

A STRATEGY FOR THE IDENTIFICATION OF A CANDIDATE GENE FOR DROUGHT INDUCED STRESS IN PENDUCULATE OAK (*QUERCUS ROBUR* L. (*Q. PEDUNCULATA* EHRH.)), *FAGACEAE*

B. TRUDIC, V. GALOVIC, S. ORLOVIC, P. PAP and S. PEKEC

¹ University of Novi Sad, Institute of Lowland Forestry and Environment, 21000 Novi Sad, R. Serbia

Abstract

TRUDIC, B., V. GALOVIC, S. ORLOVIC, P. PAP and S. PEKEC, 2013. A strategy for the identification of a candidate gene for drought induced stress in penduculate oak (*Quercus robur* L. (*Q. pedunculata* Ehrh.)), *Fagaceae*. *Bulg. J. Agric. Sci.*, 19: 338-346

In this paper a problem associated with drying of some penduculate oak populations (*Quercus robur* L. (*Q. pedunculata* Ehrh.)), *Fagaceae* from the area of Srem was systematically approached, and a theoretical explanation of the phenomenon of resistance of wooden species at a molecular level was given. The most important segment of this paper represents a strategic proposal for approaching this problem of gene expression at the molecular level in order to obtain the first applicable insights into the future protection of this species in the area of Srem, Republic of Serbia and in other parts of Europe, where oak trees are exposed to constant climatic changes and soil water deficit.

Key words: DNA, gene expression, (m)RNA, penduculate oak, senescence, drought induced stress

Introduction

About the species of interest for molecular analyses

Quercus robur L. (*Q. Pedunculata* Ehrh.), fam. *Fagaceae*, the Penduculate oak is a deciduous species that extends from western Asia to Europe. It is a tree of exceptional size reaching the height of some 50m, stem width of 3 m, and age of up to 1000 years. As the English yew, it can also attain the greatest age among the European tree species. Its bark is deeply cracked in old age, furrowed with longitudinal and transverse shallow furrows. The crown is heavily branched, wide spreading and massive, with bright, irregular, curved and knee shaped branches. The fruit of the oak is an acorn, elongated or oval nut, the base of which is surrounded by an indurate scaly cup. As an indigenous species, it grows along the valleys of the major rivers. Primary tree species of oak forest is penduculate oak, or mixed communities of hornbeam, ash and elms. There are significant ecological, first of all, soil differences among penduculate oak forests, which are re-

flected in a specially isolated penduculate oak plant communities (phytocenosis). The best penduculate oak forest in Slavonia and Srem are well known all over the world for its valued and quality wood. The wood is used for making furniture, construction material etc. Given the wide range (penduculate oak is a polymorphic species) a large number of lower systematic categories are isolated and described, among which the *Q. robur* L. ssp. *Asimetrica* is singled out, and it can be found in the botanical garden „Jevremovac“ in Belgrade (Jovanović-Juga, 2005).

Penduculate oak forests in Serbia- populations of individuals of interest for sampling and molecular analyses

In the valley of the river Sava between Belgrade (Serbia), Zagreb (Croatia) and Pecs (Hungary) the most beautiful penduculate oak forests of the central Europe are found in the area of some 300 000 ha and altitude of 70 to 120 m. Observed per hectare, the penduculate oak forest are economically the most valuable forests in Serbia. These forests

have long been developed under the influence of biotic and abiotic factors (floods, insect calamities, epiphytotic diseases). Regardless of the influences of various biotic and abiotic factors, it is evident that the greatest number of trees in these forests is characterized by a good phenotype, which was the reason for permanent acorn collection, and its export especially to Eastern Europe (Orlović et al., 2008).

Since English acorn is an endangered species and that the efforts are made for its preservation through various forms of *ex situ* and *in situ* conservation, present penduculate oak stands represent the best forms of preservation of penduculate oak genetic diversity. New efforts should be constantly made in order to preserve these forests (Orlović et al., 2008).

Until the first half of the 19 century, huge complexes of penduculate oak forests covered the area of Posavina. These were the real rainforests regularly flooded by river Sava, as it still floods the existing forests today in unprotected part of Srem. Besides oak, the dominant tree species were elm and ash. These two species were more frequent, often much more frequent than the oak itself. Participation of oak in these forests usually ranged between 10-15 trees, rarely between 20-30 trees, and only exceptionally 40 trees per one acre. 30 to 50 trees (Fekete, 1890) commonly represented dominant tree species gathered in the same area. Such structural composition of dominant trees species in the forests was the result of strong competition for light and space.

