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Intra- and interspecific diversity in the response to waterlogging of two co-occurring white oak species (*Quercus robur* and *Q. petraea*)

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Summary *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. are sympatric oak species with different ecological requirements. *Quercus robur* is more tolerant to waterlogging than *Q. petraea*. This ecological divergence may play a role in the maintenance of the two species despite the absence of an insurmountable reproductive barrier between them. We predicted that the genetic architecture of traits related to waterlogging tolerance differs between the species. To gain insight into this architecture in the absence of genetic markers for waterlogging tolerance, we compared populations of seedlings of each species for diversity in the expression of quantitative phenotypic traits induced by severe hypoxia. To determine the capacity for hypertrophied lenticel formation, we applied gas-impermeable mastic to stems of seedlings. Two months after application, the mastic treatment had induced the formation of $3 (\pm 2) \text{ cm}^{-2}$ hypertrophied lenticels in the absence of root hypoxia. Leaf epinasty during root hypoxia was an early predictor of seedling mortality. Four weeks of waterlogging resulted in greater epinasty in *Q. petraea* than in *Q. robur*, but fewer hypertrophied lenticels (16 ± 6 versus $21 \pm 9 \text{ cm}^{-2}$) and adventitious roots (2.7 ± 4.7 versus 5.2 ± 5.9). Differences between species in these traits were associated with differences in the frequencies of extreme phenotypes rather than with a generally higher tolerance to waterlogging in *Q. robur* seedlings.

Keywords: *adventitious roots, epinasty, hypertrophied lenticel, hypoxia, variability.*

Introduction

Quercus robur L. and *Q. petraea* (Matt.) Liebl. are sympatric oak species of temperate Europe. They can be differentiated based on a set of polymorphic molecular markers (Bodénès et al. 1997, Muir et al. 2000), several phenotypic traits such as leaf and fruit morphology, or on water-use efficiency (Dupouey and Badeau 1993, Kremer et al. 2002, Ponton et al. 2002). Although these species are interfertile and can exchange genes, they maintain distinct phenotypes in all tested

forest stands (Kremer et al. 2002). Different ecological requirements likely explain the persistence of these distinct species despite interspecific gene flow (Petit et al. 2004, Lexer et al. 2006). Neutral nuclear markers can differentiate between the species based on a combination of loci (Bodénès et al. 1997, Muir et al. 2000). With such markers greater genetic diversity has been recorded in *Q. petraea* than in *Q. robur* (Mariette et al. 2002), but it is not known if this tendency also holds for adaptive traits subject to natural selection (Doebeli et al. 2005, Lexer et al. 2005).

The local distribution of *Q. robur* and *Q. petraea* in old-growth forests is highly constrained by soil type: *Q. petraea* is found on deep and well drained acidic soils, whereas *Q. robur* is more common on deep and fertile bottomland soils (Lévy et al. 1992). This distribution reflects the different ecological requirements of the species: *Q. petraea* is known to be more tolerant to drought (Bréda et al. 1992, 1993, Cochard et al. 1992), whereas *Q. robur* displays a higher tolerance to waterlogging and root hypoxia (Dreyer et al. 1991, Dreyer 1994, Wagner and Dreyer 1997, Schnull and Thomas 2000, Parelle et al. 2006). Tolerance to waterlogging may therefore play a key role in controlling the local distribution of each species, which would imply that waterlogging is one of the selective forces maintaining species integrity in mixed stands.

Earlier studies have shown that the degree of waterlogging tolerance of *Q. petraea* and *Q. robur* probably depends on several characteristics such as metabolism (fermentation, sucrose degradation, nitrate metabolism; Alaoui-Sossé et al. 2005, Parelle et al. 2006) and morphological traits (Colin-Belgrand et al. 1991, Schnull and Thomas 2000, Parelle et al. 2006). These traits may be under selective pressure and could serve as candidate markers for interspecific differentiation. However, no data are available on the intraspecific variability of such traits, and it has not been demonstrated that they are subject to natural selection.

