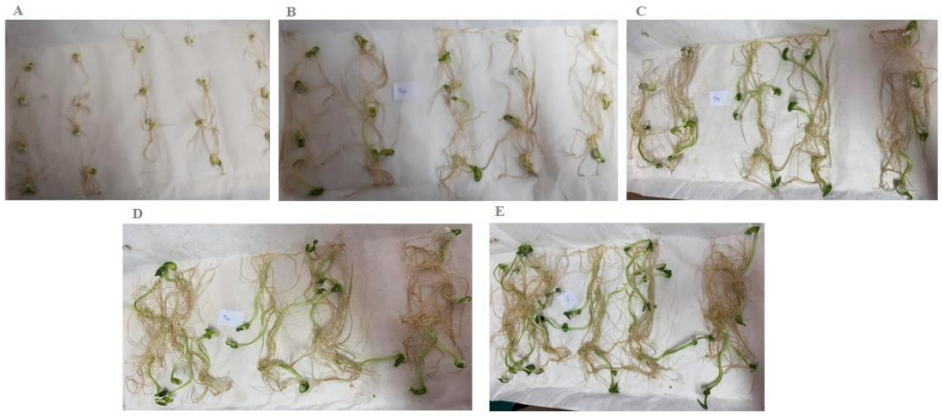


Figure 3.1. Stages of growth and development of sample SVGB-5513 after germination: A -2 days after germination of the samples (10 days from the time the samples were germinated); B - 5 days after germination of the samples (13 days from the time the samples were germinated); C - 7 days after the germination of the samples (15 days from the time the samples were germinated); D - 9 days after the germination of the samples (17 days from the time the samples were germinated); E - 12 days after the germination of the samples (20 days from the time the samples were germinated).

3.2. QUANTITATIVE AND QUALITATIVE ASSESSMENT OF DNA

3.2.1. DNA spectrophotometric quantification - quantitative and qualitative assessment

The genomic DNA concentration ranged from 347.4 ng/µl (SVGB-3076, individual 2) to 605.7 ng/µl (SVGB-2087, individual 1). The A260/A280 ratio for genomic DNA ranged from 1.70-1.89; with small exceptions, when the ratio value was below the minimum

threshold of 1.7 (1.6-1.69); on the other hand, the A260/A230 ratio ranged from 1.81-2.05. PCR amplicon concentration ranged from 9.79-51.2 ng/ µl. In this case the A260/A280 ratio ranged from 1.72-1.92 and that of A260/230 ranged from 1.80-2.07.

3.2.2. Qualitative assessment of DNA by agarose gel electrophoresis

To test the integrity and purity of genomic DNA, agarose gel migration was performed. Using this method, fragment size was assessed by comparison with a molecular marker of known weight (MW-1 kb) (Figure 3.2).

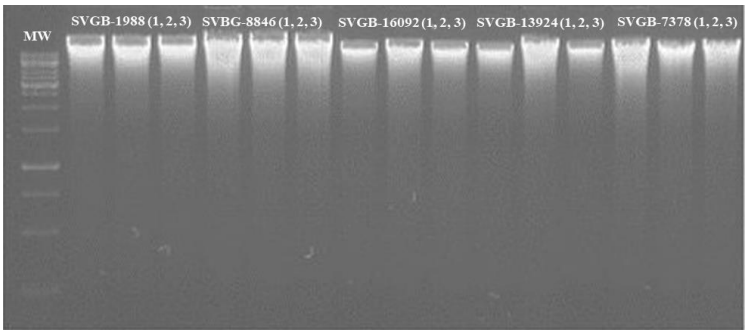


Figure 3.2. Genomic DNA electrophoregram, three individuals from 5 populations of *Phaseolus vulgaris* L. (SVGB-1988, SVBG-8846, SVGB-16092, SVGB-13924, SVGB-7378); agarose gel concentration 1.5%; migration - 120 min, 70V; 5 µl M.W. - 1 kb; 2 µl L.D. +5 µl DNA

3.3. PCR AMPLIFICATION OF SEQUENCES AND VALIDATION OF AMPLICONS BY ELECTROPHORESIS

Amplification of the six regions, from the chloroplast genome (trnL-trnT, rpoC1-rpoC2, PvRPS4) and from the nuclear genome (*PvDREB5A, PvDREB6B, PvLEA3*) was performed according to the procedures exemplified in Chapter 2, Materials and Methods. The amplification reaction yielded only the DNA fragments of interest, *PvDREB5A, PvDRE6B, PvLEA3, trnL-trnT, rpoC1-rpoC2* and *PvRPS4*, and non-specific products were excluded (Figure 3.3).

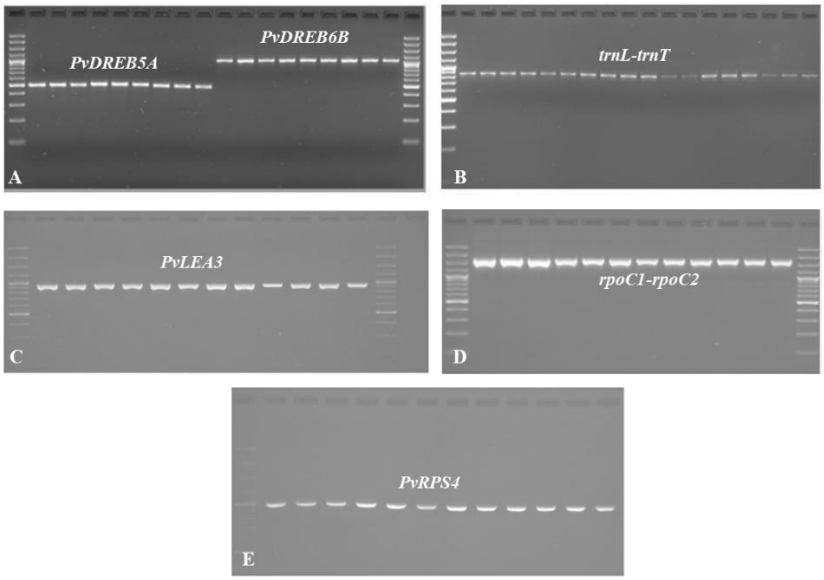
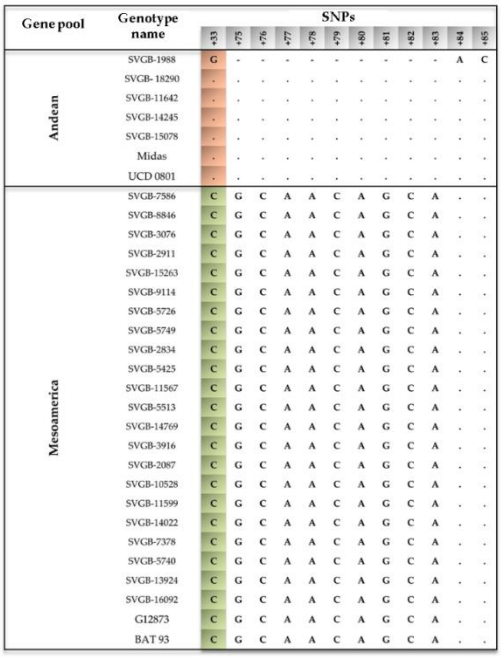


Figure 3.3. PCR amplicon electrophoregrams for three individuals from different populations of *Phaseolus vulgaris* L.: **A** -PvDREB5A and PvDREB6B (SVGB-1988, SVGB-11599, SVGB-2087); **B** -trnL-trnT (SVGB-1988, SVGB-11599, SVGB-2087, SVGB-10528, SVGB-11642, SVGB-9114); **C** - PvLEA3 (SVGB-1988, SVGB-11599, SVGB-2087, SVGB-10528); **D** - rpoC1-rpoC2 (SVGB-1988, SVGB-11599, SVGB-2087, SVGB-10528); **E** - PvRPS4 (SVGB-1988, SVGB-11599, SVGB-2087, SVGB-10528); agarose gel concentration 2.5%; PCR amplicon migration 120 min, 55V,
5 µl M.W. - 1 kb; 2 µl L.D. +5 µl DNA

3.4. *PvDREB5A GENE SEQUENCE ANALYSIS*

The *PvDREB5A* sequence is a short coding region in the nuclear genome that can provide information on the geographical origin of bean samples (483 bp - length is characteristic of *Phaseolus vulgaris* L. samples originating from the Mesoamerican geographical region and 474 bp - length is characteristic of *Phaseolus vulgaris* L. samples originating from the Andean geographical region) (Konzen et al., 2019). As a result of the sequencing reaction, for the 81 individuals (27 populations) belonging to the species *Phaseolus vulgaris* L., from the 27 counties of Romania, the following results were obtained:

- 81.5% of the populations considered in the study are of Mesoamerican origin (22 of the populations analyzed) (Galan et al., 2023);
- 5 populations (SVGB-1988, SVGB-18290, SVGB-11642, SVGB-14245, SVGB-15078) among those analyzed are of Andean origin, with a sequence length of 474 bp (Figure 3.4).



Gene pool	Genotype name	SNPs											
		+33	+75	+76	+77	+78	+79	+80	+81	+82	+83	+84	+85
Andean	SVGB-1988	G	-	-	-	-	-	-	-	-	-	A	C
	SVGB- 18290	.	.	.	.	.	.	.	.	.	.	.	.
	SVGB-11642	.	.	.	.	.	.	.	.	.	.	.	.
	SVGB-14245	.	.	.	.	.	.	.	.	.	.	.	.
	SVGB-15078	.	.	.	.	.	.	.	.	.	.	.	.
	Midas	.	.	.	.	.	.	.	.	.	.	.	.
	UCD 0801	.	.	.	.	.	.	.	.	.	.	.	.
Mesoamerica	SVGB-7586	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-8846	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-3076	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-2911	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-15263	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-9114	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-5726	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-5749	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-2834	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-5425	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-11567	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-5513	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-14769	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-3916	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-2087	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-10528	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-11599	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-14022	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-7378	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-5740	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-13924	C	G	C	A	A	C	A	G	C	A	.	.
	SVGB-16092	C	G	C	A	A	C	A	G	C	A	.	.
	G12873	C	G	C	A	A	C	A	G	C	A	.	.
	BAT 93	C	G	C	A	A	C	A	G	C	A	.	.

Figure 3.4. *PvDREB5A* nucleotide sequence alignments for the 27 local populations of *Phaseolus vulgaris* L. Midas, UCD 0801 genotypes are of Andean origin, and G12873 and BAT 93 are of Mesoamerica basin origin (Konzen et al., 2019). SNP-type variations specific to the Andean genotype were highlighted in orange, and those originating from the Mesoamerica basin are labeled in green

The spread of the 27 local populations of *Phaseolus vulgaris* L. is relatively uniform across Romania, in the case of the populations with origins in Mesoamerica; whereas the samples originating from the Andean geographical basin are concentrated in the central-northern part of Romania: Suceava (SVGB-1988), Sălaj (SVGB-15078), Alba-Iulia (SVGB-14245), Vaslui (SVGB-11642) and Harghita (SVGB-18290) (Figure 3.5).

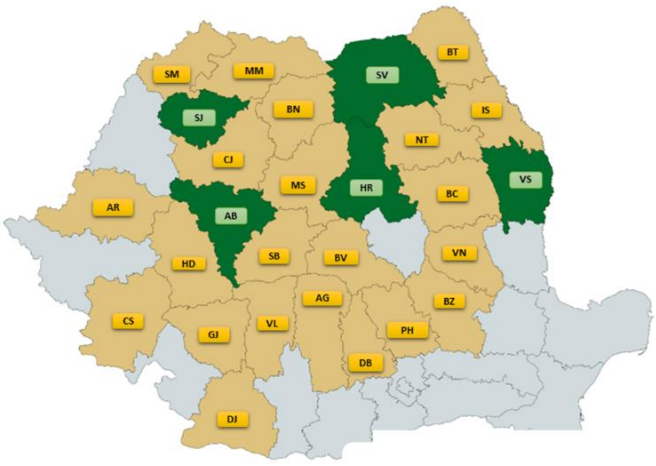


Figure 3.5. The distribution of the 27 local populations of *Phaseolus vulgaris* L. in Romania, according to geographical origin: Andean geographical basin/ Mesoamerican geographical basin. Andean genotype were highlighted in green, and those originating from the Mesoamerica basin are labeled in yellow

The cladistic differentiation and historical evolution generated by the Neighbour-Joining method for the *PvDREB5A* fragments provided information on the degree of similarity between the 27 *Phaseolus vulgaris* L populations evaluated. Two clades were generated, as follows: green color marks the group in which populations originating from the Mesoamerica geographic basin were categorized, and at the bottom of the phylogenetic tree, marked in red, bean samples originating from the Andean geographic basin were classified. Genetic distances were determined by the Maximum Composite Likelihood method and the Bootstrap statistical test (1000 replicates) was also used to determine the degree of confidence (Figure 3.6).

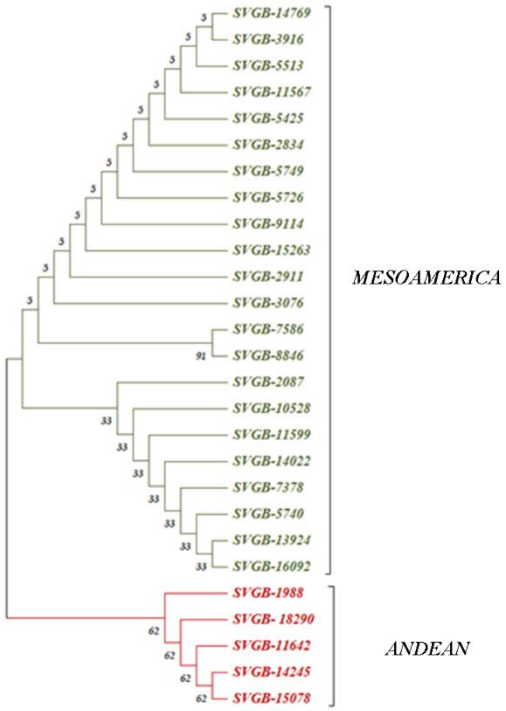


Figure 3.6. Phylogenetic tree - Neighbor-Joining method - *PvDREB5A* gene sequences. Populations labeled in red are from the Andean geographic basin and those in green are from the Mesoamerican geographic basin

3.5. *PvDREB6B* GENE SEQUENCE ANALYSIS

The gene encoding *DREB6B* (*Dehydration-Responsive Element-Binding Protien*) is a transcription factor in plants, and its main role is to activate the expression of genes directly involved in water stress resistance.

