PhD THESIS

Genetic characterization of several provenances of Silver fir (*Abies alba* Mill.) from Romania and their response to abiotic stress factors

(SUMMARY OF THE PhD THESIS)

PhD student: **Irina Maria Todea (Morar)**

Scientific supervisors: **Professor Radu E. Sestraș, PhD**
Professor Monica Boscaiu, PhD

INTRODUCTION

Climate change has been a growing problem for both the environment and humanity in recent years. Over time, plants have been exposed to different types of stress, which limit their growth and development, as well as their productive capacity and yield (NARESHKUMAR *et al*., 2020).

The forests decline is steadily increasing worldwide, and forest species with long life cycles cannot adapt to the rapid effects of climate change. Coniferous forests are the ecosystems considered most sensitive to the effects of global warming. There are conifer species that have a high genetic diversity, as well as a phenotypic plasticity that allows existing populations to tolerate climate change that occurs in their natural habitats. Otherwise, there is a risk that those populations which are unable to cope with the new environmental conditions will suffer greatly and disappear over time.

Drought and soil salinity are considered the most adverse and critical environmental factors for plants, causing massive losses in agricultural production worldwide and, at the same time, substantially affecting the distribution of wild species in nature (RAZA *et al*., 2019; ZHOU *et al*., 2019). Plants can respond to abiotic stress, such as water deficiency or saline stress, by activating certain physiological, biochemical, and molecular reactions, even when their metabolism, growth and development are affected (BARTELS and RAMANJULU, 2005; MBARKI *et al*., 2018). The study of these responses and the mechanisms of drought and salinity tolerance is currently one of the major topics of research in plant biology.

The genus *Abies* belongs to the family Pinaceae, subfamily Abietoideae. In the past this genus has been widely studied from the point of view of taxonomy (PARDUCCI *et al*., 1996) and it includes over 40 species of trees widespread especially in the mountain regions of the northern hemisphere (CLINOVSCHI, 2005). *Abies alba* Mill. is a species described for the first time in 1759 by a Scottish botanist, Philip Miller (1691-1771). The species is known as the European silver fir or common fir, or *abeto* in Spanish. Silver fir is one of the most important forest trees in Central Europe, especially in the mountainous regions, being a very important species for economic and ecological reasons (DOBROWOLSKA, 2008). Silver fir – as other conifers - does not grow naturally in saline environments, but can be affected by relatively high salt concentrations in stands near mountain roads, due to the common practice in several European countries and in Romania, of road de-icing in winter, using large amounts of NaCl (FLÜCKIGER and BRAUN, 1981; SCHIOP *et al*., 2015).

Because of the identification and evaluation of reliable biochemical stress markers will significantly contribute to elucidating the mechanisms of drought and

salinity tolerance, this doctoral study aims to identify stress indicators associated with the general responses of the silver fir to water stress and salt stress, based on plants evaluation at a very young stage, in the one-year seedling stage.

In this proposed study there were analysed the genetic diversity of nine Romanian silver fir (*A. alba*) populations, as well as the response to water and salt stress of one-year-old silver fir seedlings. Information related the genetic diversity of silver fir populations was obtained using SSR markers.

STRUCTURE OF THE THESIS

The PhD thesis entitled *"Genetic characterization of several provenances of Silver fir (Abies alba Mill.) from Romania and their response to abiotic stress factors",* counts 160 pages and is written in compliance with the rules of elaboration and writing style imposed for doctoral theses at national level and the internal norms of USAMV-CN. The thesis is structured in two parts, containing 8 chapters, 15 tables, 41 figures and graphs and 322 bibliographic references.

The first part of the doctoral thesis, focused on the current state of knowledge in the issues pursued in the thesis, is structured in 3 important chapters. The **first chapter** summarizes the information on the evolution of the species *Abies alba* and its spread in Europe and in our country. **Chapter 2** includes general aspects of the *Abies alba* species, respectively the systematic classification, botanical description, ecology of the species, stress factors, but also the genetic characterization of this species. **Chapter 3** presents aspects regarding the impact of abiotic factors and their ecological effect on the fir, respectively the plants response to abiotic stress and the main biochemical parameters that indicate the reaction and tolerance of plants under stress conditions.