The objectives of our study were to: (1) determine if the diversity of expression of traits related to waterlogging tolerance differs between *Q. petraea* and *Q. robur*; (2) assess if any such

differences are correlated to mortality (i.e., subject to natural selection); and (3) test whether *Q. petraea* displays greater diversity in these traits than *Q. robur*, as reported for neutral molecular markers (Mariette et al. 2002). To assess the diversity of waterlogging responses within a population of seedlings of each species, we first tested the ability of seedlings to form hypertrophied lenticels independent of root hypoxia by applying a gas-impermeable mastic to seedling stems to induce a local hypoxia. Then, on the same seedlings, we determined the degree of variability in the formation of hypertrophied lenticels and adventitious roots during waterlogging. We determined the correlation of all the investigated traits with seedling mortality after 4 weeks of waterlogging. The time course of leaf epinasty, which is known to be a highly specific response to hypoxia and an indicator of the severity of hypoxia (Vartapetian and Jackson 1997, Else and Jackson 1998, Kamaluddin and Zwiazek 2002), was established to test its potential as a hypoxia marker, and to determine the variability of leaf epinasty within each seedling population. We sought answers to four questions. (1) Does local induction of hypoxia in stems provide a means to quantify the ability of seedlings to produce hypertrophied lenticels? (2) Do the mean values of the investigated traits differ between the species? (3) Are the selected traits correlated with the risk of mortality in individuals and therefore subject to natural selection? (4) Is variance in these traits higher for *Q. petraea* than for *Q. robur*, thus corresponding to findings with neutral molecular markers?

Material and methods

Plant material

Acorns were collected during October 2004 in the State Forest of Compiègne, France (2°49' E, 49°25' N), an old-growth forest of pedunculate oaks (*Q. robur*) that includes a well-drained zone supporting sessile oak (*Q. petraea*). About 10 adults of each species were selected based on the morphological markers described by Dupouey and Badeau (1993) and Kremer et al. (2002) and acorns were collected below these trees. Eighty seedlings of each species were grown in 4-l pots containing a peat and sand mixture (2:1 v/v). Seedlings were grown in a greenhouse from March to June 2005 and fertilized each week with commercial liquid fertilizer (N,P,K 5,5,7). At the beginning of June, when seedlings of both species had a mean root collar diameter of 5.5 mm, individuals were randomly assigned to 20 independent blocks comprising four individual pots of each species. Block effects were taken into account during the statistical analyses.

Inducing the formation of hypertrophied lenticels

A 2-cm-long piece of gas-impermeable mastic (Terostat VII, Henkel Teroson, Heidelberg, Germany) was applied to seedling stems below the insertion point of the first basal leaf. The surface covered by the mastic was $1.05 \pm 0.20 \text{ cm}^2$. The mastic was applied before the 8-week waterlogging treatment (from

April 24 to June 22), and the number of hypertrophied lenticels formed was counted after the mastic was removed.

Waterlogging treatment

The seedlings were subjected to waterlogging for 4 weeks from June 27 to July 23. Each block of seedlings was placed in a large container in which a permanent water table was maintained 5 cm above the growth substrate surface. The area of submerged stem was determined for each seedling by measuring stem length and diameter. The O_2 concentration of the water was kept below 3 mg l^{-1} by bubbling N_2 through the water. The O_2 concentrations in the free water and in the piezometric tubes installed in the soil of each pot were measured with a dissolved-oxygen meter (MO-128, Mettler, Toledo). Dissolved O_2 concentrations were lower in the piezometric tubes than in the free water. A gradient from outside ($4.3 \pm 1.1 \text{ mg l}^{-1}$) to inside ($3.2 \pm 1.1 \text{ mg l}^{-1}$) the pots was a result of O_2 consumption in the rhizosphere, probably resulting in a lower O_2 concentration in close vicinity to the roots. Lack of differences in means and variances between the two populations (Figure 1), and lack of correlation with any of the analyzed traits (not shown), indicated stable hypoxic conditions across populations. Fluctuations with time were associated with the difficulty in maintaining a constant hypoxic environment, mainly because temperature variations in the greenhouse changed the solubility of O_2 in water.

