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Étude de la réponse à l'ennoyage chez le chêne sessile (*Quercus petraea*) et le chêne pédonculé (*Quercus robur*): Implication de l'hémoglobine non-symbiotique

Claire Parent

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UNIVERSITÉ DE FRANCHE-COMTÉ
U.F.R. SCIENCES, TECHNIQUES
Laboratoire de Chrono-environnement (UMR UFC/CNRS 6249 USC INRA)

THÈSE

Présentée en vue de l'obtention du grade de
DOCTEUR DE L'UNIVERSITÉ DE FRANCHE-COMTÉ
Spécialité : Sciences de la vie
École doctorale : Homme, Environnement, Santé

Étude de la réponse à l'ennoyage chez le chêne sessile
(*Quercus petraea*) et le chêne pédonculé (*Quercus robur*):
Implication de l'hémoglobine non-symbiotique

par

Claire PARENT

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to my sailor

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Introduction

A l'échelle du globe, les sols, à hydromorphie temporaire, c'est-à-dire subissant un excès d'eau (ennoyage), occupent des surfaces de plus en plus importantes. Ces sols, souvent mal drainés se rencontrent fréquemment et le plus souvent dans nos régions tempérées, mais pas seulement. Les régions relativement arides peuvent elles-mêmes présenter des excès d'eau dans le sol lors de fortes précipitations occasionnelles.

On peut affirmer presque avec certitude, qu'au cours des prochaines décennies, les inondations seront plus marquées et plus fréquentes en raison du réchauffement climatique. En effet, les prévisions fournies par les climatologues regroupés au sein du GIEC (Groupe d'experts Intergouvernemental sur l'Evolution du Climat) indiquent une augmentation des épisodes de fortes précipitations même dans les régions évoluant globalement vers la sécheresse (Fig 1; GIEC, 2007). Aux causes naturelles et aux modifications climatiques globales, on peut ajouter l'anthropisation : l'imperméabilisation des sols due à l'urbanisation croissante, le terrassement, l'absence de drainage et l'irrigation intensive des cultures. Ces divers éléments génèrent des accumulations d'eau dans le sol. Ce sont des zones non constructibles car référencées inondables, et difficilement valorisables par l'agriculture. Elles sont donc le plus souvent laissées à l'exploitation forestière. En France, près de deux millions d'hectares de sols forestiers sont soumis à l'ennoyage temporaire (Lévy & Lefèvre, 2001). La présence d'une nappe d'eau temporaire ou continue a des conséquences sur la végétation et sur la diversité des écosystèmes forestiers. De plus, elle modifie la répartition des espèces et leur régénération (Siebel & Blom 1998, Lopez & Kursar 1999, Lavabre & Andreassian 2000, Parolin *et al.* 2004, Vreugdenhil *et al.* 2006).

La tolérance des plantes ligneuses à l'ennoyage dépendra des caractéristiques du site (type de sol, altitude, exposition, température...) mais surtout de l'espèce considérée, de ses réserves glucidiques et de son stade de développement. En effet, les jeunes plants sont

Phenomenon and direction to trend	Likelihood of future trends based on projections for 21 st century using SRES scenarios	Examples of major projected impacts by sector			
		Agriculture, forestry and ecosystems	Water resources	Human health	Industry, settlement and society
Heavy precipitation events. Frequency increases over most area	Very likely	Damages to crops: soil erosion, inability to cultivate land due to waterlogging of soils	Adverse effects on quality of surface and groundwater; contamination of water supply; water scarcity may be relieved	Increased risk of deaths, injuries and infectious, respiratory and skin diseases	Disruption of settlements, commerce, transport and societies due to flooding: pressures on urban and rural infrastructures; loss of property

Tableau.1. : Exemples des impacts dus aux changements climatiques basés sur des projections pour le milieu du 21^{ème} siècle. Extrait de « Climate Change 2007: Synthesis Report. An Assessment of the Intergovernmental Panel on Climate Change »

particulièrement vulnérables à l'excès d'eau et les précipitations étant particulièrement abondantes au printemps, au moment de la germination des graines, l'engorgement exerce une forte sélection intra et interspécifique sur les espèces ligneuses (Streng *et al.* 1989, Angelov *et al.* 1996, Guo *et al.* 1998, Kozłowski & Pallardy 2002, Walls *et al.* 2005). Les conséquences néfastes de l'excès d'eau ne s'appliquent pas seulement aux semis. En effet, la saturation de la macroporosité du sol par l'eau a des conséquences très importantes puisqu'elle est l'un des principaux facteurs limitant la productivité végétale (Kozłowski 1997).

La contrainte majeure liée à l'excès d'eau dans les sols est sans doute la réduction drastique des échanges gazeux au niveau du compartiment racinaire (Drew 1997). Les pores du sol, permettant l'aération de la rhizosphère, sont rapidement saturés par l'eau. Le peu d'oxygène encore présent est rapidement consommé par les végétaux mais aussi les microorganismes du sol. Pour les plantes, l'engorgement représente donc principalement un déficit en oxygène au niveau des racines (hypoxie racinaire). Les racines ne disposant plus d'assez d'oxygène pour la respiration, elles doivent trouver d'autres voies alternatives pour leur métabolisme énergétique cellulaire (Dat *et al.* 2004). Pour survivre à ces conditions de stress, les plantes doivent mettre en place des stratégies de tolérance, soit en s'adaptant aux conditions anaérobies et en favorisant le métabolisme fermentaire, soit en essayant de maintenir le métabolisme aérobie en développant des adaptations morphologiques permettant de fournir de l'oxygène aux racines engorgées (Drew 1997, Vartapetian & Jackson 1997, Blom 1999, Gibbs & Greenway 2003). Parmi ces adaptations, on peut citer les aerenchymes, réseaux de lacunes gazeuses se formant dans le cortex des tissus et qui offrent à la plante une alternative pour suppléer le déficit en oxygène (Drew *et al.* 2000, Jackson & Ricard 2002). Au niveau de la base de la tige, chez de nombreux ligneux, le développement de lenticelles hypertrophiées favoriserait la diffusion de l'oxygène mais aussi l'évacuation des produits phytotoxiques produits par le métabolisme anaérobie. Autre adaptation caractéristique de la réponse à l'engorgement, les racines d'adaptation ou racines adventives se développent à proximité de l'interface sol/air, là où la concentration en oxygène est la plus élevée. Elles permettent également l'absorption d'eau qui n'est plus assurée par le système racinaire initial qui devient nécrosé.

A l'échelle cellulaire et moléculaire, plusieurs hypothèses sont avancées pour tenter d'expliquer l'initiation de la cascade d'événements conduisant à une réponse adaptée. Les changements de potentiel redox au niveau de la rhizosphère et/ou la réduction du pH cytosolique comptent parmi les premiers signaux déclenchant la réponse à l'engorgement (Dat *et*

al. 2004). Cependant l'hypothèse retenue par le plus grand nombre d'études est une baisse de la teneur intracellulaire en oxygène qui pourrait être perçue rapidement grâce à certaines molécules qui joueraient un rôle de senseur (Drew 1997). Cependant à ce jour, aucune molécule fonctionnelle de ce type n'a été identifiée chez les plantes supérieures. Chez certains micro-organismes, les variations de la concentration en oxygène du milieu sont perçues grâce à des protéines hémiques dont l'hémoglobine fait partie (Drew 1997). Chez les plantes, on distingue deux types d'hémoglobine dont l'hémoglobine non symbiotique de classe 1 qui présente une forte affinité pour l'oxygène. Plusieurs travaux de recherche ont montré qu'elle est synthétisée dans les racines en conditions d'hypoxie (Taylor *et al.* 1994, Trevaskis *et al.* 1997, Lira Ruan *et al.* 2001). Même si sa fonction n'a pas encore été clairement établie, plusieurs rôles autres que celui de senseur d'oxygène ont été proposés. Elle pourrait jouer un rôle très important dans la régulation du monoxyde d'azote et par réaction avec celui-ci maintenir l'homéostasie énergétique de la cellule.