In the current study, the *PvDREB6B* region was amplified and sequenced for the 27 populations (3 individuals/population). Data obtained from nucleotide sequence analysis revealed the following:

- within the *PvDREB6B* sequence, a large number of polymorphic sites were identified, 16, some of them group-specific (Andean/Mesoamerican) (Figure 3.7);
- a part of the identified point mutations in the *PvDREREB6B* sequence produce changes in the protein structure, through modifications of the encoded amino acids. Ten SNPs resulted in amino acid changes in the protein structure, depending on the geographical origin of *Phaseolus vulgaris* L.; thus, isoleucine and glutamine were identified for local bean populations originating from the Andean geographical basin, and histidine and leucine were confirmed for populations originating from the Mesoamerican basin. For the remaining eight nonsynonymous mutations, differences within and between the Andean and Mesoamerican groups (+103, +232, +241, +252, +261 and +262);

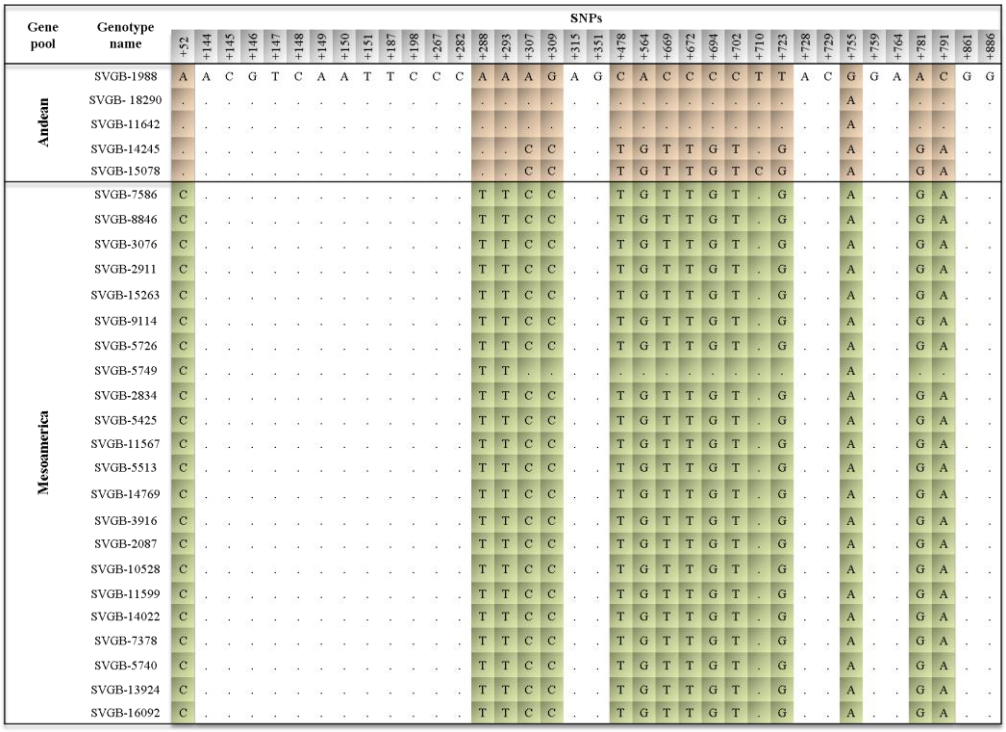


Figure 3.7. Nucleotide sequence alignments of the *PvDREB6B* gene for the 27 local populations of *Phaseolus vulgaris* L. evaluated in the study. SNP variations specific to the Andean genofond were highlighted in orange and those originating from the Mesoamerica basin were marked in green

- on the other hand, a particular aspect is related to the nucleotide sequence pattern of SVGB-14245 and SVGB-15078 populations originating from the Andean gene pool and SVGB-5749 with Mesoamerican origins. At certain loci (+307, + 309, +478, +564, +564, +669, +672, +694, +702, +723,+781, +791) the Andean-origin SVGB-14245 and SVGB-15078 populations show the same type of point mutation as the Mesoamerican populations. And again, the population with Mesoamerican origins,

SVGB-5749, shows SNPs identical to those of populations originating from the Andean geographical basin. This can be explained by the fact that the three populations were formed by the process of hybridization of populations from the two regions of America and thus hybrids that incorporate traits from both genomes were produced.

The cladistic arrangement of the studied samples, according to the *PvDREB6B* sequences, was based on geographical origin (Figure 3.8). Two main clades were generated, the first one being divided into two subclades: the main one consisting of populations originating from the Mesoamerican geographic basin, and the second subclade containing two populations with Andean origins. In the second clade, the populations were divided into two subclades, one of them containing the population SVGB-5749 with Mesoamerican origins and the second subclade containing three populations with Andean origins (SVGB-8290, SVGB-1988, SVGB-11642). The evolutionary history was carried out by the Neighbor-Joining method, genetic distances were determined by the Maximum Composite Likelihood method, and the Bootstrap statistical test (1000 replicates) was applied to determine the degree of confidence.

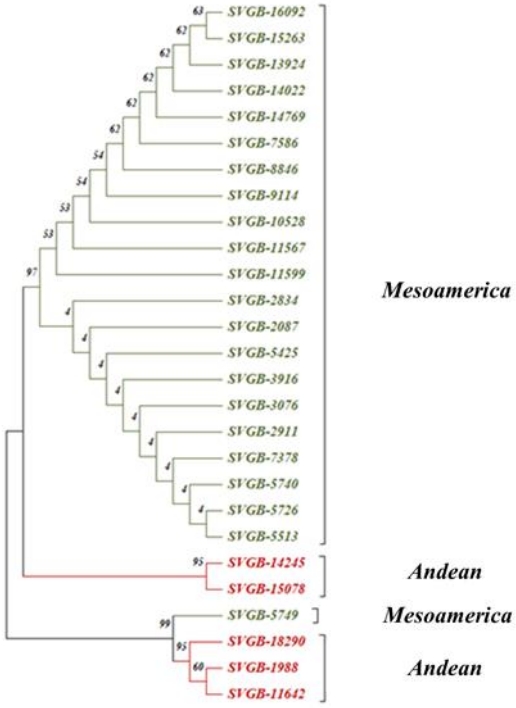


Figure 3.8. Phylogenetic tree - Neighbor-Joining method - *PvDREB6B* gene sequences. Populations labeled in red are from the Andean geographic basin and those in green are from the Mesoamerican geographic

3.6. *PvRPS4 GENE SEQUENCE ANALYSIS*

The PvRPS4 (ribosomal protein S4) gene encodes a protein, the concentration of which increases when the plant grows under water stress.

Following the sequencing reaction, for the PvRPS4 coding sequences, the presence of thymine was identified at the +297 locus for part of the populations evaluated, and adenine was detected for the other populations. By associating these data with the results previously obtained (following sequencing of the PvDREB5A region), it was found that thymine (T) is present in the populations SVGB-1988, SVGB-18290, SVGB-14245, SVGB-1578, SVGB-11642, with origin in the Andean geographical basin. In the same locus, populations originating from the Mesoamerica region show the adenine nucleotide (A). On the other hand, this identified SNP is a nonsynonymous point mutation that causes the modification of the encoded amino acid in the protein sequence. Further studies could show whether this mutation has an effect on the level of resistance of plants to less favorable environmental conditions, such as lack of water (Figure 3.9).

Gene pool	Genotype name	SNP			Amino acid
		+382	+383	+384	+128
Andean	SVGB-1988	T	T	T	F
	SVGB- 18290	.	.	.	.
	SVGB-11642	.	.	.	.
	SVGB-14245	.	.	.	.
	SVGB-15078	.	.	.	.
Mesoamerica	SVGB-7586	.	.	A	L
	SVGB-8846	.	.	A	L
	SVGB-3076	.	.	A	L
	SVGB-2911	.	.	A	L
	SVGB-15263	.	.	A	L
	SVGB-9114	.	.	A	L
	SVGB-5726	.	.	A	L
	SVGB-5749	.	.	A	L
	SVGB-2834	.	.	A	L
	SVGB-5425	.	.	A	L
	SVGB-11567	.	.	A	L
	SVGB-5513	.	.	A	L
	SVGB-14769	.	.	A	L
	SVGB-3916	.	.	A	L
	SVGB-2087	.	.	A	L
	SVGB-10528	.	.	A	L
	SVGB-11599	.	.	A	L
	SVGB-14022	.	.	A	L
	SVGB-7378	.	.	A	L
	SVGB-5740	.	.	A	L
	SVGB-13924	.	.	A	L
	SVGB-16092	.	.	A	L

Figure 3.9. Alignment of *PvRPS4* gene nucleotide sequences and protein sequence amino acids for the 27 local populations of *Phaseolus vulgaris* L. evaluated in the study. The nucleotide/amino acid patterns of the Andean populations were highlighted in orange and those originating from the Mesoamerica basin were labeled in green (L- leucine and F- phenylalanine)

The phylogenetic tree of the 27 populations of *Phaseolus vulgaris* L., based on PvRPS4 sequences, consists of 2 main clades, the first one being formed by populations originating from the Mesoamerica geographic basin, and the second group contains the 5 populations with Andean origins. The evolutionary history was determined by the Neighbor-Joining method, and genetic distances were calculated by the Maximum Composite Likelihood method, also, the Bootstrap statistical test (1000 replicates) was applied to determine the degree of confidence (Figure 3.10).

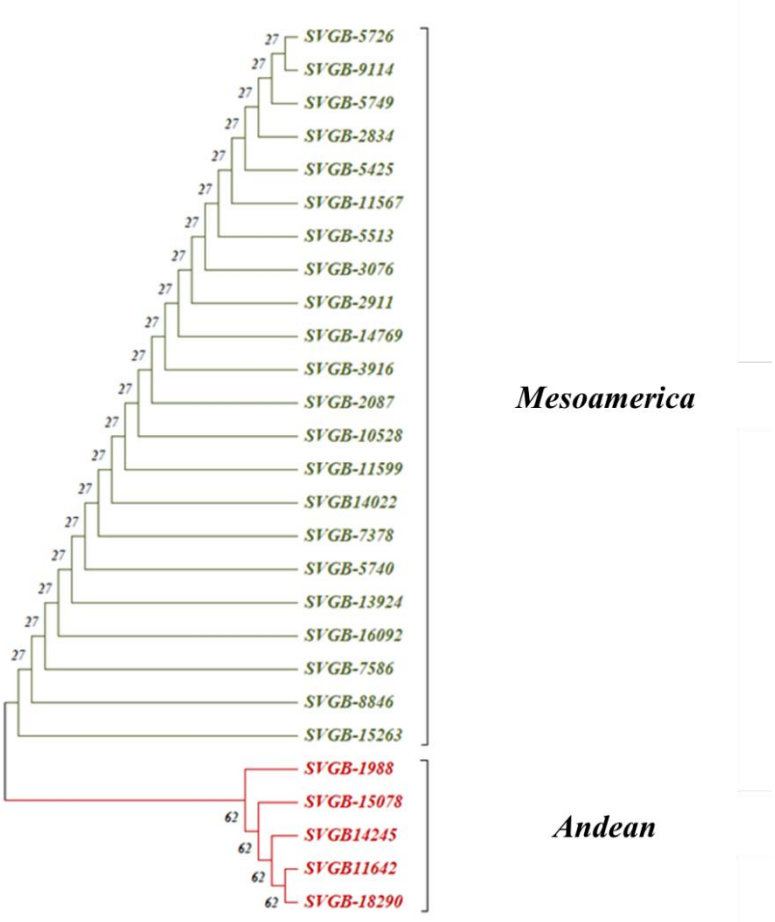


Figure 3.10. Phylogenetic tree - Neighbor-Joining method - *PvRPS4* gene sequences. Populations labeled in red are from the Andean geographic basin and those in green are from the Mesoamerica geographic basin

3.7. *PvLEA3 GENE SEQUENCE ANALYSIS*

The *PvLEA3* sequence is a coding region in the nuclear genome. The data obtained allowed the identification of uninucleotide polymorphisms within the *PvLEA3* coding sequence in *Phaseolus vulgaris* L., as follows (Figure 3.11):

- a single uninucleotide polymorphism could allow the differentiation of populations originating from Mesoamerica from those originating from the Andean geographical

basin. Thus, at locus +38, samples of Andean origin show cytosine, whereas those from the Mesoamerican geographical basin show the nucleotide thymine;

- another SNP was identified at the +23 locus; adenine is present in Andean populations, but also in samples of Mesoamerican origin. The presence of thymine is reported at locus +23 for 50% of Mesoamerican populations;
- two other polymorphisms identified were for loci +847 and +848, only in group originating from the Mesoamerica geographical gene pool;

Gene pool	Genotype name	SNP			
		+23	+38	+847	+848
Andean	SVGB-1988	A	C	G	A
	SVGB- 18290	.	.	.	.
	SVGB-11642	.	.	.	.
	SVGB-14245	.	.	.	.
	SVGB-15078	.	.	.	.
Mesoamerica	SVGB-16092	.	T	.	.
	SVGB-15263	.	T	.	.
	SVGB-14769	.	T	.	G
	SVGB-11567	.	T	.	.
	SVGB-13924	.	T	.	.
	SVGB-5740	.	T	.	.
	SVGB-3916	.	T	A	G
	SVGB-2834	.	T	.	.
	SVGB-3076	.	T	.	.
	SVGB-2911	.	T	.	.
	SVGB-2087	.	T	.	G
	SVGB-14022	T	T	.	.
	SVGB-11599	T	T	A	G
	SVGB-10528	T	T	.	.
	SVGB-9114	T	T	.	.
	SVGB-8846	T	T	.	.
	SVGB-7586	T	T	.	.
	SVGB-7378	T	T	.	.
	SVGB-5749	T	T	.	.
	SVGB-5726	T	T	.	.
	SVGB-5513	T	T	.	.
	SVGB-5425	T	T	.	.

Figure 3.11. Alignment of the nucleotide sequences of the *PvLEA3* gene, for the 27 local populations of *Phaseolus vulgaris* L. Nucleotide patterns of the Andean populations have been highlighted in orange, and those originating from the Mesoamerica basin have been marked in green

The evolutionary history analysis for the 27 populations of *Phaseolus vulgaris* L., based on the PvLEA3 sequence, led to the separation of the samples according to geographical origin. Two main clusters were obtained, the first (lower part of the phylogenetic tree) consisting of populations originating from the Mesoamerican geographic

basin, and the second cluster containing two subclades with genotypes originating from both geographic basins. The evolutionary history was determined by the Neighbor-Joining method and the genetic distances were calculated by the Maximum Composite Likelihood method, and the Bootstrap statistical test (1000 replicates) was applied to determine the degree of confidence (Figure 3.12).