Tree dimensions, especially those of the penduculate oak were excellent due to the optimal habitat conditions. They can be hardly achieved under present conditions. The following other species were also present: hornbeam, maple, poplar, linden, willow, alder and beech (Jodal, 2008).

Actual strategies regarding preservation/breeding of Penduculate oak genepool in Srem

During the first stage of penduculate oak breeding, the seed stands were isolated. They were isolated based on tree phenotype depending on the variety the most trees in the stand belonged to. All isolated stands were found on the area managed by the Lumber camp "Sremska Mitrovica". Up to now, 8 stands of the total area of 692.82 ha are registered and acorn collected from all of them, which can be seen from the Table 1. The greatest number of seed stands is found in geographical unit "Vinična-Žeravinac-Puk" – Forest Directorate in Morović.

The basic problems occurred about management of the seed stands were the need for constant intervention by the removal of the dry trees and the impossibility of implementation of protection measures against diseases and harmful insects (Orlović et al., 2008).

Seed stands were isolated according to a good phenotype. Prior to isolation, the management was aimed at producing high quality timber. In addition to that, the yield in the seed stands occurred on the average every fifth year. Due to that, the development of the project for founda-

Table 1
Seed stands of the penduculate oak (*Q. robur*) in Srem (Orlović et al., 2008)

Registration number	Forest management	Management unit	department	Area, ha	altitude	Type of soil	Number of trees/ha	Remarks
S 02.02.01.08	Višnjićevo	Varadin	41 i 42	57.69	80	humogley	153	
	Morović	Blata	19 i 32	44.60	80	meadow black soil	151	
S 02.02.01.09	Morović	Varoš	44 i 45	76.54	83	meadow black soil	92	
S 02.02.01.06	Morović	Vinična	14,15,16,20	162.54	82	meadow black soil	113	
S 02.02.01.05	Morović	Vinična	18,30,31	126.74	82	meadow black soil	65	<i>ssp. tardissima</i>
S 02.02.01.03	Morović	Vinična	34, 43	98.19	83	meadow black soil	65	
S 02.02.01.13	Morović	Rađenovci	3,13,14,15	98.81	83	meadow black soil	77	<i>ssp. tardissima</i>
S 02.02.01.04	Kupinovo	Lošinci	8,9	27.91	75	meadow black soil	240	
Total				692.82				

tion of intended stands for penduculate oak production (*Q. robur*) acorn i.e. seed plantation was approached. Experiences of some researchers revealed that by foundation of the seed plantations it is possible to diminish problems regarding the periodicity in the yield (Vidaković, 1996; Orlović et al., 2001). Works on choosing the “plus” trees started in 1968 and ended in 1978, when from the area of 42.000 ha 86 “plus” trees-genotypes aged 110 and above were chosen. This separation lasted way too long, mainly because the area from which “plus” genotypes were chosen was large, and the need to determine the phenophases of leafing and flowering of the chosen genotypes. The basic criteria for isolation of these trees were the following: dimensions (the speed of growth), straightness of the trunk, branching (monopodially) and sensitivity to Oak powdery mildew (*Microsphaera alphitoides*).

All four varieties occurring naturally along river Sava were presented in the seed stand: early sprouting pedunculate oak (*Q. robur* var. *praecox*), typical oak (*Q. robur* var. *typica*), and two varieties of late penduculate oak (*Q. robur* var. *Tardiflora* and *Q. robur* var. *tardissima*). Table 2 shows the stand structure according to the variety (Orlović et al., 2008).

