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Proteins as Gene Markers of Tolerance to Drought in Austrian Pine (*Pinus nigra* ARNOLD)

By

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With 8 Figures

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Summary

MATARUGA M., ISAJEV V., KONSTANTINOV K., MLADENOVIĆ-DRINIĆ S. & BALLIAN D. 2012. Proteins as gene markers of tolerance to drought in Austrian pine (*Pinus nigra* ARNOLD). – Phyton (Horn, Austria) 52 (2): 263–280, with 8 figures.

The aim of the research was to determine the variability of protein content in seeds and seedlings at the level of the studied provenances, populations and open-pollinated trees. Studies have included 40 trees that originated from 5 provenances in the Balkan Peninsula. Within each provenance, seeds were collected from five trees growing in extremely harsh habitat (rocky terrain population) and three trees growing in a favorable habitat (control population). The specifics of the studied provenances, populations and open-pollinated trees have been confirmed in the analysis of salt soluble protein with molecular weight from 12.42 kDa to 240kDa. Variability and stability of proteins were observed on the seed, and then on 9-day-old seedlings obtained on a 4% sucrose solution (drought conditions) and distilled water (control). The analysis of seed protein complex shows the most significant differences between the analyzed provenances, whereas the differences between populations and habitat

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conditions are insignificant. Also, there is a high intra- and inter- open-polinated trees variability of the analyzed proteins. At the same time, protein complex in the seedlings obtained in drought and controlled conditions points to the differences among the analyzed populations, ie. it shows a different gene expression depending on the differences in habitats where the seed were collected (rocky-control). This research is important for practical application in the production of seeds and planting materials which could be used for afforestation in extreme habitats.

Zusammenfassung

MATARUGA M., ISAJEV V., KONSTANTINOV K., MLADENOVIĆ-DRINIĆ S. & BALLIAN D. 2012. Proteins as gene markers of tolerance to drought in Austrian pine (*Pinus nigra* ARNOLD). [Proteine als Gen-Marker für die Trockentolerenz der Schwarzföhre (*Pinus nigra* ARNOLD)]. – Phyton (Horn, Austria) 52 (2): 263–280, mit 8 Abbildungen.

In dieser Arbeit wurden Unterschiede des Proteingehalts von Samen und von Keimpflanzen hinsichtlich ihrer Herkunft, ihrer Population und der natürlichen Bestäubung der Bäume untersucht. Als Stichproben dienten 40 Bäume aus 5 Herkunftsgebieten der Balkanhalbinsel. Von jedem dieser Herkunftsgebiete wurden Samen von 5 Bäumen extrem widriger Standorte (Felsgelände-Gruppe) und Samen von drei Bäumen eines günstigen Standortes (Kontrollgruppe) geprüft. Mit Hilfe der Analyse salzlöslicher Proteine mit einem Molekulargewicht von 12,42 kDa bis 240 kDa wurden die Merkmale der untersuchten Herkunftsgebiete, der Populationen und der natürlichen Bestäubung der Bäume charakterisiert. Unterschiede bzw. Ähnlichkeiten der Proteinmuster wurden zuerst in den Samen und anschließend in 9 Tage alten Keimlingen, die in 4% Sucrose-Lösung (Trockenheitsbedingung) bzw. im destillierten Wasser (Kontrolle) gezogen wurden, untersucht. Die Analyse der Samen-Proteinmuster zeigte signifikante Unterschiede zwischen den untersuchten Herkünften, während die Unterschiede zwischen Population und Standort nicht signifikant waren. Durch die natürliche Bestäubung der Bäume war eine große Variabilität bei den untersuchten Proteinen, sowohl innerhalb der Stichproben, als auch zwischen den einzelnen Stichproben zu beobachten. Gleichzeitig waren zwischen den Populationen Unterschiede im Proteinmuster zu erkennen, je nachdem, ob die Samen unter Trockenheitsbedingungen oder unter Kontroll-Bedingungen gezogen wurden, d. h. sie zeigten unterschiedliche Genexpression, abhängig von den Standorten, an denen die Samen gesammelt wurden (Felsgelände oder Kontrolle). Für eine praktische Anwendung bei der Aufforstung extremer Standorte ist diese Untersuchung wertvoll, um zu entscheiden zu können, welches Material bei der Samenproduktion und bei der Anzucht von Pflanzmaterial verwendet wird.