Epinasty, hypertrophied lenticels and adventitious roots

The degree of epinasty (E) was estimated as the angle that the adaxial face of the petiole formed with stems after 9, 13, 17 and 29 days of treatment. Three ordinal classes were defined: beyond perpendicular, perpendicular and less than perpendicular to the stem axis, and assigned numerical values of 120, 90 and 60°, respectively, to obtain quantitative values. For dead plants, E was recorded just before death.

Hypertrophied lenticels were counted on the submerged portion of each stem 8 and 15 days after waterlogging, before neighboring lenticels merged. The mean of the two estimates was used after confirming that no significant increase occurred between the dates. Hypertrophied lenticel developmental stages were quantified after 27 days of waterlogging (DS) based on an ordinal scale from 1 to 14. This scale extends from the appearance of a black disk visible on the bark, corresponding to the start of hypertrophy, to splitting of the bark, and finally to the appearance of totally white lenticels that expand and gradually merge.

Adventitious root dry biomass (M_{adv}) and root number (N_{adv}) were determined for each seedling at the end of the waterlogging treatment. Adventitious roots were identified as the white and plagiotropic lateral roots inserted on the main stem or at the base of the tap root.

Statistical analysis

Because of the large number of individuals, tests were considered significant when $P < 0.01$. Differences in mortality between populations were analyzed by a χ^2 test. Rank correlation

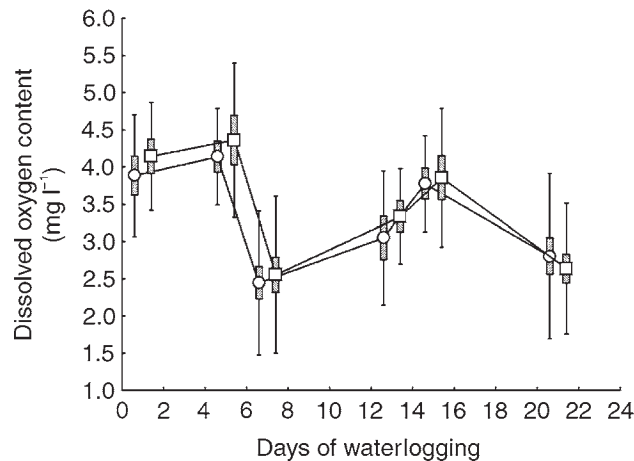


Figure 1. Time course of mean dissolved oxygen concentration (the shaded rectangle indicates standard deviation and the whiskers indicate the 95% confidence interval) in piezometric tubes installed in pots containing *Quercus robur* (□) and *Q. petraea* (○) seedlings. No significant difference was observed between the species for means or variances.

between the dates of death and E was tested with a Spearman rank correlation test. Individuals that survived the treatment were included in the last rank of the date of observation of death.

The significance of the differences in means between species, free water and piezometric tubes (for oxygen concentration measurements) and survival rates were tested with a linear model:

$$Y_{ij} = \mu + C_i + b_j + \varepsilon_{ij}$$

where Y_{ij} is the observed value of the trait, μ is the overall mean, C_i is the fixed effect of the group i tested, b_j is the random effect of block j and ε_{ij} is the error term. All correlations among variables were tested with the same linear model, with C_i the fixed effect of the quantitative variable. To determine the interaction between species and mortality, we added to the model a term of interaction (Cb_{ij}). The time course of epinasty was tested with a similar model by adding a fixed effect of time. For all comparisons between species, a further test was performed by removing individuals that died during the experiment.

Densities of hypertrophied lenticels (DHL) were log transformed to improve homoscedasticity. In the case of M_{adv} and N_{adv} , none of the tested transformations proved efficient. As a consequence, differences between species were tested with a Mann and Whitney U test. To test the interaction between species and mortality by non-parametric analyses, we tested independently the differences between dying and surviving individuals for each species. The developmental stage of hypertrophied lenticels, estimated by an ordinal scale of colors, was considered a quantitative variable as all postulates for an ANOVA were respected. Differences in variance between the species were tested with the Bartlett test. A principal compo-

nent analysis (PCA) was performed with R 2.0.1 software (www.r-project.org). The principal component factors calculated were linear combinations of the original variables that were constructed so that they displayed the largest variance between individuals. Traits included in the analysis were: (1) the density of hypertrophied lenticels induced by mastic (DHL_m); (2) DS and the density of hypertrophied lenticels formed below the water table after 4 weeks of waterlogging (DHL_{wl}); (3) M_{adv} and N_{adv} ; and (4) E .