Abiotic drought stress as constantly present problem endangering sustainable development of seed stands and natural populations of Penduculate oak in Srem

Occurrence of tree drying in these forests was caused by biological age of trees, with first symptoms manifested as dry tips or as the result of competition, i.e. biological differentiation of trees in which suppressed trees were retarded and gradually dried up. The opinion of the most of authors engaged in these studies is that forest drying in Posavina started at the end of the first decade of the 20th century. According to Manojlović (1926), drying of oaks in forest east of Morović started in 1910 and lasted until 1925. Majority of drying occurred in 1911, 1916, 1917, 1919 and 1924. The most severe drying occurred in Naklo,

Varadin and Đepuš forests. When drying of penduculate oak started on territory of some forest managements, the survey was conducted by Forest Directorate in Vinkovac. The survey was constructed as questionnaire, divided in 18 sections. According to that, Đurđić (1932) mentioned the following data: “Starting year of drying varied, some mentioned 1920, others 1916, and yet others 1927, and an isolated case occurred in Klenak forest management in which it was stated that drying started in 1928 and 1929”. Djurdic believes that these responses are quite uncertain. However, data agrees with the fact that the higher degree of drying occurred in pure stands of oak, in wet soil, and that the highest drying occurred in the middle-aged stands.

Edaphic factors played significant role in drying, thus pedological studies have become very important. First papers in this field were published by Stebut (1925a and b). According to him, the reason for drying should be looked for in soils where processes such as podzolization and gleying are taking place. Physical and chemical properties of soils are disturbed as the consequence of podzolization. It should be mentioned that these conclusions, according to forest breeding experts, are contradictory with forest science statements, that quality of soil should be evaluated according to particulate indicators of stand development. These indicators were good, even the best until the moment of drying. Thus, it cannot be accepted that the reason for drying should be looked for in the mentioned stands.

Half a century later, Škorić and Vranković (1974) revealed results of their studies published in regard to dynamic of individual soil components significant for estimation of the intensity of forest drying. These were the results: 1) podzolization and soil salinization are not significant as the causes of forest drying; 2) tree drying increases from pseudogleic to gleic soil; 3) intensity of drying on gleic soils varies and for now a final response as to which degree of gleying influences this phenomena cannot be given; 4) analysis of surface stagnant waters, water from waterways and probes 7 m deep revealed that water pollution cannot be cause of forest drying. The studies were conducted in Žutica and Lipovljani in forests with pronounced drying.

Table 2
Taxonomic status of genotypes in a clone seed orchard “Banov brod” (Orlović et al., 2008)

Taxonomic status	Flowering	
	Starts:	Ends:
<i>Q. robur</i> var. <i>praecox</i>	April, 17th	April, 21th
<i>Q. robur</i> var. <i>typica</i>	April, 21th	April, 29th
<i>Q. robur</i> var. <i>tardiflora</i>	May, 2nd	May, 10th
<i>Q. robur</i> var. <i>tardissima</i>	May, 15th	May, 18th

A special study is devoted to the role of climatic factors in forest drying

Vajda (1948) concluded (1948) based on measures and analysis of climatic data that during the period of 37 years (starting from 1908) mean annual temperatures were in-

creased on the average by 0.4°C in comparison to multi-year average, prior to the occurrence of drying. Distribution of rainfalls during drying period became more unfavorable, extremely dry years doubled in frequency, and their distribution was such that two such years followed each other almost regularly. Such climatic changes caused physiological weakening of forest. Former climate favored gradation of gipsy moth and other harmful insects, and accelerated spreading and epidemic of powdery mildew. Vajda concluded that the climatic changes, which started at the beginning of the 20th century was in indirect but a narrow casual relation with occurrence of forest drying. According the more recent data, Androić (1974) stated: “*Climatic changes... continued and the periods with dry springs and summers following each other had become more frequent. In these periods, very low water levels of the rivers Sava, Drava, and Dunube were observed... Due to that the level of lower waters dropped ...*”

Pure oak forests irrespective of the manner of their origin represented unstable forest biocenoses lacking the necessary forest breeding works. The stand structure was kept until the end of the rotation period. As the result of that – especially in artificially formed stand – the drying of trees occurred, which became more pronounced at about 60 years of age, and it continued later on at older age. The highest proportions of abnormal drying were observed in these stands (Jodal, 2008).

Drought resistance mechanism

Drought resistance mechanism in plants develops depending on plant species and individual plant organs. Poikilohydric plants can tolerate drought during vegetation period by decreasing growth intensity and all physiological processes to a minimum, which is characteristic of dormancy in plants, and more or less expressed seed specificity. One of the ways to survive is the tissue ability to accumulate moisture, as in succulents, where the main role is played by the tissue water-retention abilities, and morphological changes of the aboveground parts in terms of reducing losses by transpiration and root development in depth towards water rich soil layers. Physiological defense mechanism against drying is based upon increased concentration of osmotic active ions and molecules so called osmoprotectants, which prevent dehydration and denaturation of proteins, i.e. maintain colloid structure of protoplasm. Compatible osmolits, therefore, maintain balance of water potential within the cell (Stevanović and Janković, 2001). Known organic molecules showing such action are prolines, sorbitol, ectoine and glycine-betain,

and so called LEA proteins, from which dehydrines are the most common.