Introduction

Large areas of bare mountainous terrain and degraded habitats within the sub-Mediterranean region need to be afforestated as soon as possible to mitigate deforestation (STEFANOVIĆ & al. 1977, RANKOVIĆ 2009) and global warming. Afforestation activities should involve species suitable for these modified environmental conditions, and the Austrian pine (*Pinus nigra* Arnold) is one of them. Austrian pine is popularly used for afforestation due to its commercial viability, but the genetic structure of the population is little known and current planting strategies could threaten its genetic diversity (BOGUNIĆ & al. 2011, NAYDENOV & al. 2006).

Austrian pine properties disjunctive range of distribution, high level of genetic variability, compound population structure, plasticity and the ability to grow in harsh conditions make it an attractive species for planting in these types of habitats (ISAJEV & al. 2004). Its ability to grow in poor habitats, to protect and improve land functions and its high wood mass production make it among the most significant species in Bosnia and Herzegovina (MATARUGA 1997, 2003). Disjunctive range of distribution together with the distinguishing environmental features of the isolated Austrian pine populations has resulted in high intra-species variability as a result of the influence of specific genetic mechanisms (VIDAKOVIĆ 1974, BASSIOTIS 1967, MATZIRIS 1983, 1986, BARBERO & al. 1998). Despite the large number of published scientific results, there has been little research activities on those species which are difficult on to access, such as on steep cliffs and in some river canyons (Fig. 1a), where there is little or no surface substrate (MATARUGA 2006). Additionally, there is no detailed information on genetic potential and diversity of the local populations and genotypes taken from these localities. Thus we lack information on the prospects of their utilization in terms of producing planting stock for extremely dry habitats.

Markers based on protein polymorphism may be useful in this respect, since proteins act directly on biochemical processes, and thus must be closer to the "build-up" of the phenotype than DNA. Protein variation could therefore be more informative about the variability expressed at the organism level than DNA variation, and provide markers directly affecting quantitative traits (GERBER & al. 1998). Polymorphic molecular markers (NAYDENOV & al. 2006, ZARA & DOOD 2007), alongside with morphological and cytogenetic ones (BOGUNIĆ & al. 2006, 2011), were used to assess the variability, whereas monoterpens and proteins were used at gene product level (KONSTANTINOV & al. 2000, 2005, NAHÁLKOVÁ & al. 1999). So far many research papers dealing with possibilities of applying protein markers within *Pinus* genus were published (ALVAREZ & al. 2004, COSTA & al. 1998, 1999, 2000, COSTA & PLOMION 1999, GERBER & al. 1993, 1998, NIKOLIĆ 1982, PLOMION & al. 1995, 1997, HAJDUCH & al. 2001, MILOVANOVIĆ & ŠIJAČIĆ-NIKOLIĆ 2008, ISAJEV & al. 2008, VIENNE & al. 1996).

The positional polymorphism of polypeptides is analysed through the use of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in a lot of species. All obtained results show that 2-D PAGE is a quite abundant and cheap source of good-quality genetic markers. Genetic mapping showed that the loci involved are well distributed on the chromosomes. In the context of the genome mapping projects, such markers are physiologically relevant in that they reveal loci whose transcripts are translated in the organ analysed (VIENNE & al. 1996).

The research goal was to define the Austrian pine genetic variability by analyzing the protein in seed originating from habitats of extreme properties. At the same time, the analysis of proteins in seedlings at the level of open pollinated trees which germinated in assumed drought stress environment will help to identify their individual reaction. This research aimed to monitor the occurrence of variations at protein level as a response to water deficit in seed germination conditions. Also, this research casts some light on the significance of the background information of genetic potential of the Austrian pine growing in extreme conditions habitats. According to the above mentioned facts, this research is of great significance for practical use in seed production and stock planting, which will be used in the process of reforestation and establishing forests in habitats of extreme conditions.

Material and Methods

Site Selection and Seed Collection

Material obtained from natural populations was used in the research activities. Seed was collected from a total of 40 open-pollinated trees originating from five provenances (Table 1). Two populations were selected from each provenance (Fig. 1a and 1b): five open-pollinated trees (1–5) present a population growing in extremely

Table 1. Location, elevation, and soil conditions at each seed collection site.