Objectifs généraux et plan de la thèse

L'objectif de mon travail de recherche a été de caractériser, par une approche intégrée, la réponse à l'ennoyage de deux espèces de chênes, le chêne sessile (*Quercus petraea* L.) et le chêne pédonculé (*Quercus robur* L.). Le chêne est l'espèce ligneuse la plus répandue en Europe (IFN 2005) et les deux espèces étudiées, bien que génétiquement très proches, présentent une différence de tolérance à l'ennoyage. En effet, le chêne sessile préfère les sols bien drainés alors que le chêne pédonculé prospère sur des sols bien alimentés en eau.

L'étude du comportement de ces deux espèces en conditions d'ennoyage, vise à mieux comprendre les mécanismes de la réponse aux conditions d'hypoxie racinaire mais aussi à identifier certains éléments clés de l'adaptation à ce stress. Dans ce contexte, nous avons choisi de nous intéresser à plusieurs réponses dans le cadre d'une étude intégrée : moléculaire, cellulaire, physiologique et morphologique. Nous nous sommes ainsi penchés sur les mécanismes intervenant rapidement lors de l'application du stress et en réponse à une durée courte d'ennoyage, et ensuite, sur les adaptations mises en place lorsque l'ennoyage perdure.

Nous nous sommes tout d'abord proposés d'analyser quelles étaient les différences au niveau de la croissance et du développement chez les deux espèces en réponse à l'ennoyage. Pour cela, nous avons analysé différents paramètres de croissance (biomasse, longueur des tiges, surface foliaire, nombre de feuilles...) afin de bien comprendre l'impact de l'ennoyage sur le développement du chêne.

Ces différences sont la conséquence de modifications métaboliques, cellulaires et moléculaires relatives au stress subi. Pour relier ces résultats aux différences observées à d'autres niveaux, nous avons entrepris l'étude des modifications de différents paramètres physiologiques pendant l'hypoxie racinaire. Nous avons ainsi suivi la conductance stomatique, la photosynthèse et le potentiel hydrique. D'autre part, nous nous sommes demandé comment le système racinaire, premier organe à subir l'ennoyage, était affecté par l'hypoxie. Pour cela, nous avons étudié l'augmentation de la porosité racinaire chez les deux espèces au cours du traitement. Les résultats obtenus nous ont amenés à nous intéresser aux modifications susceptibles d'expliquer la tolérance accrue du chêne pédonculé par rapport au chêne sessile. L'hémoglobine non-symbiotique pourrait être un marqueur moléculaire de la tolérance à l'hypoxie et semble jouer un rôle prépondérant dans la réponse à ce stress. Nous avons donc entrepris le clonage de ce gène chez le chêne et ainsi confirmé sa présence dans son génome. Ce gène, *QpHb1*, a été séquencé, comparé à celui d'autres espèces et ensuite caractérisé en conditions témoins : nous avons analysé son expression par *Northern blotting*

dans les différents organes mais également sa distribution au niveau tissulaire dans les racines par hybridation *in situ*. Ces expériences nous ont permis de démontrer une distribution hétérogène des transcrits dans les différents tissus de la racine suggérant un rôle constitutif pour l'hémoglobine non-symbiotique. Par la suite, nous avons cherché à mettre en relation la différence de tolérance à l'engorgement observée chez les deux espèces de chêne et une éventuelle différence dans l'expression de *QpHb1*. Pour cela, nous avons réalisé les expériences de *Northern* aux différents temps de traitement ainsi que l'hybridation *in situ* des transcrits après 14 jours d'engorgement. Enfin, nous avons comparé les adaptations morphologiques mises en place par les deux espèces notamment le développement de racines adventives ainsi que l'expression de l'hémoglobine dans ces racines d'adaptation.

Les résultats obtenus durant ces trois années de thèse ont été intégrés dans ce manuscrit sous la forme d'articles de recherche dans la partie « Résultats ». La première partie, composée de deux articles, se concentre sur la réponse rapide à l'engorgement et la caractérisation du gène d'hémoglobine non-symbiotique cloné chez le chêne sessile, *QpHb1*. La deuxième partie, composée d'un article en préparation, porte sur l'analyse intégrée des réponses lors d'un engorgement prolongé de 28 jours mais également sur l'implication de l'hémoglobine non-symbiotique dans la tolérance et la mise en place des adaptations morphologiques (aérenchymes, racines adventives).

Le présent manuscrit est organisé en trois volets, comportant chacun plusieurs parties.

Le premier volet correspond à une synthèse bibliographique et à l'état des connaissances actuelles relatives à notre sujet d'étude. Il comporte (i) une première partie sur le chêne qui est suivie par deux autres parties traitant respectivement (ii) de la réponse des plantes à l'excès d'eau et (iii) des voies de signalisation mises en place en réponse à ce stress et notamment au rôle joué par l'hémoglobine. Ces parties ont fait l'objet de publications sous forme de *review* et sont intégrées comme telles.

Le second volet, comme annoncé ci-dessus est présenté sous forme de publications (acceptées ou en préparation). Il constitue le corps du manuscrit et présente en détail les matériels et méthodes employés ainsi que les résultats des expérimentations réalisées, qui sont ensuite discutés.

Le dernier volet reprend les principales connaissances tirées de notre étude afin de les analyser dans une discussion générale et de présenter les différentes perspectives envisageables.

Synthèse

Bibliographique

A. Le chêne

1. Le chêne au sein de la forêt

a) Importance de la forêt

Parmi les écosystèmes terrestres, les forêts sont ceux qui concentrent la diversité biologique la plus riche. Elles participent ainsi au maintien de la biodiversité, de la qualité et de la gestion des ressources en eau (Kremar *et al.* 2005). Elles constituent le refuge d'une majorité d'espèces, face à l'emprise croissante des zones urbanisées et à la banalisation des espaces agricoles. Le rôle des forêts dans les grands équilibres écologiques, en liaison avec le cycle de l'eau, représente un enjeu majeur pour le développement durable des ressources naturelles de la planète. Plus que jamais les eaux et forêts sont indissociables (Rapport de développement durable, gestion 2006, ONF).

La forêt représente environ 30% des terres émergées du globe avec un peu moins de 4 milliards d'hectares (FAO 2007). En Europe, la superficie des forêts augmente dans la plupart des pays et était de 193 millions d'hectares en 2005. En termes de surface, la France se place à la 3^{ème} position, derrière la Suède et la Finlande, avec 15 millions d'hectares de forêt soit 28,3% de son territoire (Fig.1). La forêt française est surtout composée de feuillus, environ 63%, et par sa diversité géographique, géologique et climatique, elle possède une biodiversité particulièrement importante (Cinotti 1996).

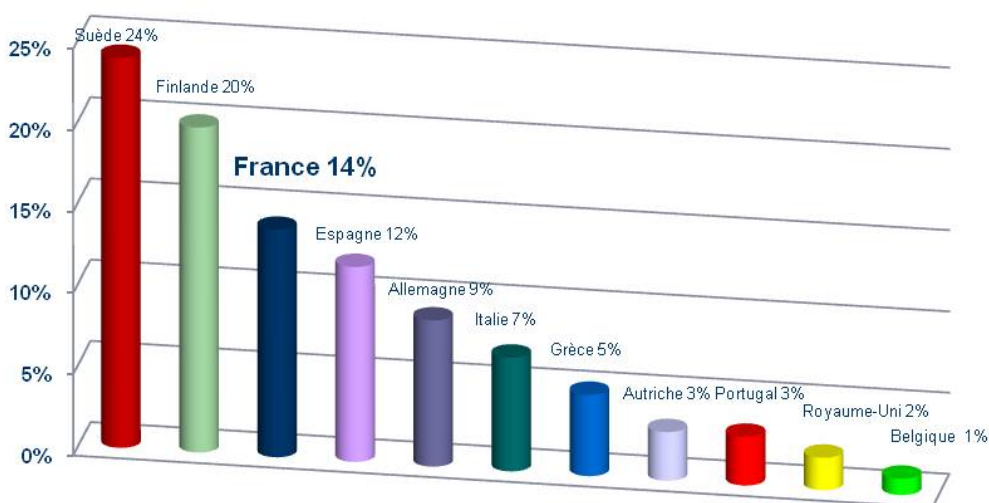


Fig.1 Répartition de la surface forestière en Europe par pays. FAO, 2007.

b) Importance du chêne

Les chênes appartiennent à la famille des Fagacées, de l'ordre des Fagales, ce sont des angiospermes dicotylédones du genre *Quercus*. Ils sont présents dans de très nombreuses régions du globe, notamment dans l'hémisphère nord (Kleinschmit 1993, Nixon 1993b).

