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To cite this version:

Julien Parelle, Oliver Brendel, Catherine Bodenes-Brezard Bodénès, Daniel Berveiller, Pierre P. Dizengremel, et al.. Differences in morphological and physiological responses to water-logging between two sympatric oak species (Q. Petraea [Matt.] Lieb., Q. Robur L.). Annals of Forest Science, 2006, 63 (8), pp.849-859. 10.1051/forest:2006068 . hal-02656239

HAL Id: hal-02656239

https://hal.inrae.fr/hal-02656239

Submitted on 29 May 2020

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Ann. For. Sci. 63 (2006) 849–859
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DOI: 10.1051/forest:2006068

Original article

Differences in morphological and physiological responses to water-logging between two sympatric oak species (*Quercus petraea* [Matt.] Liebl., *Quercus robur* L.)

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(Received 24 October 2005; accepted 1 February 2006)

Abstract – Pedunculate (*Quercus robur* L.) and sessile (*Q. petraea* [Matt.] Liebl.) oaks are known to display different ecological requirements, particularly relative to root hypoxia induced by water-logging. *Q. robur* is more tolerant to hypoxia than *Q. petraea*. We designed an experiment aiming at identifying morphological and physiological responses to root hypoxia that might differ between the two species. Potted seedlings were submitted during seven weeks to a water-logging treatment with O_2 concentrations below 3 mg L^{-1} in the vicinity of roots. The treatment induced growth cessation in both species. *Q. petraea* displayed a lower tolerance to hypoxia as demonstrated by the higher number of seedlings suffering shoot dieback and leaf chlorosis as compared to *Q. robur*. This difference should be related to the high number of adventitious roots and hypertrophied lenticels that were formed in *Q. robur*, compared to *Q. petraea*. In the fine roots of the two species, the activity of pyruvate decarboxylase (PDC), the key enzyme of the fermentative pathway, was stimulated after 24 h of water-logging. Transcripts of PDC increased after 48 h of water-logging in *Q. robur* and not in *Q. petraea*. Interestingly, transcripts of haemoglobin (Hb) (possibly involved in the putative nitric oxide cycle) followed the same pattern of response than those of PDC. Enzymes of the sucrose degradation pathway displayed decreased activities after 3 weeks of water-logging, probably due to decreased carbohydrate availability. Alcohol dehydrogenase (ADH), sucrose synthase (Susy), and pyruvate kinase (PK) activities were higher in *Q. robur* after 3 weeks of water-logging. This study provided a set of markers characterizing the differences of tolerance to hypoxia between the two species for further studies on intra and inter-specific diversity.

water-logging / hypoxia / adventitious root / hypertrophied lenticel / carbon metabolism

Résumé – Différences de réponses morphologiques et physiologiques à l'ennoyage entre deux espèces sympatriques de chêne (*Quercus petraea* [Matt.] Liebl., *Quercus robur* L.). Les chênes pédonculé (*Quercus robur* L.) et sessile (*Quercus petraea* [Matt.] Liebl.) présentent des différences de tolérance à l'hypoxie racinaire induite par ennoyage, *Q. robur* étant plus tolérant que *Q. petraea*. Nous avons mené une expérience visant à identifier des différences inter-spécifiques dans les réponses morphologiques et physiologiques à l'hypoxie racinaire. Des semis en pots ont été soumis à un ennoyage de 7 semaines avec une concentration en O_2 maintenue en dessous de 3 mg L^{-1} au voisinage des racines. Le traitement a provoqué un arrêt de croissance chez les deux espèces. *Q. petraea* a montré une plus faible tolérance que *Q robur*, avec un nombre plus élevé de plants présentant un dessèchement de l'appareil aérien ainsi qu'une plus forte chlorose des feuilles. Cette différence pourrait être due au plus grand nombre de racines adventives et de lenticelles hypertrophiées formées au collet de *Q. robur*. Dans les racines fines des deux espèces, l'activité pyruvate décarboxylase (PDC), enzyme clef de la fermentation alcoolique, a été stimulée après 24 h d'ennoyage. Les transcrits de PDC ont augmenté après 48 h d'ennoyage uniquement chez *Q. robur*. De façon intéressante, les transcrits d'hémoglobine (Hb) (qui pourrait être impliquée dans la voie de signalisation de l'oxyde nitreux), ont suivi le même profil de réponse que ceux de la PDC. Les enzymes du catabolisme du saccharose ont présenté une diminution d'activité après 3 semaines d'ennoyage, probablement consécutivement à une baisse de la disponibilité en hydrates de carbone. Les activités alcool-déshydrogénase (ADH), saccharose-synthase (Susy), et pyruvate-kinase (PK), ont été plus fortes après 3 semaines d'ennoyage. Cette étude a fourni des marqueurs caractérisant des différences inter-spécifiques de tolérance, qui pourront être utilisés lors d'études ultérieures de diversité intra et inter-spécifique de traits liés la tolérance à l'hypoxie racinaire.

ennoyage / hypoxie / racine adventive / lenticelle hypertrophiée / métabolisme du carbone

1. INTRODUCTION

Quercus robur L. and *Quercus petraea* [Matt.] Liebl. are two sympatric oak species of temperate Europe. While phenotypic traits like leaf and fruit morphology consistently differentiate the two species [25, 42], a clear-cut genetic differentiation based on molecular markers has still not been evidenced [7, 50]. The search for candidate genes controlling the functional traits that differ between the two species is expected to be an efficient strategy for the identification of potential genetic markers of inter-specific differences. As a first step in such a strategy, we developed an experiment aiming

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Article published by EDP Sciences and available at http://www.edpsciences.org/forest or http://dx.doi.org/10.1051/forest:2006068

at identifying some functional markers of the differences between the two species.

The local distribution of the two species in old growth forests is highly constrained by the soil properties: *Q. petraea* is found on deep and well drained and rather acidic soils while *Q. robur* favours deep and fertile bottomland soils with sometimes large levels of hydromorphia [44]. This distribution reflects different ecological requirements: *Q. petraea* is known to be more tolerant to drought [10, 11, 14], whereas *Q. robur* displays a larger tolerance to water-logging and associated root hypoxia [22, 23, 53, 60]. This difference of tolerance to water-logging between the two oaks was used as a starting point to identify some functional markers for inter-specific differentiation.