Synthesis of the mentioned molecules is proven even at different forms of abiotic stress, such as salinity (soil salinity), and more recently more and more evidence points out to the protective role of proline in detoxification of free oxygen radicals that occur under increased concentration of oxidative stress. Protective role of sugar and potassium, which increase osmotic value of protoplasm and thus increase the content of bound water, is also important for plant resistance to frost (Stevanović and Janković, 2001, Popović et al., 2001).

Plant senescence - the natural biological phenomenon

Deterioration processes that lead to aging and eventually to death of organs and organism itself are called senescence or aging. Although meristems do not age, and are potentially immortal, all differentiated cells originating from meristem have a limited life span. Hence, the senescence occurs in all non-meristematic cells, but at various times. Senescence is induced by numerous internal and external environmental factors. The most important of the internal factors are the age and correlative relationships between vegetative and reproductive organs and those of the external are the water regime, mineral nutrition, light, temperature, and influence of various pathogens. According to whether the senescence is observed at the level of the whole organism or only individual parts, we determine different **monocarpic and sequential senescence**.

Sequential senescence

Polycarpic species include perennial herbaceous and wooden species, in which the senescence is mainly limited to reproductive organs and leaves. The example of sequential senescence is observed through gradual aging and leaf fall from the base upwards. It is assumed that sequential senescence occurs due to the competition for metabolites and nutritive matters between lower (older) and younger leaves in the upper parts of the plant developing rapidly. Sequential leaf senescence is particularly expressed under condition of diminished light intensity, lack of nutritive elements, and other unfavorable environmental factors (Stanković et al., 2006).

Molecular basis of senescence in plants

Senescence occurs at different levels: at the level of cells, tissue, organs and the whole plant. Loss of green color is the most obvious sign of leaf senescence. Photosynthesis is involved in the first major step of shutting down,

due to changes occurring in chloroplast structure and in their chemical composition. Instead of photosynthesis, the catabolism of chlorophyll, protein, nucleic acids and membrane lipids occurs in chloroplasts. Photosystem II and chlorophylls a and b are the first to undergo degradation. Since almost the whole chlorophyll b is located in the photosystem II the senescent leaves are characterized by a high ratio of chlorophyll a to chlorophyll b. Degradation of chlorophyll begins under the influence of chlorophyllase activity, which removes phytol, and then Mg-dechelataase acts on chlorophyllid extracting Mg from the porphyrinic nucleus. Hence the pheophorbide is formed in which the porphyrinic ring is broken down under the influence of oxygenase and the color is lost. Pheophorbide oxygenase enzyme is formed only in senescence. Other products of chlorophyll degradation are not degraded, do not exhibit fluorescence, and are accumulated in vacuole. In senescence, the mitochondria remain active and increased breathing is observed at an early stage of senescence. Number of peroxisomes is increased approx. four times compared to young leaves. However, their enzyme composition changes, and instead of being used in photorespiration (characteristic of photosynthesis) they are used in fatty acids oxidation and glyoxylate cycle (take over glyoxysome function). Nucleus in yellow leaves remain intact. Vacuoles survive and receive degraded products of many cell components. The level of lipids is decreased in senescence. Carbon hydrates are not formed due to lack of photosynthesis, and the fatty acids are the main substrate of increased respiration. Break down of macromolecules prior to leaf falling, and translocation of saccharose, glutamine and asparagine into other plant parts is of enormous importance for plant growth during the following season.

The level of total RNA decreases in leaf senescence, synthesis of many genes whose products participate in photosynthesis also shuts down, but new quantities of mRNA and proteins do occur. In aging leaves, new transcripts are selected, and at least 40 genes are cloned. Detailed analysis is done using leaves of *A. thaliana*. Genes activated during senescence are called genes associated with senescence or SAG genes (*senescence associated genes*). SAG12 is the most numerous gene completely lacking in young leaves (Pariasca et al., 2001; Gepstein et al., 2003).