C'est un arbre à bois très dur et sa forte teneur en tanins lui confère une résistance naturelle aux pathogènes. Le bois de chêne est très important économiquement : il est utilisé comme énergie en bois de chauffage, pour la construction marine et dans le bâtiment, la menuiserie, la tonnellerie, etc.

Les espèces de chênes sont nombreuses (le genre *Quercus* compte 250 espèces) et variées à feuillage caduc, semi-persistant ou persistant. Une dizaine d'espèces seulement sont présentes en France. Après les deux espèces les plus courantes que sont le chêne sessile (*Quercus petraea*) et le chêne pédonculé (*Quercus robur*), on trouve le chêne chevelu (*Quercus cerris*), le chêne vert (*Quercus ilex*), le chêne des marais (*Quercus palustris*), le chêne pubescent (*Quercus pubescens*), le chêne rouge d'Amérique (*Quercus rubra*), le chêne liège (*Quercus suber*) et le chêne écarlate (*Quercus coccinea*).

Le chêne est l'arbre le plus répandu en France, avant le pin. Il représente 40% de la surface boisée (Preney *et al.* 1997). Le chêne pédonculé et le chêne sessile occupent une place prépondérante (Fig.2). A elles seules, ces deux espèces forment la majorité des chênaies françaises.

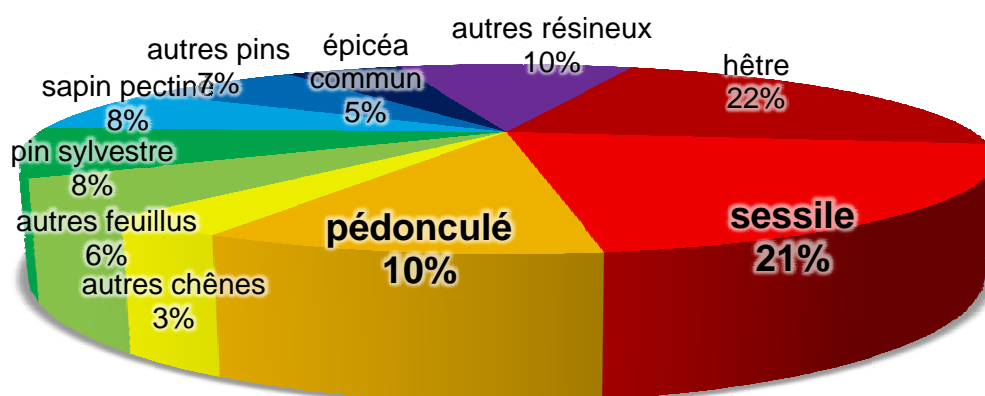


Fig.2 Répartition de la surface boisée pour la production par essence prépondérante du peuplement sur l'ensemble des forêts domaniales françaises. IFN, 2005

2. Le chêne sessile et le chêne pédonculé

a) *Ecologie*

Le chêne sessile et le chêne pédonculé sont présents en plaine mais peuvent aussi se développer jusqu'à 1000m d'altitude, et à l'exception de la bordure méditerranéenne où le climat est trop sec, on peut les rencontrer sur l'ensemble de l'hexagone. Le chêne pédonculé occupe la plus grande partie de l'Europe tempérée depuis l'Oural et la Volga jusqu'à l'océan Atlantique et de l'Espagne jusqu'à la Scandinavie (Fig3a). L'aire de répartition du chêne sessile est incluse dans celle du chêne pédonculé et comprend toute l'Europe occidentale excepté le Portugal et une grande partie de l'Espagne (Fig3b).

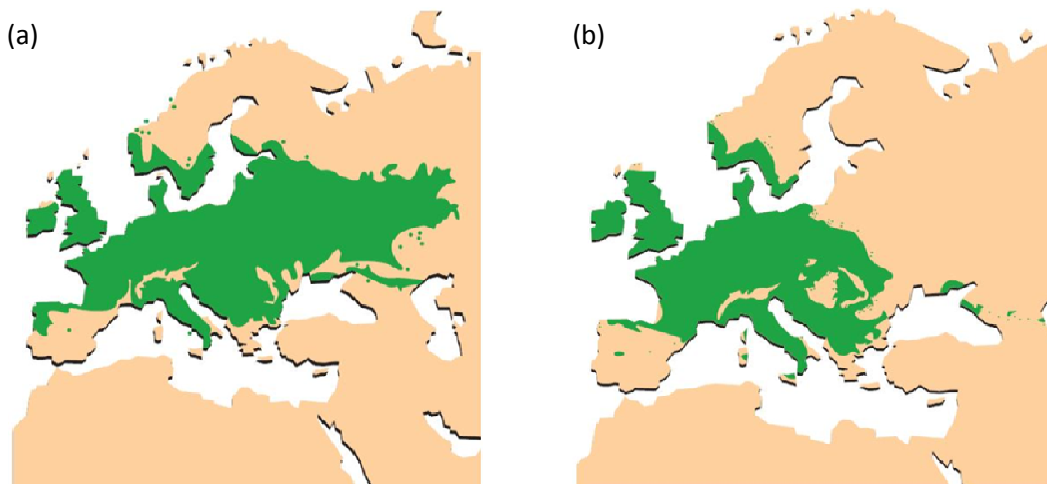


Fig.3 Répartition du chêne pédonculé(a) et du chêne sessile(b) en Europe. IDF, 2004.

En France, le chêne pédonculé se rencontre partout sauf dans les Alpes du Sud et la région méditerranéenne. Il est présent en plaine, à l'étage collinéen et jusqu'à 1 300 m dans les Pyrénées (Fig.4a). Le chêne sessile, lui, est indigène sur l'ensemble du territoire. Il est présent partout en plaine, disséminé dans le Sud-Ouest mais rare en région méditerranéenne. On le rencontre dans tous les massifs montagneux jusqu'à 1 600 m. Il est toutefois rare dans les Alpes du Nord et quasi absent des Alpes du Sud et de la Corse (Fig.4b). Quand on parle de chêne en France, on pense avant tout aux chênes sessiles et pédonculés, ils sont les plus grands et ce sont ceux exploités pour le bois d'œuvre. Il est facile de les distinguer des autres espèces de chênes, cependant différencier les deux espèces entre elles s'avère plus complexe. Les chênes sessiles et pédonculés n'ont effectivement pas les mêmes exigences en matière de lumière, sol, nutrition et taux d'humidité et n'occupent par conséquent pas les mêmes niches

écologiques. Le chêne pédonculé pousse préférentiellement sur des sols calcaires, et bien alimentés en eau. Les stations humides, voire inondées pendant quelques mois, lui conviennent, il est donc caractérisé comme espèce tolérante à l'inondation, alors qu'on trouve le chêne sessile sur des sols profonds, bien drainés et plutôt acides (Lévy *et al.* 1992).