Provenance	Habitat	Latitude	Longitude	Altitude (m)	Geological substrate	Soil type	Soil depth
Sutjeska	rocky	43 19’20”	18 42’46”	1400	limestone	no surface soil	–
	control	43 19’19”	18 39’33”	1300	limestone	colluvium	A-C horizon – approx. 2m
Viegrad	rocky	43 45’20”	19 24’04”	550	limestone	no surface soil	–
	control	43 51’26”	19 14’24”	475	serpentinite	eutric cambisol	A-(B)-R horizon – greater than 0.5m
Tara	rocky	43 52’50”	19 24’20”	800	limestone	no surface soil	–
	control	43 53’20”	19 32’40”	1050	serpentinite	eutric cambisol	A-(B)-R horizon – greater than 0.5m
Teslić	rocky	44 34’30”	17 43’28”	510	serpentinite	eutric leptosol – ranker	A-C horizon – up to 0.5 m
	control	44 34’03”	17 43’34”	470	serpentinite	Albeluvisol – (Pseudogley)	A-g-C horizon – greater than 0.5m
Durmitor	rocky	43 09’12”	19 15’53”	1240	limestone	Leptosol – (Melanosol)	A-R horizon – up to 0.3m
	control	43 00’29”	19 25’29”	1320	limestone	Calco-cambisol	A-(B)-R horizon – greater than 1m

harsh habitat (rocky) and three open-pollinated trees (6–8) present a population growing in the most favourable habitat (control). Steep slope terrains, very warm south and south eastern expositions, very shallow soil with extreme or absolute rocky surface are distinctive characteristics of extreme habitat conditions. High average temperatures of air and soil, and adverse amounts and distribution of precipitation during the vegetative period have been recorded in these habitats (STEFANOVIĆ & al. 1983).

Cones were collected from individual trees during winter 2000 (January-Mart). Trees were selected based on the presence of cones and accessibility in their specific habitat. Approximately 200 cones from each tree were collected. Cones were dried initially in a room (20°C), then placed in a drying oven (45°C) for 24–48h. Seeds were extracted from cones and placed into sealed plastic bags in cold storage (4°C) until laboratory testing. All tests were conducted in a laboratory at the Maize Institute in Zemun Polje-Belgrade.

Proteins in Seed

The protein complex was isolated from seed according to WANG & al. 1994. Proteins were extracted from haploid megagametophyte in all forty (families) of open pollination trees. Altogether 1200 seeds were analyzed by taking 10 seeds (3 replicates x 10 seed = 30 seeds per open-pollinated tree) in each of the replicates. Proteins were separated according to their molecular weight by polyacrylamide gel electrophoresis. 19 polypeptides (12.42kDa – polypeptide 1, until 240kDa – polypeptide 19) of different molecular weights with genotype identification were analysed based on the presence or absence of polypeptides. Qualitative differences in the concentration of protein fractions were not analyzed any further.

Proteins in 9-Day-Old Seedlings

In addition to protein analyses in seed of open-pollinated trees, we also performed a protein analysis from 9-day-old seedlings. Protein analysis in seedlings was performed on samples taken from three provenances (Višegrad, Teslić, Durmitor), with altogether 24 open-pollinated trees. Within the analysed provenances, the selected ones are the furthest and characterized with the greatest variations in vertical and horizontal distribution. Moreover, populations with the greatest (Višegrad) and the slightest (Teslić, Durmitor) inter-population variations regarding habitat conditions were selected from these provenances.

Seeds were germinated in distilled water (mimicing normal conditions) and in 4% sucrose solution (mimicing drought conditions). Sucrose solutions create a water deficit condition by lowering water potential and thereby decreases water uptake capability. Four replications of seed (100 seeds each) from each tree and from each habitat within three provenances (Višegrad, Teslić, Durmitor) were placed in Petri dishes (12 cm diameter). Treatments were applied by adding 7ml of either distilled water or 4% sucrose solution to each Petri dish.

The protein complex was isolated according to WANG & al. 1994 from 9-day-old seedlings. Qualitative analysis consisted of marking 22 polypeptides with numbers from 1 to 22 (12.42kDa – 240dDa). Regarding their presence in the analyzed samples, protein fractions (polypeptides) were classified into four groups: 1) unstable polypeptides; 2) stable polypeptides; 3) polypeptides which appear in the normal condi-