The role of plant hormones in the process of plant senescence

The role of cytokinin in aging is confirmed by genetically transformed plants, which is incorporated into the genome of *lpt* gene. *lpt* is a bacterial gene coding the en-

zyme called isopentenyl transferase and it enables transgenic plants to synthesize large quantity of cytokinin. Such plants show various symptoms characteristic for cytokinin over dose application. Senescence of their leaves is slowed down. In one experiment, the *lpt* gene was linked to promoter of SAG12 gene from *A. thaliana*, which is active only in senescence. When this construct was introduced into tobacco genome, the plants did not differ from the control until the senescence occurred. Only then, the SAG12 became active, which led to an excellent cytokinin synthesis, and the interruption of the senescence. The leaves of transgenic plants remained green 30 days longer than leaves of the control plants (Gepstein et al., 2003). In individual plants, gibberellins have similar effect in senescence postponing, which was proved by longer retention of chlorophyll in cut leaves. There are characteristic plant mutants. For example, pea mutant is characterized by apical part of stem aging only during long day hours, and during short hours it grows and produces new leaves and flowers. It is because during short days there is a 10-fold increase in concentration of gibberellins found in plant tips (GA1). It was also confirmed that gibberellins added exogenously prevented senescence.

Ethylene is the major participant in senescence after pollination. In addition to that, it induces fruit maturation, after which they become senescent. Ethylene plays no key role in leaf aging, although it acts as cytokinin antagonist. It was proven that exogenous ethylene accelerated senescence, and inhibitors of ethylene synthesis slowed down that process. From the results obtained on mutant and transgenic plants it was concluded that the senescence depends first on internal factors associated with leaf aging, and that ethylene affects only the time of its occurrence. Numerous ethylene insensitive mutants of *A. thaliana* are studied and identified. They retain chlorophyll in senescence much longer than a wild type, but at the end they also develop the senescence (Stanković et al., 2006; Stevanović and Janković, 2001; Gepstein et al., 2003).

The Aim of the Strategy

Reference data and reports from forest managements revealed that senescence occurred in some parts of penduculate oak population in Srem induced by abiotic drought stress. The aim of this strategy is to determine genetic basis of senescence induced by drought, through system of molecular analyses and to give recommendations for development of new strategies for penduculate oak genepool preservation from Srem provenance. More precisely, the aim of the experiment would be to target candidate genes

responsible for penduculate oak senescence occurrence in Srem, induced by abiotic drought stress.

Methodological Approach

A. Detailed reference review -

- a-Biochemical pathways and metabolites included in the process of oak drying.
- b-Candidate genes and sequences with homology to known candidate genes from another species.

Advanced search and literature review in regard to above mentioned topics will be done through the on line database (KobSON, PubMed, ScienceDirect etc.) search mechanisms and other sources and will provide the basis for further development and the experimental confirmation of the null hypothesis. It will provide the prerequisites for finding the most appropriate experimental protocols that could be further adapted to our plant species aimed at exploration.

B. Detailed experiment setting according to the key points based on found literature and confirmed null hypothesis. Confirmation of critical experimental points and definite formulation of initial hypothesis – definition of target genes in penduculate oak and their expression.

C. Sampling of plant material - leaves and roots from the individuals obtained from seed and vegetative stands of Srem provenance. Sampling of our plant material must be done under strictly controlled condition – using sterile equipment and gloves, and physical integrity of the plant tissue must be preserved. This reduces the risk of potential contamination by other bio-molecules and preserves intactness of our genomic DNA and total RNA. Transfer of plant tissue to the testing laboratory must be done in sterile plastic or metal boxes or containers with added silica gel.

D. Isolation of total, genomic DNA and RNA from collected plant material. At this step it is essential to choose the method of isolations of both types of bio-molecules – using a standard protocol from the literature or laboratory practice or from available kits. Well-chosen method for isolation of both types of nucleic acids will enable more efficient, easier and more reliable laboratory analysis.

E. Control of the quality and quantity of yield isolation of nucleic acids from the sample – spectrometry and electrophoresis on 1% agarose. This step enables control of the quality and quantity of our isolation of nucleic acid from the plant sample. Spectrometry is

used to reveal the final concentration of our isolated nucleic acids in the solution, and the degree of purity, and electrophoresis is used to visually prove the level of purity (presence or absence of protein presence or nucleic acid degradation).