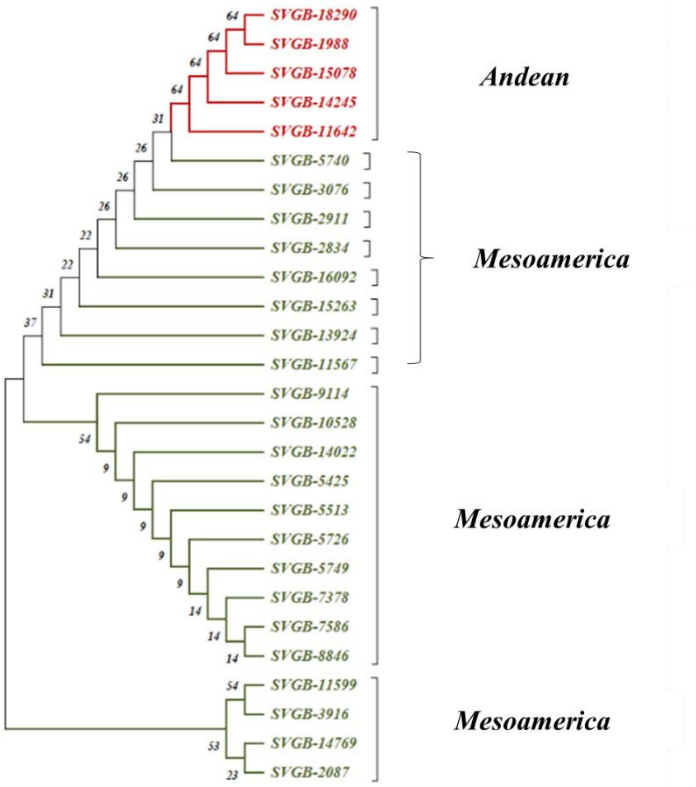


Figure 3.12. Phylogenetic tree - Neighbor-Joining method - *PvLEA3* gene sequences, populations marked in red come from the Andean geographic basin, and those marked in green from the Mesoamerican geographic gene pool

3.8. *rpoC1-rpoC2* INTERGENIC REGION ANALYSIS

The *rpoC1-rpoC2* intergenic region is present in the chloroplast genome and is flanked by the *rpoC1* and *rpoC2* genes. Sanger reaction revealed the following:

- the final length of the sequenced fragment is 930 base pairs;
- seven uninucleotide polymorphisms were identified in the *rpoC1-rpoC2* region (Figure 3.13);
- six of the total SNPs allow the identification of populations with different geographical origins.

Gene pool	Genotype name	SNPs						
		+231	+252	+253	+254	+368	+456	+444
Andean	SVGB-1988	A	T	A	T	T	A	C
	SVGB- 18290	.	.	.	.	.	.	.
	SVGB-11642	.	.	.	.	C	.	.
	SVGB-14245	.	.	.	.	.	.	.
	SVGB-15078	.	.	.	.	.	.	.
Mesoamerica	SVGB-16092	T	A	T	A	.	T	T
	SVGB-15263	T	A	T	A	.	T	T
	SVGB-14769	T	A	T	A	.	T	T
	SVGB-11567	T	A	T	A	.	T	T
	SVGB-13924	T	A	T	A	.	T	T
	SVGB-5740	T	A	T	A	.	T	T
	SVGB-3916	T	A	T	A	.	T	T
	SVGB-2834	T	A	T	A	C	T	T
	SVGB-3076	T	A	T	A	.	T	T
	SVGB-2911	T	A	T	A	.	T	T
	SVGB-2087	T	A	T	A	.	T	T
	SVGB-14022	T	A	T	A	.	T	T
	SVGB-11599	T	A	T	A	.	T	T
	SVGB-10528	T	A	T	A	.	T	T
	SVGB-9114	T	A	T	A	.	T	T
	SVGB-8846	T	A	T	A	.	T	T
	SVGB-7586	T	A	T	A	.	T	T
	SVGB-7378	T	A	T	A	.	T	T
	SVGB-5749	T	A	T	A	.	T	T
	SVGB-5726	T	A	T	A	.	T	T
	SVGB-5513	T	A	T	A	.	T	T
	SVGB-5425	T	A	T	A	.	T	T

Figure 3.13. Nucleotide sequence analysis, for the *rpoC1-rpoC2* intergenic region, for the 27 local populations of *Phaseolus vulgaris* L. SNP Andin were highlighted in orange, and those originating from the Mesoamerica basin are marked in green

The assessed genotypes were separated according to their geographical origin in a phylogenetic tree, thus 2 main groups were obtained. The evolutionary history was determined by the Neighbor-Joining method, the genetic distances were calculated by the Maximum Composite Likelihood method and the Bootstrap statistical test (1000 replicates) was applied to determine the degree of confidence (Figure 3.14).

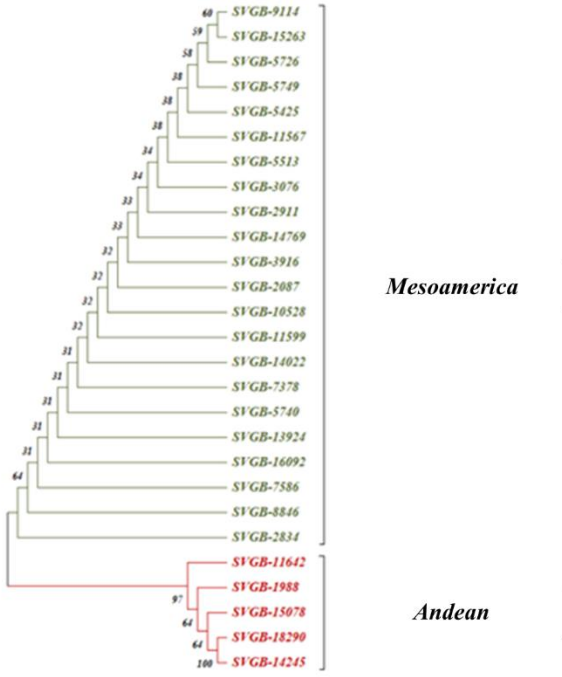


Figure 3.14. Phylogenetic tree- Neighbor-Joining method - *rpoC1-rpoC2* intergenic region sequences, populations marked in red are from the Andean geographic basin, and those marked in green are from the Mesoamerican geographic basin

3.9. *trnL-trnT INTERGENIC REGION ANALYSIS*

The *trnL-trnT* non-coding sequence is an intergenic region in the chloroplast genome flanked by the trnL and trnT genes, respectively. 3 uninucleotide polymorphisms were identified by Sanger sequencing; at+78 locus , populations from the Andean gene pool reported the presence of adenine, and at the same locus, populations from the Central American region reported the presence of thymine. The second uninucleotide polymorphism identified, in the non-coding sequence *trnL-trnT* is at +79 locus , and the third uninucleotide polymorphism revealed is at locus +136 and does not allow differentiation of *Phaseolus vulgaris* L. populations according to geographical origin (Figure 3.15).

Gene pool	Genotype name	SNP		
		+78	+79	+136
Andean	SVGB-1988	A	T	A
	SVGB- 18290	.	.	.
	SVGB-11642	.	.	.
	SVGB-14245	.	.	.
	SVGB-15078	.	.	.
Mesoamerica	SVGB-16092	T	A	.
	SVGB-15263	T	A	.
	SVGB-14769	T	A	.
	SVGB-11567	T	A	.
	SVGB-13924	T	A	.
	SVGB-5740	T	A	.
	SVGB-3916	T	A	.
	SVGB-2834	T	A	.
	SVGB-3076	T	A	C
	SVGB-2911	T	A	.
	SVGB-2087	T	A	C
	SVGB-14022	T	A	.
	SVGB-11599	T	A	.
	SVGB-10528	T	A	.
	SVGB-9114	T	A	.
	SVGB-8846	T	A	.
	SVGB-7586	T	A	.
	SVGB-7378	T	A	C
	SVGB-5749	T	A	.
	SVGB-5726	T	A	.
	SVGB-5513	T	A	.
	SVGB-5425	T	A	.

Figure 3.15. Nucleotide sequence alignments of the *trnL-trnT* intergenic region for the 27 local populations of *Phaseolus vulgaris* L. Andean SNP were highlighted in orange, and those originating from the Mesoamerica basin are marked in green

The evolutionary history of the 27 genotypes belonging to the species *Phaseolus vulgaris* L. can also be deduced by analyzing the phylogenetic trees. The evaluated populations were separated according to their geographical origin into two main groups, the Andean and the Mesoamerican group. The evolutionary history was determined by the Neighbor-Joining method, genetic distances were calculated by the Maximum Composite

Likelihood method, and the Bootstrap statistical test (1000 replicates) was applied to determine the degree of confidence (Figure 3.16).

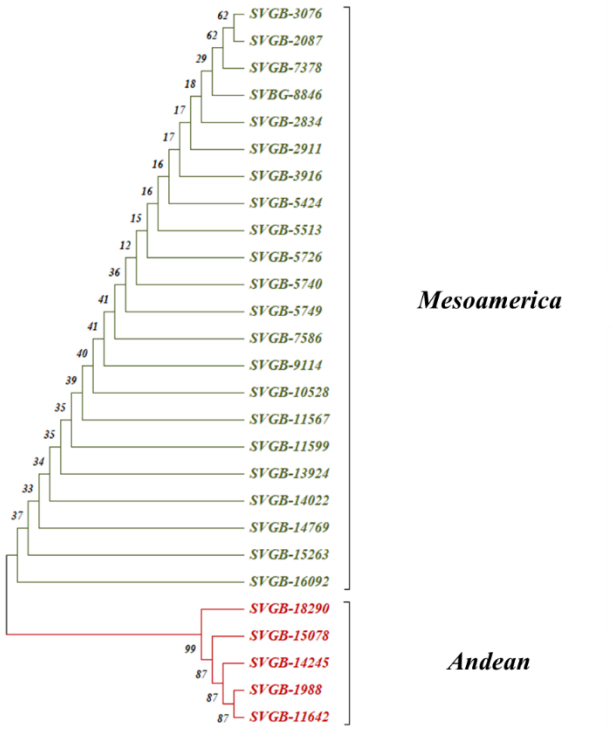


Figure 3.16. Phylogenetic tree - Neighbor-Joining method - *TrnL-trnT* intergenic region sequences, populations marked in red come from the Andean geographic basin and those marked in green from the Mesoamerican geographic gene pool

3.10. PHYLOGEOGRAPHICAL ANALYSIS OF THE *PHASEOLUS VULGARIS* L. SPECIES USING MORPHOLOGICAL PARAMETERS

After weighing M100 seeds, for each of the 27 populations, the following were obtained:

- 29.62% of the populations are represented by large seeds (mass of 100 seeds > 40 g);
- 62.96% are considered medium seeds (the weight of 100 seeds varies between 25-40 g);
- 7.40% of the analyzed populations have small seeds (weight of 100 seeds < 25 g).

If belonging to a geographical area were to be achieved only on to according on morphological assessments, samples SVGB-18290 (M100 = 22.95 g), SVGB-3076 (M100 = 24.21 g) would originate from Mesoamerica (7.40% of the analyzed populations would have Mesoamerican origins), and 92.30% of the populations would have Andean origins (25

populations out of the 27 studied). Establishing the belonging of *Phaseolus vulgaris* L. samples to a certain geographical region can also be achieved by assessing the shape of the seeds, thus those with a cubic (SVGB-16092) and reniform (SVGB-11642) shape are characteristic of the Mesoamerica geographical region. Based only on these characteristics, no relevant statements can be made regarding the geographical origin of the populations of *Phaseolus vulgaris* L. However, the studies carried out at the molecular level, by sequencing specific regions, can confirm or deny the results from the morphological level. Based on the morphological parameters of the plant germplasm, differences were identified between the two groups (Andean/Mesoamerican) (Table 3.1).

Table 3.1. Average values of morphological parameters, for the two groups of *Phaseolus vulgaris* L.

The morphological parameter	Range (Min-Max)	Mean ± SD	Standard error of the mean	The coefficient of variation
Andean gene pool				
Length (mm)	5.19 (11.61 - 16.8)	14.04 ± 2.25	1.01	16.09%
Width (mm)	0.96 (4.55 - 5.51)	509 ± 0.36	0.16	7.25%
Height (mm)	2.57 (5.66 - 8.23)	7.09 ± 1.2	0.54	17.03%
Weight (mm)	0.33 (0.23 - 0.56)	0.38 ± 0.14	0.06	36.53%
Flatness index	0.19 (1.26 - 1.45)	1.36 ± 0.06	0.03	5.01%
The eccentricity index	1.33 (1.84 - 3.17)	2.58 ± 0.53	0.23	20.60%
M100 (g)	27.52 (22.95 - 50.48)	37.74 ± 10.32	4.61	27.35%
Mesoamerica gene pool				
Length (mm)	5.94 (9.86 – 15.8)	12.42 ± 1.9	0.4	15.29%
Width (mm)	2.15 (4.39 – 6.54)	5.26 ± 0.61	0.13	11.61%
Height (mm)	4.16 (6.56 – 10.72)	7.83 ± 1	0.21	12.81%
Weight (mm)	1.71 (0.24 – 1.95)	0.43 ± 0.35	0.07	80.09%
Flatness index	0.24 (1.05 – 1.29)	1.15 ± 0.06	0.01	5.49%
The eccentricity index	2.12 (1.27 – 3.39)	2.3 ± 0.61	0.13	26.73%
M100 (g)	36.79 (24.21 – 61)	34.44 ± 9.19	1.96	26.69%

3.11. MORPHOLOGICAL EVALUATION OF *PHASEOLUS VULGARIS* L. PLANTS DEVELOPED IN DROUGHT CONDITIONS

Drought periods stress plant organisms to take different measures, which can reduce water loss. This is also manifested by changes in tissues such as leaves and roots. An example of this is the movement of the leaves. The leaf roll phenomenon is very common at *Phaseolus vulgaris* L. and is a mechanism for adapting the species to stress (Kadioglu et al., 2012; Lizana et al., 2006; Pastenes et al., 2005). In this case, the leaf roll phenomenon and paraheliotroph movement (grouping of trifoliated leaves) were observed (Figure 3.18).

