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Physiology

Osmotic stress responses of individual white oak (*Quercus* section, *Quercus* subgenus) genotypes cultured *in vitro*Zita Demeter^a, Péter Kanalas^a, Csaba Máthé^{a,*}, Klára Cseke^b, Erzsébet Szöllösi^a,
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ABSTRACT

White oaks (*Quercus* section, *Quercus* subgenus) are widely distributed in Europe. *Quercus petraea* (sessile oak), an economically important species is predicted to be affected by climate change. *Q. pubescens* (pubescent oak) and *Q. virgiliana* (Italian pubescent oak) are economically less important, drought tolerant species. Frequent hybridization of white oaks was observed and currently the introgression of *Q. pubescens* and *Q. virgiliana* in non-mediterranean regions of Europe has been reported. Our goal was to use tissue cultures established from individual trees of the above taxa and their putative hybrids, all present in the forest stand of Síkfőkút LTER Research Area (NE Hungary) as simple experimental model systems for studying drought/osmotic stress tolerance. Tissue cultures are more suitable models for such studies, than seedlings, because they are genetically identical to the parent plants. Polyethylene glycol (PEG6000) treatments were used for this purpose. The identification of taxa was based on leaf morphological traits and microsatellite analysis and showed that *Q. petraea* is genetically distinct to all other taxa examined. We established six callus lines of *Quercus*. As expected, in *Q. petraea* cultures PEG6000 induced severe loss of fresh weight and the ability to recover after removal of the osmoticum, which was not characteristic for *Q. pubescens* and *Q. virgiliana*. Putative hybrids exhibited an intermediate response to osmotic stress. Activity gels showed the increase of single-strand preferring (SSP) nuclease and no significant change of guaiacol-peroxidase activities in drought-sensitive genotypes/cultures and no significant increase of SSP nuclease activities accompanied with increases of guaiacol-peroxidase activities in drought-tolerant ones. This indicates that drought/osmotic stress tolerance is associated to increased capacity of scavenging reactive oxygen species and hence less susceptibility to DNA damage. Our results confirm that tissue cultures of oak are suitable model systems for studying drought/osmotic stress responses.

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Introduction

Oaks (*Quercus* spp.) are widespread in Europe and play an important ecological and silvicultural role. High genetic and morphological variability has been reported for the *Quercus* section (=Lepidobalanus; or white oaks sensu Nixon, 1993) of the *Quercus* subgenus (Herzog, 1996; Gailing et al., 2007). The three main white oak species are *Quercus robur* L., *Q. petraea* (Matt.) Liebl and *Q. pubescens* Willd. Several other oak taxa have also been distinguished from the European broadleaved forests, of which *Q. dalechampii* Ten., *Quercus polycarpa* Schur, and *Q. virgiliana* Ten. are important. The two former ones resemble to *Q. petraea* sensu stricto and are usually included in the aggregate of *Q. petraea* sensu

lato, while *Q. virgiliana* belongs to the aggregate of *Q. pubescens* sensu lato (Schwarz, 1936a,b; Bordács et al., 2002). Interspecific hybridization is very common between white oak species (Curtu et al., 2007; Lepais et al., 2009; Salvini et al., 2009; Lepais and Gerber, 2011) which increases the genetic diversity in natural populations (Borovics et al., 1998; Gömöry and Schmidtová, 2007; Kanalas et al., 2008).

European white oaks differ in the preference for ecological conditions and grow in various habitats. The distribution of oaks is mostly dependent on their capacity to resist drought or excess of water in the soil or even the two phenomena successively (Jones, 1959; Johnson et al., 2002). Among the three main white oak species *Q. pubescens* is the most drought tolerant one and occupies warm and xeric sites in Europe (Borovics et al., 1998; Yurukov and Zhelev, 2001; Thomas et al., 2002; Gallé et al., 2007; Siam et al., 2009). *Q. petraea* grows predominantly on mesic or relatively dry sites on lower altitude slopes and ridges, whereas *Q. robur* can populate lowland sites with wet and temporarily waterlogged soils (Jones,

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Table 1
Identity of *Quercus* taxa used in this study, based on 16 leaf quantitative traits measured according to Borovics (2000) and Kanalas et al. (2008, 2009).

Explant code number	Identity
A149	<i>Q. petraea</i>
D137	<i>Q. petraea</i> × <i>Q. dalechampii</i>
D89	<i>Q. petraea</i> × <i>Q. pubescens</i>
A211	<i>Q. virgiliana</i> × <i>Q. polycarpa</i>
B50	<i>Q. virgiliana</i>
A75	<i>Q. pubescens</i>

1959; Aas, 1998). *Q. dalechampii* Ten. (Theodoropoulos et al., 1995) and *Quercus polycarpa* Schur (Matula, 2009) are distinct from *Q. petraea* sensu stricto in ecological requirements. Both are more drought tolerant and grow in warmer sites. *Q. dalechampii* Ten. and *Q. virgiliana* have been described from arid sites of southern Europe (Ofletea et al., 2011). Those oak species or their hybrids that are more capable to stand dry and hot summer periods could be important tools for the future forestry as global warming and frequent drought events are predicted to increase the pressures to European oak forests (IPCC, 2007).

In Hungary mixed forests of sessile oak and Turkey oak (*Quercus petraea*-*cerris*) cover the largest part of forested area. The Síkfőkút Long-term Ecological Research Site (LTER) (Bükk Mountains, north-eastern Hungary) represents this forest type (Jakucs, 1985) and has international reputation as a former IBP and MAB area and current LTER Europe network member. The area is situated in the transition between forest and forest-steppe zone (47°55' N, 20°26' E, 320–340 m a.s.l.) and is vulnerable to the climate change (Mészáros et al., 2007). Intense forest monitoring research has been running for 40 years in the site. The forest stand is currently characterized by the dominance of taxa from *Quercus* section and *Q. cerris* (Mészáros et al., 2007; Kanalas et al., 2008, 2009). Based on leaf morphological traits the assignment of trees to white oak species showed that this forest stand is mostly composed of *Q. petraea* sensu stricto (70.2%). 1% of sampled trees was assigned to *Q. polycarpa*, 2.5% was assigned to *Q. virgiliana* and 4% was assigned to *Q. pubescens*. 22, 3% of trees were considered as putative hybrids: *Q. petraea* × *Q. dalechampii* (5.1%), *Q. petraea* × *Q. polycarpa* (2.5%), *Q. petraea* × *Q. pubescens* (5.6%), *Q. petraea* × *Q. virgiliana* (7.1%) and *Q. virgiliana* × *Q. pubescens* (2%) (Kanalas et al., 2008, 2009). The percentage of hybrids is relatively high as compared to oak communities from other European regions (see Curtu et al., 2007; Gugerli et al., 2007 for examples). The oak decline observed in the 1980s in Europe approached this site too, the die-back of trees occurred primarily in population of *Quercus petraea* sensu lato.