Responses of trees to water-logging have been the subject of numerous studies [41, 43]. The primary effect of water-logging is the development of hypoxic conditions in the rhizosphere, induced by restricted diffusion of O_2 through water-logged soil layers. The tolerance to hypoxia has been ascribed to short term responses (mainly adjustments in carbon metabolism in roots) as well as to long term acclimations (mainly the development of tissues enabling the transfer of O_2 to roots). As both types of processes are potentially involved in the inter-specific difference of tolerance, we tested some markers that could be relevant to explain the occurrence of such differences. With this aim, *Quercus* seedlings were submitted to water-logging during seven weeks, and changes in root metabolism as well as in morphology were recorded.

Short-term metabolic adjustments to hypoxia have been described in detail (see Drew [20] for a review). At cell level, metabolic responses include modifications of the sucrose degradation and of the fermentative metabolism pathways [20, 38]. These modifications contribute to the maintenance of energetic status and redox potential of cells in the reductive environment induced by hypoxia. However no data is yet available on *Q. robur* or *Q. petraea* for these aspects. The regulation of activity and transcript levels of pyruvate decarboxuylase (PDC) is thought to be central in this process as PDC is the key enzyme for the fermentative pathway [55], and as its transcription and activity are known to be modulated by O_2 availability [16]. Hexokinases (HK) and, to a lesser extent, neutral invertases (INV-7.5), are known to play a key role in sugar sensing under hypoxia [38]. Moreover, HK activity in anoxic maize roots is a major limiting step of the glycolysis-fermentative pathways [8]. Potential differences in the capacity to mobilize carbon for fermentative metabolism, as well in the short as in the long term (24 h to several weeks of hypoxia), could be markers for inter-specific differences in hypoxia tolerance. Susy and PK activities as well as ADH activity, he latter known to be the most responsive enzyme to hypoxia [20], might be involved in such differences.

Another potential pathway to maintain the energetic status of cells during hypoxia has been evidenced recently (see review by Igamberdiev [33]): it is the nitrate-nitric oxide cycle coupled to an oxydo-reduction of a haemoglobin that displays a very high affinity to O_2 and is able to cope with very low O_2 concentrations. Haemoglobin has been found to be highly induced by hypoxia in roots of several plant species [37].

Finally, Gravatt and Kirby [30] suggested that starch accumulation could be a predictor for the tolerance level of a given species: water-logging-tolerant plants could display a lower starch accumulation in the leaves due to the maintenance of an effective phloem transport [58, 59], as reported for *Nyssia aquatica*, *Quercus alba*, and *Quercus nigra* [30].

Long term responses in tolerant plants include the development of structures expected to contribute to hypoxia avoidance by favouring O_2 diffusion to the root tips, such as adventitious roots [5, 35, 39, 48, 49], aerenchyma [5, 20, 26, 32, 39] or hypertrophied lenticels [34, 39, 40]. In order to test whether the two oaks differed in their capacity to enhance diffusion of O_2 through plant tissues, we monitored lenticel formation and adventitious roots biomass from 24 h to 7 weeks of hypoxia. We also searched for aerenchyma in adventitious roots in order to test if these roots potentially had a high porosity to gas.

2. MATERIALS AND METHODS

2.1. Plant material

Acorns were sampled during the end of October 2002 in the Domain Forest of Compiègne (France, 02° 49' E, 49° 25' N). Adult oaks of the two species were selected based on morphological markers as described by Sigaud [54] and acorns were collected below these trees. Seedlings were grown in a greenhouse in 4 L pots containing a peat/sand mixture (2/1 v/v) from March to June 2003. Fifty-one, four months old seedlings from each species were submitted to water-logging by a total submergence of their roots, and 41 were used as controls. Water-logging was imposed during 7 weeks on 4 months old seedlings. Sampling was done according to following schedule: control and stressed plants from each species were sampled after 24 h, 48 h, 1, 2, 4 and 7 weeks of water-logging. Five plants were collected for each condition, except at 24 h (only 3 controls) and at 48 h (no control).

2.2. Water-logging treatment

Potted oak seedlings were placed into large plastic containers by groups of 8 pots. Root hypoxia was imposed by maintaining a permanent water table in the containers, adjusted daily at 2 cm above the substrate level. Water used for water-logging was deoxygenated by bubbling with N_2, in order to maintain the O_2 concentration below 5 mg L^{-1}. O_2 was measured in the free water and in piezometric tubes installed in the middle of each pot with a dissolved-oxygen Meter MO-128 Mettler Toledo. Lower dissolved O_2 concentrations were recorded in the piezometric tubes (1.5 to 3 mg L^{-1} during the overall treatment) as compared to the free water (4.5 to 6.5 mg L^{-1} during the overall treatment). In spite of some heterogeneity among piezometric tubes, dissolved O_2 never exceeded 3 mg L^{-1}, which corresponds to hypoxic conditions as compared to tap water (8.5 mg L^{-1} at similar temperature). The gradient from outside to inside the pots was due to O_2 consumption in the rhizosphere, resulting probably in an even lower concentration in close proximity to the roots.

2.3. Growth and shoot status

Main stem height and leaf chlorophyll content were monitored on all plants twice a week during the experiment. Chlorophyll content

was recorded with a Chlorophyll Content Meter (CCM, Optic Science, Tyngsboro USA) on mature fully expanded leaves. In parallel, occurrence of shoot dieback (i.e. leaf senescence and shedding) was recorded on the seedlings.

2.4. Biomass, hypertrophied lenticels, and adventitious roots

At each sampling date, roots were washed with tap water. Leaves of each flush and fine roots were immediately frozen in liquid N_2. In order to minimize the effects of potential diurnal variations in the recorded parameters, seedlings were randomly sampled between 14:00 and 20:00 h. Fine roots were defined as non-lignified roots, which could be easily separated from the main roots. Adventitious roots were identified as the white and plagiotropic lateral roots inserted on the main-stem or at the basis of the tap-root, and were harvested separately. After sampling, the fresh weight of fine and adventitious roots was measured separately. Fine roots were kept frozen for further physiological measurements. For observation under an optical microscope, adventitious roots were conserved in a glutharaldehyde 0.5%, paraformaldehyde 2%, 25 mM Phosphate buffer (pH 7.2). Fine sections were cut with a razor blade and coloured with a green crimson dye. Hypertrophied lenticels at collar were counted using a visual ordinal scale: 0: no hypertrophied lenticels, 1: less than 15–20 hypertrophied lenticels, 2: more than 15–20 hypertrophied lenticels, 3: large number of merged and uncountable lenticels. Dry biomass of the main root was directly measured, fine and adventitious root biomass were derived from fresh mass based on water content measurement with several trees.