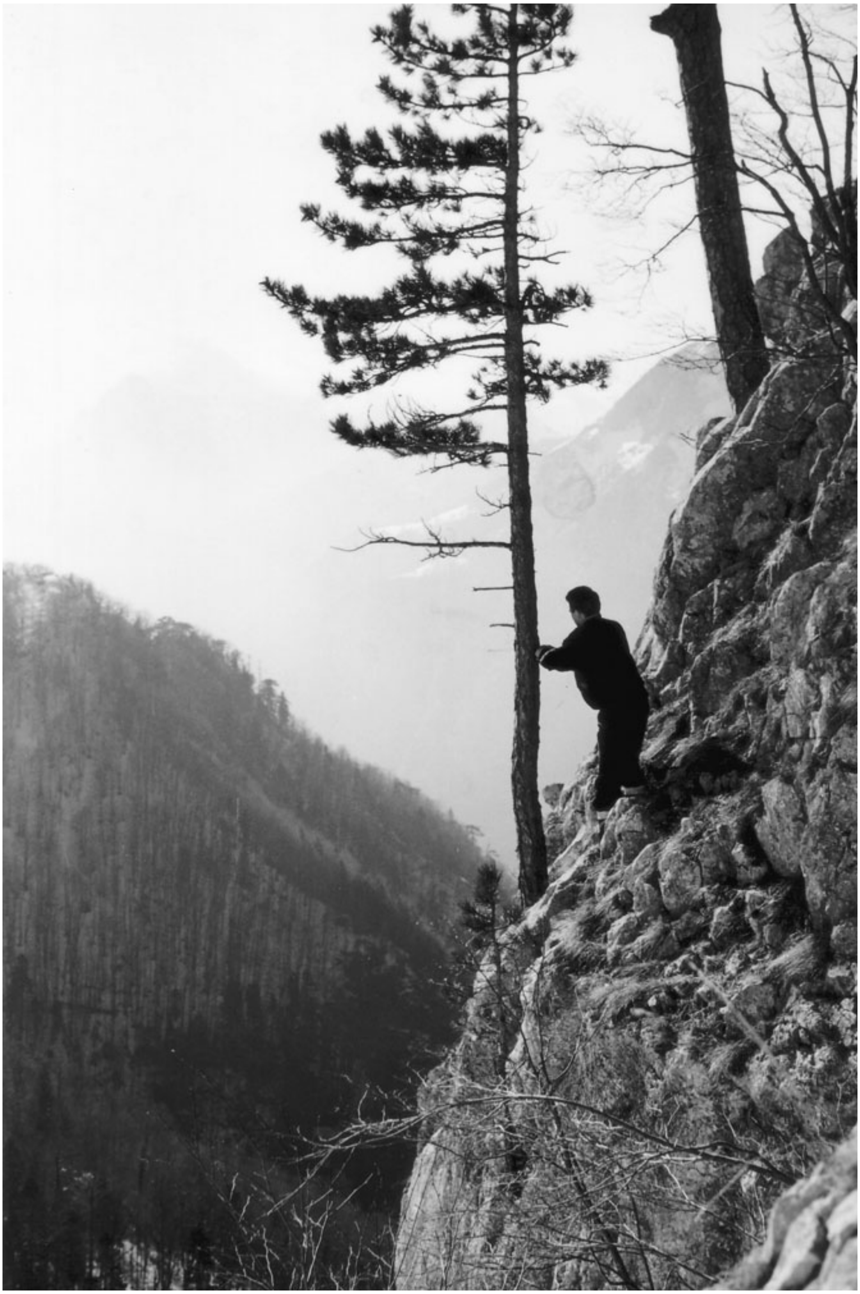

Fig. 1a. Typical habitats where Austrian pine (*Pinus nigra* Arnold) seeds were collected for this research – rocky.

Fig. 1b. Typical habitats where Austrian pine (*Pinus nigra* Arnold) seeds were collected for this research – control.

tions of germination and 4) polypeptides which appear in the drought conditions of germination.

Statistical Analyses

Data were analysed at multiple levels: open-pollinated tree, population (habitat), and provenance. Cluster analyses were performed to examine genetic affinity and diversity. We analysed quantitatively the existence of protein (present – not present). The cluster analysis of protein in 9-old-day seedling included the results obtained in both groups of seedlings obtained from distilled water and 4% sucrose solution. Complete linkage distance was used. Statistical software Statistica 6.0 and SPSS 12.0 was applied to process the data.

Results

Proteins in Seed

The analyses of proteins isolated from the seeds show that all studied genotypes have a specific protein pattern. Protein analysis in the seed showed the widest variations among provenances (Fig. 2). Dendrogram cluster analysis, indicates (with slight variations) that open-pollinated trees were arranged into two groups. The first group mainly includes trees from Sutjeska and Višegrad provenances (these provenances are near each other), while the second group includes trees from the other three prove-