Plant tissue culture techniques including callus and cell suspension cultures offer many advantages – e.g. they provide fully controllable systems – for physiological/biochemical studies. Consequently they were applied for oak species as well, e.g. for studying the role of ABA in the maturation of *Q. ilex* embryos (Mauri and Manzanera, 2004) or of dehydrin proteins in *Q. robur* somatic embryos (Šunderlíková et al., 2009). Even though *in vitro* cultures represent different developmental stages and gene expression patterns than mature plants, cell and callus cultures of sessile oak and pedunculate oak proved to be excellent models and are currently used for the study of osmotic stress-related transcriptional changes and other physiological responses. They are thought to be suitable for physiological studies at tissue, cell and molecular level (Gleeson et al., 2004; Porth et al., 2005; Šunderlíková et al., 2009).

In our study we have selected trees of several putative drought-sensitive and -tolerant taxa belonging to *Quercus* section co-occurring in the forest stand of Síkfőkút Project LTER site (Jakucs, 1985) for establishing *in vitro* cultures (Table 1). What could be the importance of tissue cultures in this respect? Instead of studying the effect of environmental stress in mature trees we can examine

physiological responses in *in vitro* cultures under controlled conditions. *In vitro* cultures established from vegetative tissues are more likely to reflect the genetic background of original plants/explants, than e.g. seedlings with uncertain genetic origin. Our principal aim was to study drought/osmotic stress tolerance of different oak genotypes in a model *in vitro* culture system. For this purpose, we needed to establish a procedure for creating stable tissue cultures since there were no literature data or culture ready-to-use for those oak genotypes. The use of axenic cultures allowed us to avoid potential superimposing effects of multiple environmental conditions.

Peroxidases (E.C. 1.11.1.7) play a role in the scavenging of reactive oxygen species (ROS) known for elevated levels during drought. The presence of scavenging systems is a good indicator of drought tolerance (Reddy et al., 2004). In the absence of protection against oxidative stress, DNA and RNA damage occurs. This may be accompanied by increases in the activity of nucleases, among them, single strand preferring (SSP) nucleases (EC 3.1.30.1) (Reddy et al., 2004; Roldán-Arjona and Ariza, 2009). Therefore, we studied PEG6000 induced changes in stress enzyme – peroxidase, nuclease – activities that accompanied alterations of fresh weight and recovery of oak tissue cultures. PEG6000 is widely used for modeling osmotic stress in higher plants, due to minimal side-effects and the incapability of plant cells to metabolize it (see Hohl and Schopfer, 1991; Guóth et al., 2010 for examples). Our basic hypothesis was that osmotic stress responses will be different for *in vitro* cultures established from explants derived from individuals of drought sensitive (*Q. petraea* sensu stricto) from those of drought tolerant species (*Q. pubescens*, *Q. virgiliana*). Since *Q. dalechampii* is closely related to *Q. petraea* (Borovics et al., 1998), thus the sensitivity to drought of tissue cultures of individuals representing hybrids between them (*Q. petraea* × *Q. dalechampii*) was expected to be close to drought sensitive *Q. petraea*. In contrast, cultures of *Q. virgiliana* × *Q. polycarpa* hybrids were expected to be more tolerant to osmotic stress than *Q. polycarpa*, due to the higher drought tolerance of *Q. virgiliana* parent. Cultures of *Q. petraea* × *Q. pubescens* hybrid were presumably intermediary between the two parents with respect to drought tolerance. If *in vitro* experiments confirm the expected responses for individuals of non-hybrid taxa (i.e. drought sensitivity of *Q. petraea* and tolerance of *Q. pubescens* and *Q. virgiliana*), they can be used for testing of cultures derived from *Quercus* genotypes (e.g. putative hybrids) with unknown drought sensitivity.

The main goal of this study was to offer a model system based on *in vitro* cultures of selected individual white oak trees for the estimation of their genotype-dependent drought tolerance.

Materials and methods

Plant material and tissue culture

Plant material was collected in the oak forest of Síkfőkút Research Area, North-Eastern Hungary (47°55'N, 20°26'E, 320–340 m a.s.l.) in early spring before leaf flush. Young (2–4 years old) shoots were cut from the lower canopy of 105–110 years old mature trees of *Quercus petraea* (Mattuschka) Lieblein, *Q. pubescens* Willd., *Q. virgiliana* Ten. and of putative hybrids and transferred to laboratory. Each explant was collected from a selected single tree per genotype listed in Table 1 and used for establishment of tissue culture and subsequent physiological experiments. All explants originated from the same ecological conditions. After collection shoots were kept under conditions of 22 ± 2 °C, 20 μmol m⁻² s⁻¹ PFD and 12/12 h photoperiod until the development of young leaves (12 ± 4 mm length). These young leaves were used as explants for the induction of tissue cultures. The induction and maintenance of callus production

Table 2

Key parameters for the SSR markers used for microsatellite analysis of white oak trees used in this study.

SSR primer pair	Repeat motif	Primer sequence	No. of alleles/ no. of trees	Observed heterozygosity	Reference for the method of microsatellite analysis
ZAG 1/5 F ZAG 1/5 R	(GT)5(GA)9	GCTTGAGAGTTGAGATTTGT GCAACACCCCTTAACTACCA	7/6	0.500	Steinkellner et al. (1997)
ZAG 9 F ZAG 9 R	(AG)12	GCAATTACAGGCTAGGCTGG GTCTGGACCTAGCCCTCATG	7/6	0.833	Steinkellner et al. (1997)
ZAG 110 F ZAG 110 R	(AG)15	GGAGGCTTCCTCAACTACT GATCTCTTGCTGCTGTATTT	9/6	1.000	Steinkellner et al. (1997)
ZAG 11 F ZAG 11 R	(TC)22	CCTTGAACCTCGAAGGTGTCCTT GTAGGTC A AAACATTGGTTGACT	4/6	0.833	Kampfer et al. (1998)
ZAG 96 F ZAG 96 R	(TC)20	CCCAGTCACATCCACTACTGTCC GTTTGGGAAAAGGAGATCAGA	7/6	1.000	Kampfer et al. (1998)
ZAG 112 F ZAG 112 R	(GA)32	TCTTGCTTTGGTGGCGC GTGGTCAGAG ACTCGGAAGTATTC	2/6	0.167	Kampfer et al. (1998)

was achieved on WPM medium (Woody Plant Medium, Lloyd and McCown, 1980) solidified with 0.8% (w/v) agar (Difco, Lawrence, KS, USA). The plant growth regulators (PGRs) used were 0.05–4 mg L⁻¹ (0.53–21.5 μM) α-naphthaleneacetic acid (NAA) and 0.05–1 mg L⁻¹ (0.25–0.5 μM) indole-3-butyric acid (IBA) as auxins and 0.1–4 mg L⁻¹ (0.44–17.7 μM) N⁶-benzyladenine (BA) as a cytokinin. All PGRs were from Sigma-Aldrich, Budapest, Hungary. The design of PGR content of tissue culture media was based on the methods of Seckinger et al. (1979), Tanaka et al. (1995), Cuenca et al. (1999) and Toribio et al. (2004). Growth conditions for *in vitro* culture were: 14/10 h photoperiod with a photon fluence rate of 10 μmol m⁻² s⁻¹ during the light period and temperatures of 22 ± 2 °C/18 ± 2 °C.