2.5. Starch extraction and determination

Soluble sugars were extracted from leaves (equal mix sample of different growth flushes) by boiling 20 mg of dry matter in 80% ethanol. Starch quantification was done on the residue by enzymatic digestion (α-amylase and amyloglucosidase), followed by a colorimetric measurement (450 nm) of glucose hydrolysate with a peroxidase glucose-oxidase/ortho-dianisidine reagent after adding HCl 2 N [13]. Absorbance was calibrated against standards of known glucose concentrations.

2.6. Protein extraction and quantification

Proteins were extracted from fine roots. No extraction was done from adventitious roots due to the small amounts of material. Extraction was made according to Alaoui-Sossé [2] with some modifications, particularly by adding Triton-X100 in order to solubilise membrane bound proteins. Frozen fine roots (500 mg) were homogenized in a mortar with liquid nitrogen and 250 mg PVPP. Proteins were extracted with 6 mL buffer (see Appendix 1). Extracts were centrifuged 30 min at 18 000 g at 4 °C, and then desalted on Sephadex G-25 column (Amersham). Samples were stored at –80 °C. Total proteins (soluble and membrane proteins) were quantified using the protocol of Bradford [9].

2.7. Enzymatic assays

For all enzymatic assays, 10% (v/v) protein extracts/assay buffer were used, and absorbance was measured using a microplate spectrometer ALx808 BIO-TEK Instruments, INC. A control was obtained in the absence of substrate, except for the ADH assay. ADH and PDC activities were determined according to Kimmerer [36] with slightly modified reaction buffers (see Appendix 2). For PK activity we used the protocol described by Zervoudakis [62], with slight modifications. HK activity was determined according to the protocol of Bouny [8], slightly modified, by a reaction coupled to G6PDH (Glucose-6-P dehydrogenase). INV-7.5 and Susy activities were assayed with the same protocol [8] by adding hexokinase. For Susy activity, an assay was done without co-factors (UDP and NaPPi) in order to remove the residual invertase activity. The composition of all reaction buffers is given in Appendix 2.

2.8. Real-time RT-PCR

Total RNA was extracted from fine roots according to Chang [12]. We used a homogenous mix of roots from the seedlings of each species, treatment and date (3 extraction repetitions). No extraction was performed after 7 weeks water-logging because of the small amount of tissue available due to root necrosis. RNA quality was controlled at 260 and 280 nm. Reverse transcription was done with a M-MULV reverse transcriptase (Ozyme/Finnyme), following factory protocol. cDNA was stored at –20 °C. All RT products were controlled by a PCR assay of PDC transcript without RT enzyme, to check the absence of DNA contamination. The sequence of PDC transcript was identified by an AFLP assay during a short term (24 h) hypoxia experiment with oak (Bodénès, unpublished data, EMBL accession number: CR942275). A data basis of oak bud burst EST yielded Hb and GAPDH sequences (Derory, unpublished data, EMBL accession numbers: Hb: CR627830, GAPDH: CR628241). GAPDH was used as housekeeping gene. This choice was suggested by its known stable expression within cells as well as during stresses [57]. It allowed us to compute the data as percent of a transcript of the glycolysis pathway that is expressed constitutively.

Real time PCR was performed on Roche light-cycler under following conditions: cDNA 1/100 diluted (1/50 for GAPDH transcripts), 0.03 mM of each primer (Tab. I), $MgCl_2$ 2.5mM and 10% (v/v) Roche Syber-Green Mix. We used the following annealing temperatures: PDC 55 °C, Hb 52 °C, and GAPDH 50 °C. Final products were confirmed by melting curves, and, for several samples, by length after electrophoresis on agarose gel.

2.9. Statistical analyses

Statistical analysis was performed with Statistica 7 software (Statsoft, 2004, Tulsa USA). For root biomass, stem height, chlorophyll content, leaf starch content, and transcript levels, the effects of species, treatment and time course were tested with a linear model procedure, followed by Tukey-Kramer mean comparison tests (for transcript level, repetition were only technical, biological variance being removed by homogenisation of fine root powder). For dissolved O_2, time course and piezometric versus free water effects were tested with a linear model procedure, followed by Tukey-Kramer mean comparison tests. For shoot dieback data, we were interested in difference of precocity of the phenomenon between species, thus for each plant the difference between species for the earliest date of observation of shoot dieback was tested using a Student t-test.

For enzymatic activities and adventitious root formation, postulates of a linear model procedure (homoscedasticity and normality of residuals) were not respected and no transformation of data was possible, therefore non-parametric analyses were used. On account of the ordinal scale for lenticel formation, non-parametric analysis was also

Table I. Primers pairs for PCR amplification of PDC, Hb and GAPDH transcripts.

Transcript	Forward primer	Reverse primer
PDC	5'-GCAGCCTCTAATCCCATCTG-3'	5'-CAAGAGCTTCGGTGTTTTCAG-3'
Hb	5'-ACCTCGGAAGTGATCACAGG-3'	5'-GCATGGGATTTAAGCTTTGG-3'
GAPDH	5'-CCATTGAGCTCCTTCTCAGC-3'	5'-TGTCCTGCCATCACTTAAAGG-3'