F. Isolation of iRNA from isolated total RNA. The choice of a standard protocol (trisol). Preservation of intact isolated RNA is the precondition for successful analysis of gene expression. Sample of our cellular iRNA must be decontaminated from the residues of genomic DNA to avoid nonspecific amplification.

G. Process of reverse transcription and construction of cDNA sequence. Reverse transcription is the greatest source of variability in RT-qPCR reaction (Pfaffl, 2003). Primers could be nonspecific or gene-specific. If specific primers are used the reaction is carried out at higher temperatures, reducing possibility of nonspecific transcription, but it is necessary to set separate RT reactions for each of the primer pairs, which increases variability between samples. Nonspecific primers could be hexamers, octamers or decamers primers, as well as the poly-dT primers for total mRNA isolation. If we use nonspecific primers, one reaction of reverse transcription would be enough, and the synthesized cDNA could be divided in such way as to set separate PCR reactions for each of the studied genes. As it leaves only RT reaction the variability of the results, which is the consequence of various reaction efficacy of the reverse transcription is avoided. **It was confirmed experimentally that the best efficacy of RT reaction is achieved with nonspecific-hexamer primers, then with oligo-dT, and the least with gene-specific primers.** Various detection systems are based on the principle of fluorescent labelled probes for qPCR or emission of fluorescent dyes, nonspecifically linked to double stranded DNA molecule (SYBR Green) (Vidović, 2009). Apparatus for quantitative PCR is equipped with optical system enabling fluorescence detection. Samples can be normalized in respect to one reference gene – endogenous control or relative to total cellular RNA (no. of molecules/g of total DNA/RNA). Concept of normalization based on total cellular RNA is very common, although the data on total RNA quantity in a cell is incomplete. It is believed that the optimal choice is normalization in relation to rRNA, despite the fact that ratio rRNA/mRNA varies between samples and that they were transcribed using different polymerases (Pfaffl, 2003). Absolute quantification (AQ-PCR) involves quantification of products of PCR reaction based on

constructed calibration curve. Precisely constructed calibration curve provides sensitivity, specificity and reproducibility of the obtained results. Reliability of the method also depends on the choice of the material, used as a standard. Standards are designed in such a way as to ensure stability for a long period of time. Recombinant plasmid DNA (recDNA), genomic DNA, RT-PCR products, in vitro transcribed recombinant RNA (recRNA), and synthetic oligonucleotides can be used as standards (Pfaffl, 2003). The choice of standard depends on the type of the experiment. Construction of calibration curve is performed for each individual gene. The results obtained by absolute qualification are expressed in relation to the quantity of total RNA, number of cells or tissue quantity.

H. Choice of stable endogenous control for qPCR analysis.

The choice of control is one of the critical moments in setting the experiment. There is no an ideal gene for endogenous control. While choosing endogenous control the literature data should be consulted and the most appropriate gene for the tested tissue and the type of treatment should be selected. The most important criteria while making choice is the fact that expression of endogenous control remained unchanged due to treatment or pathological state. The most often as endogenous control the mRNA are used:

- glyceraldehyde-3-phosphate dehydrogenase
- β -actin- this would serve as endogenous control in our case
- MHC I (major histocompatibility complex I)
- cyclophilins
- ribosomal RNA: 28S and 18S
- Arbp-acid ribosomal phosphoprotein PO (Vidović, 2009).

I. Reconstruction of 5' and 3' gene ends, responsible for senescence and resistance to drought, for *Quercus* genera using RACE method. RACE (eng. *Rapid Amplification of cDNA Ends*) is the technique used in studies of molecular biology in order to obtain complete RNA sequence or target cells. The results of this technique is the production of cDNA copies of targeted RNA sequence, obtained by the process of reverse transcription, followed by RT-PCR amplification. Amplified cDNA were then sequenced and if they were long enough they should be overlapping complementary with target RNA sequence. RACE could provide sequence of RNA transcript from a small source-sequence, starting from 5' (5' RACE-PCR) end to 3' end (3' RACE-PCR) RNA molecule (Sambrook and Rusell, 2001).