Determination of taxonomical status of white oak individuals by leaf morphological traits

Leaf morphological traits were assessed in all trees selected for establishment of *in vitro* cultures in a former comprehensive taxonomic survey of 198 white oak trees in the forest stand (Kanalas et al., 2008, 2009). Briefly, in this survey five leaves were collected from the lower canopy of trees. Altogether means of 16 leaf quantitative traits were used in numeric classification analysis worked out previously for each taxon (Borovics, 2000; Kanalas et al., 2008, 2009). This analysis allowed to classify the individual trees as oak species *sensu stricto* and hybrids.

Microsatellite (SSR) analysis

For DNA extraction winter bud samples of the individual trees listed in Table 1 were used. After grinding with liquid nitrogen the extraction was carried out by the Qiagen Plant Mini Kit (BioMarker, Gödöllő, Hungary). DNA concentration of extracts was checked by gel electrophoresis on a 0.5% agarose (Roth Roti[®] garose NEEO, RK Tech, Budapest, Hungary) gel. Polymerase chain reactions were performed with the following SSR markers (fluorescent dyes at the 5'-ends are indicated in brackets): ZAG 1/5 (6-FAM), ZAG 9 (6-FAM), ZAG 110 (HEX) (Steinkellner et al., 1997), ZAG 11 (TET), ZAG 96 (TET), ZAG 112 (HEX) (Kampfer et al., 1998). Key parameters for SSR markers are presented in Table 2. PCR mastermixes and optimisation procedures were made up according to Steinkellner et al. (1997) and Kampfer et al. (1998) and comprised the following components for 15 μL final reaction volume: 5× buffer (PromegaGoTaq Flexi) 4 μL; MgCl₂ 1 mM (0.6 μL) in case of markers ZAG 1/5 and ZAG 9, 2 mM (1.2 μL) in case of the other markers; Primer F and R (Roth Roti[®] garose NEEO, RK Tech, Budapest, Hungary): 0.25 pM (0.375 μL) each for ZAG 9, 0.75 pM (1.125 μL) for ZAG 1/5 and 0.34 pM (0.5 μL) for the remaining markers; dNTPmix (Promega 10 mM) 0.4 μL; polymerase enzyme (PromegaGoTaq Flexi) 0.4 U;

DNA sample 1 μL (approx. 10 ng/μL). For the PCRs an Eppendorf Mastercycler Gradient thermocycler was used with the following program: in case of ZAG 1/5 and ZAG 9 initial denaturation 95 °C 15 min, denaturation 95 °C 50 s, primer annealing 55 °C/ZAG 9 and 65 °C/ZAG 1/5 50 s, elongation 72 °C 1 min 45 s, repetition of last three steps in 35 cycles, final elongation 72 °C 10 min. In case of the other four markers: initial denaturation 95 °C 15 min, denaturation 95 °C 30 s, primer annealing 50 °C 30 s, elongation 65 °C 1 min 30 s, repetition of last three steps in 35 cycles, final elongation 65 °C 15 min. Fragment analyses were carried out by an ABI Prism 310 genetic analyser (Applied Biosystems Life Technologies, Budapest, Hungary) in multiplexed runs. C matrix set and TAMRA 500 size standard were applied (Applied Biosystems Life Technologies). The evaluation of fragment sizes was done by the GeneMapper software.

The raw genotype data set with the SSR fragment length sizes was analyzed by the GenAlEx 6.4 (Peakall and Smouse, 2006) population genetic software. The genetic distances between pairs of individuals were calculated based on the shared allele frequencies. The genetic relationship among samples was represented on a dendrogram constructed with the unweighted pair-group average amalgamation method (UPGMA) (Sneath and Sokal, 1973). For this purpose the Cluster Analyses option of Statistica 6.0 software was used.

PEG treatments and recovery experiments

Callus cultures were grown on solidified WPM medium (see Results section for PGR content). They were transferred on liquid medium of the same composition (2 mL culture medium in 10 mL sterile plastic flasks, Labsystem, Budapest, Hungary) and treated for 24 h with 0, 5, 10, 20 and 40% (w/v) polyethylene glycol 6000 (PEG6000, VWR, Leuven, Belgium). PEG6000 solutions corresponded to osmotic potentials of -0.05, -0.15, -0.49 and -1.75 MPa. During treatments with the osmoticum, cultures were gently shaken (100 rpm) on a rotatory shaker (E. Bühler KS-15, E. Bühler GmbH, Hechingen, Germany). Fresh weight (FW) of calli was measured at the start and the end of PEG6000 treatments by means of an analytical balance (Model AA 200 DS, accuracy ±10 μg, Denver Instrument Co., Arvada). FW was in the range of 20–50 mg. Before FW measurement at the start of experiments, excess liquid medium was removed by gentle centrifugation without affecting callus growth and viability (1000 rpm, 5 s on a Heraeus Biofuge, Kendro Laboratory Products, Harau, Germany). Enzyme activities (see below) were measured directly after treatments with the osmoticum. Percentage of FW increase was calculated on the basis of the difference between FW at the end and at the start of experiments. Recovery experiments were performed as follows: following PEG6000 treatments, cultures from PEG containing

medium were washed out two times by gentle shaking (100 rpm) in the presence of liquid culture medium lacking the osmoticum followed by further culture on agar-solidified medium for 30 days. Fresh weight of recovered calli was also measured as described earlier. Beside FW measurements at the start and end of culture period, the presence of viable, green callus tissue at the end of culture was monitored as well.