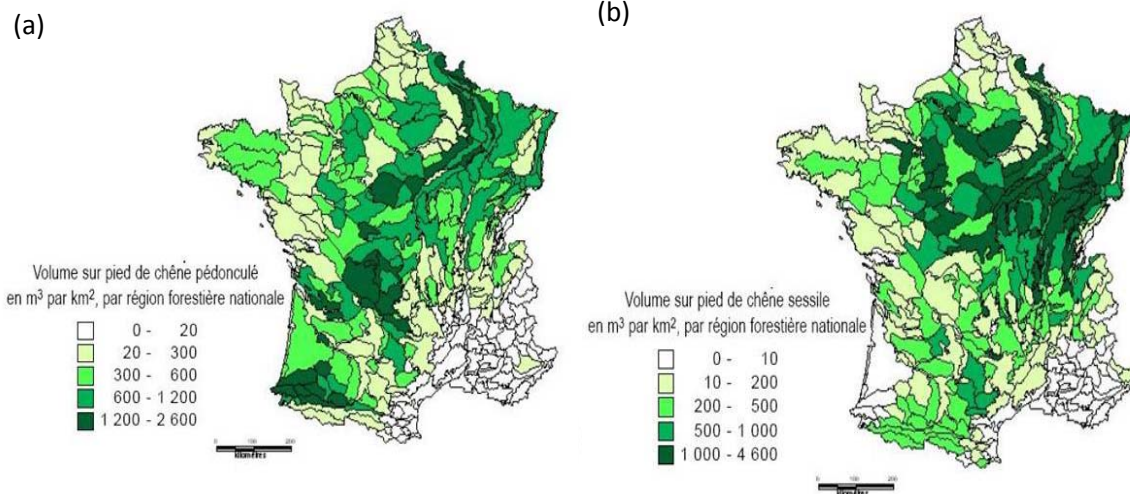


Fig.4 Répartition du chêne pédonculé (a) et du chêne sessile (b) en France. IFN, 2001.

Différenciation des deux espèces

Ce sont les différences phénotypiques qui ont d'abord permis de distinguer les deux espèces. Ainsi le tableau 1, nous montre qu'au niveau du fruit, la longueur du pédoncule, supérieure à 18 mm chez le chêne pédonculé et inférieure à 6mm chez le chêne sessile, est un caractère qui discrimine bien ces deux espèces, et c'est d'ailleurs ce pédoncule long qui a donné son nom au chêne pédonculé. Pour les feuilles, c'est l'inverse, elles possèdent un pétiole assez long et bien visible chez le chêne sessile alors qu'il est très court chez le chêne pédonculé (Fig.5).

Leurs caractéristiques anatomiques et écologiques discriminantes leur octroient le nom d'espèce, même si au niveau génotypique, le chêne sessile et le chêne pédonculé présentent beaucoup de similitudes. En effet, la taille de leur génome est semblable, l'organisation génomique est conservée et il n'existe que très peu de marqueurs moléculaires les différenciant (Kelleher *et al.* 2005).

Caractère	<i>Q. petraea</i>	<i>Q. robur</i>
Fruit		
Longueur du pédoncule	Court ou absent (< 6mm)	Long (entre 18 et 90mm)
Stries du gland	Pas de stries	Strié
Pubescence du pédoncule	Poils regroupés	Aucune
Forme du gland	Ovoïde et trapu	Allongé
Feuilles		
Longueur du limbe	Grand (approx. >10 cm)	Petit (approx. < 10 cm)
Longueur du pétiole	Long (>12 mm)	Court (< 7 mm)
Lobes à la base de la feuille	Absents	Bien développés
Pilosité de la feuille	Importante	Réduite
Forme de la feuille	Ovale - plus large au milieu	Obovale - plus large au milieu
Veines	Aucunes	Présentes
Paires de lobe	>6	<6
Largeur du lobe	Étroit	Large
Pollen		
Taille du pollen	Grand	Petit

Tableau.1. Principaux caractères morphologiques discriminant *Quercus petraea* et *Quercus robur*. Adapté de Bodénès *et al.* 1997b et Kelleher *et al.* 2004.

Le développement, ces dernières années, de marqueurs moléculaires permet d'avoir maintenant d'avoir accès à une partie de l'information génétique jusqu'alors inaccessible. Les différentes approches ont permis d'obtenir des marqueurs spécifiques à chacune des deux espèces, mais pas exclusifs à l'une ou l'autre. Plus récemment, les recherches se sont orientées vers les marqueurs ciblés, c'est-à-dire des gènes qui pourraient différencier les deux chênes étudiés. En l'occurrence, c'est la réponse à l'ennoyage des racines qui a été retenue sachant qu'il s'agit d'un caractère adaptatif important que les deux chênes expriment différemment (Bodénès *et al.* 1997a, Muir *et al.* 2000, Kremer *et al.* 2002).

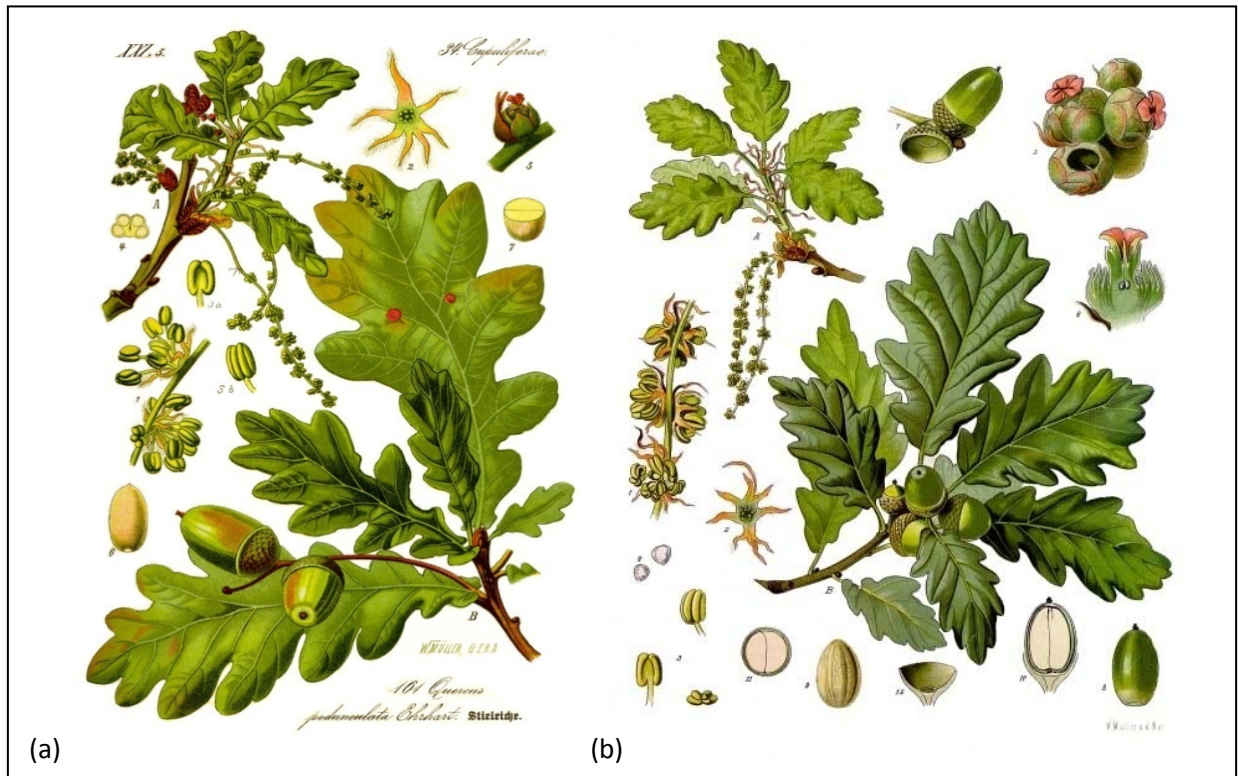


Fig.5 Planche illustrant la morphologie générale des feuilles et fruits du chêne pédonculé (a) et chêne sessile (b). Source : « Flora von Deutschland, & Ouml ; sterreich und der Schweiz », 1885.

Nous avons donc choisi d'étudier le chêne sessile et le chêne pédonculé, pour leur importance écologique, économique et dans le but d'identifier des marqueurs de discrimination entre les deux espèces lors de leurs réponses aux stress d'origine abiotique. Cette étude vise à faire avancer nos connaissances fondamentales sur l'origine des différences de tolérance à l'engorgement entre les deux espèces mais aussi à caractériser les réponses mises en place pendant l'hypoxie.