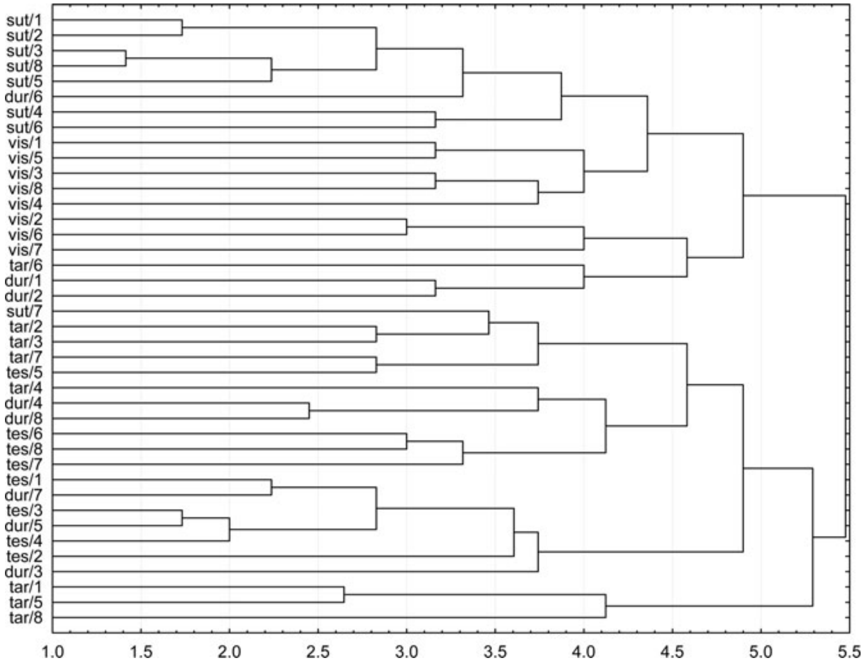

Fig. 2. Cluster analysis of 40 open-pollinated trees on the basis of 19 polypeptides in seed.

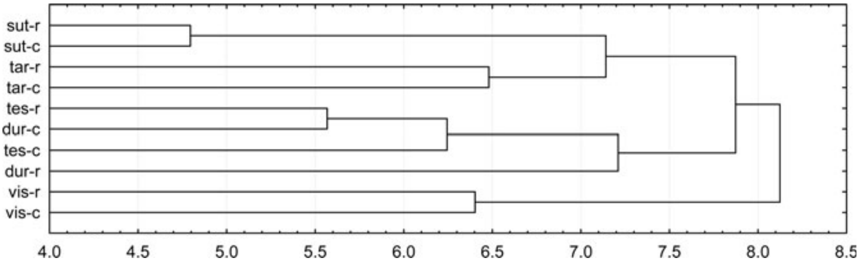

Fig. 3. Cluster analysis of 10 populations (habitats) on the basis of 19 polypeptides in seed.

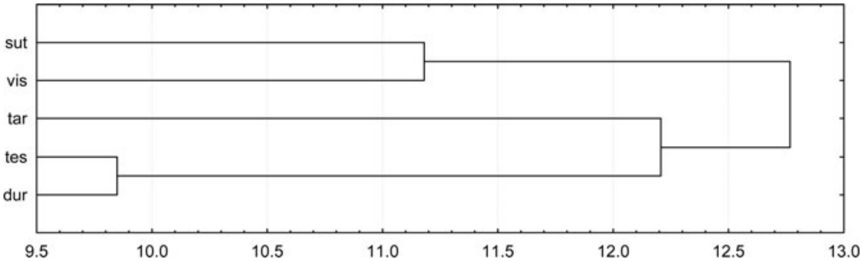

Fig. 4. Cluster analysis of 5 provenances on the basis of 19 polypeptides in seed.

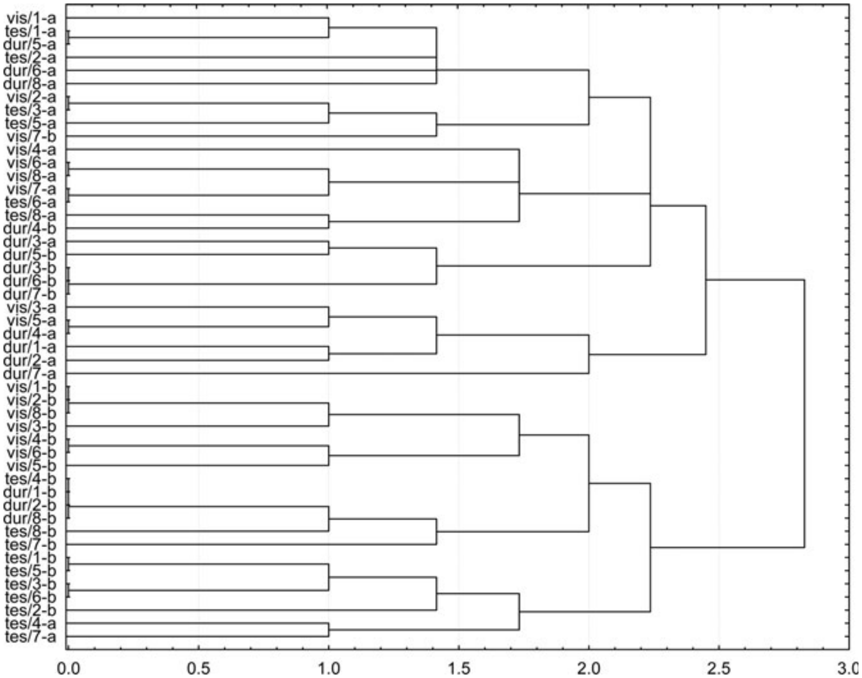

Fig. 5. Cluster analysis of 40 open-pollinated trees on the basis of 9-day-old seedlings proteins (a- in distilled water; b- in 4% sucrose solution).