Results

Local hypoxia induced with mastic

Application of mastic to seedling stems induced local hypoxia as indicated by the formation of hypertrophied lenticels on all individuals irrespective of species and position of the mastic on the stem (Figure 2). The density of hypertrophied lenticels induced by local hypoxia was about one-seventh that induced by waterlogging, and was significantly lower in the *Q. robur* population than in the *Q. petraea* population (Figure 2). The variance in DHL_m was larger in *Q. petraea* than in *Q. robur*. Extreme phenotypes with high DHL_m were detected in the *Q. petraea* population (Figure 2). Application of mastic did not induce the formation of adventitious roots on any of the seedlings.

Waterlogging and hypertrophied lenticel formation

The density of hypertrophied lenticels induced by waterlogging was higher than that induced by mastic. In contrast to the hypertrophied lenticels induced by mastic, those induced by waterlogging were present at a significantly higher density and at a more advanced developmental stage in *Q. robur* than in *Q. petraea* (Figure 2). Hypertrophied lenticels appeared rapidly after the onset of waterlogging and a threshold density was reached after between 8 and 15 days, beyond which time density remained stable but lenticels merged and developed. Variances of DHL_{wl} and DS were significantly higher in the *Q. robur* population than in the *Q. petraea* population (Figure 2). Extreme phenotypes with high DHL_{wl} were detected in the *Q. robur* population. Advanced DS were reached by a few individuals in both populations, and many *Q. petraea* seedlings had hypertrophied lenticels that remained at less advanced DS (Figure 2).

Waterlogging and degree of epinasty

There was greater epinasty in *Q. petraea* than in *Q. robur* but variances were similar between populations (Figure 2). The similar range of values for the populations was not an artifact associated with the small number of classes, because the scale used covered the entire range of E exhibited by the seedlings during the study.

Waterlogging and adventitious root formation

At the end of the treatment, N_{adv} and M_{adv} were significantly higher in *Q. robur* than in *Q. petraea*, as were the variances of the two traits (Figure 2). A few extremely high values of N_{adv}