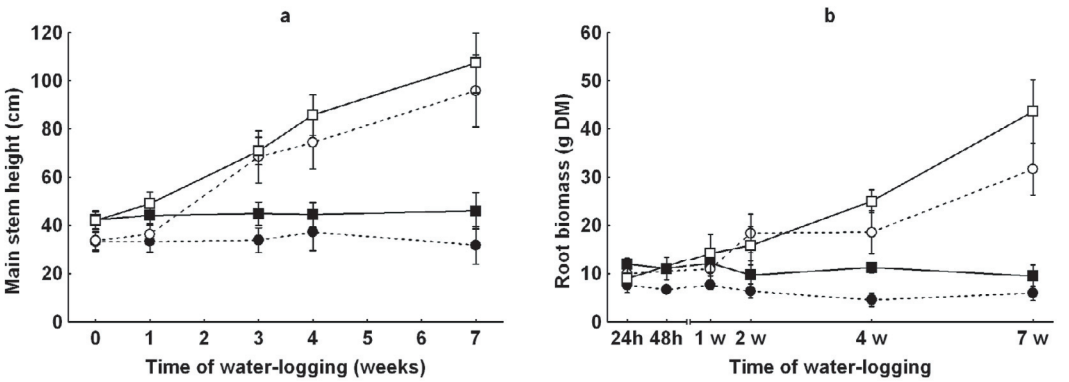

Figure 1. Time course of main stem height and total root biomass during water-logging. (Open squares) *Q. robur* control, (closed squares) *Q. robur* hypoxia, (open circles) *Q. petraea* control, (closed circles) *Q. petraea* hypoxia. (a) Main stem height (cm, means and SEM). (b) Total root biomass (g DM, means and SEM, $n = 5$ for control and treated except for control at 24 h: $n = 3$).

used for this trait. Kruskal-Wallis test was used for multiple comparison of time evolution and Mann-Withney ranked sum test (U test) for species or treatment comparison. When no significant species variation could be detected, we pooled data from the two species for treatment comparison tests. To test differences between seedlings showing or not shoot dieback, we pooled all data from all dates (after having tested that no significant time-shift could be detected), and compared the amount of adventitious roots and lenticels with Mann-Withney ranked sum test (U test). The variance heterogeneity of enzymatic activities and leaf starch content between species or treatments was tested with the Cochran test. All differences were considered significant when p value was below 0.05.

3. RESULTS

3.1. Growth, chlorophyll content, and shoot dieback

In the two species, main stem and total root biomass growth stopped within the first week after water-logging while both root and shoot growth remained very active in controls (Fig. 1). *Q. robur* seedlings displayed significantly larger main stem high and larger root biomass than *Q. petraea* (Fig. 1 and Tab. II). In response to water-logging, the number of seedlings displaying total shoot dieback increased with time to a much larger extent for *Q. petraea* than for *Q. robur* (Fig. 2a and Tab. II). In parallel, leaf chlorophyll content decreased in the water-logged individuals of the two species, with however an earlier and more severe decline in *Q. petraea* (Fig. 2b and Tab. II).

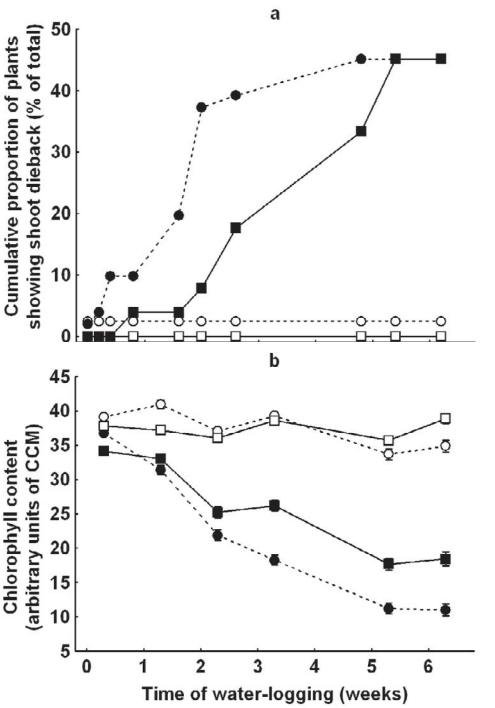

Figure 2. Effects of hypoxia on shoot dieback, (a) cumulative fraction of seedlings displaying shoot dieback and on chlorophyll content, (b) arbitrary units of chlorophyll content Meter (CCM), means and SEM. Same symbols as in Figure 1.

Table II. Statistical analysis of the effects of water-logging on different functional traits in seedlings of *Q. robur* and *Q. petraea*. Time, treatment, and species effects of each variable, significant effect: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: no significant effect, –: test not done, (1) significant differences on the 3 first dates only, (2) significant differences on the 3 latest dates only, (3) technical repetitions.

	Type of analysis	Time effect				Water-logging effect		Species effect	
		Water-logged		Control		*Q. robur*	*Q. petraea*	Water-logged	Control
		Q. robur	*Q. petraea*	*Q. robur*	*Q. petraea*				
Growth									
Main stem height	Parametric	ns	ns	*	*	*	*	*	
Root mass	Parametric	ns	ns	***	***	***	***	**	
Chlorophyll content	Parametric	***	***	ns	ns	***	***	***	
Shoot dieback	Parametric	-	-	-	-	*	*	-	-
Leaf starch content	Parametric	ns	**	ns	ns	ns	*	ns	ns
Transcripts									
PDC	Parametric	***(3)	ns	ns	ns	***(3)	ns	***(3)	ns
Hb	Parametric	***(3)	ns	ns	ns	***(3)	ns	***(3)	ns
Enzyme activities									
ADH	Non-parametric	ns	*	ns	ns	***	*(1)	**(2)	ns
PDC	Non-parametric	ns	ns	ns	ns	ns	*	ns	*
Susy	Non-parametric	ns	***	ns	ns	ns	*	*	ns
INV-7.5	Non-parametric	*	**	ns	ns	*		ns	ns
GK	Non-parametric	ns	*	ns	ns	*		ns	ns
FK	Non-parametric	*	**	ns	ns	***		ns	ns
PK	Non-parametric	ns	**	ns	ns	ns	ns ($p = 0.06$)	*	ns
Morphology									
Adventitious root mass	Non-parametric	ns	ns	ns	ns	*(2)	ns	*	ns
Hypertrophied lenticels	Non-parametric	ns	ns	ns	ns	*	*	*	ns

3.2. PDC and haemoglobin transcripts in fine roots

Absolute levels of GAPDH transcripts and their variations with time (Fig. 3a) were small when compared to PDC and haemoglobin (Hb) transcripts (1.25 million of copies/µRNA, for GAPDH compared to over 220 for Hb and PDC). Moreover no significant variation among treatments were detected (Tab. II). Transcript levels of PDC were higher after 48 h of hypoxia than in controls for *Q. robur*. They later decreased down to control levels after 4 weeks of hypoxia (Fig. 3b and Tab. II). In *Q. petraea*, no hypoxia-induced change occurred (Fig. 3b and Tab. II). Transcript levels of Hb followed very similar patterns (Fig. 3d and Tab. II).