nances. Within the second group, open-pollinated trees from Tara was arranged in two separate units, whereas trees from Teslić and Durmitor provenances did not have any patterns in grouping (activities). Nevertheless, some trees deviate from the above mentioned rules, and these are: Sutjeska/7 tree which already showed a higher level of affinity with the trees from Tara provenance, and Durmitor/6 tree which groups with the trees from Sutjeska provenance at a shorter distance.

Grouping activity regarding habitat conditions (rocky – control) of the test trees was not noticed. There is a distinctive grouping activity at the population level within individual provenances at the lowest distances, which confirms slight differences in habitat conditions and differences occurring among populations and highlights the significant role of provenances (Fig. 3). An interesting fact was noticed at provenance level (Fig. 4) where tree grouping betwen Teslić and Durmitor provenances was observed at the shortest distance, although the distance between these two provenances is the largest compared to the distances between other provenances (in vertical and horizontal terms).

Proteins in 9-Day-Old Seedlings

Cluster analysis (Fig. 5) distinctly separated two homogeneous groups according to the type of the substrate the seed germinated on (distilled water or 4% sucrose solution). Significant deviations were noticed in seedlings germinated in distilled water Teslić/4 and Teslić/7 open-pollinated trees, which are classified into the group of seedlings germinated in 4% sucrose solution considering their protein complex. Moreover, seedlings germinated in drought stress conditions – the lines Višegrad/7; Durmitor/3, 4, 5, 6, 7 – were classified into the group of seedlings germinated in control – distilled water conditions considering their protein structure.

The grouping activity at open-pollinated trees level (Fig. 6) is almost regular in the provenances they originate from (except Durmitor/4). At population level (rocky-control) the grouping activity of populations from Višegrad provenances (Fig. 7) were recorded at slightly higher distances. Populations from Teslić and Durmitor provenances were grouped at shorter distances. The above mentioned facts indicate inter population differences at protein level in seedlings germinated in water and in 4% sucrose solution conditions. The results confirm the significance of the population differences within Višegrad provenance. In later experiments these differences also manifested in direct exposure of seedlings to drought effects (data not shown).

The analysis of the individual polypeptides indicate that gene expression resulted from various germination conditions and open pollinated trees (Fig. 8). Protein fractions were recorded in all lines, as well as the fractions whose presence is conditioned by open pollination trees, popu-

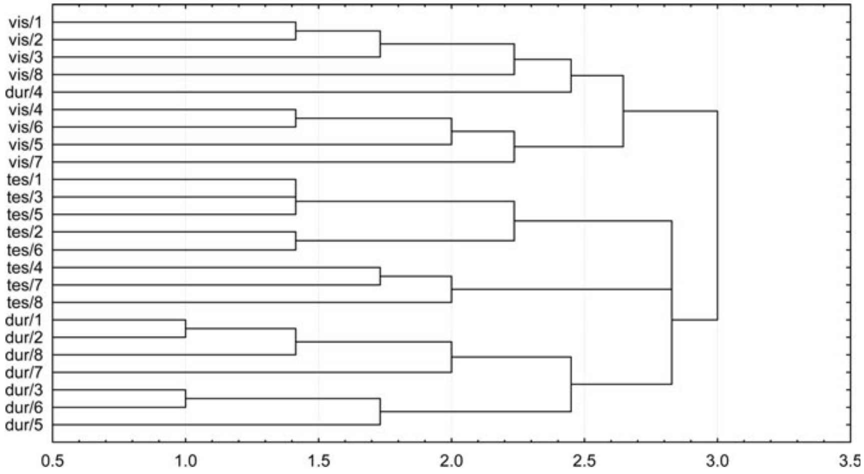

Fig. 6. Cluster analysis of 24 open-pollinated trees on the basis 22 polypeptides of 9-day-old seedlings.