La partie bibliographique qui suit fait l'état des lieux des éléments déjà connus sur les réponses des plantes à l'excès d'eau et apporte des réponses aux questions suivantes :

*Quelles sont les modifications du milieu rhizosphérique consécutives à l'engorgement ?
Quels changements métaboliques sont observés durant les conditions d'hypoxie racinaire ?
Quelles sont les réponses physiologiques, morphologiques et anatomiques mises en place par la plante pour survivre dans ces conditions de stress ?*

*B. Les réponses
des plantes à l'excès d'eau*

An overview of plant responses to soil waterlogging

Cette partie a fait l'objet d'une publication acceptée le 13 avril 2008 dans la revue *Plant stress*. Les auteurs sont C. Parent, N. Capelli, A. Berger, M. Crèvecoeur, J.F. Dat.

Résumé:

Dans leur milieu naturel, les plantes sont fréquemment soumises à l'ennoyage temporaire ou permanent du sol. Les propriétés physico-chimiques du sol sont fortement modifiées par l'ennoyage, plus particulièrement le potentiel redox, le pH et le taux d'oxygène. Les conditions d'hypoxie voire d'anoxie qui en découlent sont fréquemment imposées au système racinaire de la plante. Le manque d'oxygène se répercute sur la croissance, le développement et la survie de la plante. Une des réponses les mieux connues durant les conditions d'ennoyage est le changement de métabolisme, qui se traduit par le passage de la respiration aérobie à la fermentation anaérobie. La plupart des protéines dont la synthèse est induite durant l'hypoxie sont des enzymes impliquées dans la mise en place de ce métabolisme fermentaire. Afin de pouvoir continuer à produire de l'ATP, d'autres accepteurs d'électrons que l'oxygène sont utilisés. Par ailleurs, la mise en place de la glycolyse suivie de la fermentation éthanolique est un élément indispensable à la survie de la cellule en conditions d'ennoyage. Les réponses de la plante impliquent également une réduction de la conductance stomatique et de la photosynthèse ainsi que de la conductivité hydraulique racinaire. Ces modifications physiologiques se répercutent sur les réserves glucidiques et leur translocation à travers la plante. D'ailleurs, une gestion efficace des réserves glucidiques s'avère très importante car elle pourrait être impliquée dans la différence de tolérance entre les espèces. Parmi les autres adaptations observées, des changements morphologiques apparaissent, notamment le développement de lenticelles hypertrophiées, l'initiation de racines adventives et la formation d'aérenchymes. Les approches génomiques et protéomiques développées récemment ont amélioré nos connaissances sur les mécanismes d'adaptation des plantes en réponse à l'ennoyage racinaire, cependant la diversité de ces réponses et la complexité de leurs relations méritent d'être soulignées. Cette *review* actualise les connaissances sur les réponses au niveau métaboliques, physiologiques et morphologiques et les adaptations des plantes en réponse à l'ennoyage.

An Overview of Plant Responses to Soil Waterlogging

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ABSTRACT

Under natural conditions, plants are frequently exposed to transient or permanent soil waterlogging. Flooding drastically influences the soil physico-chemical properties, most notably soil redox potential, pH and O₂ level. Thus, conditions of hypoxia or anoxia are commonly encountered by plant root systems. These O₂ restrictive conditions dramatically affect plant growth, development and survival. One of the best characterised plant responses to soil waterlogging is the metabolic switch from aerobic respiration to anaerobic fermentation. In fact, most proteins induced during hypoxic conditions are enzymes involved in the establishment of this fermentative pathway. Because the plant cells need to keep a continuous ATP supply, the use of alternative electron acceptors and/or alternative pathways may be key elements of survival under soil waterlogging. The plant response may also include a reduction in stomatal conductance and photosynthesis, as well as root hydraulic conductivity. These physiological modifications may in turn affect carbohydrate reserves and translocation. In fact, efficient use of carbohydrates may discriminate between tolerant and intolerant species. Other observed adaptations include morphological changes which comprise the formation of hypertrophied lenticels, the initiation of adventitious roots and/or the development of aerenchyma. Our knowledge of the basic adaptive mechanisms of plants to soil waterlogging has benefited from large scale genomic and proteomic approaches, however, the diversity of the adaptive responses involved underlines the difficulty when studying this stress. This update reviews our current comprehension of the metabolic, physiological and morphological responses and adaptations of plants to soil waterlogging.

Keywords: anoxia, adaptation, hypoxia, roots, soil waterlogging

Abbreviations: ABA, abscisic acid; ADH, alcohol dehydrogenase; ANPs, anaerobic proteins; Eh, redox potential; Hb, hemoglobin; IAA, auxin; LDH, lactate dehydrogenase; Lp, hydraulic conductivity; NO, nitric oxide; PDC, pyruvate decarboxylase; PIPs, plasma membrane intrinsic proteins

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INTRODUCTION

Soil waterlogging has long been identified as a major abiotic stress and the constraints it imposes on roots have marked effects on plant growth and development. When such events take place in the spring, they can greatly reduce seed germination and seedling establishment. Thus, soil waterlogging is an important factor affecting the growth, development and survival of numerous plant species, not only in natural ecosystems but also in agricultural and horticultural systems (Dat *et al.* 2006).

Rapid changes in soil properties take place following soil waterlogging. As water saturates the soil pores, gases are displaced, a reduction in gas diffusion occurs and phytotoxic compounds accumulate as anaerobic conditions prevail. All these changes greatly affect the capacity of a plant to survive such conditions. In response, the stomatal resistance increases, photosynthesis and root hydraulic conductivity decline, and the translocation of photoassimilates is reduced.

However, one of the best characterised plant adaptations to hypoxia/anoxia includes a switch in biochemical and metabolic processes commonly observed when O₂ availability becomes limiting (Dat *et al.* 2004). The selective synthesis of a set of about 20 anaerobic stress proteins (ANPs) enables oxygen-independent energy generating metabolic processes under conditions unfavourable for aerobic energy production (Subbaiah and Sachs 2003). Other observed adaptations include morphological and anatomical changes which comprise the formation of hypertrophied lenticels, the initiation of adventitious roots and the development of aerenchyma (Vartapetian and Jackson 1997; Jackson and Colmer 2005; Folzer *et al.* 2006).

This review details the different plant stress responses to hypoxia/anoxia, induced by soil waterlogging/flooding and examines some of the key metabolic, physiological and morphological adaptive features.

CHANGES IN THE ROOT ENVIRONMENT DURING SOIL WATERLOGGING

As water saturates the soil, air spaces are filled, leading to the modification of several soil physico-chemical characteristics (Kirk *et al.* 2003; Dat *et al.* 2004). The first event that takes place is in fact the increased presence of H₂O: soil water saturation characterises flooding. Nevertheless, the mechanisms which trigger a plant response are often presumed by-products of root zone flooding (i.e. changes in soil redox and pH; a decline in O₂ level ...).

Soil redox potential (Eh) is often considered the most appropriate indicator of the chemical changes taking place during soil flooding (Pezeshki and DeLaune 1998). Eh generally declines during soil waterlogging (Pezeshki and DeLaune 1998; Pezeshki 2001; Boivin *et al.* 2002; Lu *et al.* 2004). It is not only an indicator of O₂ level (Eh around +350 mV under anaerobic conditions) (Pezeshki and De-Laune 1998) as reducing conditions lead to a high competitive demand for O₂, it also critically affects the availability and concentration of different plant nutrients (Pezeshki 2001). However, changes in Eh are influenced by the presence of organic matter as well as Fe and Mn (Lu *et al.* 2004). Soil reduction induces the release of cations and phosphorous through adsorption of ferrous ion and dissolution of oxides (Boivin *et al.* 2002). Soil reducing conditions also favour the production of ethanol, lactic acid, acetaldehyde and acetic and formic acid.