J. Control of gene expression intensity for „key“ metabolite- qPCR. Using the (Real-Time) qPCR method we would first perform the DNA/cDNA quantification from the isolated, target mRNA molecules and, of course, it happens during the amplification reaction, which has some advantages over the traditional PCR method. (*Measurements of the sample quantity in traditional PCR method are performed by agarose electrophoresis after completed PCR reaction, when products concentration corresponds to concentrations in the plateau phase of the reaction*). Quantitative chain polymerase reaction (qPCR) proceeded by the reverse transcription (RT-PCR) method was developed for analysis of gene expression, i.e. transcriptome of interest (Vidović, 2009).

K. Sequencing the fragments or the whole genes potentially responsible for the senescence of penduculate oak exposed to drought stress. Sanger methods of sequencing use the natural way of DNA replication as the basis for obtaining the precise distribution of nucleotides on DNA molecule/gene. This method is fairly automated today on so called sequencers of different resolution, speed and sequence capacity that it can receive. This method is based upon dideoxi principle, which can be most simple explained as follows: DNA replication is performed using DNA polymerase, which “is incorporated into the growing second DNA chain as opposed to lying polynucleotide chain (matrix) “. However, if in the replication procedure 2'3' ddNTP is used as a precursor instead of a natural nucleotide, then after its incorporation the series of replications will be completed. At this point the chain termination will occur. For each basis G,T,C, or A there is one corresponding 2'3' ddNTP (<http://www.nature.com/scitable/topicpage/the-order-of-nucleotides-in-a-gene-6525806>).

L. Bioinformatics applications for automated data processing through adequate analytical programs for gene expression control and analyses of obtained sequences; comparison of obtained results from on line database. **Bioinformatics applications for automated data and results processing would be performed throughout experimental activities.** Processing of data obtained by RT-qPCR method is supplied with great number of available program packages. Softwares are adapted to qPCR apparatus. Their designs enable users to analyze obtained results in a very simple way - REST (Relative Quantification Software), DART-PCR (Data Analysis for Real-Time PCR), Q-Gene, SoFAR and qBASE (Vidović, 2009).

M. Choice of appropriate expression (plasmid) vectors; transient transformation of penduculate oak in vitro culture with gene fragments or the whole gene responsible for drought resistance, which originate from other plant species (targeted by on line search of gene pool basis). The method involves only introduction and expression of foreign genes, without their integration into genome. This procedure is fast and reproducible, and gene products can be detected only few hours after DNA introduction, unlike the strategy of stable transformation, where months are needed for regeneration of transformants. Experiment of transient expression could as well be the pilot experiments for projects of stable transformation, used to control functionality of gene constructs prior their incorporation into genome by some other method. Transient expression is often used for gene expression testing during cellular cycle etc. (Simonović, 2011).

N. Control of penduculate oak transformation from in vitro culture -PCR and qPCR. Control of the presence and the level of expression of transgene of interest (targeted construct) in penduculate oak from *in vitro* culture using standard PCR and qPCR methods

Conclusion

Construction of the adequate strategy for molecular testing of identification and gene expression in all plant species enables a reliable approach to more efficient design of experiments. The most important is that prompt strategies enable time saving, which otherwise molecular genetic engineering may require. Oak is a wooden species of high economic and biological value in forestry. penduculate oak seed stands in Srem represent a resource of European importance, which allows the natural oak populations in Serbia and beyond to become sustainable and vital through climatic changes, which are inevitable and constantly present. Development of gene banks responsible for various aspects of resistance of oak and other tree species to various abiotic factors should become an imperative for sustainable forestry that can respond to various ecological challenges it is facing today. This paper has answered to a major part of the need for development of new gene banks responsible for drought resistance in penduculate oak in the Western Balkan.

Acknowledgements

This paper was realized as a part of the project “*Studying climate change and its influence on the environment:*

impacts, adaptation and mitigation” (III 43007) financed by the Ministry of Education and Science of the Republic of Serbia within the framework of integrated and interdisciplinary research for the period 2011–2014.