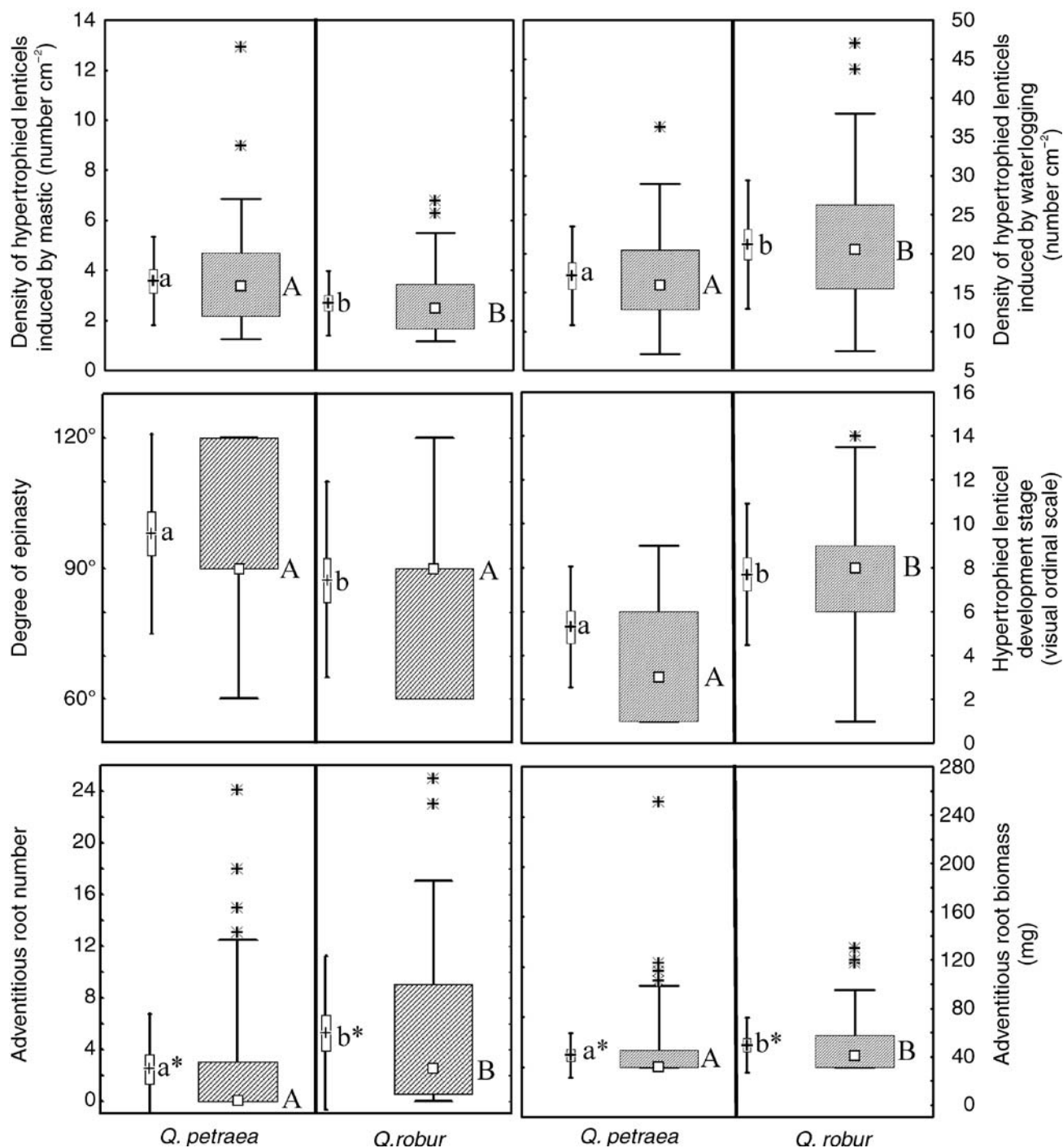


Figure 2. Differences in means and variances between *Quercus petraea* and *Q. robur* seedlings. Data were plotted in two forms. Left side of each panel: means (+), 95% confidence interval of the means (boxes) and standard deviation (whiskers). Significant differences between means are indicated by different lowercase letters (Students *t*-test; an asterisk (*) indicates that differences were tested on medians by the Mann and Witney *U* test). Right side of each panel: medians (□), quartiles (boxes), 95th percentiles (whiskers) and extreme values (*). Significant differences of variances are indicated by different uppercase letters (Bartlett test).

and M_{adv} were observed in both species.

Waterlogging and mortality

Quercus petraea experienced significantly higher mortality during waterlogging than *Q. robur* (32.5 and 10.0%, respec-

tively; $P = 0.0005$). Except for *E*, differences between species remained significant when dead individuals were excluded from the analyses.

The degree of epinasty was higher in *Q. petraea* than in *Q. robur*; however, no difference between populations was de-

tected in *E* if only seedlings surviving at the end of the treatment were considered. Epinasty was detected both in declining and in surviving individuals; however, a close correlation was detected between *E* and mortality (Table 1); the time course of *E* differed among individuals and was a function of the date of mortality, *E* increased markedly before death (Figure 3) and there was a significant rank correlation between date of death and *E*.

The time course of development of hypertrophied lenticels differed between plants that died and plants that survived (independent of species, see Table 1). Most individuals that displayed shoot die-back failed to develop hypertrophied lenticels beyond Stage 1. In contrast, DHL_m or DHL_{wl}, and *N*_{adv} and *M*_{adv}, did not differ between dying and surviving individuals (Table 1).

Principal component analysis and correlations among traits

Principal component factors 1 and 2 (PC 1 and 2) represented 54% of the total variability (29 and 24%, respectively). The two species were significantly discriminated along PC 1 only (data not shown). On the factorial plan (Figure 4a), correlations among traits were similar to those found for the overall correlation analysis (Table 2). No correlations were found among traits characterizing hypertrophied lenticels and traits characterizing adventitious roots. A positive correlation was detected between *N*_{adv} and *M*_{adv}, and there was a significant negative correlation between *E* and DS (Table 2). On the left of the projection plan (Figure 4b), several *Q. robur* seedlings lay outside the main cloud of the scatter-plot. These individuals correspond to seedlings with high DS, *N*_{adv} and *M*_{adv}, and low *E*. Most individuals scattered on the right of the plot corresponded to *Q. petraea* seedlings having high *E*.