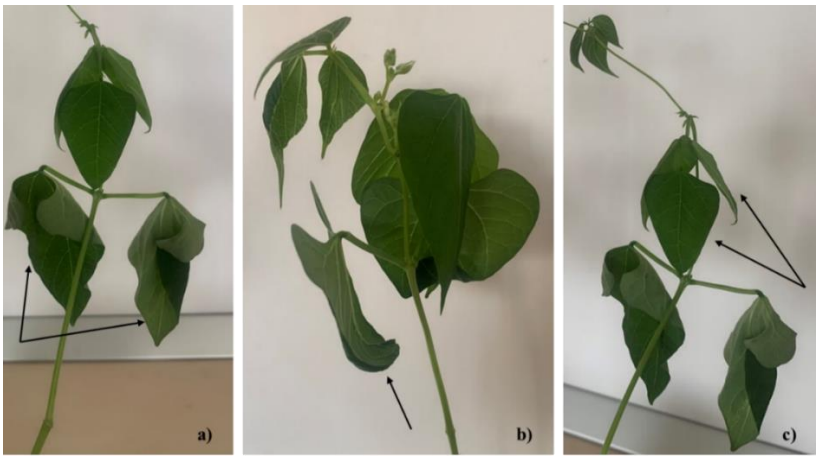


Figure 3.18. Images captured at different stages of the experiment, presenting the changes occurring at the foliar tissue level: a) highlighting the phenomenon of senescence captured on the leaves of the lower floor of the plant, SVGB-2087 variety, after 12 days of water stress; b) leaf rolling, at SVGB-1988 variety, after 12 days of drought; c) paraheliotropic leaf movement of SVGB-2087 variety, after 12 days of drought

Morphological analysis of plants of the SVGB-1988 variety (originating from the Andean geographical gene pool) revealed the presence of flowers, both in the case of irrigated plants, as well as for non-irrigated samples for 8 and 12 days (Figure 3.19). This is a classic phenomenon of adaptation to the lack of water, called “escape from drought”, the plant channels all its energy and all nutrients to complete the cell cycle, allowing the development of the flower and subsequently the fruit (Fang & Xiong, 2015; Shavrukov et al., 2017).

Figure 3.19. The phenotypic study of the SVGB-1988 variety (Andean): a) the control group, irrigated for 30 days; b) the hydric stressed lot, 8 days; c) the control group, irrigated for 34 days; d) the hydrated lot, 12 days

For SVGB-2087 landraces, a less pronounced development of seedlings in the drought stress group was noted for 8 and 12 days, respectively, compared to samples irrigated throughout the experiment (Figure 3.20).

Figure 3.20. Phenotypic study of the SVGB-2087 variety (Mesoamerica): a) control group, irrigated 30 days; b) water-stressed lot, 8 days; c) irrigated control group, 34 days; d) the hydrated lot, 12 days

Although the Lechinta variety is known to have increased drought resistance, by simple morphological analysis of the plants, one can observe a poor development of the unirrigated seedlings compared to those in the control lots (Figure 3.21), where the flower is also developed, and the number of trifoliate leaves is much higher.

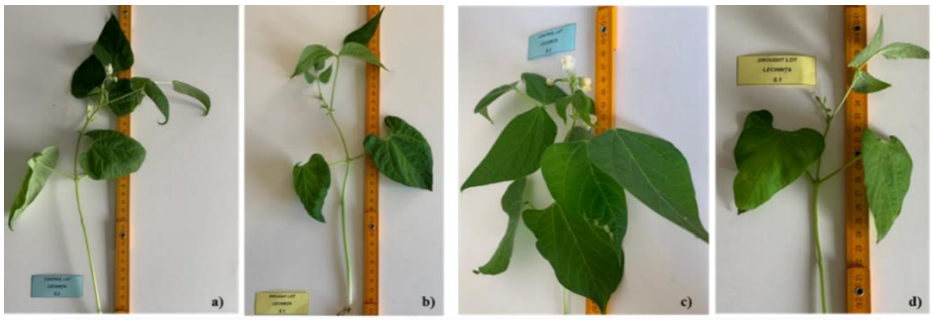


Figure 3.21. Phenotypic study of the Lechinta variety: a control group, irrigated 30 days; b) water-stressed lot, 8 days; c) control lot, irrigated 34 days; d) water-stressed lot, 12 days

Following the determination of some phenotypic parameters for samples of the three variants, differences were observed between the irrigated and non-irrigated lots, greater for the landrace, originating from the Mesoamerican basin, SVGB-2087 and smaller for the population with Andean origins, SVGB-1988 (Figure 3.22).

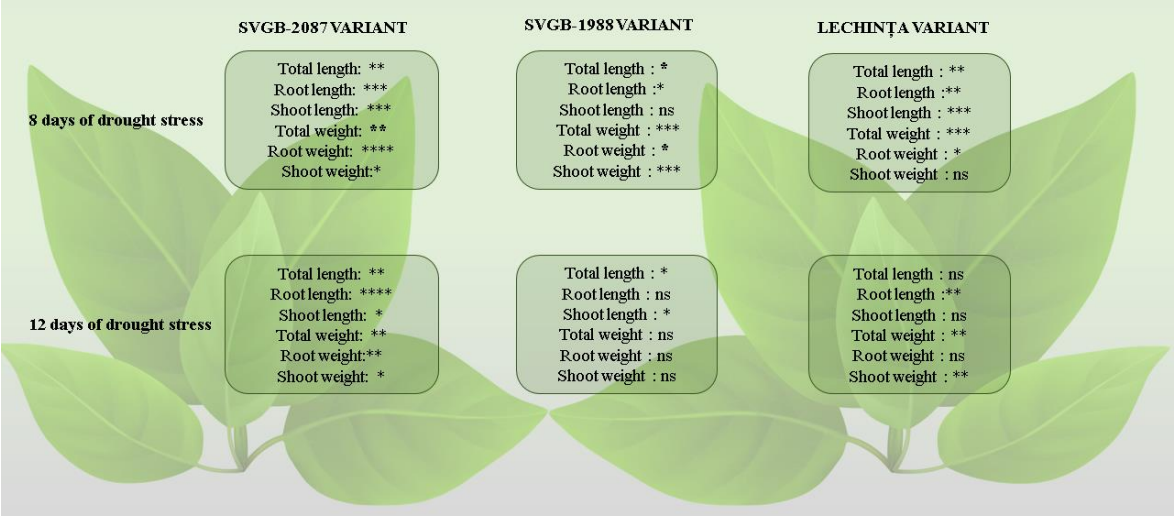


Figure 3.22. The differences obtained between the irrigated and non-irrigated lots of the 3 varieties of the *Phaseolus vulgaris* L. species, following the determination of some morphological parameters and highlighting the statistical significance

3.12. ANALYSES OF ANATOMICAL HYPOCOTYL AND EPICOTYL STRUCTURES

Phenotypic changes, generated by drought stress, are the result of deep physiological and metabolic changes. Thus, the morphoanatomical study of plant tissues can provide valuable information regarding the degree of resistance of *Phaseolus vulgaris* L. varieties to the drought stress. Morphoanatomical evaluations can be used as identification markers for genotypes with superior drought resistance traits.

3.12.1. Analyses of anatomical structures for the SVGB-2087 genotype

In the transverse section through hypocotyl, some differences were identified (Figure 3.23). The differences between the two variants (irrigated and water stressed for 8 days) are quantitative. For the irrigated individual, several secondary free elements were found (marked with yellow arrows), while in the non-irrigated variety the histogenesis process of the conducting tissues stagnates, the supposed reason being the lack of water.

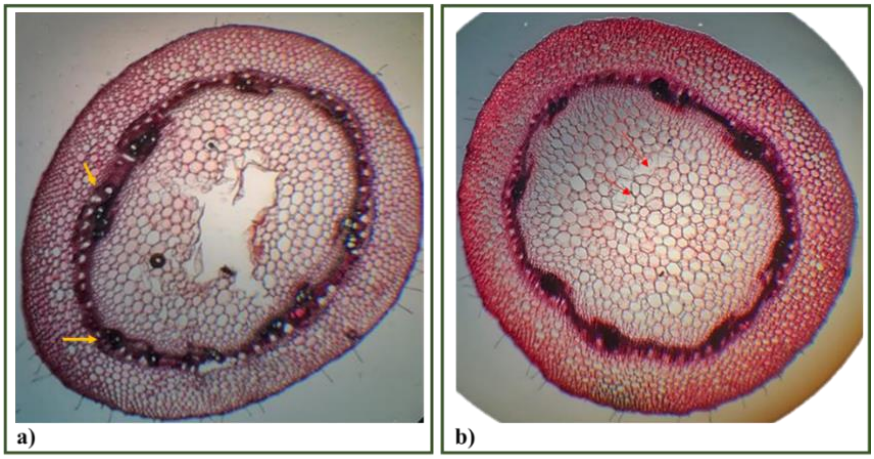


Figure 3.23. Transverse section through the hypocotyl: a) SVGB-2087, control lot (x20); b) SVGB-2087, drought stress lot 8 days (x20)

Furthermore, differences between irrigated and non-irrigates samples were also observed in transversal sections from the epicotyl axis (Figure 3.24). The outline of the cross-sections is similar, in the case of irrigated and drought stressed seedlings, hexagonal-irregular, with 6 ridges alternating with 6 valleys. Conductive tissues are organized mainly in the form of primary free-wooden fascicles, open collateral type, arranged in a circle (20-24 fascicles). The wood vessels in the fascicles of the control lot are visibly larger than those of the fascicles of the non-irrigated lot (marked with yellow arrows); their size is correlated with raw sap flow.

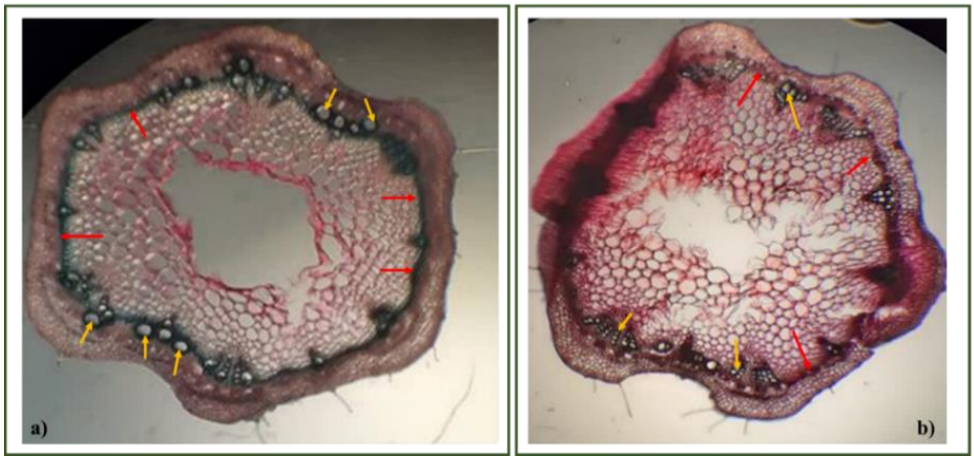


Figure 3.24. Transverse section through the epicotyl: a) SVGB-2087, control lot (x20); b) SVGB-2087, drought stress lot 8 days (x20)

In the transverse section through the axis of the hypocotyl, with regard to the variant SVGB-2087, differences were observed between the control group and the 12-day drought

stressed lot. Thus, the interfascicular cambium is being formed, it is not active, it does not generate anything; the structure is less developed than that of the drought stressed lot, although the plant is morphologically better developed. The interfascicular cambium is very active and the structure of the central cylinder is histologically more advanced than that of the control, although the plant is morphologically less developed (Figure 3.24).

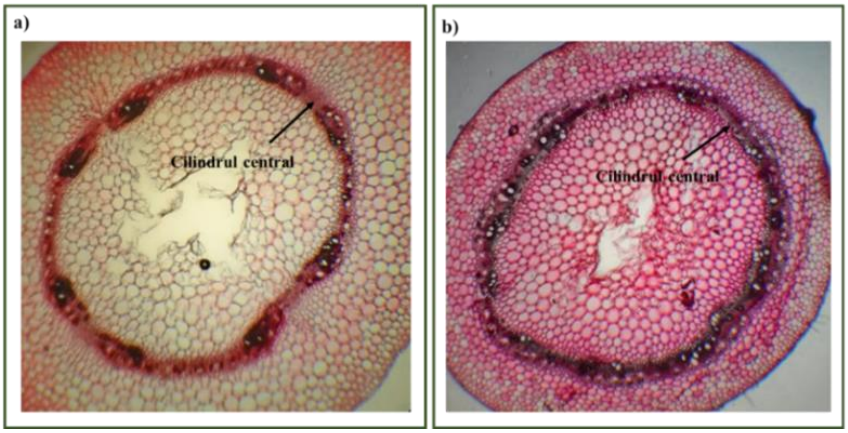


Figure 3.24. Transverse section through the hypocotyl : a) SVGB-2087, control lot (x20); b) SVGB-2087, drought stress lot, 12 days (x20)

A specific phenomenon (Figure 3.25) that can be correlated with water stress is the field of plant response manifested by the generation of adventitious roots, of endogenous origin. Even if it can be supposed objectively, that in the absence of water the plant has no reason to develop its root system, the aspect is valid for what grows in the soil; instead, roots may appear from the aerial, adventitious hypocotyl, as an active strategy to identify possible water resources.

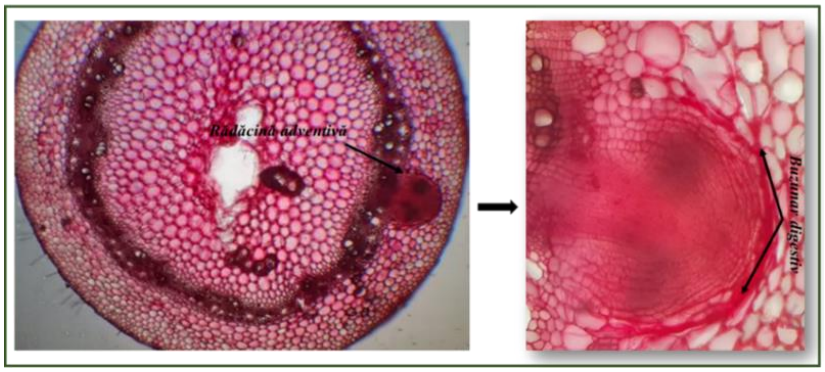


Figure 3.25. Transverse section through the hypocotyl: SVGB-2087, left of the image: batch water stress, 12 days; the formation of an adventitious root (x20); right of the image: drought stress lot, 12 days; formation of an adventitious root (x200)

In the transversal section through the epicotyl axis, a various differences were observed between the irrigated sample (34 days) and the drought stress plants (12 days) (Figure 3.26). The interfascicular cambium is more active in the water-stressed group, generating free secondary elements on the outside, and libriform on the inside. With regard to the development, the drought stress group has the epicotyl axis more advanced from the histological point of view (it is as if they are in a hurry with the edification of the vegetative part in order to have time for the formation of the flower, fruit, seeds). The SVGB-2087 genotype indicates through the structural behavior of the water-stressed variants that it has resistance potential through characteristic structural and morphological responses. răspunsuri structurale și morfologice caracteristice.