SSP nuclease activity gels

The activity of SSP nucleases (EC 3.1.30.1) was assayed on polyacrylamide gels, basically as described before (Jámbrik et al., 2011). In brief, PEG6000 treated calli were extracted with 10 mM Tris-HCl (Sigma-Aldrich, Budapest, Hungary), pH 8.0, 150 mM NaCl (Reanal, Budapest, Hungary), 14.6 mM 2-mercaptoethanol (Sigma-Aldrich) and 2% (w/v) polyvinyl-pyrrolidone (PVP, Merck, Darmstadt, Germany). Protein extracts (20 µg/well) were loaded on SDS- and single-stranded DNA containing polyacrylamide gels along with a molecular weight marker (Sigma-Aldrich). Protein content of extracts was determined according to Bradford (1976). After renaturation of enzymes, gels were incubated in Tris-HCl, pH 6.8 (14 h, 39 °C) for assaying their activity, stained with 0.5 µg mL⁻¹ ethidium bromide (Sigma-Aldrich, Budapest, Hungary) and examined with an UV transilluminator. SSP nuclease activities appeared as clear bands, not stained with ethidium-bromide. Total nuclease activities on gels were quantified with the aid of CpAtlas[®] software and expressed as relative band intensities, where the value of control activities was 1. The molecular weight of ssDNase isoenzymes was estimated with the UVI-TEC[®] software.

Peroxidase activity gels

PEG6000 treated calli were extracted at 4 °C with a buffer containing 100 mM KH₂PO₄/K₂HPO₄ (VWR International Ltd., Debrecen, Hungary), pH 7.2, 8 mM MgCl₂ (Reanal, Budapest, Hungary), 4 mM dithiothreitol (DTT, Sigma-Aldrich), 1% (v/v) Triton X-100 (Reanal), 2% (w/v) PVP (Merck). After centrifugation (two times for 30 min) at 15,000 × g with a Heraeus Biofuge, protein content of supernatants was assayed by the method of Bradford (1976). 10 µg protein was loaded onto each well of native 7.5% (w/v) polyacrylamide gels. Electrophoresis was performed at 4 °C, followed by gel staining for 30–60 min in a buffer containing 100 mM sodium acetate (VWR), 10% (v/v) hydrogen peroxide and 1 mM guaiacol. Peroxidase (E.C. 1.11.1.7) activity was visible due to dark-colored tetraguaiacol bands (Dixit et al., 2011). Guaiacol-peroxidase activities were quantified with the aid of CpAtlas[®] software, and expressed as for SSP nucleases.

Data analysis

All experiments were performed at least four times with six parallel callus samples per experiment for each genotype and representative data are presented in the Results section. The mean ± SE of quantitative data was calculated and plotted with the aid of Sigma Plot 10.0 software, where it was appropriate. Plots represent mean ± SE values for different calli/the respective individual genotype. Quantitative data were subjected to statistical analysis by two-way ANOVA. This involved All Pairwise Multiple Comparison Procedures (Holm-Sidak method) with an overall significance level of 0.05. This method allowed to analyze the effects of PEG concentrations within a single genotype as well as differences between genotypes within a single PEG concentration. Differences were considered significant at $P < 0.05$.

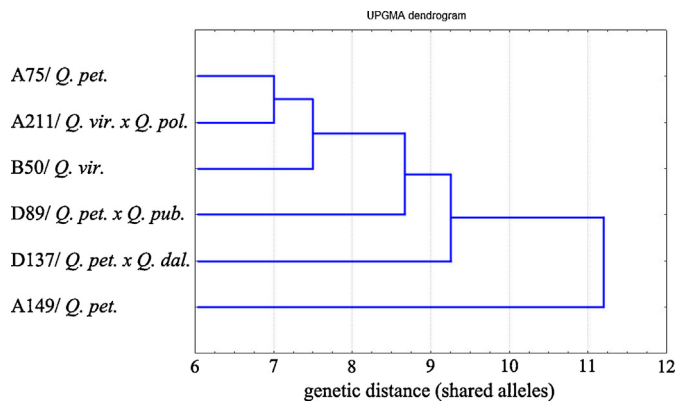


Fig. 1. Cluster dendrogram showing genetic distances between *Quercus* taxa involved in this study.

Results

Genetic relationships between *Quercus* explants studied

Explants used in this study originated from six individual mature trees from the *Quercus* (=Lepidobalanus) section co-occurring at the Síkfökút Research Site (Bükk Mts., Hungary). Leaf morphological traits of the individual trees were studied in order to estimate their taxonomical identity. Based on this, we identified three non-hybrid and three hybrid taxa (Table 1). In order to estimate the genetic relatedness of selected trees a microsatellite analysis was applied. Then genetic distances were calculated between individuals based on the shared allele content of multilocus genotypes derived from the six SSR loci analyzed (Fig. 1). This revealed that A149 (*Q. petraea*) is genetically distinct to all other individuals examined, including A75 (*Q. pubescens*) and B50 (*Q. virgiliana*). D89, a putative *Q. petraea* × *Q. pubescens* hybrid and A211, a putative hybrid between *Q. virgiliana* and *Q. polycarpa*, appeared to be in the same cluster with B50 and A75. D137, a putative hybrid between *Q. petraea* and *Q. dalechampii* was genetically distant to A149 and appeared to be in the same cluster, but at a relatively high distance to all other individuals (Fig. 1).

The establishment and maintenance of tissue cultures from white oak explants

We have induced and stabilized callus cultures for the first time from six *Quercus* genotypes originating from Síkfökút Research Area (Table 1). A wide range of growth regulator concentrations was tested (see Materials and methods section). Calli appeared after 30 ± 3 days of culture of young leaf explants. PGR combinations for callus induction and maintenance as well as embryogenesis and organogenesis were genotype dependent (data not shown). This is a general rule for tissue cultures of related, but genetically different plant taxa (see Duncan et al., 1985; Máthé et al., 2012 for examples). However, we have found a PGR combination, where all callus samples originating from different oak genotypes were of similar morphology (undifferentiated state), growth rate and viability. This was WPM medium containing 4 mg L⁻¹ NAA and 0.5 mg L⁻¹ BA. At this PGR content, growth of all stable cultures proved to be constant since they were initiated (a time period of at least one year), therefore they were suitable for osmotic stress experiments.