The second part of the thesis is allocated to personal contribution, being structured in five chapters. In this part, the main results obtained during the doctoral studies are presented, which have been exploited by publishing in specialized journal. Three articles were published in peer-reviewed journals, indexed in recognized academic databases, including Clarivate (ISI WoS), all with impact factor (IF), two of which were in ISI journals in Q1 and Q2. In **Chapter 4** is presented the purpose and objectives pursued in the research. The aim of this research was to identify useful information on genetic differences among silver fir populations with different geographical origins from our country and to identify certain physiological and biochemical mechanisms underlying silver fir resistance or tolerance to water stress and salt excess in the soil.

Considering the purpose of the research, the following objectives were established: the analysis of genetic diversity, respectively genotyping of nine *A. alba* populations

from different geographical areas of Romania, using SSR molecular markers; evaluation of the morphological, physiological and biochemical reaction of silver fir seedlings to water and salt stress; identification of the main physiological and biochemical mechanisms underlying the resistance or tolerance of fir seedlings to water and salt stress and identification of possible genetic resources with appropriate response to the action of abiotic stress factors, represented by the drought and salt excess in the soil.

Analysis of the morphological, physiological, and biochemical response to water and salt stress of *A. alba* Mill. seedlings were performed through the following parameters:

a) The main elements of plant growth.

b) Water content and soil electroconductivity.

c) Genotype-induced changes (silver fir populations) and applied stress treatments (water and salt) on photosynthetic pigments: chlorophyll a, chlorophyll b and total carotenoids.

d) Ion content in roots and needles: calcium, sodium, potassium, and chlorine.

e) Osmolytes quantification: proline and total sugars.

f) Malondialdehyde (MDA).

g) Non-enzymatic antioxidants: phenols and flavonoids.

h) Enzymatic activity of antioxidants: superoxide dismutase, catalase, ascorbate peroxidase and specific glutathione reductase activities.

Chapter 5 presents the materials and methods used to identify the morphological and biochemical response of fir seedlings to water and salt stress, the influence of vernalization on the germination capacity of fir seeds, and the methodology used to analyse genetic diversity, respectively genotyping nine populations of *A. alba* from different geographical areas of Romania, using SSR molecular markers.

RESULTS AND DISCUSSIONS

Chapter 6 presents the results obtained in doctoral research, in the form of scientific articles published in specialized journals. The chapter consists of three subchapters, each representing a research issue according to the proposed objectives and materialized in the form of an article published in an ISI journal.

The first topic referred to the genetic relationships between different Romanian fir populations (*Abies alba* Mill.), their reproductive features and their implications on the sustainable management of forest resources. The researches highlighted the existence of a significant variability for the morphological characters of the cones and seeds from the nine studied populations. An obvious variation was registered among the populations also regarding the germination capacity of the seeds, revealing the fact that

the genotype significantly influences the germination capacity of silver seeds. In addition, germination was influenced by the way in which the seeds were vernalized before sowing. There were no significant differences between the genetic distances between populations and the geographical distances between them, even if the regression line had an upward trend. AMOVA analysis showed that there is little differentiation between populations, with only 9% of the total variance attributable to population differences.

Responses to water deficit and salt stress in silver fir seedling was a research whose results were published in the second article. For most plant species, the determination of growth parameters is often used to assess the effects of stress on them. This approach is not as useful for slow-growing species as conifers, including fir, especially in the first years of life (TODEA *et al*., 2019; GANANÇA *et al*., 2018). Nevertheless, after one month of water and salt stress treatments, the research detected a significant inhibition of growth in one-year-old silver fir seedlings. Water and salt stress caused a significant reduction in chlorophyll a and b in silver fir needles. Salt stress caused the accumulation of relatively high concentrations of Na^+, Cl^- and Ca_2^+ in roots and needles, in parallel to the increasing NaCl concentration in the irrigation solution. A significant increase in proline concentration was also detected in response to saline stress, even though the differences from the control plants were relatively small. Therefore, the contribution of proline to osmotic adjustment under saline stress conditions may be relatively small. However, this does not exclude the role of proline in the salt tolerance mechanisms of *A. alba* seedlings, based on its additional "osmoprotective" functions. On the contrary, regarding water stress, proline does not seem to play an important role, at least under our experimental conditions.