References

- Androić, M.**, 1974. Preliminary results of the team research causes drying in Slavonian oak forests. Proceedings of the hundredth anniversary of the forest southeast of Slavonia, Vinkovci-Slavonski Brod, pp. 59-76 (Cro).
- Durđić, T.**, 1932. Causes of drying of oak stands and measures taken in the Directorate of Forest Vinkovic, *Šumarški list*, pp. 438-459 (Cro).
- Fekete, L.**, 1890. Horvát-Szlavónország erdészeti viszonyai III, (Condition and relations in forestry in Croatia and Slavonia), *Erdészeti Lapok*, pp. 583-585, Budapest (Hu).
- Gepstein, Sh., Sabehi, G., Carp, Marie-Jeanne, Hajouj, T., Neshier, M.F.O., Yariy, I., Dor, C., Bassani, M.** 2003. Large-scale identification of leaf senescence-associated genes. *The Plant Journal*, **36**: 629-642.
- Jodal, I.**, 2008. The drying of oak forests in Posavina, Monograph: 250 years of forestry Ravni Srem, JP Vojvodinašume (Sr).
- Jovanović-Juga, S.**, 2005. The wonderful world of plants in botanical garden “Jevremovac” dendroflora Guide-Biology, University of Belgrade (Sr).
- Manojlović, P.**, 1926. The current situation in Slavonia oak forest. *Half a Century of Forestry*, pp. 372-385, Zagreb (Cro).
- Orlović, S., Erdeši, J., Radivojević, S., Obućina, Z., Janjatović, G.** 2001. Seed orchard of oak (*Quercus robur* L.) - a basis for further improvement in Srem. *Šumarstvo*, **1**: 1-9 (Sr).
- Orlović, S., D. Šimunovački, Z. Đorđević, A. Pilipović and N. Radosavljević**, 2008. The preservation of the gene pool and the production of seeds of oak (*Quercus robur* L.), Monograph: 250 Years of Forestry Ravni Srem, JP Vojvodinašume (Sr).
- Pariasca, J., A. T., Sunaga, A., Miyazaki, T., Hisaka, H., Sonoda, M., Nakagawa, H. and T. Sato**, 2001. Cloning of cDNAs encoding senescence-associated genes, ACC synthase and ACC oxidase from stored snow pea pods (*Pisum sativum* L. var *saccharatum*) and their expression during pod storage. *Postharvest Biology and Technology*, **22** (3): 239-247.
- Pfaffl, M. W.**, 2003. Habilitation: Livestock Transcriptomics: Quantitative mRNA Analytics in Molecular Endocrinology and Physiology (<http://gene-quantification.com/habilitation.html>)
- Popović, Ž., Purać, J., Kojić, D., Pamer L., E., Worland, R., Blagojević, D. and G. Grubor-Lajšić**, 2011. Lea Protein Expression During Cold-induced Dehydration in the Arctic

- Collembola *Megaphorura Arctica*. *Archives of Biological Sciences*, **63** (3): 681-683.
- Sambrook, J. and D. W. Russell**, 2001. Rapid amplification of 5' cDNA ends, in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
- Simonović, A.**, 2011. *Biotechnology and Genetic Engineering of Plants*. Beograd (Sr).
- Škorić, A. and A. Vranković**, 1974. Soil investigation in connection with the drying of pedunculate oak forests, Symposium: One Hundred Years of scientific and organized approach to forest southeast of Slavonia, Proceedings, pp. 403-411, Vinkovci (Sr).
- Stanković, Ž., B. Krstić, M. Petrović and Z. Erić**, 2006. *Plant Physiology*, Faculty of Sciences in Novi Sad, University of Novi Sad (Sr).
- Stebut, A.**, 1925a. A Contribution to a Cause of Drying Slavonian Oak. Beograd (Sr).
- Stebut, A.**, 1925b. More on Oak Drying in Slavonia. Beograd (Sr).
- Stevanović, M., B. and M. M. Janković**, 2001. *Ecology of plants with the basics of plant physiological ecology*. NNK International, Beograd (Sr).
- Vajda, Z.**, 1948. Impact of climate fluctuations on the drying of oak in Posavina and in lower Podravina lowland forests, p. 154, Zagreb (Cro).
- Vidaković, M.**, 1996. Raising the clone seed plantation of oak. In: Red Oak (*Quercus robur L.*) oak. CASA Center for Scientific Work, Vinkovci (Cro).
- Vidović, D.**, 2009. Standardization of conditions for the use of polymerase chain reaction analysis of gene expression in the example of Actb, and Tspo Hsd3b, The final paper, Faculty of natural sciences, Novi Sad (Sr).
<http://www.nature.com/scitable/topicpage/the-order-of-nucleotides-in-a-gene-6525806>

Received February, 2, 2012; accepted for printing December, 2, 2012.