Discussion

Lenticel formation in response to local hypoxia

We applied gas-impermeable mastic to the stems of seedlings

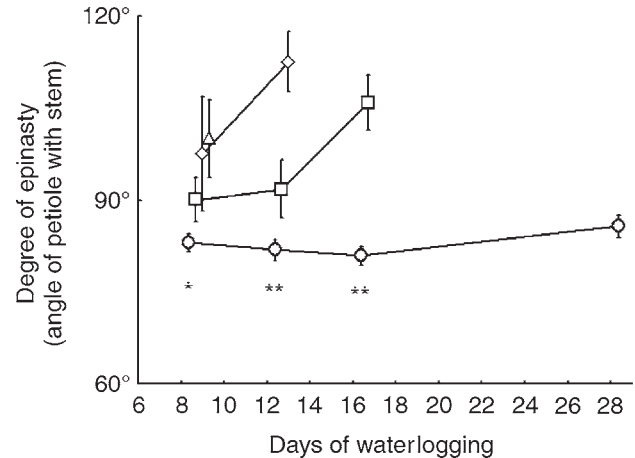


Figure 3. Development of epinasty (*E*) in two *Quercus* populations subjected to waterlogging and correlation of *E* with mortality. Seedlings were clustered as a function of the number of days recorded between the start of the treatment and death: 13 days after treatment (Δ), 17 days after treatment (◇), 29 days after treatment (□) or survivors (○). Differences among the clusters were tested by Spearman rank correlation tests for each date. Asterisks (*) indicate significant differences between groups: *, $P < 0.05$; and **, $P < 0.01$.

to test for potential inter- and intraspecific diversity in the ability to produce hypertrophied lenticels in response to hypoxia. The finding that mastic induced the formation of hypertrophied lenticels in all individuals confirms the usefulness of the test; however, it did not fully mimic the effect of waterlogging on lenticel formation. The density of induced lenticels was much lower, and species differences were smaller than those induced by waterlogging. Local hypoxia induced lenticel formation independent of root hypoxia. Hypertrophied lenticels occur mainly or solely on the submerged segment of the stem during waterlogging (Newsome et al. 1982, Tang and Kozlowski 1982a, 1982b, 1984, Nunez-Elisea et al. 1999), sup-

Table 1. Differences between dying or surviving individuals in *Quercus petraea* and *Q. robur* populations (means $\pm$ standard deviation). Significant differences between species, mortality and interaction are indicated as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ns, not significant. None of the species $\times$ mortality interactions were significant. Abbreviations: DHL_m and DHL_{wl}, density of hypertrophied lenticels induced by mastic or waterlogging, respectively; *E*, degree of epinasty after 4 weeks of treatment; DS, development stage of hypertrophied lenticels after 4 weeks of treatment; *N*_{adv}, number of adventitious roots after 4 weeks of treatment; and *M*_{adv}, dry mass of adventitious roots after 4 weeks of treatment. Only *E* and DS were significantly different among plants with respect to mortality; these differences were similar for the two species.

	DHL _m (number cm ⁻²)	DHL _{wl} (number cm ⁻²)	<i>E</i> (°)	DS	<i>N</i> _{adv}	<i>M</i> _{adv} (mg)
<i>Q. petraea</i>						
Surviving seedlings	3.6 $\pm$ 1.7	17.1 $\pm$ 6.3	89 $\pm$ 21	5.3 $\pm$ 2.8	2.5 $\pm$ 4.3	11.6 $\pm$ 22.6
Dying seedlings	4.0 $\pm$ 2.4	15.3 $\pm$ 6.5	118 $\pm$ 12	1.4 $\pm$ 1.6	3.1 $\pm$ 5.5	27.0 $\pm$ 55.9
<i>Q. robur</i>						
Surviving seedlings	2.7 $\pm$ 1.3	21.2 $\pm$ 8.3	84 $\pm$ 21	7.7 $\pm$ 3.2	5.3 $\pm$ 5.6	21.9 $\pm$ 27.3
Dying seedlings	2.7 $\pm$ 1.4	22.2 $\pm$ 13.8	120 $\pm$ 0	3.1 $\pm$ 3.1	4.5 $\pm$ 5.5	12.9 $\pm$ 13.7
Significance of factors						
Species	**	*	ns	**	***	**
Mortality	ns	ns	***	***	ns	ns