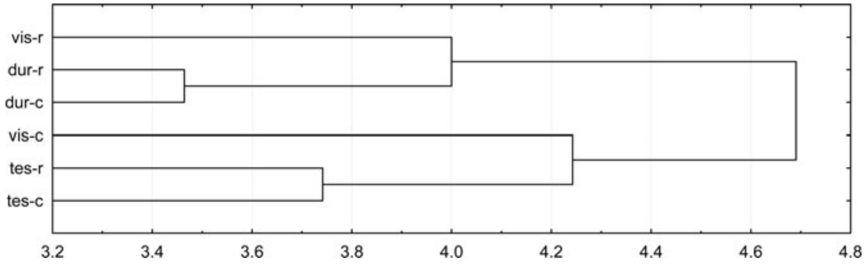

Fig. 7. Cluster analysis of 6 populations (habitats) on the basis 22 polypeptides of 9-day-old seedlings.

lations or provenances. “Conservative quality” can be noticed in certain polypeptides i.e. they are present in all of the open pollination lines which were analyzed. On the other hand, the results determined variable protein fractions at open pollination line level, as well as those protein fractions whose presence vary relative to the the provenance.

The results of the polypeptide analysis reveal the presence of:

1. unstable polypeptides – present in a minority of open pollinated trees, regardless of the habitat conditions that seedlings originate from – (polypeptide marked with number 1 or 4,6% of total number analysed polypeptides);

2. stable polypeptides – present in all open pollinated trees regardless of the habitat conditions that seedlings originate from (polypeptides marked with numbers 2, 4, 6, 7, 8, 9, 11, 13, 14, 15, 16, 20, 21, 22 or 63.6% of total number of analyzed polypeptides);

3. polypeptides which, in most cases, exist in 9-day-old seedlings germinated in normal conditions – distilled water (polypeptides marked with numbers 12 and 17 or or 9,10% of total number of analyzed polypeptides);

4. polypeptides which, in most cases, exist in 9-day-old seedlings germinated in drought conditions –4% sucrose solution (polypeptides marked with numbers 3, 5, 10, 18 and 19 or 22,70% of total number of analyzed polypeptides);

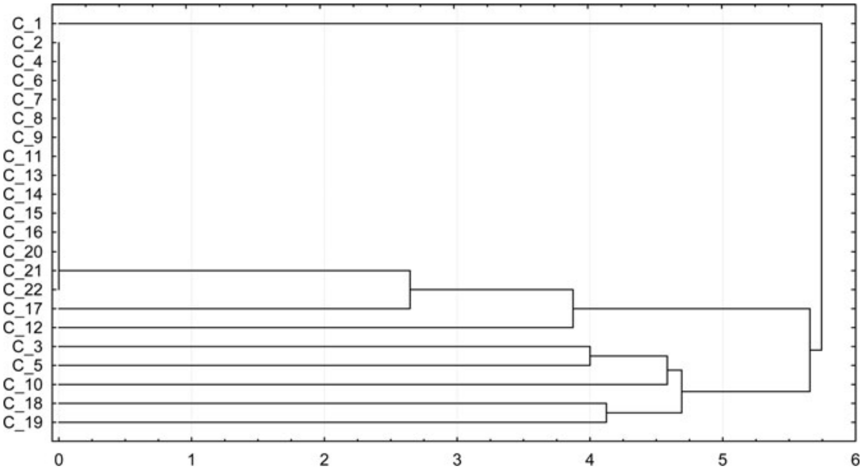

Fig. 8. Cluster analysis of 22 polypeptides on the basis of 9-day-old seedlings in distilled water and 4% sucrose solution.

The above mentioned facts indicate that some polypeptides are present/absent regardless of germination conditions. They also indicate the polypeptide group whose presence/absence depends on germination conditions and reaction of the open pollinated trees to actual conditions.

Discussions

Protein in Seed

Habitat significantly influences seed structure, cone size, and needle morphology of Austrian pine (Mataruga 2003, Mataruga & al. 2003). Seed from within the five Balkan provenances showed some differences in water uptake when comparing those from harsh, arid sites to those from favorable environmental conditions. However, water uptake was not correlated with subsequent germination. With the exception of the Višegrad provenance, seed from control habitats had higher germination percentages than those from rocky habitats on both the 4% sucrose solution and distilled water (Mataruga & al. 2010). In these studies, “the specificity” of the Višegrad provenance with respect to the protein complex on the 9-day-old seedlings obtained in different drought conditions was noted. Seed coat, in particular, influences water uptake and is affected by hereditary factors (BARNETT 1998), but probably not by protein complex.

Protein complex in seed is usually described as stable regarding the habitat conditions the seed originates from i.e. variations are attributed to inter provenance differences. Nevertheless, its analysis at seedling level which germinated in stressful environments revealed different results.