3.3. Enzyme activities in the alcoholic fermentative pathway in fine roots

The activity of enzymes of the alcoholic fermentative pathway remained stable with time in control fine roots (Fig. 4), with significantly higher PDC activity in *Q petraea* than in *Q robur*. The activity of ADH increased immediately at the onset of water-logging (24 h, 48 h and one week of hypoxic treatment) in the two species (Fig. 4a and Tab. II). It remained up-regulated during the course of the treatment in *Q. robur*, but decreased to control levels after 2 weeks in *Q. petraea* (Fig. 4a and Tab. II). Compared to ADH, the activity of PDC displayed a different pattern in response to water-logging, similar level were reached in *Q. robur* and *in Q; petraea* (Fig. 4b). PDC activity was higher in hypoxia-treated *Q petraea* than in controls, while a non significant increase was observed for *Q. robur*. Water-logging resulted in an increased variability in PDC and ADH activities among individuals of both species, for PDC this variability being larger in *Q. robur* than in *Q. petraea*.

3.4. Activities of carbohydrate catabolism enzymes in fine roots

The activity of enzymes involved in sucrose degradation, like Susy (Fig. 5a), INV-7.5 (Fig. 5b), GK (Fig. 5c), and FK (Fig. 5d) remained close to control during the first days of hypoxia (Tab. II). Afterwards, all activities declined in *Q. petraea*, whereas in *Q. robur* only INV-7.5 and FK were affected (Tab. II). A larger Susy activity was recorded in water-logged *Q. robur* than *Q. petraea*, whereas no inter-specific difference was recorded in the controls (Tab. II). For the other enzymes related to sucrose degradation, activities did not differ between the two species (Tab. II). The activity of PK, last enzyme of the glycolytic pathway (Fig. 5e and Tab. II) decreased after one week of water-logging in *Q. petraea,* while there was no significant variation in *Q. robur*. For all enzymes,

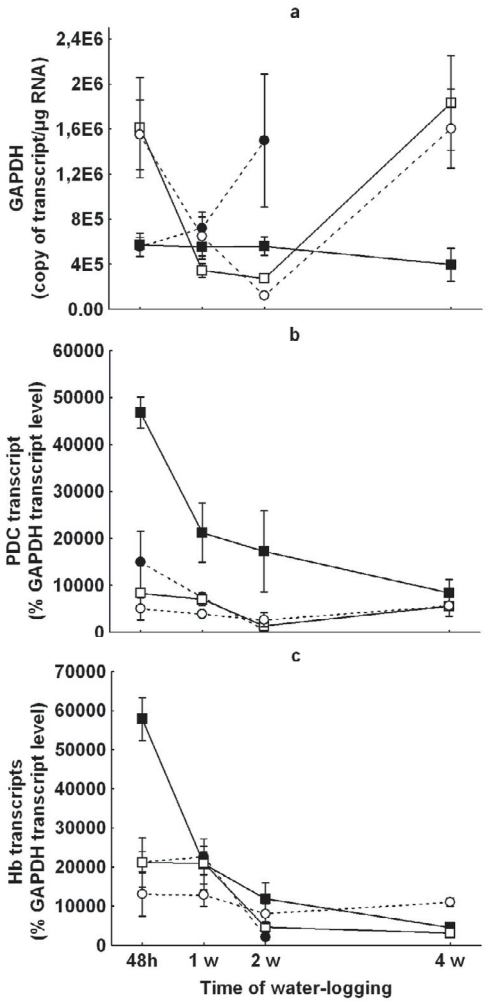

Figure 3. Time course of activity and transcripts of PDC and of transcript of Hb. (a) Housekeeping gene: GAPDH transcript level (copies/µgARN, means and SEM). (b) PDC transcript level. (related in per cent of the GAPDH transcript level, means and SEM). (c) PDC activity in fine roots (nkatal mg^{-1} protein, means and SEM, $n = 5$ for control and treated, except for control at 24 h: $n = 3$). (d) Hb transcript level (related in per cent of the GAPDH transcript level, means and SEM). w: weeks; same symbols as in Figure 1.

the inter-individual variability of responses was high whatever the species.

3.5. Leaf starch content

Leaf starch content significantly decreased during water-logging in *Q. petraea* seedlings while no significant variation was recorded in *Q. robur* (Fig. 6 and Tab. II). However, in the latter species, the variability higher in water-logged than in control samples: some water-logged individuals of *Q. robur* displayed the same response than *Q. petraea*, with lower leaf starch contents than the controls, while others showed an accumulation of starch (twice that of control level).

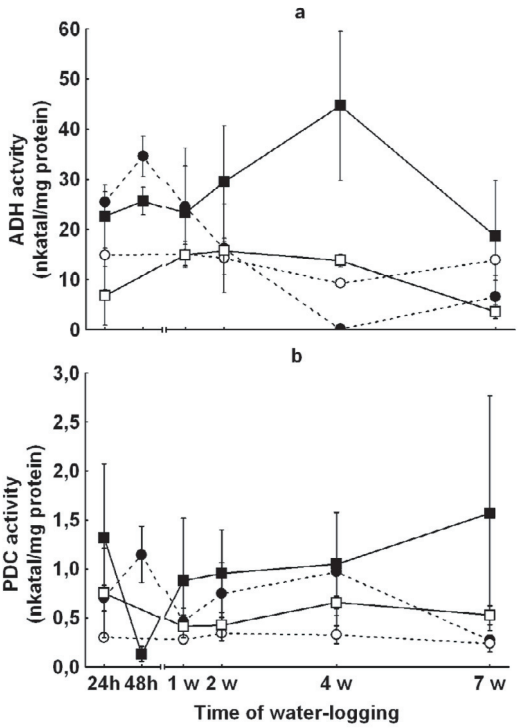

Figure 4. Specific enzymatic activities of the fermentative pathway in fine roots (nkatal mg^{-1} protein, means and SEM, $n = 5$ except for control at 24 h: $n = 3$). (a) ADH activity. (b) PDC activity. w: weeks; same symbols as in Figure 1.