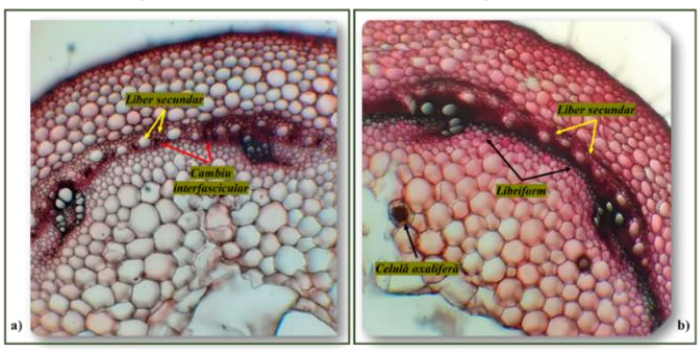


Figure 3.26. Transverse section through the epicotyl: a) SVGB-2087, control lot (x100); b) SVGB-2087, drought stress lot, 12 days (x100)

3.12.2. Analyses of anatomical structures for the SVGB-1988 genotypes

The development of some adventitious roots was found, both in the case of the irrigated sample and drought stress one, which have the role of contributing to the improvement of the hydration status of the plant (Figure 3.27). The genesis of adventitious roots originates in the interfascicular cambium with the participation of secondary free and pericyclic elements.

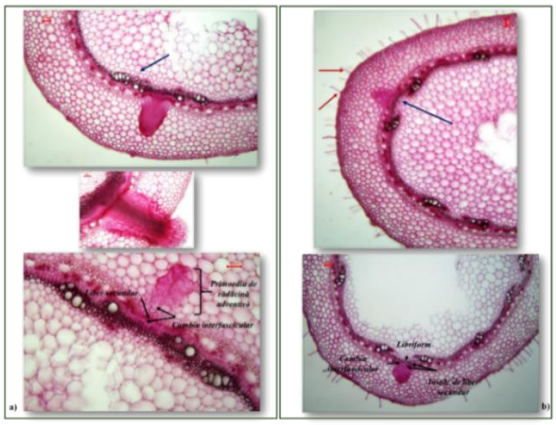


Figure 3.27. Transverse section through the hypocotyl: a) SVGB-1988, control lot (x20, x100); b) SVGB-1988, drought stress lot, 8 days (x20, x100)

In the transversal section of the epicotyl, a different arrangement of the free-woody bundles was observed, for the irrigated and the non-irrigated seedlings. Thus, for the genotype irrigated for 30 days, the initial, heteromorphic primary free-woody bundles present a disposition of the wood vessels in a uniseriate manner. On the other hand, in the non-irrigated variety, for 8 days, the initial, primary free-wooden bundle has a disposition of the wood vessels in the shape of the character V (Figure 3.28).

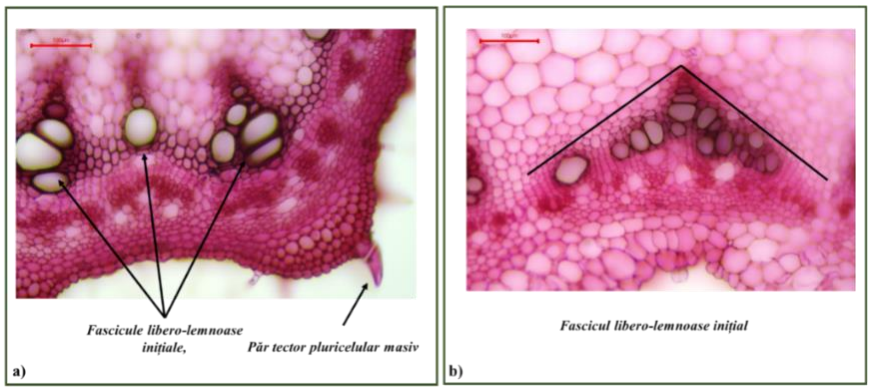


Figure 3.28. Transverse section through the epicotyl: a) SVGB-1988, control lot (x100); b) SVGB-1988, drought stress lot, 8 days (x100)

In the transversal section, the morphoanatomical study showed numerous differences, between the irrigated and non-irrigated variants, at the level of the hypocotyl (Figure 3.29). With regard with the irrigated SVGB-1988 genotype, a tendency to disorganization of cells in the parenchymal cortex was observed. This phenomenon was determined by the accumulation of additional tissues from the inside, produced by interfascicular cambium and intrafascicular cambium. Regarding the non-irrigated genotype, different aspects were highlighted in Figure 3.29 b) compared to those of the control group. Blue arrows mark

disorganized parenchymal cortex cells, while yellow arrows represent elements of the non-lignified sclerenchymatous pericycle.

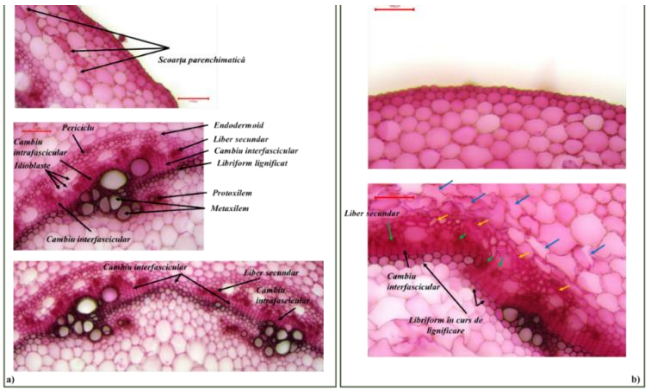


Figure 3.29. Transverse section through the hypocotyl: a) SVGB-1988, control lot (x100, x200); b) SVGB-1988, drought stress lot, 12 days (x100, x200)

Differences were also identified at the epicotyl level for the two variants of the SVGB-1988 genotype (Figure 3.30). In the control variant, the green arrows (a and b) indicate the formation of secondary wood vessels by the intrafascicular cambium, located in the in close proximity of the metaxylem vessel in the primary wood. Elsewhere, the interfascicular cambium is the most active, producing outwardly secondary free islands and secretory idioblasts, and inwardly, lignified libriform. In the drought stress variant (d), the green arrow indicates the formation by the intrafascicular cambium of secondary wood vessels, located in the extension of the last large metaxylem vessel, slightly flattened vessels and with slightly thickened but non-lignified walls. In places (blue arrow), vessels of secondary wood produced by the interfascicular cambium appear, next to the lignified libriform. Through the above, we can state that the SVGB-1988 genotype responds very well to water stress.

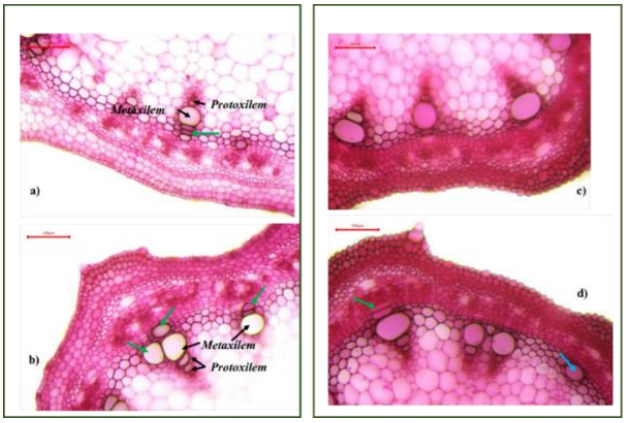


Figure 3.30. Transverse section through the epicotyl: a) SVGB-1988, control lot (x100); b) SVGB-1988, drought stress lot, 12 days (x100)

3.12.3. Analyses of anatomical structures for the Lechința genotype

Regard with the not irrigated variant for 8 days, differences and aspects were reported that suggest that the Lechința genotype is resistant to drought, through numerous adaptation mechanisms that it achieves. In Figure 3.31 a), the interfascicular cambium is particularly active in the process of tracheogenesis, even if it is a matter of water stress. It can be considered as a special feature of the Lechința genotype, which reacts well to water stress. The central air cavity is smaller, basically, the cells are kept as a source of water (with vacuolar localization).

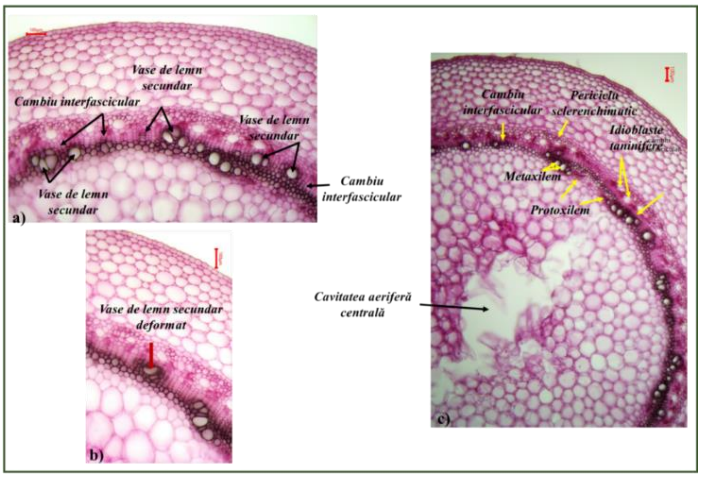


Figure 3.31. Transverse section through the hypocotyl: a) Lechința, drought stress 8 days, (x100); b) Lechința, drought stress 8 days, (x100); c) Lechința, drought stress 8 days (x100);

The transverse sections through the structure of the epicotyl allowed the highlighting of some points iregard with the irrigated and non-irrigated geotypes (Figure 3.32). The outline of the cross-sections is circular-ovate in the control version and circular-ribbed in the hydraulically stressed version. Similarly, the non-irrigated genotype has very long, multicellular peri tectors at the level of the epidermis; in the control variant they are rarer. The number of secretory idioblasts is much lower in the water stressed variant than in the control. The marrow disorganizes in both variants, almost equally strongly and intensely.

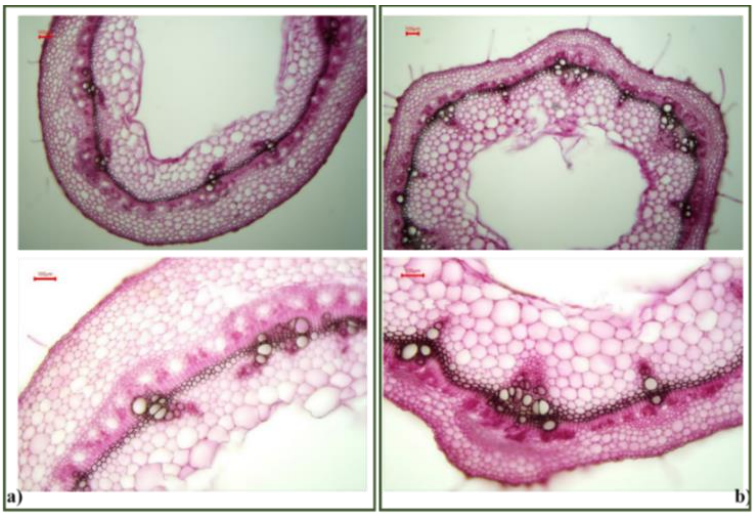


Figure 3.32. Transverse section through the epicotyl: a) Lechința, control lot, (x20); b) Lechința, drought stress lot, 8 days (x20)

In Figure 3.33, the black arrows highlight the difference between the libriform produced by the interfascicular cambium of the control variant and the drought stress lot one: a pluristratified, intensely lignified libriform in the control variant and a uni-bilayered, weakly lignified libriform in the water-stressed variant.

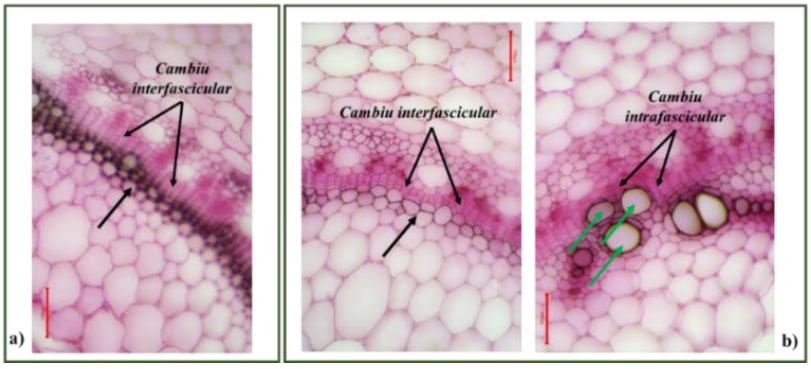


Figure 3.33. Transverse section through the hypocotyl: a) Lechința, control lot (x200); b) Lechința, drought stress lot, 12 days (x200)

In Figure 3.34 the red arrows indicate the area of interfascicular cambium which is very poorly represented and which differentiates almost entirely outwards in free secondary. Conductive fascicles are entirely of primary structure, as generated by procambium having wood vessels (protoxylem and metaxylem) and wood parenchyma cells with thin cellulose-pectic walls.

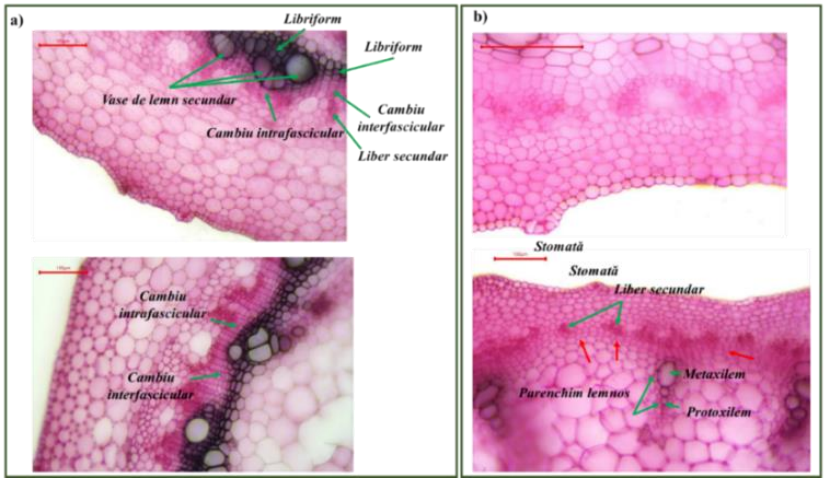


Figure 3.34. Transverse section through the epicotyl: a) Lechința, control lot; b) Lechința, drought stress lot, 12 days (x100)

3.13. ANALYSES OF THE CONTENT OF ASSIMILATORY PIGMENTS

When plants do not have a sufficient water, they will undergo a series of changes, not only at the morphological level, but also at the biochemical level. In this category can be included the changes occurring during photosynthesis processes, changes in the concentration of some substances involved in the osmotic regulation mechanisms, as well as some antioxidant enzymes and phytohormones. In this case, the concentration of chlorophyll a, chlorophyll b and carotenoids in the leaf tissue of the three genotypes was determined. The data showed that the largest differences between the experimental groups were reported for the landraces originating from the Mesoamerica geographic basin, SVGB-2087, and the smallest differences, in the case of the Lechința genotype (Figure 3.35).