The results confirmed that habitat does not influence the structure of the protein complex, which was revealed by FORREST 1994. Moreover, the research previously carried out by BAHRMAN & a1. 1997, BAHRMANN & PETIT 1995 confirmed the variability among spatially isolated populations.

The polymorphism of protein markers on the selected genotypes originating from six populations (seed stands) in Serbia was analyzed by LUČIĆ & al. 2008. Based on the electrophoregram, the degree of genetic variation among the provenances was higher than the variation within the provenances. There was also a high genetic variation in seed proteins within the analyzed populations. The results of these authors are almost the same as our results.

According to SCALTSOYIANNES & al. 1994, *Pinus nigra* is characterised by a high total variation because of the high intra-population genetic variation, which points to a high genetic variation in local populations and the possibility that the same alleles are distributed throughout the entire range of this species.

Similar results came from NAYDENOV & al. 2006. In that study they investigated the genetic structure and genetic diversity of Austrian pine populations in Bulgaria using chloroplast microsatellite markers and terpene analysis. A total of 22 size variants at the 3 loci were identified, that were combined in 68 different haplotypes, of which 7 represent 39.81% of the genetic structure. ANOVA analysis revealed that 6.06% of the variation was found among populations, while 93.94% was found within populations.

Protein in 9-Day-Old Seedlings

Analysis of 22 polypeptides in 9-day-old seedlings which grew up in drought conditions (4% sucrose solution) and normal conditions (distilled water) could be a powerful method of studying plant responses to stress at the protein level. We found: 4.6% “unstable” polypeptides; 63.6% – “stable” polypeptides; 9,10% polypeptides which occur in distilled water and 22.70% polypeptides which occur in drought conditions. This is probably due to the fact that the polypeptides, which could be directly related to actual variations in habitat conditions of the seed intended to be used in analysis (population – provenance), were not identified. These studies provide fundamental data for further research on responses to drought and high temperatures, two major factors limiting the growth of forest trees during the summer or under recent climatic warming.

The results obtained in the change of protein complexes were in favor of already published results of a great number of authors. Kozlowski & Pallardy 2002 found that at the molecular level, plants respond to water stress by synthesis of certain new proteins and increased levels of synthesis of some proteins produced under well-watered conditions. Evidence has been obtained for enhanced synthesis under water stress of water-channel proteins and other proteins that may protect membranes and other important macromolecules from damage and denaturation as cells dehydrate.

In order to study stress-responsive molecular mechanisms for Populus × euramericana cv. '74/76', He & al. 2008 analyzed the response of 2-year-old cuttings of Populus × euramericana cv. '74/76' to drought and high temperature using two-dimensional gel electrophoresis. More than 1,000 reproducible leaf proteins were detected in the controls and treatments, and 26 proteins were found to change considerably in abundance. They identified 13 proteins affected by drought stress and 11 proteins affected by high temperature.

In the experiment of short-term response of protein of Scots pine (*Pinus sylvestris* L.) seedlings to low temperature, Kontunen-Soppela & al. 2002 found no change in specific proteins or in their quantity, as determined by SDS-PAGE. Immunoblots using a dehydrin antibody detected a 50-kDa protein in all samples, as well as a 42-kDa-dehydrin that was missing from the day 1 and 14 samples. The results of this study indicate that protein and carbohydrate metabolism of Scots pine adjust to low temperature in less than ten days. Neither soluble protein patterns nor dehydrin-proteins of needles are markedly altered by cold treatment.

Conclusions

The analysis of salt soluble seed proteins indicated a specific protein pattern for all tested genotypes, while the greatest differences were indicated at provenance level. The identical protein structure in the replicates of the same open pollinated trees was recorded in a small number of cases, which supports high intra-line variability which can be the result of the effect of the father, as well as of heterozygosity of mother trees.

Unlike the protein complex in seed which can be described as stable regarding habitat conditions (rocky-control), where the seeds originate from, the analysis in seedlings which germinated in stressful conditions reveals some different results. Variations in gene expressions are noticed when plants are exposed to drought conditions, which can be attributed to variations identified at population (habitat) level, which is especially true for the Višegrad provenance.

Protein analysis in 9-day-old seedlings germinated in normal (distilled water) and in drought condition (4% sucrose solution) indicates that certain proteins exist in all open pollinated trees (regardless of germination conditions), certain proteins are present or absent depending on germination condition, while the presence of certain proteins significantly depends on open pollinated trees, populations (habitats) or provenances.

These studies provide basic data for future research on the responses of Austrian pine from extremely different site condition to drought, with two major factors limiting the growth of forest trees during summer under recent climatic warming.

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