Another soil chemical characteristic which is strongly

affected by soil waterlogging conditions is soil pH, which is negatively correlated with Eh (Singh 2001; Zarate-Valde *et al.* 2006). The soil pH generally tends to increase towards neutrality upon waterlogging (Lu *et al.* 2004). The increase in pH may be explained by the dissolution of carbonate and bicarbonate early during waterlogging (Lu *et al.* 2004). Soil pH also affects the turnover of soil organic matter and processes such as mineralization, nitrification and urea hydrolysis (Probert and Keating 2000).

Overall, however, one of the main effects of flooding is a lower pool of available O₂ in the submerged plant part, as gases diffuse 10,000 faster in air than in water. The effect of O₂ limitation on cellular metabolism is concentration dependent and the gradual decline in O₂ availability in the root environment has varying effects on plant metabolism: i) normoxia allows aerobic respiration and metabolism to proceed normally and most of the ATP is generated via oxidative phosphorylation, ii) hypoxia occurs when the reduction in available O₂ starts to be a limiting factor for ATP production through oxidative phosphorylation and, iii) anoxia when ATP is only produced through fermentative glycolysis, as no more O₂ is available. Thus, as anaerobic conditions develop in the waterlogged soil, there is an increasing amount of by-products of fermentative metabolism accumulating in the root environment and the levels of CO₂, methane and volatile fatty acids increase (Pezeshki 2001). The decline in available energy has dramatic consequences on cellular processes, leading to water and nutrient imbalances and/or

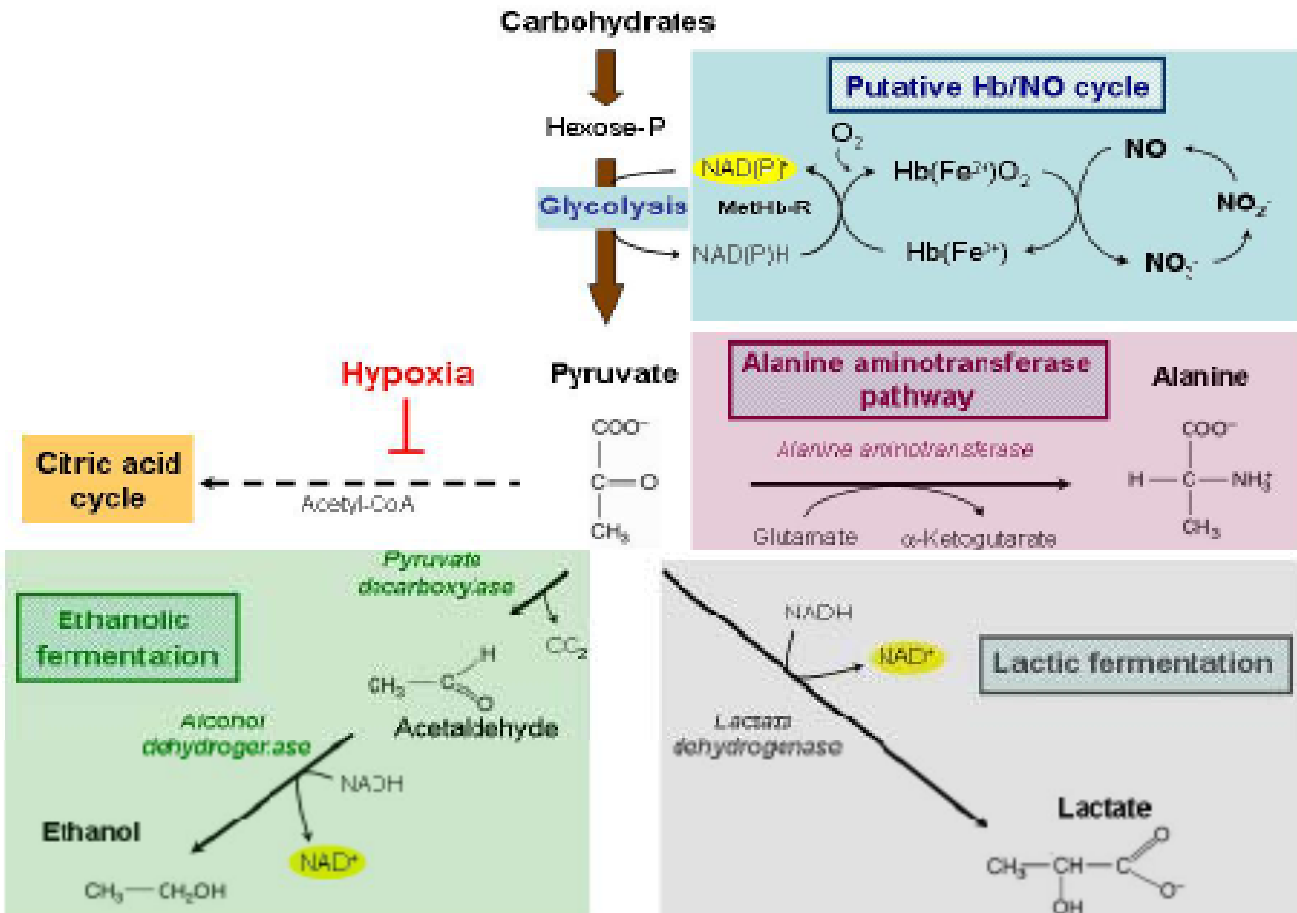


Fig. 1 Schematic diagram of the main metabolic pathways proposed during plant flooding stress. Hypoxia causes a decrease in mitochondrial respiration, which is partly compensated by increases in both the glycolytic flux and fermentation pathways. Nitrate has been proposed as an intermediate electron acceptor under low O₂ tensions and may participate in NAD(P)H oxidation during hypoxia (Igamberdiev *et al.* 2005). NO can be oxygenated to nitrate with the tightly bound O₂ of class-1 hemoglobin [Hb(Fe²⁺)O₂], which is oxidized to metHb [Hb(Fe³⁺)]. The alanine aminotransferase enzyme which converts pyruvate to alanine is strongly induced in hypoxic conditions. However, unlike ethanol formation, there is no consumption of NAD(P)H in the process (Gibbs and Greenway 2003). MetHb-R: methemoglobin reductase; NO: nitric oxide.

deficiency (Dat *et al.* 2006). In addition, these environmental changes may also make the plant more prone to other stresses, more particularly to pathogen infection (Munkvold and Yang 1995; Yanar *et al.* 1997; Balardi *et al.* 2003).

METABOLIC RESPONSES AND ADAPTATIONS TO HYPOXIA AND ANOXIA

The immediate consequence of soil waterlogging is a period of hypoxia, followed by a strong decline in O₂ leading to anoxic conditions (Blom and Voeselek 1996). Indeed, cellular oxygen deficiency is termed “hypoxic” as soon as oxygen levels limit mitochondrial respiration and “anoxic” when respiration is completely inhibited. As respiration declines, the electron flow through the respiratory pathway is reduced, thus diminishing ATP production. Consequently, chemical oxidising power (i.e. nicotinamide adenine dinucleotide, NAD⁺) must be generated via alternative pathways that do not use O₂ as terminal electron acceptor (Roberts *et al.* 1984; Drew *et al.* 1994; Drew 1997; Summers *et al.* 2000). As adenosine diphosphate (ADP) oxidative phosphorylation becomes limiting, plants shift their metabolism from aerobic respiration to anaerobic fermentation (**Fig. 1**) (Peng *et al.* 2001; Fukao and Bailey-Serres 2004). The fermentative pathway serves as a metabolic safe route and includes two steps: carboxylation of pyruvate to acetaldehyde (catalysed by pyruvate decarboxylase, PDC) and the subsequent reduction of acetaldehyde to ethanol with concomitant oxidation of NAD(P)H to NAD(P)⁺, catalysed by alcohol dehydrogenase (ADH) (Vartapetian and Jackson 1997; Kingston-Smith and Theodorou 2000; Nakazono *et al.* 2000). The fermentative metabolic route allows the synthesis of only 2 moles of ATP against 36 per mole of glucose produced during aerobic respiration. To compensate the deficit in energy, glycolysis is accelerated, leading to the depletion of carbohydrate reserves (“Pasteur effect”). Not surprisingly, the enzymes that participate in the fermentation pathway (see above PDC and ADH) belong to a group of approximately 20 ANPs, selectively induced during hypoxic stress, whereas overall protein synthesis is reduced (Sachs *et al.* 1980; Chang *et al.* 2000). ANPs which are induced mainly under hypoxia include enzymes of glycolysis, ethanolic fermentation, processes related to carbohydrate metabolism but also others involved in aerenchyma formation (xyloglucans endotransglycosylase) and cytoplasmic pH control (Vartapetian 2006). Species tolerant to soil waterlogging are generally considered those able to maintain