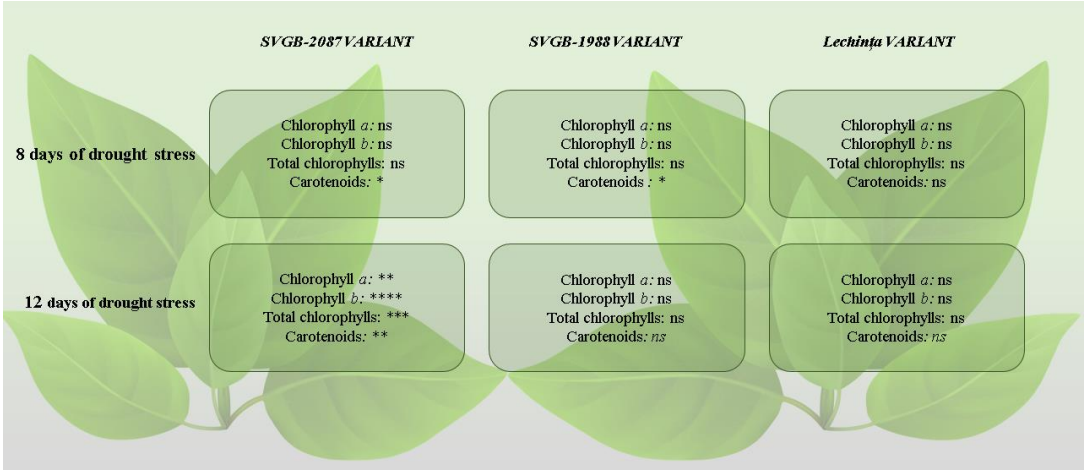


Figure 3.35. Differences obtained between the irrigated and non-irrigated lots of the 3 *Phaseolus vulgaris* L. varieties, following the determination of some biochemical parameters and highlighting the statistical significance

3.14. ANALYSES OF PHYSIOLOGICAL PARAMETERS OF COMMON BEAN SAMPLES EXPOSED TO DROUGHT

The determination of a few plant physiological parameters , such as the relative water content (RWC) and the relative growth rate, can be exploited in order to identify the varieties of the *Phaseolus vulgaris* L. species with increased resistance to the water stress factor. The results obtained in this study were different depending on the variety evaluated, but also depending on the intensity of the applied stimulus (Figure 3.36).

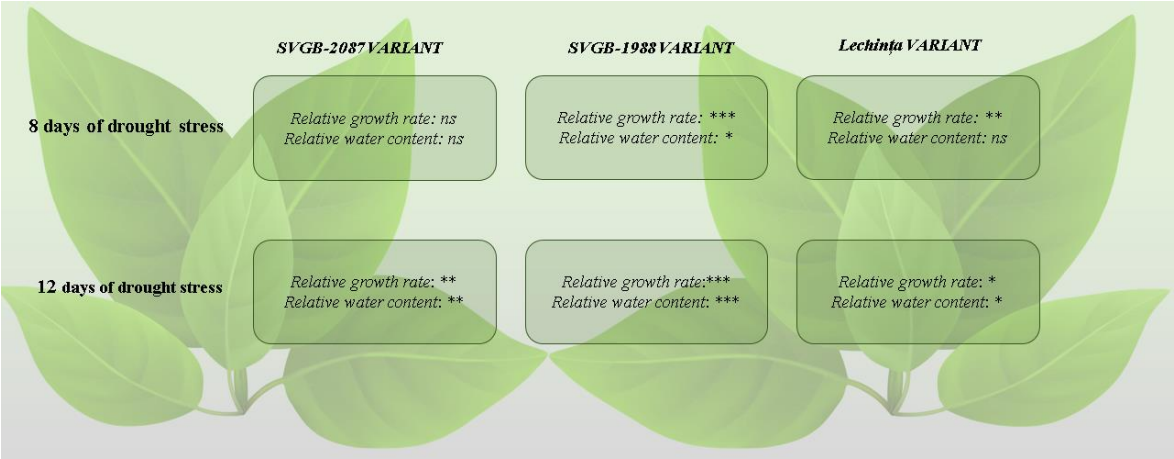


Figure 3.36. Differences achieved between the irrigated and non-irrigated lots of the 3 Phaseolus vulgaris L. varieties, following the determination of some physiological parameters and highlighting the statistical significance

3.15. ANALYSES OF GENE EXPRESSION

In the current study, the expression of 14 genes was analyzed, of which 13 are involved in growth and development, but also in the response to water stress of the *Phaseolus vulgaris* L. species and a reference (housekeeping) gene: *PvABA'8H, PvDREB1, PvDREB2, PvDREB6B, PvERF, PvLEA3, PvMYB03, PvMYB07, PvMYC, PvP5CS10, PvPP2C-12, PvWRKY53, PvWRKY57* and *PvACT* (housekeeping).

3.15.1. Analyses of functional genes involved in drought stress response

Genes involved in the response to water stress are classified into two categories: functional genes and regulatory genes. In the first category are included genes in this synthesis product is directly involved in the response to stress.

3.15.1.1. Relative expression of the *PvLEA3* gene

After 8 days of drought stress, differences were reported between the experimental groups, thus the *PvLEA3* gene was more intensely expressed in the root tissue, compared to the leaf tissue, for all the variants studied. By exposing the plants to 12 days of drought stress, higher values of *PvLEA3* gene expression were observed in the leaf, for the SVGB-2087 variant, and in the root, for the Lechința genotype. In the root tissue, after 12 days of drought, the relative expression of *PvLEA3* gene was downregulated for the SVGB-2087 population (Figure 3.36).

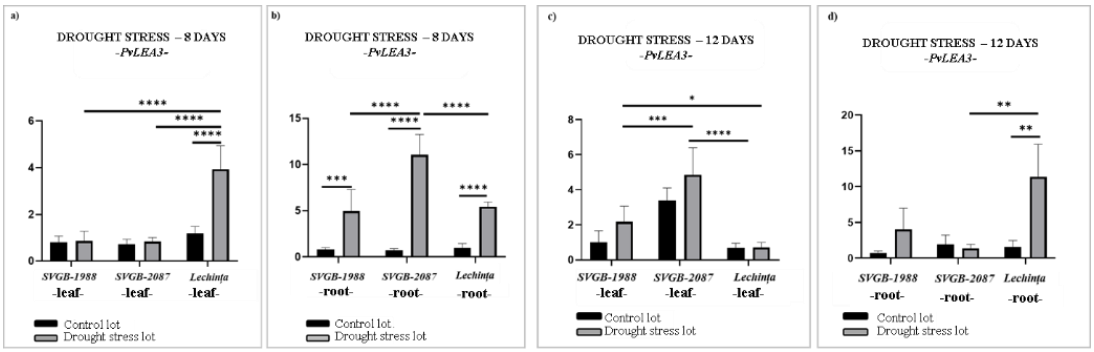


Figure 3.36. The relative expression of the *PvLEA3* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: $p>0.05$ (ns); $p\leq0.05$ (*); $p\leq0.01$ (**); $p\leq0.001$ (***); $p\leq0.0001$ (****)

3.15.1.2. Relative expression of the *PvP5CS10* gene

Δ1-Pyrroline-5-carboxylate synthase or *P5CS10* is a functional gene controlled by the ABA-dependent pathway, and its expression increases under abiotic stress conditions. After 8 days of drought, higher *PvP5CS10* gene expression values were recorded in both leaf and root tissue for the SVGB-2087 genotype. On the other hand, the 12 days of water stress allowed the recording of different expression values, higher in the leaf for the SVGB-2087 population and for the Lechința genotype in the root tissue (Figure 3.37).

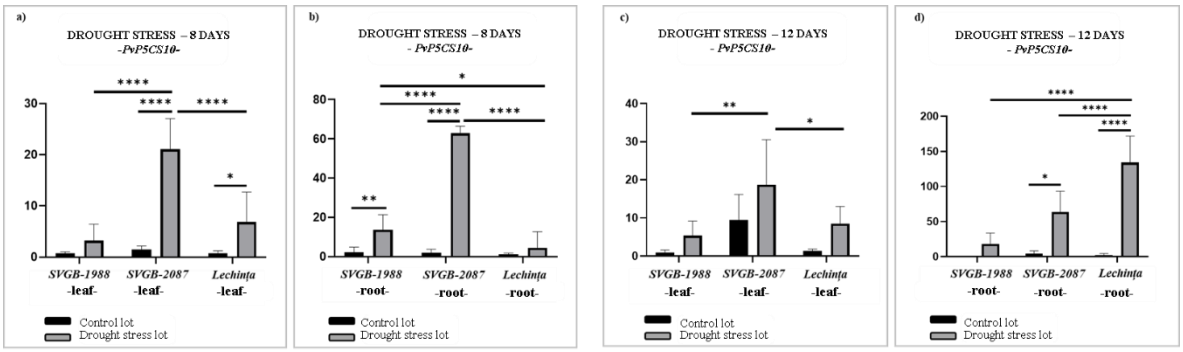


Figure 3.37. The relative expression of the *PvP5CS10* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2. Analyses of regulatory genes involved in drought stress response

When plants grow and develop in a less favorable environment, they trigger certain mechanisms that allow them to survive. Thus, at the molecular level, the activity of functional and regulatory genes becomes more intense. Regulatory genes are represented by synthesis products responsible for signal transmission, but also transcription factors, which are involved in the activation of some functional genes. The two categories of genes are indirectly involved in the response to abiotic stress.

3.15.2.1. Relative expression of the *PvABA'8H* gene

ABA or abscisic acid is a phytohormone with numerous implications in plant physiological processes. During periods of water stress, abscisic can produce various changes at the physiological, molecular or biochemical level, through which the plant can adapt and survive in the absence of water. The *PvABA'8H* gene encodes abscisic acid 8-hydroxylase, an enzyme that participates in the abscisic acid catabolism reaction. The relative gene expression of *PvABA'8H* was upregulated in the leaf tissue, compared to the root tissue, for all three variants studied, after the 8 days of water stress. After 12 days of drought, the expression of the gene was more pronounced regard the SVGB-1988 genotype, both in the leaf tissue and in the root, the lowest values being observed for the variety Lechința in the leaf and for SVGB-2087 in the root (Figure 3.38).

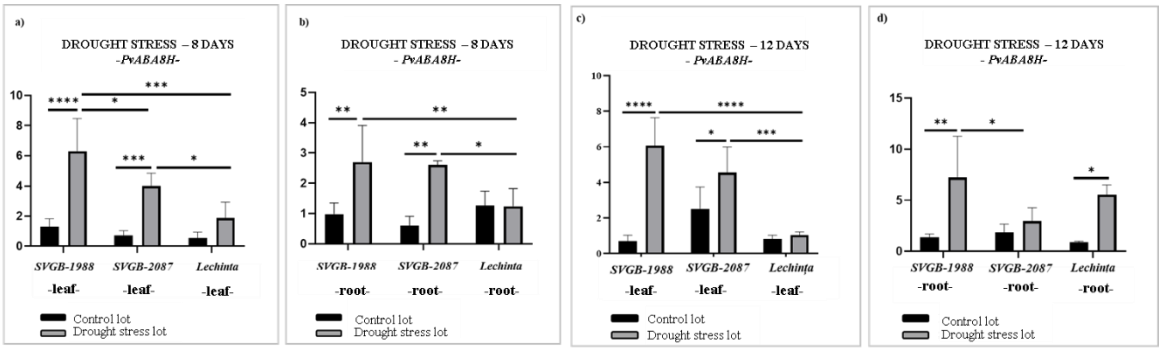


Figure 3.38. The relative expression of the *PvABA'8H* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.2. Relative expression of the *PvPP2C-12* gene

PP2C phosphatase is known to negatively regulate the ABA-mediated response. By exposing the three genotypes to 8 days of water stress, a decrease in expression was observed for the Lechința genotype in both tissues (leaf/root) and an increase in the SVGB-1988 and SVGB-2087 variants in the leaf and in the root for SVGB -1988. By intensifying the water stress (12 days of drought), the expression of the *PvPP2C*-12 gene was downregulated for the three genotypes, in the leaf tissue and increased in the root tissue for Lechința and SVGB-2087 (Figure 3.39).

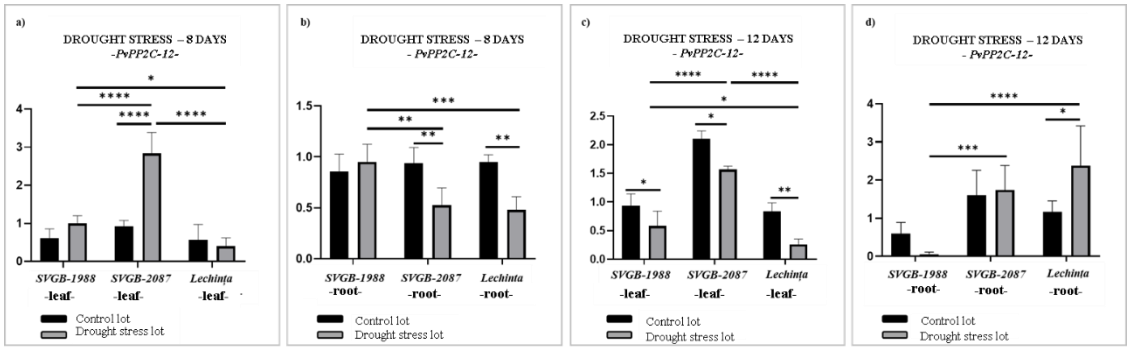


Figure 3.39. The relative expression of the *PvPP2C-12* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.3. Relative expression of the *PvDREB1* gene

DREB1 (*Dehydration Responsive Element Binding*) is a transcription factor whose expression increases when plants are exposed to abiotic stress. In this study, the expression of the *PvDREB1* gene was analyzed for the three variants, so after 8 days of drought, increases in the expression in the leaf tissue and decreases in the root tissue were recorded, for all genotypes studied. By extending the drought period, the *PvDREB1* gene expression value was downregulated, again, for all three variants in the root and upregulated in the leaf tissue for the SVGB-2087 and Lechința genotypes (Figure 3.40).