3.6. Hypertrophied lenticels, adventitious roots and aerenchyma

No hypertrophied lenticels were detected in control seedlings of any of the two species during the course of the experiment. During hypoxia, a larger number of lenticels was present in *Q. robur* compared to *Q. petraea* (Fig. 7 and Tab. II). The water-logged treatment resulted in an accumulation of adventitious roots relative to control in *Q. robur,* and no detectable change in *Q. petraea*. Thus, large inter-specific differences were found in the formation of adventitious roots under hypoxia. In addition none of the individuals with hypertrophied lenticels suffered any sign of shoot dieback (Tab. III). However, there was no significant difference in adventitious root biomass among plants displaying severe or no shoot dieback. Fine sections of adventitious roots revealed no structured aerenchyma, we only observed some larger intercellular space in a few samples (data not shown).

4. DISCUSSION

4.1. Higher tolerance to water-logging of *Q. robur* than *Q. petraea*

The O_2 concentrations measured in the vicinity of the rhizosphere of water-logged *Q. robur* and *Q. petraea* seedlings,

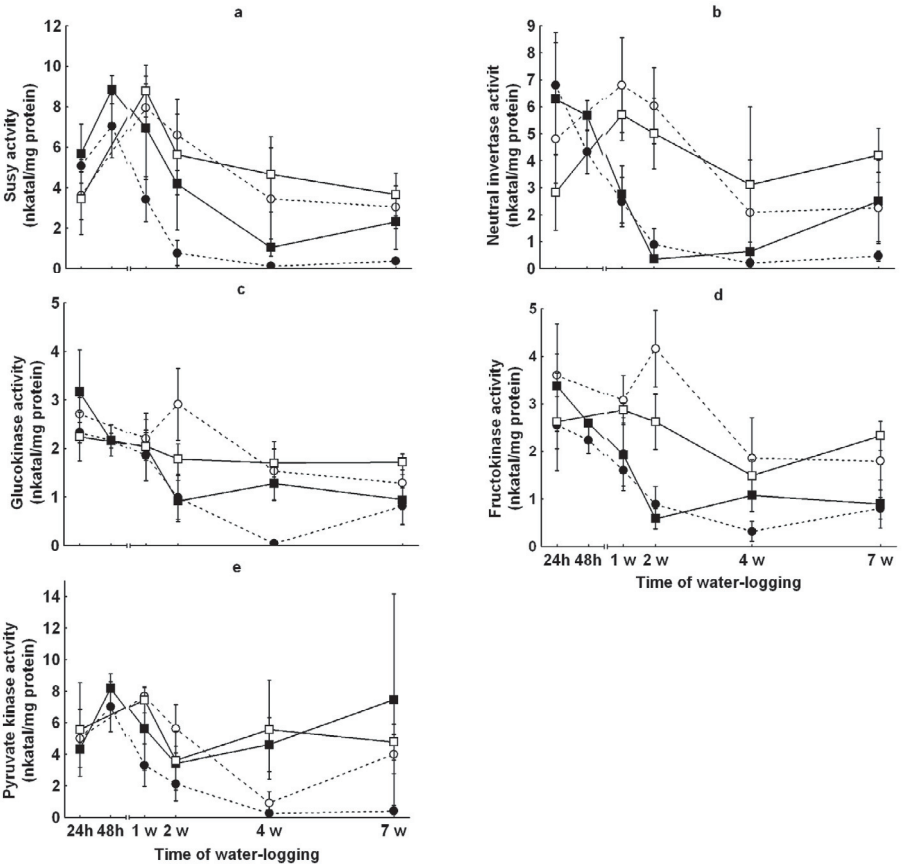

Figure 5. Specific enzymatic activities of the carbon catabolism in fine roots (nkatal mg^{-1} protein, means and SEM, $n = 5$ except for control at 24 h: $n = 3$). (a) Susy activity. (b) INV-7.5 activity. (c) GK activity. (d) FK activity. (e) PK activity. w: weeks; same symbols as in Figure 1.

Table III. Fraction of plants displaying shoot dieback as a function of the presence or the absence of hypertrophied lenticels or adventitious roots (including *Q. robur* and *Q. petraea* data).

	Proportion of plants showing shoot dieback
Hypetrophied lenticels	
None	100%
Up to 0	0%
Adventitious roots	
None	27.3%
Up to 0	16.2%

were 3 times lower than in O_2 saturated water. As expected, these low O_2 concentrations were sufficient to induce a large difference in the response of the two oak species. The occurrence of a severe shoot dieback in many *Q. petraea* seedlings in comparison to the small number of affected *Q. robur* seedlings clearly confirmed that *Q. petraea* is more sensitive to water-logging than *Q. robur*. This observation is strengthened by the larger decline in leaf chlorophyll content observed in *Q. petraea*. Causes of the observed shoot dieback can be multiple. Water relations of hypoxia-sensitive species are severely affected by root hypoxia. Alaoui-Sossé [1] found a decrease of shoot water potential after 15 days of water-logging Predawn leaf water potential decreased in the sensitive *Q. rubra* to a much larger extent than in the tolerant *Q. robur* [22]. Stomatal conductance declines severely in almost all reported hypoxia cases [23, 53, 60], in parallel with root hydraulic conductivity. Stomatal conductance declined more severely in *Q. petraea* than in *Q. robur* [53]. All these observations on different oak species suggest the occurrence of a water deficit in the shoots of seedlings exposed to root hypoxia.