their energy status via fermentation. In addition to their ability to keep an appropriate energy level, maintenance of cytosolic pH is critical. When hypoxia or anoxia occur the pH of the cytoplasm shows an early decrease that is attributed to an initial production of lactic acid by fermentation. According to the “Davies-Roberts pH-stat theory”, the decline in pH permits the switch from lactate to ethanol fermentation by inhibition of lactate dehydrogenase (LDH) and activation of ADH (Chang *et al.* 2000). Because acidosis can induce cell necrosis, the switch taking place maintains pH at approximately 6.8, thus allowing cell survival. Although this hypothesis has been verified in some cases, there are numerous reports which question this model (Tadege *et al.* 1998; Kato-Noguchi 2000b). Indeed, it is obvious today that the correlation between lactate and cytoplasmic acidification is not ubiquitous in all tissues and plants studied (Felle 2005). Because O₂ is lacking under hypoxic conditions, it has to be substituted by alternative electron acceptors. In fact, nitrate has long been considered as a terminal electron acceptor for plant mitochondria under hypoxic or anoxic conditions (Vartapetian and Polyakova 1998; Vartapetian *et al.* 2003). More recently nitrate reduction has been investigated as an alternative respiratory pathway, and it could be crucial for the maintenance of redox and energy homeostasis of the cell under limiting oxygen conditions (Igamberdiev and Hill 2004). This sequence of reactions, referred to as the Hb/NO cycle, in which NO (nitric oxide) is oxidized to nitrate, involves a class I non-symbiotic hemoglobin which is induced under hypoxia (**Fig. 1**) (Dordas *et al.* 2003; 2004; Perazzolli *et al.* 2004; Parent *et al.* 2008a). The postulated Hb/NO cycle was very recently demonstrated in hypoxic roots and in addition to being important during the plant flooding response it could also play a role early during seed germination (Hebelstrup *et al.* 2007).

PHYSIOLOGICAL RESPONSES TO SOIL WATERLOGGING

One of the earliest plant physiological responses to soil flooding is a reduction in stomatal conductance (**Fig. 2**) (Sena Gomes and Kozlowski 1980; Pezeshki and Chambers 1985; Folzer *et al.* 2006). Soil waterlogging may not only increase stomatal resistance but also limit water uptake, thus in term leading to internal water deficit (Jackson and Hall 1987; Ismail and Noor 1996; Pezeshki *et al.* 1996; Pezeshki 2001; Nicolas *et al.* 2005; Folzer *et al.* 2006; Parent *et al.* 2008a).

Low O₂ levels may also reduce hydraulic

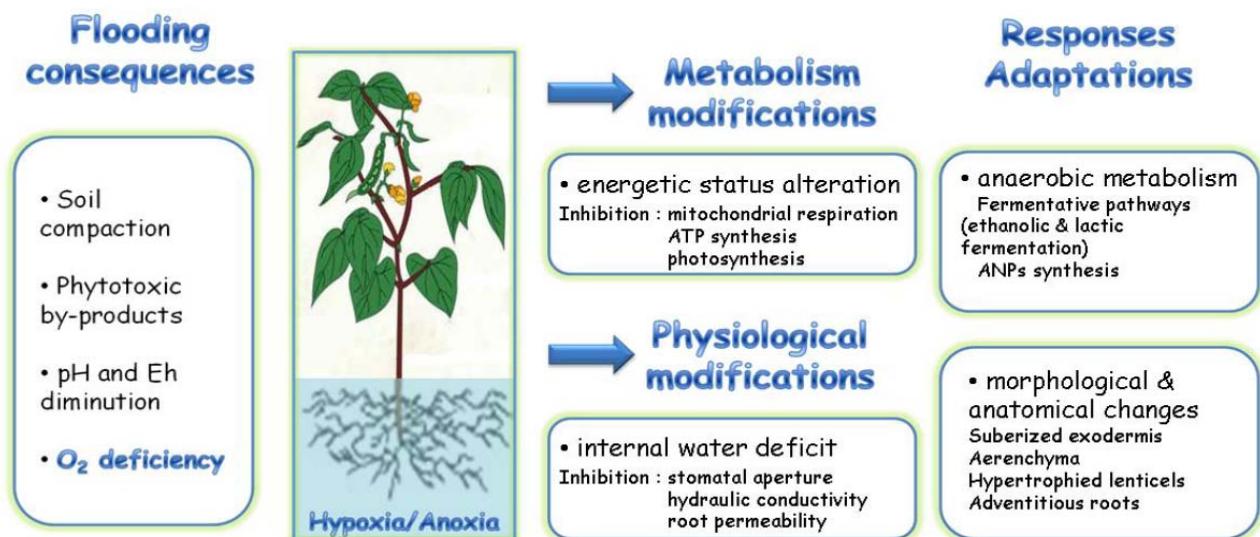


Fig. 2 Main physico-chemical events taking place in the rhizosphere during soil waterlogging and the resulting modifications in plant metabolism and physiology followed by the initiation of adaptive responses.

conductivity (**Fig. 2**; *Lp*) consequent to a decrease in root permeability (Clarkson *et al.* 2000; Else *et al.* 2001). The decrease in *Lp* may be linked to aquaporin gating by cytosolic pH (Tournaire-Roux *et al.* 2003). Evidence suggests that the regulation of plasma membrane intrinsic proteins (PIPs) by pH is especially relevant under anoxic conditions (Postaire *et al.* 2007), as a conserved histidine residue at position 197 in the intracellular Loop D has been identified to be the major pH-sensing site under physiological conditions (Tournaire-Roux *et al.* 2003; Kaldenhoff and Fischer 2006; Secchi *et al.* 2007). In fact, downregulation of aquaporin genes is commonly associated with a decline in root *Lp* as aquaporins control radial water movement in the roots (North *et al.* 2004; Vandeleur *et al.* 2005). Thus, it seems that the reduced *Lp* throughout the plant under soil waterlogging conditions is most probably linked to inhibition of water transport by aquaporins, though in depth studies on the effect of aquaporin on whole plant water regulation during soil waterlogging are still lacking. Furthermore, the reduction in radial water movement may in part be explained by the presence of cross-sectional oxygen gradients in the root tissue. Indeed, there is clear evidence that in flooded soils, an O₂ gradient exists between the stele, which may be under anoxic conditions, and the cortical cells which may only be under hypoxic conditions (Thomson and Greenway 1991; Colmer 2003). Thus, these differences in tissue microenvironment may also contribute to cross-sectional differences in cellular energy levels and subsequent declines in root *Lp*.

O₂ deficiency generally induces a rapid reduction in the rate of photosynthesis in flood-intolerant plants which is generally considered a result of reduced stomatal aperture (Huang *et al.* 1997; Gravatt and Kirby 1998; Pezeshki and DeLaune 1998; Malik *et al.* 2001). Other factors such as a decrease in leaf chlorophyll content, early leaf senescence and a reduction in leaf area may also contribute to inhibition of photosynthesis at a later stage (Sena Gomes and Kozlowski 1980; Cao and Conner 1999).