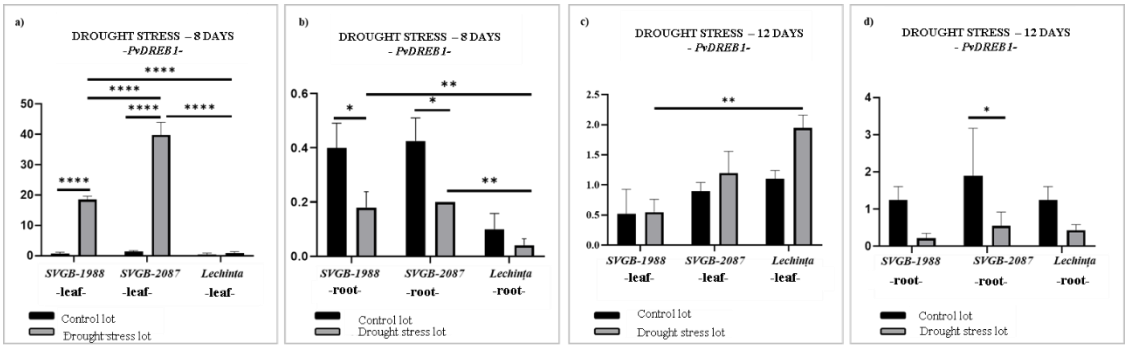


Figure 40. The relative expression of the *PvDREB1* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: $p>0.05$ (ns); $p\leq0.05$ (*); $p\leq0.01$ (**); $p\leq0.001$ (***); $p\leq0.0001$ (****)

3.15.2.4. Relative expression of the *PvDREB2* gene

After 8 days of drought stress, different values of *PvDREB2* gene expression were recorded for the three genotypes, depending on the evaluated tissue. Increased expression was observed in the leaf and lower expression values in the root tissue. The 12 days of drought had a different effect on *PvDREB2* gene expression, especially at the root level, where the relative expression of gene was upregulated for SVGB-2087 and Lechința genotypes (Figure 3.41).

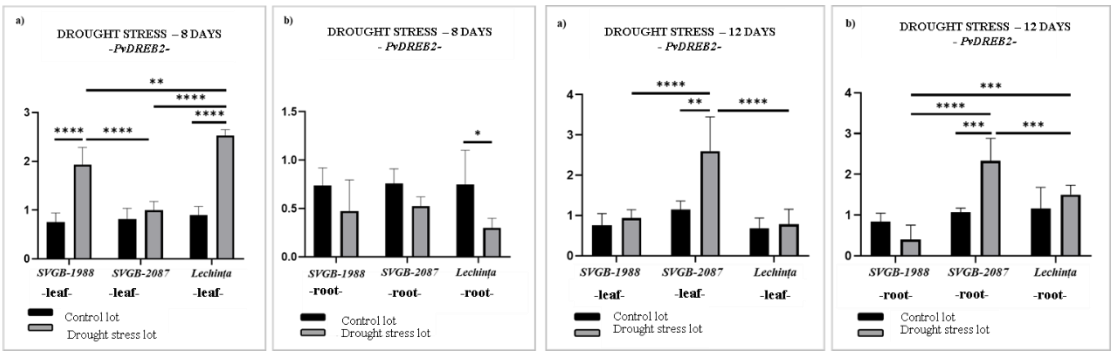


Figure 3.41. The relative expression of the *PvDREB2* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.5. Relative expression of the *PvDREB6* gene

PvDREB6 gene expression was quantified after 8 and 12 days of water stress in both leaf and root tissue. Thus, after 8 days of water stress, the expression of the *PvDREB6* gene was downregulated for all the variants studied in the root and leaf tissue, except for the Lechința variety, which has a slight upregulated in expression at the leaf level. For the second experiment (12 days of drought), decreases in the expression of the *PvDREB6* gene were observed for the three genotypes in the leaf and root tissue, with the exception of the Lechința variant, which recorded a significant increase in the expression of the *PvDREB6* gene in the root (Figure 3.42).

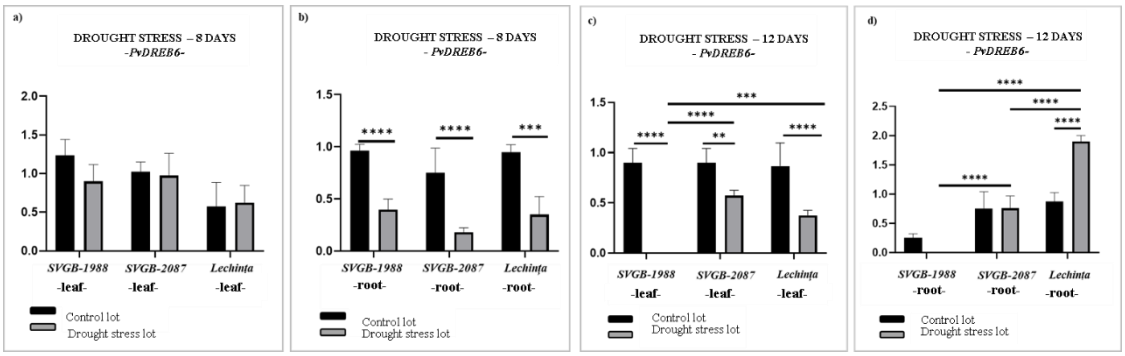


Figure 3.42. The relative expression of the *PvDREB6* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.6. Relative expression of the *PvERF* gene

Ethylene Responsive Factor (ERF) is a transcription factor involved in various physiological processes, plant growth and development, but also in the processes of resistance to abiotic and biotic stress factors (Fatma et al., 2022). After 8 days of drought, the expression of the *PvERF* gene was downregulated in the root tissue for the three genotypes and upregulated in the leaf tissue level for the varieties Lechința and SVGB-1988. Similar results could also be observed by exposing the plants to 12 days of water stress, generally reporting decreases in expression, except for the Lechința variety, in the leaf tissue (Figure 3.43).

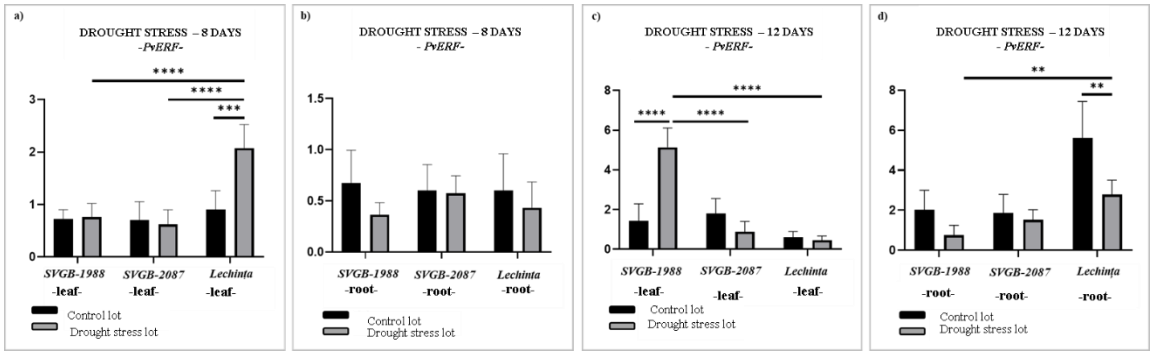


Figure 3.43. The relative expression of the *PvERF* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: $p>0.05$ (ns); $p\leq0.05$ (*); $p\leq0.01$ (**); $p\leq0.001$ (***); $p\leq0.0001$ (****)

3.15.2.7. Relative expression of the *PvMYB03* gene

MYB transcription factors have an essential role in the processes of development, senescence, but also in the response of plants to abiotic stress factors, such as drought (Li et al., 2019). By exposing the 3 varieties of the *Phaseolus vulgaris* L. species to water stress for 8 days, the expression of the transcription factor *PvMYB03* was downregulated in the root and leaf tissue level for the SVGB-1988 variety and upregulated in the leaf level for the Lechința genotypes. SVGB-2087. By intensifying the stimulus (12 days of drought), increases in the expression values of the *PvMYB03* gene were recorded, both in leaf and root tissue, with small exceptions, regarding the Lechința and SVGB-2087 populations, at leaf and root level (Figure 3.44).

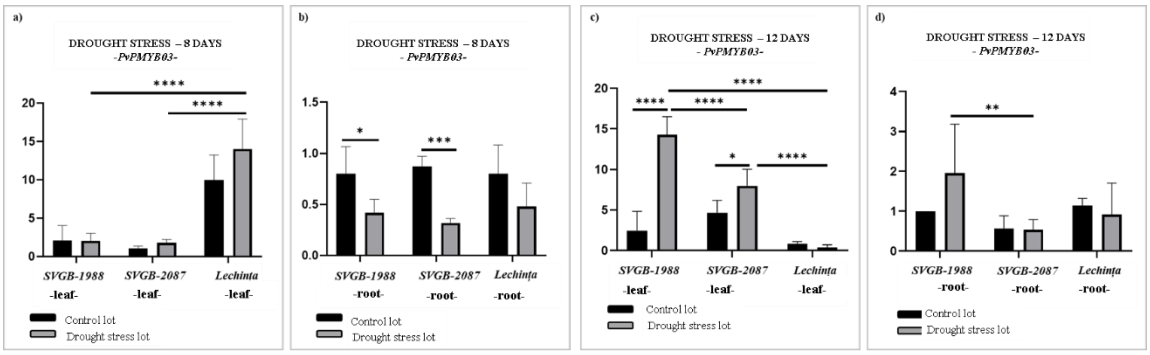


Figure 3.44. The relative expression of the *PvMYB03* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.8. Relative expression of the *PvMYB07* gene

After exposing the samples to 8 days of water stress, an upregulated expression of the *PvMYB07* gene was observed for all three genotypes except SVGB-2087 and Lechința at the root level. Distinct results were obtained after extending the drought period up to 12 days. The relative expression of *PvMYB07* gene expression was upregulated in both tissues, leaf and root (Figure 3.45).

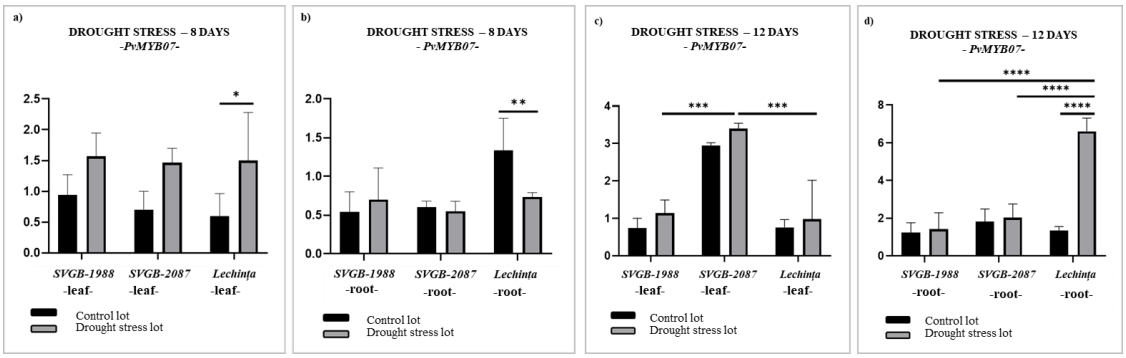


Figure 3.45 The relative expression of the *PvMYB07* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.9. Relative expression of the *PvMYC* gene

MYC or *Myelocytomatosis oncogenes* is a transcription factor that is responsible for regulating numerous physiological processes in plants, such as growth and development, senescence process in leaf tissue, root elongation, stamen development, seed production, protein accumulation in seeds, and chlorophyll degradation process (Chen et al., 2019). After 8 days of drought stress the expression of the transcription factor *PvMYC* was downregulated for all three genotypes in root and upregulated in leaf tissue for SVGB-1988 and SVGB-2087 variants. The 12 days of drought had a different impact on *PvMYC* gene expression, with increases only in the Lechința genotype (Figure 3.46).

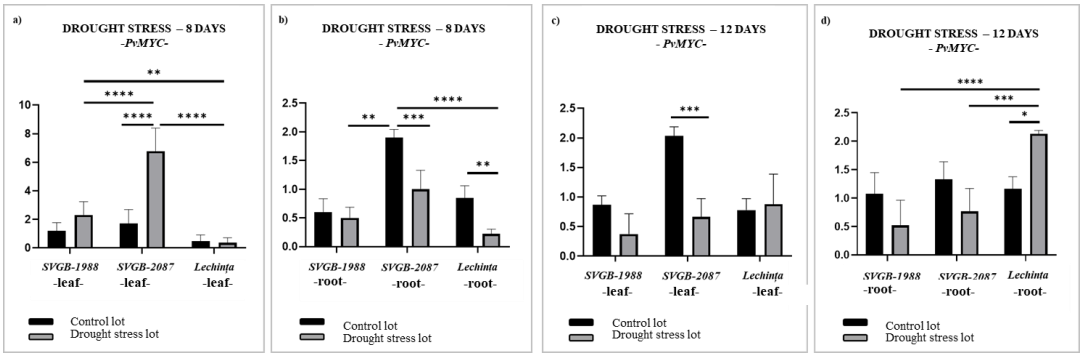


Figure 3.46. The relative expression of the *PvMYC* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.10. Relative expression of the *PvWRKY53* gene

WRKY is a large family of genes encoding WRKY-like proteins involved in various biological processes. Following 8 days of water stress, *PvWWRKY53* gene expression for SVGB-2087, Lechința and SVGB-1988 variants was upregulated in leaf and root tissue, except for the Lechința genotype, for which expression was downregulated in root tissue. After 12 days of drought, there was a decrease in expression in both root and leaf tissue in the two local populations studied, except for the Lechința variety, where *PvWRKY53* gene expression increased in both tissues (Figure 3.47).

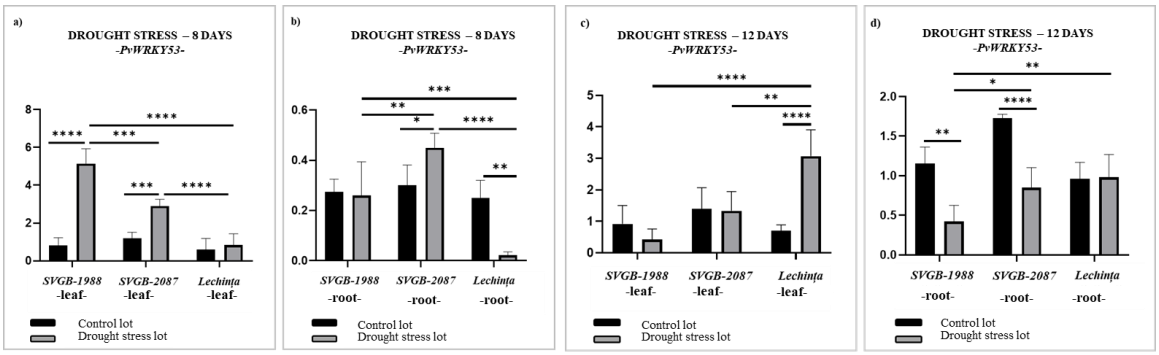


Figure 3.47. The relative expression of the *PvWRKY53* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

3.15.2.11. Relative expression of the *PvWRKY57* gene

Subjecting the three variants of *Phaseolus vulgaris* L. to 8 days of drought had a different impact on *PvWRKY57* gene expression, depending on the tissue evaluated. Thus, there was an increase in expression at leaf tissue level and a decrease at root level for all variants. Intensification of the applied stress (12 days drought) induced different expression values, depending on the studied variant and the analyzed tissue. Increased expression was reported for genotype SVGB-2087 in leaf tissue and Lechința in root tissue; in the other cases *PvWWRKY57* gene expression decreased (Figure 3.48).