It is worth mentioning, that we could not establish an efficient micropropagation system (i.e. mass plant regeneration without an intermediary callus stage) from the aforementioned oak genotypes. However the choose of PGRs (NAA and BA instead of 2,4-D and kinetin) excluded somaclonal variability during callus induction

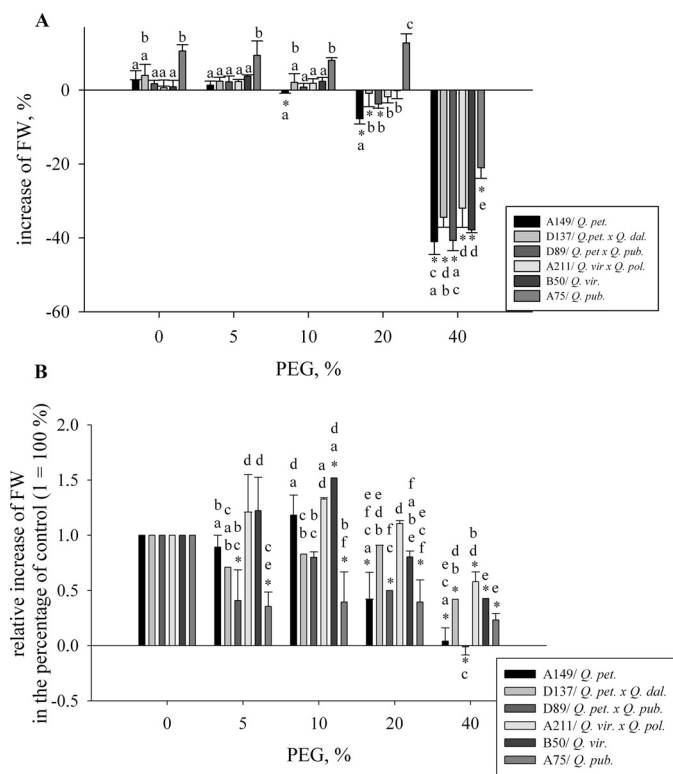


Fig. 2. The effect of PEG concentration on fresh weight changes of calli with different taxonomic/genetic origin. Cultures were grown on WPM medium containing 4 mg L^{-1} NAA and 0.5 mg L^{-1} BA. (A) FW changes after PEG treatments; (B) FW changes during recovery of PEG treated calli. Mean \pm SE values for different calli/the respective individual genotype are plotted. Asterisks represent significant differences between PEG treatments for a single genotype, while lettercodes represent differences between callus lines within a single PEG treatment. Differences were considered to be significant at $P < 0.05$.

and maintenance, thus callus cultures proved to be suitable for osmotic stress experiments.

The effects of PEG6000 on water loss and recovery of *Quercus* callus cultures

Two-way ANOVA revealed that there was a significant relationship ($P < 0.05$) between the genotype of individual oak trees and the effects of PEG treatments concerning all physiological parameters (growth, recovery and enzyme activities) studied (Figs. 2–5).

The measurement of FW after treatment with the osmoticum revealed that for A149, significant water loss occurred at 10% PEG6000 and increased progressively and significantly as PEG6000 concentrations increased. In contrast, A75 and B50 were tolerant to osmotic treatment and significant water loss occurred only at 40% PEG6000 (Fig. 2A). Putative hybrids were intermediate with respect to the effects of the osmoticum: water loss occurred at 20% PEG6000 and increased at 40% PEG6000 (Fig. 2A). At recovery after osmotic stress, changes of FW showed that for A149, callus growth was slightly stimulated by 10% PEG6000, but inhibited significantly at higher concentrations, while for A211 and B50 there was a transient stimulation at 5–10% PEG6000 (Fig. 2B). After washout of the osmoticum there was a significant inhibition in the growth of A211 calli pretreated with 40% PEG6000 and pretreatment with 20–40% PEG6000 inhibited the growth of B50 calli. Thus there was no recovery in these cases. In case of culture lines D137, D89 and A75, there was a general inhibition of callus growth by PEG6000 (Fig. 2B).

Statistical analysis of data revealed that PEG6000 induced water loss of A75 calli was significantly different to all other callus lines.

Pairwise comparison showed significant differences in PEG6000 induced water loss between A149, B50 and A211 only at higher (20–40%) concentrations (Fig. 2A). Concerning callus growth during recovery experiments, although significant differences were observed between callus lines at certain PEG6000 concentrations (Fig. 2B), significantly higher growth rate of A75 and B50 calli as compared to lines A149, D137, D89 and A211 was not detected.

Concerning the presence of viable, compact, green callus tissue during recovery experiments, we have made the following observations. For A149 (*Q. petraea*), this type of tissue was not present at pretreatment with 10–40% PEG6000: it was replaced by necrotic-like, browning tissue that suggested the accumulation of phenolic compounds as a stress reaction (Fig. 3). In contrast, for A75 (*Q. pubescens*) and B50 (*Q. virgiliana*), tissue viability persisted even at 40% PEG (Fig. 3). D89 as a putative hybrid between *Q. petraea* and *Q. pubescens*, was intermediate in this respect: callus viability was lost only at 20–40% PEG. The putative hybrids D137 and A211 behaved similarly to A75 and B50 (Fig. 3).

Osmotic stress-related enzyme activities

Activity gels revealed that SSP nuclease isoenzyme(s) with molecular weight(s) in the range of 45–55 kDa were present and active in all culture lines. One dominant band of 50 kDa was detected (Fig. 4A). In control cultures, this activity was weaker in lines A149, D137 and D89 as compared to A211, B50 and A75 (Fig. 4A). The effects of PEG6000 treatments were dependent on culture line/genotype. Except A75 (*Q. pubescens*) cultures, osmotic stress induced transient increases in nuclease activities as compared to controls, with maximal activities at 5–20% PEG6000. Significant increases were detected for D89 (5–10% PEG6000), A149 (10–20% PEG6000) and A211 (20% PEG6000). In case of B50 (*Q. virgiliana*) there was only a slight stimulation of SSP nuclease activity by PEG (Fig. 4A and B). In case of A75, PEG6000 did not increase notably the enzyme activity, but at 5–10% PEG6000, two bands with strong activities were detectable in the molecular weight range of 50 kDa (Fig. 4A and B). 5–20% PEG6000 induced the appearance of one additional band with relatively weak activity in case of B50 and A211 (Fig. 4A).

Two-way ANOVA revealed significant differences between SSP nuclease activities of PEG6000 treated A149 and D137, B50, A75, respectively. At 20% PEG, A149 was characterized by significantly higher SSP nuclease activity than the rest of callus lines. Besides A149, at 10% PEG6000, enzyme activity of A211 was significantly higher as compared to A75 (Fig. 4B).

Concerning guaiacol peroxidase activities, one band with strong activity appeared in all cultures. PEG6000 decreased this enzyme activity in A149 (*Q. petraea*), although this decrease was not significant (Fig. 5A and B). A non-significant decreasing effect was observed in the case of putative hybrids D89 and A211 as well, but transient increases were detectable at 10% and 5–10% PEG6000, respectively (Fig. 5A and B). PEG treatments increased guaiacol peroxidase activities in the putative hybrid D137, *Q. virgiliana* (B50) and *Q. pubescens* (A75) with peaks at 5% (D137, B50) and 20% PEG6000 (A75) (Fig. 5A and B). An additional band of weak peroxidase activity was observed in case of D89, A211 and A75. For A75, this band was present only after treatments with 20% PEG6000 (Fig. 5A).