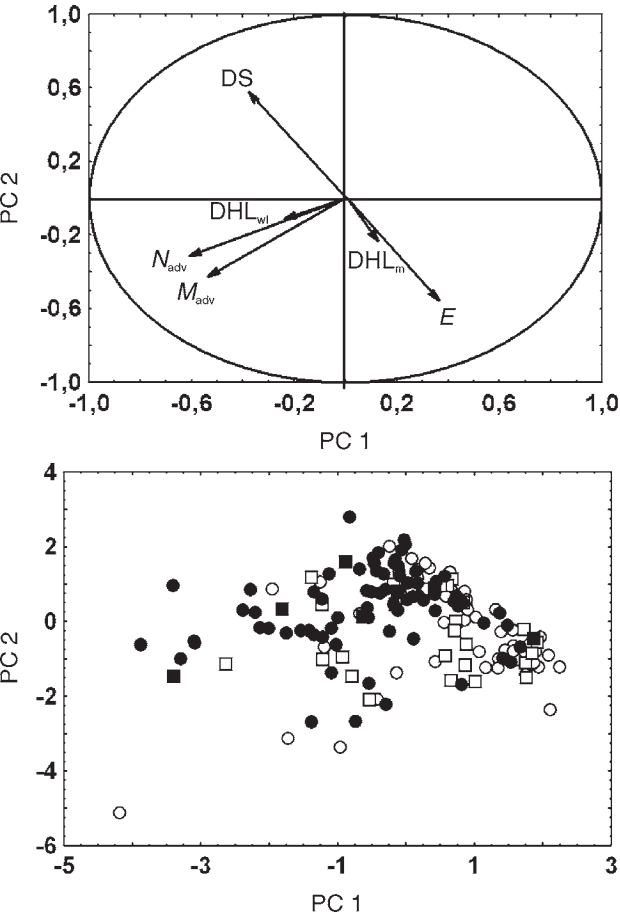


Figure 4. Principal component analysis of the response traits to waterlogging of the seedlings (upper panel) and projection of principal component factors 1 and 2 (PC 1 and PC 2; lower panel). Abbreviations: DHL_m and DHL_{wl}, density of hypertrophied lenticels induced by mastic and by waterlogging, respectively; DS, developmental stage of hypertrophied lenticels estimated by color; N_{adv} and M_{adv}, number and dry mass of adventitious roots, respectively; and E, degree of epinasty. Symbols: *Quercus robur*, ■, ●; *Q. petraea*, □, ○; surviving individuals, ○, ●; and dead individuals, □, ■.

porting the hypothesis that this process is induced by local hypoxia affecting bark tissues. The discrepancy between the induction in response to the mastic treatment and induction by

waterlogging is difficult to explain. We speculate that, although the potential response to the hypoxia signal is similar in the two species, the development of hypertrophied tissues during waterlogging of *Q. petraea* is hampered by the lack of substrate and energy, because of impaired carbohydrate transfer to roots (Kreuzwieser et al. 2004).

Interspecific trait differences

The effect of waterlogging on *E* differed between the species studied, with *Q. petraea* being affected more than *Q. robur*. In response to waterlogging, greater numbers of adventitious roots and hypertrophied lenticels formed on *Q. robur* seedlings than on *Q. petraea* seedlings, confirming earlier observations (Colin-Belgrand et al. 1991, Parelle et al. 2006). Mean values of DHL_{wl} and DS differed between the species.

Responses to waterlogging and seedling survival

The degree of epinasty was negatively correlated with the duration of survival during waterlogging; however, no species-specific difference was detected among surviving individuals. These observations illustrate the value of *E* as an index of hypoxic stress and as an early predictor of waterlogging-induced mortality. Because no functional explanation of the role of epinasty in tolerance to hypoxia is available, it can be assumed that selection for this trait may be indirect through selection of seedlings for tolerance to waterlogging.