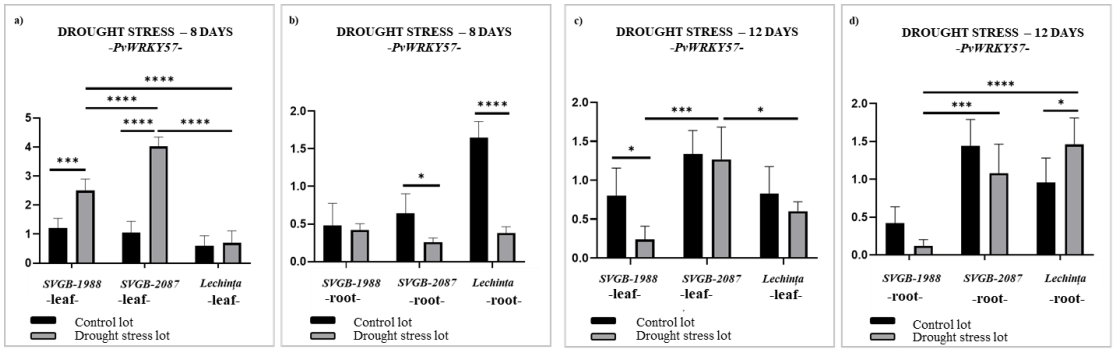


Figura 3.48. The relative expression of the *PvWRKY57* gene, after 8 and 12 days of drought - for the varieties SVGB-1988, SVGB-2087 and Lechința: a) leaf tissue; b) root tissue; c) leaf tissue; d) root tissue. The bar represents the mean value for five biological replicates, while the error bar indicates the standard deviation of the data variation. Differences between groups are expressed statistically, where: p>0.05 (ns); p≤0.05 (*); p≤0.01 (**); p≤0.001 (***); p≤0.0001 (****)

The results achieved by quantifying the expression of the 13 genes involved in growth, development and response to water stress for the three genotypes can also be seen in Figure 3.49.

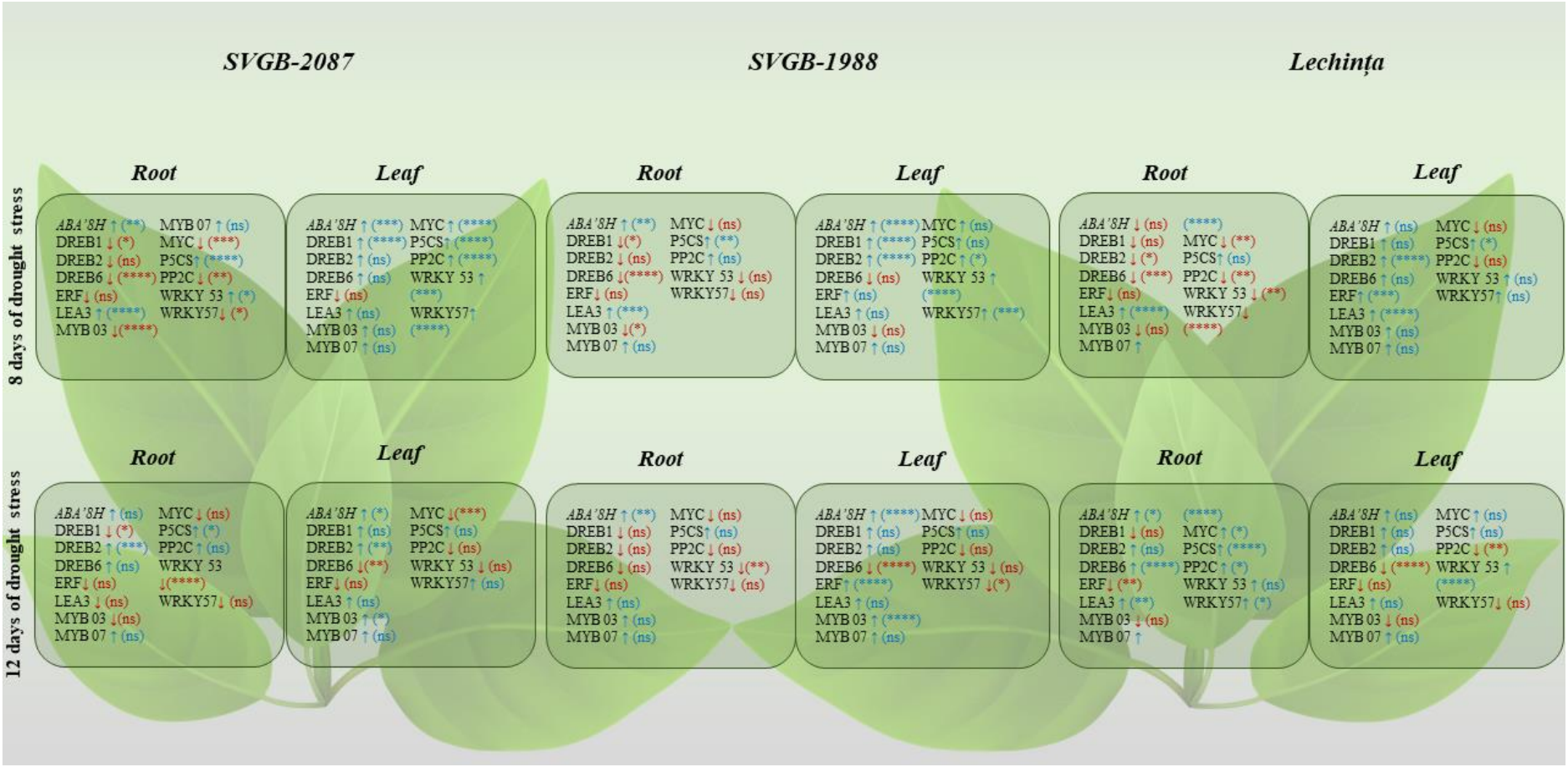


Figure 3.49. The differences obtained at molecular level between irrigated and non-irrigated lots for 8 and 12 days, following the determination of the relative expression of some genes involved in growth, development and response to drought stress factor, for the three varieties studied (SVGB-2087, SVGB-1988 and Lechința) and highlighting their statistical significance

CONCLUSIONS

The first part of the thesis involved a detailed literature review, which subsequently allowed the formulation of ideas:

1. Over the years, humankind has exploited different plants to its own advantage, selecting and adapting them to personal needs. Thus, starting with wild species, the process of evolution and selection by humans has given rise to local varieties of crops of interest; later, necessity made it necessary to create varieties and hybrids;
2. Plant biodiversity is threatened by human intervention, climate change, but also by declining people's interest in *on farm* conservation and maintaining local crop biodiversity;
3. The conservation of landraces and wild relatives of crop plants, whose survival is threatened and at risk of extinction, has become a primary activity;
4. The Food and Agriculture Organization (FAO) considers *Phaseolus vulgaris* L. to be the world's most important legume crop;
5. Drought stress is one of the most important factors limiting productivity in the agricultural sector and its effects are severe. Drought leads to losses of up to 70% of the production of *Phaseolus vulgaris* L. worldwide, thus affecting food security.

In this thesis, the research material was represented by the species *Phaseolus vulgaris* L., and the main aim of the first practical part of the study was to analysis the phylogenetic relationship between landraces collected from different counties of the Romania, as well as the phylogeographic analysis of the selected samples. The conclusions of the research work are as follows:

6. Assessment of the *PvDREB5A* sequences allowed to classify the samples according to their geographical origin. Therefore, 5 of the evaluated landrace originate from the Andean gene pool and the others show polymorphisms specific to the Mesoamerican area;
7. Through the assessment of the *PvRPS4* coding gene, a uninucleotide polymorphism (nonsynonymous mutation) specific to geographic origin was identified, which can be used in biogeographic studies as a marker to identify geographic origin. This has not been mentioned in the literature so far;
8. The *PvDREB6B* gene showed the highest nucleotide diversity, with some of the uninucleotide polymorphisms being characteristic of the geographical region. Also, some populations showed SNPs characteristic of both basins; this may suggest that, at some

point during the evolutionary processes, the hybridization phenomenon between Andean and Mesoamerican populations was realized;

9. The *trnT-trnL* and *rpoC1-rpoC2* intergenic regions are characterized by lower nucleotide variability and different according to geographical origin;

10. The *PvLEA3* region of the nuclear genome allowed the cladistic distribution of the 27 samples within a phylogenetic tree according to geographical origin;

11. Morphological studies cannot clearly and objectively reveal the geographical origin of *Phaseolus vulgaris* L. genotypes, so they must always be supported by molecular studies for confirmation and validation.

The second practical part of the thesis aimed to identify the response reactions to water stress of local populations of the species *Phaseolus vulgaris* L., with different geographical origins. The conclusions on plant resistance to water stress are:

12. Phenotypic determinations indicated that Lechința and SVGB-1988 (originating from the Andean geographical basin) are the most drought resistant cultivars, while SVGB-2087 shows a much lower adaptability to water scarcity;

13. Quantification of assimilatory pigments, such as chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids revealed that Lechința and SVGB-1988 are the most drought resistant varieties compared to SVGB-2087, a genotype with Mesoamerican origins;

14. Microscopic analysis of cellular structures showed that the Lechința genotype is the most drought resistant, followed by SVGB-1988 (from the Andean basin), while the least adaptation mechanisms to drought were reported for the variety from the Mesoamerica basin, SVGB-2087;

15. Analysis of the physiological parameters such as RGR and RWC revealed the same facts, the variety SVGB-2087 is the least resistant to water scarcity;

16. Quantification of the genes expression involved in growth, development and response to drought stress has led to a different classification of *Phaseolus vulgaris* L. genotypes according to their resistance to drought stress, thus Lechința genotype is the most resistant, followed by SVGB-2087 and SVGB-1988.

The phylogenetic relationships between distinct *Phaseolus vulgaris* L. genotypes, data on geographical origin, as well as the identification of variants with favorable response to abiotic stress factors, allow for further research on:

- Assessing a significant number of samples which belongs to *Phaseolus vulgaris* L. species and other species in the Plant Genebank Suceava collection from a phylogenetic and phylogeographic perspective;
- Correlating uninucleotide polymorphisms identified in coding regions with response to water stress;
- Assessment of production yield of variants with increased drought adaptability;
- Extending the stress resistance studies of *Phaseolus vulgaris* L.

Publications:

1. „Assessment of the Geographic Origin of Romanian Common Bean (*Phaseolus vulgaris* L.) Landraces Using Molecular Markers and Morphological Traits", by Paula-Maria Galan, Livia-Ioana Leti, Silvia Strajeru, Denisa-Elena Petrescu, Mirela-Mihaela Cimpeanu, Alina-Carmen Tanasa, Dan-Marius Sandru and Dragos-Lucian Gorgan, *Agronomy* 2023, 13(11), 2820; I.F. 3,7. https://doi.org/10.3390/agronomy13112820.
2. „Comparative Effects of Water Scarcity on the Growth and Development of Two Common Bean (*Phaseolus vulgaris* L.) Genotypes with Different Geographic Origin (Mesoamerica/Andean)", by Paula-Maria Galan, Lacramioara-Carmen Ivanescu, Livia-Ioana Leti, Maria Magdalena Zamfirache and Dragoș-Lucian Gorgan, *Plants* 2024, 13(15), 2111; I.F. 4. https://doi.org/10.3390/plants13152111.

National Scientific Events:

1. „Phylogenetic and phylogeographic study of *Phaseolus vulgaris* L. from the collection of the Plant Genetic Resources Bank "Mihai Cristea" Suceava". National Symposium "Sustainable use of agricultural biodiversity" at the Plant Genetic Resources Bank "Mihai Cristea" Suceava – 30 mai 2022. Authors: Galan Paula-Maria, Leți Livia-Ioana, Gorgan Dragoș-Lucian. Oral presentation.
2. "Phylogenetic and phylogeographic evaluation of the species *Phaseolus vulgaris* L. from the collection of the Plant Genetic Resources Bank Mihai Cristea Suceava". Annual session of scientific reports and communications - Agricultural Research and

Development Center Secuieni. 2023. Authors: Galan Paula-Maria, Leți Livia-Ioana, Gorgan Dragoș-Lucian. Oral presentation.

3. „The effects of drought stress on the development of plants belonging to P*haseolus vulgaris* L. species, with different geographical origins, Mesoamerica and Andean". Scientific Communication Session "Mihai Cristea" Plant Genetic Resources Bank Suceava - 2023. Authors: Galan Paula-Maria, Leți Livia-Ioana, Gorgan Dragoș-Lucian. Oral presentation.
4. „Identification of *Phaseolus vulgaris* L. genetic variants with different geographic origins (Mesoamerica/ Andean) showing positive response to drought under global warming". ICAR Anniversary Conference, Academy of Agricultural and Forest Sciences Gheorghe Ionescu-Șișești Bucharest, 30 mai 2024. Authors: Galan Paula-Maria, Leți Livia-Ioana, Străjeru Silvia, Gorgan Dragoș-Lucian. Oral presentation.
5. „Identification of *Phaseolus vulgaris* L. genetic variants with a favorable response to drought stressors associated with global warming",, SSFB 2023, Iași. Authors: Galan Paula-Maria, Leți Livia-Ioana, Gorgan Dragoș-Lucian, Ivănescu Lăcrămioara-Carmen, Zamfirache Maria-Magdalena, Străjeru Silvia, Petrescu Denisa-Elena. Poster presentation.

Member of national and international projects:

1. 01/09/2020 – În curs. “INCREASE - Colecții Inteligente de Resurse Genetice de Leguminoase Alimentare pentru Sistemele Europene de Produse Agroalimentare”. Proiect European.
2. 01/03/2020 – 01/12/2022. “Ameliorarea de Precizie a cultivarelor de grâu cu importanță agronomica ridicată” (PN-III-P2-2.1-PED-2019-0175). Proiect Național.
3. 01/11/2022 – 01.10.2023. “Suport educațional și formativ pentru doctoranzi și tineri cercetători în pregătirea inserției în piața muncii” (POCU/993/6/13/153322). Proiect Național.
4. 01/07/2023 – În curs. “Studiul efectelor tratamentelor cu apă activată de plasmă asupra proceselor de germinație la temperaturi scăzute și standard și dezvoltarea speciei *Zea mays*, prin evaluări morfologice, biochimice și ale expresiei genice”; ADER 1.3.4. Proiect Național.

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