Two-way ANOVA revealed significant differences between peroxidase activities of PEG6000 treated A149 and D137, B50, A75, respectively (Fig. 5B). At 5% PEG6000, the enzyme activity of B50 was significantly higher, than A75. Concerning overall effects of the osmoticum, peroxidase activities of the putative hybrid D89 did not differ significantly to A149, but it had significantly lower activities,

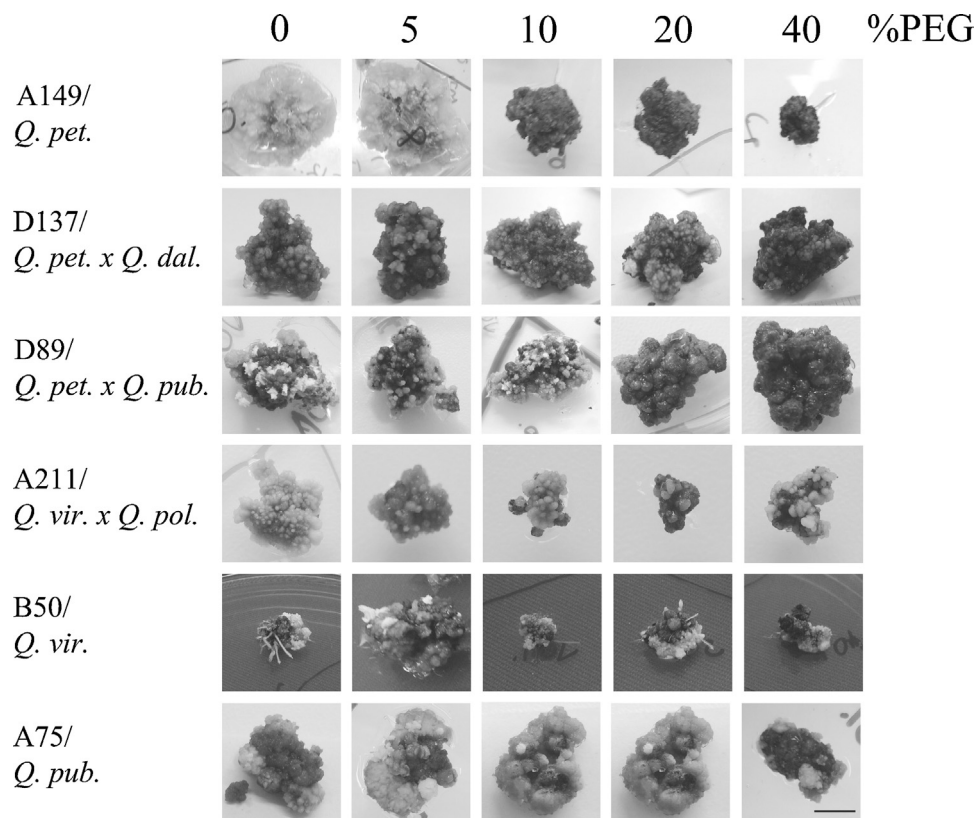


Fig. 3. Representative recovery experiments with *Quercus* tissue cultures of different taxonomic/genetic origin. PEG treatments were followed by washout of the osmoticum and further culture on a medium supplemented with 4 mg L^{-1} NAA and 0.5 mg L^{-1} BA. Scale bar: 5 mm.

than D137 and B50. A211 had significantly lower activities, than B50 and D137 (Fig. 5B).

Discussion

We have established six novel stable callus lines from oak genotypes belonging to the *Quercus* section (= *Lepidobalanus*) of *Quercus* subgenus. These cultures were suitable for comparing differences in osmotic stress responses among genotypes. Based on previous work on the production of stable tissue cultures of *Q. robur* (Cuenca et al., 1999; Toribio et al., 2004), we established the proper culture media suitable for drought stress experiments with PGR content of 4 mg L^{-1} NAA and 0.5 mg L^{-1} BA.

Clear distinction from molecular markers between *Q. petraea* and *Q. pubescens* is often difficult (Salvini et al., 2009). However, microsatellite and isoenzyme data have previously demonstrated that *Q. petraea* and *Q. pubescens* are distinct species, but crossing between them is possible (Samuel et al., 1995; Bruschi et al., 2000). By applying 6 microsatellite loci, in this study the selected *Q. petraea* individual tree could be clearly distinguished from all other *Quercus* (= *Lepidobalanus*) individuals, including *Q. pubescens* and putative hybrids (Fig. 1). Thus, significant genetic distances could be observed between individuals characterized by different leaf morphological traits. This is of particular importance, since different leaf morphologies of oaks do not necessarily reflect notable genetic differences (Curtu et al., 2007). The clear distinction of *Q. petraea* to all other individuals studied was confirmed by *in vitro* morphogenesis experiments as well: this individual explant could not regenerate roots, in contrast to all other explants studied, where efficient root production was regularly observable (to be published elsewhere). Native gels revealed a single main peroxidase activity band for all culture lines. In case of D89, A211 and A75 a minor additional band appeared. Interestingly, this band was inducible

by PEG in case of A75, that is, it was detectable only during osmotic stress (Fig. 5A, arrowheads). This additional band further supported microsatellite data: in case of the genetically more distinct lines A149 (*Q. petraea*) and D137 it was not present, while it appeared in genetically related culture lines mentioned. In case of SSP nuclease activity patterns, in the genetically related lines A211, B50 and A75, two bands appeared at PEG6000 treatments, both in the molecular weight range of 45–55 kDa (Fig. 4). It should be noted however, that many nucleases are glycoproteins and the presence or absence of glycoside residues depends on the physiological state of cells (Desai and Shankar, 2003). Therefore it is possible that double bands detected reflect a single protein with different glycosylation states.

Drought/osmotic stress, as a significant number of abiotic stresses, leads to the increase of ROS in plant cells (Mittler, 2002). Resistance to drought involves an increased capacity of scavenging ROS through superoxide dismutases (SOD), catalases and peroxidases (Wang et al., 2003). Non-enzymatic and enzymatic scavenging mechanisms are stimulated during summer midday characterized by high light exposure, temperature and water deficiency in *Q. suber* (Faria et al., 1996). In contrast, for drought and salt sensitive *Q. robur*, among ROS scavenging systems, only superoxide dismutase (SOD) activity and isoenzyme pattern was modified at exposure to NaCl (Sehmer et al., 1995). Drying of recalcitrant *Q. robur* acorns is leading to the loss of embryo viability, associated with the accumulation of ROS and low levels of scavenging enzymes (Hendry et al., 1992). In case of a drought tolerant *Q. robur* genotype, elevated levels of ROS scavenging enzyme (SOD, ascorbate peroxidase, catalase, dehydroascorbate reductase, glutathione reductase) activities were detected (Schwanz and Polle, 2001). In the absence of a proper scavenging capacity, cellular structures and macromolecules including DNA (single strand breaks) are significantly damaged (Reddy et al., 2004). Following oxidative damage