4.2. Inter-specific differences in the regulation of PDC

In response to water-logging, PDC activity in fine roots reached similar intensities in the two species. This resulted from a larger activity of water-logged *Q. petraea* with respect to controls and from a large constitutive activity in *Q. robur* controls. Enhanced PDC activities have been reported in response to water-logging in a large range of species [4, 6, 17, 24, 29, 55]. At the beginning of water-logging (48 h), transcript levels of PDC increased only for *Q. robur* and not for *Q. petraea*. Such short-term transcriptional activation of the fermentative pathway has been already described in water-logging-tolerant species [19, 24]. Dolferus [19] suggested that fermentative metabolism and glycolysis pathway

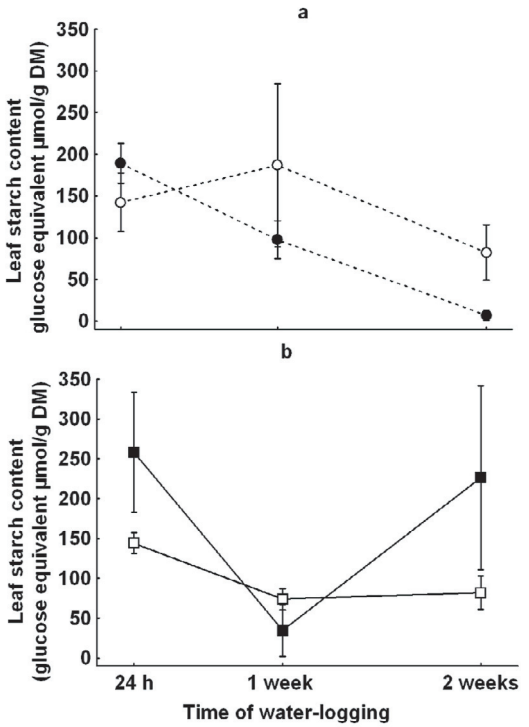

Figure 6. Leaf starch content (µmol g^{-1} Dry Mass, pool of an equal mass of leaves from each growth flush, means and SEM, $n = 5$ for control and treated except for control at 24 h: $n = 3$). (a) *Q. petrae*; (b) *Q. robur* Same symbols as in Figure 1.

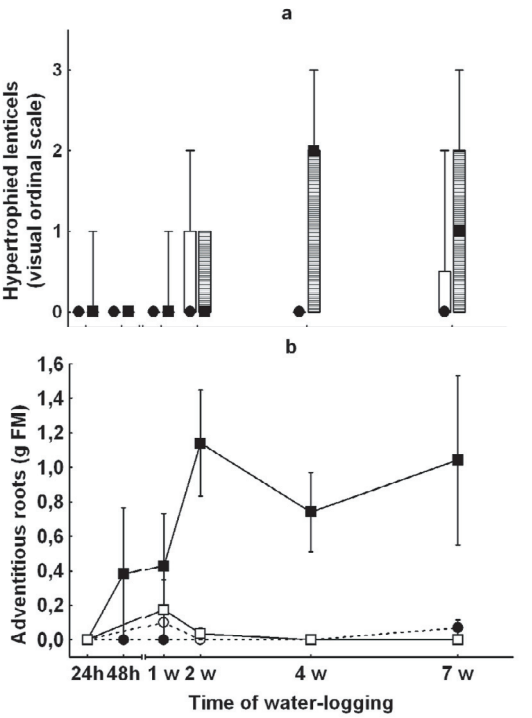

Figure 7. Formation of adaptive structures during hypoxia. (a) Hypertrophied lenticels, visual ordinal scale: 0: no hypertrophied lenticels, 1: less than 15–20 hypertrophied lenticels, 2: more than 15–20 hypertrophied lenticels; 3: large number of merged and uncountable lenticels, (medians, quartiles, minimum and maximum). *Q. petraea*, *Q. robur*. (b) Fresh mass of adventitious roots (g FM, means and SEM, $n = 5$ except for control at 24 h: $n = 3$). w: weeks; same symbols as in Figure 1.

are controlled by two sets of genes, one with a constitutive expression, and one with a low oxygen-inducible expression. In order to understand the origin of the elevated level of constitutive (control) activity in *Q. robur*, it would be interesting to differentiate the transcript levels of the two PDC genes. The observed changes in PDC transcripts resulted in only small difference of the recorded PDC activity, which could suggest a post transcriptional regulation of PDC. This point deserves further research.

4.3. Differences in induction of the putative nitric oxide pathway

Interestingly, transcripts of haemoglobin followed the same response as PDC among species and treatments. A similar co-induction by hypoxia was found in *Arabidopsis thaliana* during short term treatments (1 to 24 h) with micro-array and real-time PCR analyses [45]. The signalling pathway that triggers this activation could be similar for PDC and Hb transcripts. Hb probably plays an important role in NAD(P)H regeneration under reductive conditions via the nitric oxide cycle [33]. Further investigations of short-term modifications of this pathway may point out important differences between the two species.

4.4. An improved carbon availability in fine roots of *Q. robur* with respect *Q. petraea*

Hexokinases (HK) and, to a lesser extent, neutral invertases (INV-7.5) play a key role in sugar sensing under hypoxia [38]. Moreover, HK activity in anoxic maize roots is a major limiting step of the glycolysis-fermentative pathway [8]. During the first week of hypoxia, enzymes of the sucrose degradation pathway (Susy, INV-7.5) were maintained at a level comparable to the control seedlings for the two species. In maize, an activation of Susy was found under hypoxia in parallel to a repression of INV-7.5 activity [29, 51, 52, 61]. In our experiment, there was neither a significant activation of Susy nor a short-term repression of INV-7.5. The activity of the enzymes involved in sucrose breakdown (Susy, INV-7.5, and HK) could be restricted by carbohydrate availability as suggested by Albrecht [3]. The significantly higher Susy and PK activities in fine roots of *Q. robur* than in *Q. petraea* underline differences between the species in the long-term response. These two enzymes are known to respond positively to hypoxia in maize root tips [52]. Whereas no significant difference was recorded between species, GK activity significantly decreased only for *Q. petraea*, and FK and INV-7.5 decreases were more

significant in *Q. petraea* than *Q. robur*. All these results suggest that *Q. robur* could be less affected by the deficiency in carbohydrate availability in roots than *Q. petraea*. The inter-specific differences in ADH activity are more difficult to interpret because the alcoholic fermentation flux is assumed to be regulated by PDC activity [55]. The higher ADH activity, maintained during a longer period over two weeks of hypoxia for *Q. robur*, could play an important role during the recovery of normoxic conditions, particularly to metabolise ethanol produced at a high rate under hypoxic conditions [20]. This result suggests the occurrence of potential differences in the catabolism of ethanol between the two species.