Soluble carbohydrates play functional roles in the plant's response to abiotic stress. The experiments showed a significant increase in TSS levels under water deficit and salt stress conditions, which suggests the participation of these osmolytes in the response of fir seedlings to both drought and salinity stresses. Both water stress and salt stress seem to cause some degree of oxidative stress in silver fir seedlings. According to the results obtained, it was found that the only antioxidant system that appears to be substantially involved in the response of fir seedlings to water stress, according to the specifics of the experimental conditions used, is superoxide dismutase (SOD). The activities of the other three enzymes assayed, catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) and the content of phenols or flavonoids in the needles did not reveal significant changes in the resulting values. On the contrary, salt stress caused significant increases in the specific activity of all enzymes tested, except for CAT, as well as levels of total phenolic compounds and flavonoids. In all cases, however, the observed stress-induced activation of antioxidant systems was relatively

weak, as indicated by data correlated with the low degree of oxidative stress observed in the MDA content.

The physiological response of some silver fir (*Abies alba*) populations to water and salt stress was part of the research whose results were published in the third article. Under our experimental conditions, one-month treatments applied to the one-year-old silver fir seedlings were sufficient to determine relatively significant reductions of some important parameters for biomass, such as: stem length, root length or number of needles, compared to the control plants, not exposed to the two stress factors. A significant reduction in the weight of fresh needles, as a response to increased salt concentrations or water stress treatment, was also observed in part due to needle dehydration, caused by stress in all populations studied.

Population 6 (Valea Morii) appeared to be the most tolerant to water stress, when comparing the fresh weight of silver needles and their water content with the control plants. However, the apparently higher drought tolerance of this population should be viewed with caution, as seedlings from this source had a slow growth rate in the control treatment; probably, the effects of water stress largely depend on the size of the seedlings, smaller plants showing a smaller (relative) reduction in growth. Similarly, population 7 (Gârda Seacă) was the most tolerant to lowest concentration (100 mM NaCl).

CONCLUSIONS

Chapter 7 presents the conclusions drawn from the obtained data:

Conclusions regarding the genetic relationships and reproductive traits of Romanian populations of silver fir (*Abies alba*): implications for the sustainable management of local populations:

- A significant variability was found for the morphological characters of the silver fir cones and seeds from the nine studied populations;

- The variation also existed among populations related to the germination seeds capacity;

- The germination capacity was also affected by the way in which the seeds were stored before sowing, respectively by the type of vernalization of the seeds;

- There was no statistically assured relationship between the genetic distances among populations and their geographical distances;

- The AMOVA (analysis of molecular variance) showed that 65% of variability is among individuals and 25.9% within individuals, due to heterozygosity at individual loci. There is a small percentage of the total variation due to differences among populations, respectively only 9.1% of the total variation is attributed to these differences.

The results obtained in this research were published as follows:

TODEA (MORAR), I. M., RENSEN, S., VILANOVA, S., BOSCAIU, M., HOLONEC, L., SESTRAS, A. F., VICENTE, O., PROHENS, J., SESTRAS, R.E., PLAZAS, M., 2020, Genetic Relationships and Reproductive Traits of Romanian Populations of Silver Fir (*Abies alba*): Implications for the Sustainable Management of Local Populations, Sustainability 12(10):4199 [IF 2.576, Q2].

https://www.mdpi.com/2071-1050/12/10/4199

Conclusions regarding the effect of water deficit and salt stress on Silver fir (*Abies alba*) seedlings:

- After only one month of water and salt stress, there was identified a significant inhibition of growth in one-year-old silver fir seedlings;

- Water and salt stress caused a significant reduction in chlorophyll a and b in the silver fir seedlings;

- Saline stress caused the accumulation of relatively high concentrations of Na^+, Cl^- and Ca_2^+ in roots and needles, in parallel to the increasing NaCl concentration in the irrigation solution;

- Proline recorded a significant increase in the presence of salt stress. On the contrary to water deficit, proline does not seem to play an important role;

- The experiments performed in this study showed a significant increase in total soluble sugars (TSS) levels under applied stress conditions;

- Both, water, and salt stress appear to cause the oxidative stress, the only antioxidant system that appears to be substantially involved in the response of fir seedlings to water stress being superoxide dismutase (SOD);

- Salt stress induced significant increases in specific activity of all enzymes tested, except CAT, as well as in TPC and TF levels.

The results obtained in this research were published as follows:

TODEA (MORAR), I. M., GONZÁLEZ-ORENGA, S., BOSCAIU, M., PLAZAS, M., SESTRAS, A.F., PROHENS, J., VICENTE, O., SESTRAS, R.E., 2020, Responses to Water Deficit and Salt Stress in Silver Fir (*Abies alba* Mill.) Seedlings, Forests 11(4):395 [IF 2.221, Q1].

https://www.mdpi.com/1999-4907/11/4/395

Conclusions regarding the physiological response of several silver fir populations to water and salt stress:

- Due to the action of the analysed stress factors (drought and salinity), significant reductions were observed for the values of several important parameters for biomass, such as: stem length, root length or number of needles, compared to the control, untreated plants;

- Significant differences were noticed in the way of inhibiting growth among the investigated populations, illustrating the fact that there is a large differentiation in silver fir populations regarding their reaction to the two abiotic stress factors;

- Population 6 (Valea Morii) appeared to be the most tolerant to water stress, when comparing the fresh weight of silver needles and their water content with the control plants;

- Population 7 (Gârda Seacă) was the most tolerant to salt stress with the lowest concentration (100 mM NaCl). For treatments with 200 and 300 mM NaCl, the distances between populations were smaller and they were not so clearly separated, which indicates a more homogeneous response of silver fir seedlings to high salinity than to drought.

The results obtained in this research were published as follows:

TODEA (MORAR), I. M., GONZÁLEZ-ORENGA, S., PLAZAS, M., SESTRAS, A. F., PROHENS, J., VICENTE, O., SESTRAS, R. E., & BOSCAIU, M. (2019). Screening for Salt and Water Stress Tolerance in Fir (*Abies alba*) Populations, Notulae Botanicae Horti Agrobotanici Cluj-Napoca 47(4):1063-1072 [IF 1.168, Q3].

https://www.notulaebotanicae.ro/index.php/nbha/article/view/11348

RECOMMENDATIONS

The identification of differences among silver fir populations in terms of response to water and salt stress, followed by the selection of resistant or tolerant genotypes, their use in species improvement work and planting material production, can improve ensure appropriate measures to achieve an appropriate reproductive material for silver fir and the perpetuation of the species *Abies alba* by adequate genetic resources.

INNOVATIVE CONTRIBUTIONS OF THE TESIS

The thesis provides original information and experimental data on silver fir (*Abies alba*), an important species both economically and ecologically, and for which very little information is available regarding the response of this species to drought and especially to high salinity. The fir seedlings reaction to water and salt stress has clearly indicated how the species is affected by the two factors of abiotic stress, inhibiting the growth and development of young seedlings.

The germination capacity of silver fir seeds proved to be influenced both by the genotype (genetic resources represented by the nine Romanian populations) and by the treatment represented by the way of storing the seeds (their vernalization) before sowing.

The SSR markers allowed the evaluation of genetic diversity and genotyping of the nine silver fir populations studied.

The results may be of both theoretical and practical importance for the silver fir future improvement programs and for the afforestation and forest planting material programs, especially in the context of climate change.

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