Hypertrophied lenticels are reported to enhance oxygen diffusion into stems of herbaceous plants (Jackson and Drew 1984). This is unlikely to be the case for *Q. petraea* and *Q. robur* (Parelle et al. 2006); although they may, nevertheless, have an adaptive function. After 2 weeks of waterlogging, dead and surviving individuals displayed similar densities of hypertrophied lenticels. However, lenticels reached a more advanced stage of development in surviving seedlings than in seedlings that died. A close correlation with *E* confirms that DS is a useful indicator of seedling sensitivity to waterlogging.

Adventitious rooting is known to be involved in waterlogging tolerance (Jackson and Armstrong 1999, Armstrong et al. 1999, McDonald et al. 2001). However, our results suggest that the production of adventitious roots is not sufficient to prevent mortality because some *Q. petraea* individuals died

Table 2. Correlation matrix of waterlogging tolerance traits in seedlings (including dead individuals and both *Quercus robur* and *Q. petraea* seedlings). Significant correlations are indicated as: **, $P < 0.01$; ***, $P < 0.001$; and ns, not significant. Abbreviations: DHL_m and DHL_{wl}, density of hypertrophied lenticels induced by mastic and waterlogging, respectively; *E*, degree of epinasty after 4 weeks of waterlogging; DS, developmental stage of hypertrophied lenticels after 4 weeks of waterlogging; and N_{adv} and M_{adv}, number and dry mass of adventitious roots after 4 weeks of waterlogging, respectively.

	DHL _{wl}	<i>E</i>	DS	N _{adv}	M _{adv}
DHL _m	0.05 **	0.00 ns	0.01 ns	0.00 ns	0.00 ns
DHL _{wl}	-	0.00 ns	0.02 ns	0.04 ns	0.01 ns
<i>E</i>	-	-	0.24 ***	0.01 ns	0.00 ns
DS	-	-	-	0.01 ns	0.00 ns
N _{adv}	-	-	-	-	0.32 ***

despite having a large number of adventitious roots. Moreover, no correlation was observed between N_{adv} or M_{adv} and seedling mortality. Consequently, neither N_{adv} nor M_{adv} can be considered a measure of waterlogging tolerance in the study species.

Difference in variances between species

We observed higher variances for DHL_{wl} , M_{adv} and N_{adv} in the *Q. robur* population than in *Q. petraea* population. Because we used controlled and homogeneous environmental conditions, the variability in these traits must reflect genetic diversity. Mariette et al. (2002) found the opposite result for neutral genetic markers, with a higher genetic diversity for *Q. petraea* than for *Q. robur*, and Kremer et al. (2002) found that the variance of traits characterizing leaf morphology did not differ between these species. No difference in variance of E was detected between species, however, greater variance of DS was found in the *Q. petraea* population than in the *Q. robur* population, which is consistent with the neutral genetic diversity observed by Mariette et al. (2002).

Phenotypic variation

Quercus robur produced more highly developed lenticels and displayed less epinasty than *Q. petraea* seedlings. We observed that some individuals of both species reached similar extreme values for both traits despite a significant difference in population mean values. Consequently, the populations differed in the frequencies of the different phenotypes. More *Q. robur* seedlings displayed high DS or lower E , or both, than *Q. petraea* seedlings. The simplest explanation is that these traits are controlled by a set of genes that differ in allelic frequency between the species, as in the case of neutral molecular genetic markers (Bodénès et al. 1997, Muir et al. 2000, Muir and Schlötterer 2005).

In conclusion, the E and DS in response to waterlogging are the markers that most clearly differentiated *Q. robur* and *Q. petraea* according to their sensitivity to waterlogging. Greater waterlogging tolerance of *Q. robur* relative to *Q. petraea* was related to a difference in the frequency of extreme phenotypes. The greater frequency of extreme phenotypes in the population of *Q. robur* seedlings resulted in a greater proportion of individuals with a combination of traits associated with tolerance to root hypoxia.

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