4.5. Starch accumulation in leaves is not an efficient indicator of the degree of tolerance of the two species

Contrary to the hypothesis of Gravatt [30] which suggested that starch accumulation would be higher in less flood-tolerant species, we did not detect any larger starch accumulation in *Q. petraea* with respect to *Q. robur*. In the opposite, leaf starch content significantly declined during the course of hypoxia in *Q. petraea* but not in *Q. robur*. Indeed, leaf starch content results from a balance between carbon assimilation, phloem export and probably local consumption and therefore is not a efficient indicator of species tolerance to hypoxia.

4.6. Enhancement of O_2 diffusion towards roots in *Q. robur*

Q. robur formed more lenticels and adventitious roots in response to water-logging than *Q. petraea* as expected from earlier experiments [15]. We were searching for the occurrence of aerenchyma tissues such as those observed in maize, in which species aerenchyma readily supply O_2 to roots submitted to hypoxia [21,32]. A few air spaces were indeed visible in some of the adventitious roots of *Q. robur*. However, no large scale aerenchyma was observed in any of the roots. The observed air spaces could be the result of necrosis in adventitious roots. Adventitious roots are obviously involved in hydraulic functioning of the plant, but their number and amount was similar in individuals suffering from shoot dieback and in those presenting no such symptom. Meanwhile, all individuals that did not form hypertrophied lenticels suffered from shoot dieback. We therefore formulate the hypothesis that lenticels play a more important role in maintaining the supply of water to the shoots than adventitious roots. Moreover, we observed that the largest fraction of the lenticels was developed below the water level (data not shown), where O_2 is less available than in the air, as already observed for oak on *Q. macrocarpa* by Tang [56]. Lenticels of stems are permeable to water [31], strengthening the hypothesis that they play a significant role in water absorption. Lenticels could also play a major role in the oxygenation of shoots via import of O_2 into xylem sap, and then in the shoot via the transpiration flux [18, 27, 28, 46]. They probably are the key trait explaining the differences of tolerance among the two species, and their functional role needs to be carefully assessed.

4.7. Variability and specific differentiation

The intra-specific diversity of the response to water-logging was larger in *Q. robur* than in *Q. petraea*. This larger diversity was observed for the starch content in leaves as well as for the formation of lenticels and adventitious roots. In contrast, no significant difference of variance was observed between species for most of the enzymatic activities. Only the variance of PDC activity was significantly different between the two species. The hypothesis of a genetic origin of this diversity cannot be discarded and should be investigated. In fact neutral genetic diversity was found to be larger in *Q. petraea* than in *Q. robur* [47], while we found an opposite trend for the traits related to hypoxia tolerance.

5. CONCLUSION

The responses of the two oak species to water-logging displayed a large diversity. We observed a frequent occurrence of adaptive structures such as lenticels and adventitious roots in *Q. robur*, while they remained much less common in *Q. petraea*. At a physiological level, no inter-specific differences in PDC activities were detected. Nevertheless, some inter-specific differences were highlighted. In particular we observed differences in PDC transcripts levels. According to the level of Hb transcripts, the putative nitric oxide pathway should be differently induced between the two species. In addition to these short term responses to root hypoxia, longer term response were detected. Decreased activities of the enzymes related to carbon catabolism suggest a larger availability of carbohydrates in *Q. robur* fine roots than in *Q. petraea*. All these observations suggest that major differences in carbon economy could occur in the two species when exposed to root hypoxia.

The inter-individual diversity of responses seemed to be larger in *Q. robur*, and may point either to a higher phenotypic plasticity or to a higher genetic diversity of traits for hypoxia tolerance in this species. Future investigations should test the differences in intra-specific diversity of adaptation and its genetic origin. This knowledge is essential to explain the differences of regeneration capacity among the two oak species in water-logged forest stations.

Acknowledgements: We gratefully acknowledge the help of Jeremy Derory (INRA Bordeaux) for quantitative PCR optimisation, and for GAPDH and Haemoglobin sequences. We thank Jean-Marie Gioria (INRA Nancy) for technical support for seedling cultivation, Patrice Avias (UHP Nancy) for preliminary work on enzymatic activities, and Benjamin Faivre-Vuillin (INRA Nancy) for help in chlorophyll and O_2 content measurements. We also acknowledge the very helpful advices brought by Renaud Brouquisse (CEA Grenoble).

APPENDIX 1

Composition of the protein extraction buffer.

Hepes KOH (pH 7.5) 100 mM, $MgCl_2$ 5 mM, EGTA 5 mM, PVP-25 5 mg/mL, PEG 5.9 g L^{-1}, DTT 7 mM, Glycerol 10 % (v/v), Triton-X100 0.5 % v/v, APMSF 0.02 mM, Leupeptin 0.001 mM, Pepstatin 0.001 mM.

APPENDIX 2

Composition of the buffers for the different enzymatic assays.

A. For ADH : Mes (pH 6.25) 100 mM, DTT 1 mM, $MgCl_2$ 5 mM, NADH 0.2 mM, and 0.5 mM of Pyrazole for controls.
B. For PDC: Mes (pH 6.0) 100 mM, DTT 1 mM, $MgCl_2$ 5 mM, ADH 10 U mL^{-1}, TPP 100 mM, oxamate 250 mM, NADH 10 mM, pyruvate 10 mM.
C. For PK: Tris HCl (pH 6.9) 2.5 mM, DTT 2 mM, NADH 0.2 mM, ADP 1.5 mM, KCl 50 mM, LDH 2 U mL^{-1}, $MgCl_2$ 10 mM, Phospho-enol pyruvate10 mM.
D. For HK: Tris HCl (pH 8.5) 50 mM, G6PDH 1 U mL^{-1}, ATP 1.2 mM, NAD 2.8 mM, PGI (for fructokinase activity) 6.5 U mL^{-1}, glucose/fructose 20 mM.
E. For INV-7.5: Hepes KOH (pH 7.5) 100 mM, G6PDH 2 U mL^{-1}, HK 5 U mL^{-1}, PGI 6.5 U mL^{-1}, NAD 2.8 mM, ATP 1.2 mM, $MgCl_2$ 2 mM, sucrose 100 mM.
F. For Susy: Bis-Tris (pH 6.5) 100 mM, G6PDH 2 U mL^{-1}, HK 5 U mL^{-1}, PGI 6.5 U mL^{-1}, NAD 2.8 mM, ATP 1.2 mM, $MgCl_2$ 2 mM, sucrose 100 mM, UDP 2 mM, NaPPi 2 mM.

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