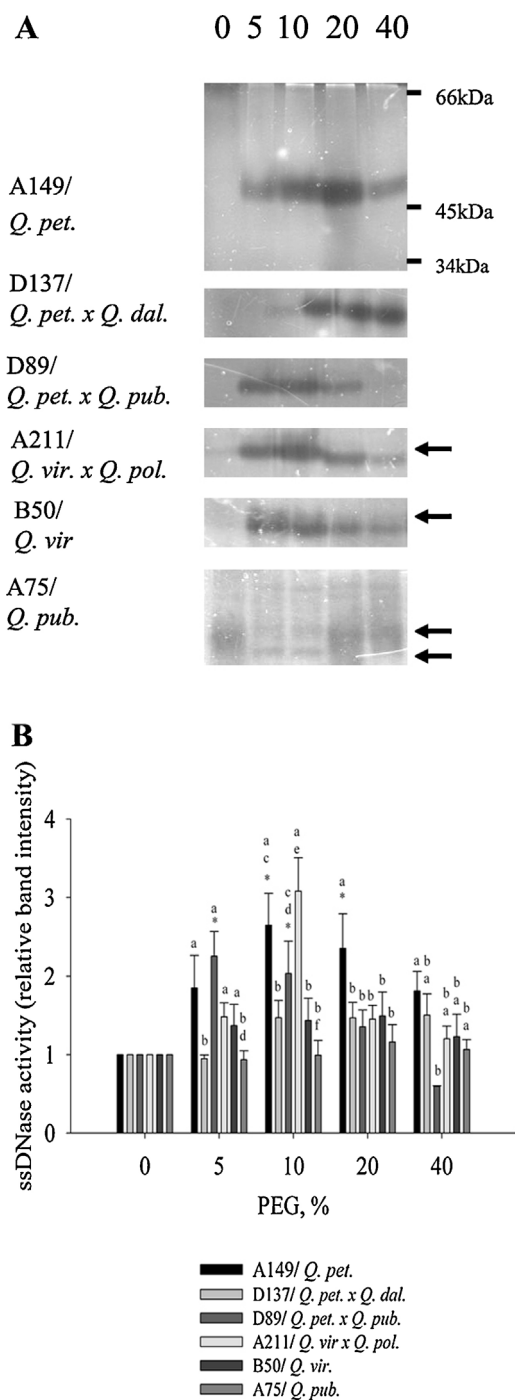


Fig. 4. The effect of PEG on ssDNAse activities of *Quercus* calli of different genetic origin, grown on a medium supplemented with 4 mg L⁻¹ NAA and 0.5 mg L⁻¹ BA. (A) Representative gels. Arrows indicate the presence of additional bands; (B) enzyme activities represented by relative band intensities as measured with CP Atlas software. Mean ± SE values for different calli/the respective individual genotype are plotted. Asterisks represent significant differences between PEG treatments for a single genotype, while lettercodes represent differences between callus lines within a single PEG treatment. Differences were considered to be significant at $P < 0.05$.

of DNA, cells are characterized by the activity of nucleases involved in repair (Roldán-Arjona and Ariza, 2009).

Several SSP nucleases inducible by drought/osmotic/salt stress have been identified. For example, barley Bnucl is a salt stress inducible type I nuclease, a putative glycoprotein slightly smaller than 36 kDa from barley (Muramoto et al., 1999). Hydrogen peroxide and drought stress induces the activity of several nuclease

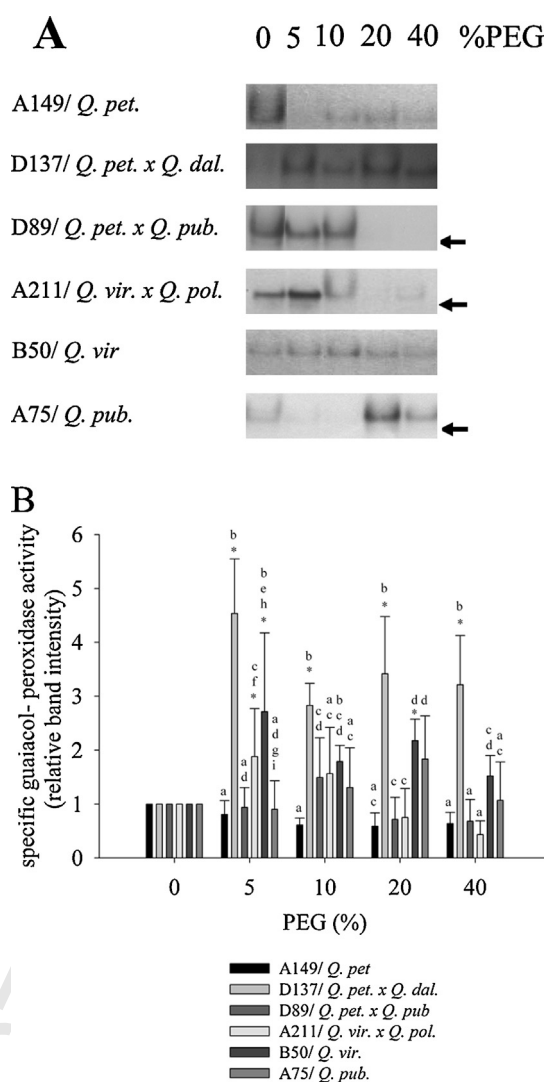


Fig. 5. The effect of PEG on guaiacol peroxidase activities of *Quercus* calli of different genetic origin, grown on a medium supplemented with 4 mg L⁻¹ NAA and 0.5 mg L⁻¹ BA. (A) Representative gels. Arrowheads indicate the presence of additional bands. (B) Enzyme activities represented by relative band intensities as measured with CP Atlas software. Mean ± SE values for different calli/the respective individual genotype are plotted. Asterisks represent significant differences between PEG treatments for a single genotype, while lettercodes represent differences between callus lines within a single PEG treatment. Differences were considered to be significant at $P < 0.05$.

isoenzymes using ssDNA as substrate of molecular weights ranging between 26 and 38 kDa in cauliflower seedlings. Two of them appeared to be the same enzyme, inducible by both stress factors (Leśniewicz et al., 2010). The main nuclease isoenzyme proved to be modulated by osmotic stress in this study appeared to have a molecular weight of 50 kDa (Fig. 4). To our best knowledge, nucleases of similar molecular weight, with changing activity at abiotic stresses are unusual in plants. It should be noted however, that a significant number of nucleases consist of subunits of lower size (see Desai and Shankar, 2003 for a review). Thus, the nature of *Quercus* nuclease of 50 kDa needs further investigation.

In case of drought sensitive individuals (A149, D89), PEG-induced increase of SSP nuclease activity was accompanied by, no significant changes or decreases of guaiacol-peroxidase activities in callus. In contrast, for drought-tolerant taxa (A75, B50), PEG treatments led to unchanged or slightly increasing SSP nuclease activities together with increases of peroxidase activities (Figs. 4 and 5, see details below). This means that ROS cannot

Table 3
Overview of potential osmotic stress/drought tolerance of PEG6000 treated oak callus lines as shown by different physiological parameters. This classification was based on significant differences in physiological responses as revealed by two-way ANOVA.

Physiological parameters	FW	Growth (recovery)	Viability (recovery)	ssDNase activity	Guaiacol-peroxidase activity
Potentially drought tolerant	B50, A75	n.d.	A211, B50, A75	B50, A75	D137, B50, A75
Potentially drought sensitive	A149, D89	n.d.	A149, D89	A149, D89, A211	A149, D89
Is the difference between A75 and B50 significant?	Only at 40% PEG6000	Yes	No	No	Yes

n.d., not detectable on the basis of available data.

be scavenged in drought-sensitive taxa, followed by DNA strand breaks leading to activity increases of nucleases probably involved in repair. Drought-tolerant taxa are expected to be able of scavenging ROS efficiently, thus DNA strand breaks occur probably with less frequency. However, changes (increases or decreases) of SSP nuclease activities were not proportional with changes of peroxidase activities for all genotypes and/or PEG concentrations (Figs. 4 and 5). This indicates that besides ROS levels, other mechanisms could modulate nuclease activities.

In case of callus line A149 (*Q. petraea*), treatments with PEG6000 induced concentration-dependent water loss, as seen by the decrease of callus fresh weight. The capacity of calli to grow and to produce green, viable tissues decreased after removal of 20–40% and 10–40% PEG6000 respectively, as shown by recovery experiments (Figs. 2 and 3). Meanwhile, peroxidase activity decreased and SSP nuclease activity increased (Figs. 4 and 5). All these effects of osmotic stress in a model experiment (Table 3) confirm previous findings (Thomas et al., 2002) and our hypothesis, that is, sessile oak is generally a drought/osmotic stress sensitive species. In contrast, cultures of A75 (*Q. pubescens*) and B50 (*Q. virgiliana*) are resistant to PEG6000 induced water loss and they are able to recover even after treatments with high concentrations of the osmoticum (even though their increase in fresh weight is inhibited by PEG in recovery experiments, Figs. 2 and 3). Osmotic stress increases peroxidase activities and induces only slight increases of SSP nuclease activities in these cultures. Overall, these results confirm previous findings and our hypothesis, that is, tissue cultures of *Q. pubescens* and *Q. virgiliana* are drought/osmotic stress tolerant (Table 3). Moreover, microsatellite data show a close relatedness between A75 and B50 (Fig. 1). For D89, PEG6000 induced water loss was intermediary as compared to A149 and A75, as shown by changes of callus fresh weight and the persistence of callus recovery after treatment with 10% PEG (Figs. 2A and 3). On the other hand, osmotic stress induced the increase of ssDNase activity and except treatments with 10% PEG, the decrease of peroxidase activity (Figs. 4 and 5). Overall, these parameters suggest that drought response of D89 is intermediary between A149 and A75, adding further proof to the idea that D89 is a hybrid between *Q. petraea* and *Q. pubescens*. However, all physiological parameters suggest that cultures of D89 are more tolerant to drought, than A149, but still can be considered as drought sensitive (Table 3). Peroxidase activities of PEG6000 treated D137 calli increased as of drought resistant genotypes (Fig. 5 and Table 3), even though we have initially hypothesized that as a hybrid between *Q. petraea* and *Q. dalechampii*, this genotype is relatively drought sensitive. Indeed, microsatellite data confirmed that D137 is genetically closer to the cluster containing *Q. pubescens*, than to *Q. petraea* (Fig. 1). On the other hand, *Q. dalechampii* lineage could also explain a higher drought tolerance. Concerning A211, its water loss and ability to recover was intermediary between drought sensitive A149 and tolerant A75 (Figs. 2A, 3 and Table 3), but PEG6000 induced SSP nuclease and peroxidase activity changes were similar to A149 (Figs. 4 and 5). Morphological and microsatellite data were not comparable for this culture line. Leaf morphology traits data suggested that A211 is a hybrid between *Q. virgiliana* and *Q. polycarpa*, but

the analysis of SSR markers suggested its close relatedness to the analyzed *Q. pubescens* tree (Table 1 and Fig. 1). Thus, even though this individual tree is putative hybrid between a drought sensitive and a drought resistant oak taxon, genes/proteins responsible for drought tolerance seem to be weakly expressed.

It should be noted that a given oak species can be characterized by high intra-populational genetic variability (Herzog, 1996) and as a consequence, differences between physiological responses of individuals may occur. Thus, intra-populational spectrum of drought tolerance of oaks in the Síkfökút LTER area needs further studies.

Table 3 shows the estimated osmotic stress/drought tolerance of the six oak culture lines studied as shown by significant physiological responses to PEG treatments. All physiological changes induced by osmotic stress indicated that drought sensitivity of *Q. petraea*, *Q. pubescens*, and *Q. virgiliana* cultures confirmed previous laboratory and field studies including ecological requirements of their populations (Cochard et al., 1992; Damesin and Rambal, 1995; Gallé et al., 2007; Siam et al., 2009; Rodríguez-Calcerrada et al., 2010; Ofletea et al., 2011) and proved our hypothesis: such *in vitro* cultures can be additional model systems for studying stress responses of taxa with unknown drought responses (D89, D137 and A211 in the present study). Interestingly, B50 (*Q. virgiliana*) and A75 (*Q. pubescens*) had significantly different responses in recovery experiments and concerning peroxidase activities (Figs. 2, 3 and 5 and Table 3), even though our data and previous findings suggest that both *Q. pubescens* and *Q. virgiliana* are drought tolerant species (see Table 3; Siam et al., 2009; Ofletea et al., 2011 for example). In general, microsatellite data confirmed relatively high genetic distances between drought sensitive and drought tolerant oak individuals.

The present work offers an *in vitro* model of studying physiological responses of oak to drought, by using tissue cultures established from selected individual trees with different taxonomic identity co-occurring in the same forest stand. Further studies both in the field and laboratory, the latter concerning tissue cultures from different individuals from the same population will reveal natural variability in drought tolerance of white oaks. We suggest that *in vitro* callus cultures as simplified experimental systems are helpful tools contributing to the modeling of drought tolerance of field grown oak plants with different genetic background and could be of general applicability for plant stress biology research.

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