When the stress is prolonged it may lead to the inhibition of photosynthetic activity of the mesophyll (Huang *et al.* 1994; Liao and Lin 1994; Pezeshki *et al.* 1996), as well as reductions in the metabolic activity and the translocation of photoassimilates (Pezeshki 1994; Drew 1997; Pezeshki 2001; Sachs and Vartapetian 2007). The outcome of a decline in photosynthesis on plant growth and development may be dramatic and it may lead to concurrent physiological dysfunctions such as the inhibition of water transport and changes in hormone balance

(Vuylsteker *et al.* 1998; Kato-Noguchi 2000a; Else *et al.* 2001; Gunawardena *et al.* 2001). In order to maintain its metabolic activity, the plant has to draw on its carbohydrate reserves. As initial carbohydrate supply is correlated with the level of tolerance to hypoxia/anoxia in many species, presumably through its involvement in providing energy during anaerobic conditions, the level of carbohydrate reserves may be a crucial factor in the tolerance to long term flooding (Setter *et al.* 1997; Ram *et al.* 2002). For instance, an increased capacity to utilize sugars through the glycolytic pathway enables rice seedlings to survive longer periods of flooding (Ito *et al.* 1999).

Although a plant may have high sugar reserves, these must however be available and converted readily through an efficient glycolytic pathway. In fact, the availability of photoassimilates to the cells under anaerobiosis has been proposed as one of the limiting steps for survival under flooding conditions (Pezeshki 2001). Indeed, waterlogged soils tend to reduce the translocation of photosynthetic products from “source” leaves to “sink” roots (Barta and Sulc 2002; Yordanova *et al.* 2004). As a result, the maintenance of photosynthetic activity and accumulation of soluble sugars to roots is clearly an important adaptation to flooding (Chen *et al.* 2005).

MORPHOLOGICAL AND ANATOMICAL ADAPTATIONS TO SOIL WATERLOGGING

The presence of hypertrophied lenticels is a common anatomical change observed in many woody species during flooding (**Fig. 3**) (Yamamoto *et al.* 1995; Kozlowski 1997). Hypertrophic growth appears as swelling of tissues at the stem base and is believed to result from radial cell division and expansion. It has long been associated with auxin (IAA) and ethylene production (Blake and Reid 1981; Kozlowski 1997). The development of hypertrophied lenticels is believed to facilitate the downward diffusion of O₂ as well as the potential venting of compounds produced in the roots as by-products of anaerobic metabolism (ethanol, CH₄, CO₂). Although there is still no clear consensus on their actual physiological role, their number has been associated with increase tolerance to flooding in *Quercus* species (Colin-Belgrand *et al.* 1991; Parelle *et al.* 2006b). In addition, hypertrophied lenticels tend to be more developed under the water surface (Tang and Kozlowski 1982; Parelle *et al.* 2006a) which does not support a role as

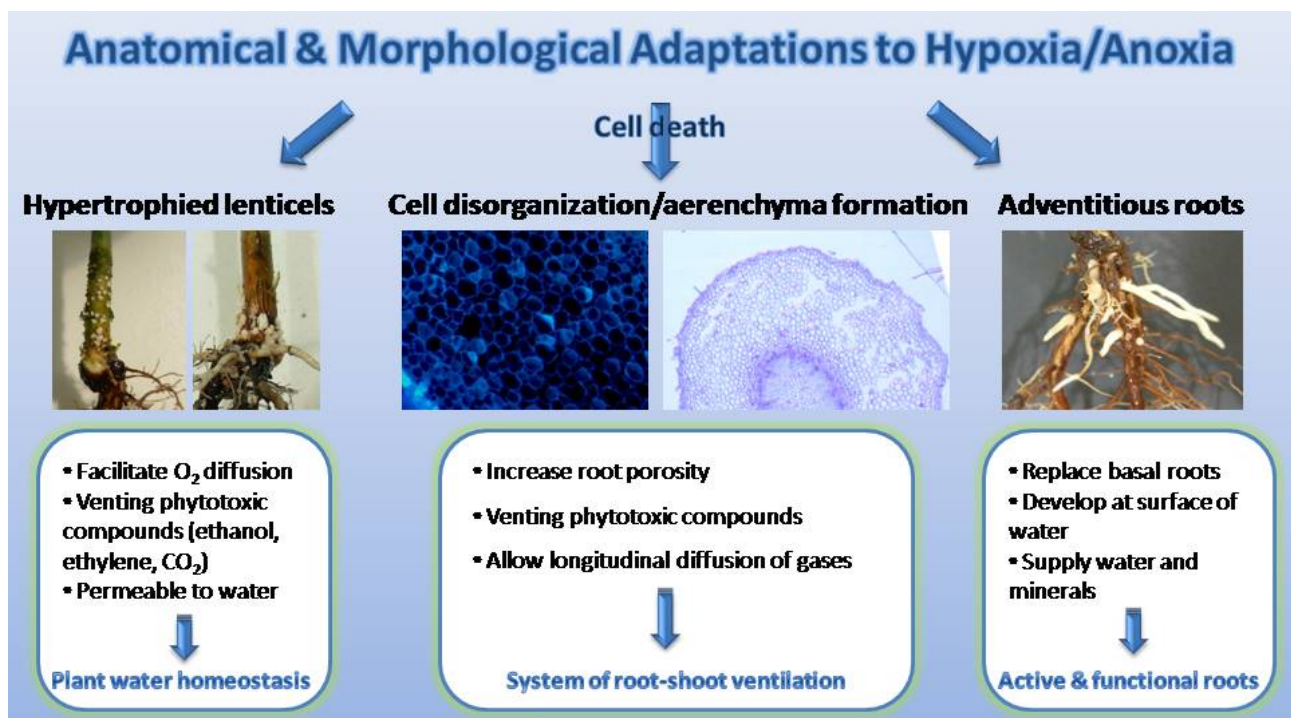


Fig. 3 Anatomical and morphological adaptations taking place during plant flooding.

important facilitators of O₂ entry and delivery toward the root system, as commonly assumed. It is thus more probable that lenticels may in fact help maintain plant water homeostasis during flooding, by partially replacing the decaying root system and providing a means of water intake for the shoot. In support for such a role, lenticels are permeable to water (Groh *et al.* 2002), the tendency for stomatal conductance to return towards control levels after a transient decrease has generally been associated with their development (Pezeshki 1996; Gravatt and Kirby 1998; Folzer *et al.* 2006), and their presence is associated with maintenance of plant water status during flooding stress in *Quercus* species (Parent *et al.* 2008a). Thus, although their function is still not clearly established, it seems that lenticels may play a crucial role during adaptation to flooding conditions in some species by helping maintain shoot water homeostasis.

Another important morphological adaptation to flooding is the development of adventitious roots (**Fig. 3**), which functionally replace basal roots (Bacanamwo and Purcell 1999; Gibberd *et al.* 2001; Malik *et al.* 2001). The formation of these specialised roots takes place when the original root system becomes incapable of supplying the shoot with the required water and minerals (Mergemann and Sauter 2000). Furthermore, decay of the main root system may be considered as a sacrifice to allow a more efficient use of energy for the development of a more adapted root system (Dat *et al.* 2006).

Adventitious roots are commonly formed near the base of the stem or in the region where lenticels are abundant, and their growth is lateral, parallel to the water/soil surface. Their presence at the interface between the water saturated soil and atmosphere reflects their importance in replacing the normal root system both underwater and following retreat of the water table. Furthermore, the ability to produce adventitious roots is commonly associated with enhanced tolerance to flooding and their development has commonly been associated with ethylene production (Voesenek *et al.* 1993; Mergemann and Sauter 2000; Steffens *et al.* 2006). More recently, other molecules have been identified as key players in their initiation (Pagnussat *et al.* 2002; 2003; 2004). Indeed, recent data indicate that NO production works downstream of IAA in the control of adventitious root formation. However, the understanding of the role of NO in the regulation of adventitious roots is in its infancy and important findings on the crucial role of NO in flooding stress tolerance may lie ahead.

Finally, one of the most important responses to waterlogging is the development of lacunae gas spaces (aerenchyma) in the root cortex (**Fig. 3**). The development of aerenchyma may be a response to flooding in both flood tolerant and flood intolerant species (Vartapetian and Jackson 1997; Schussler and Longstreth 2000; Chen *et al.* 2002; Evans 2004). On the other hand, aerenchyma formation is an adaptive response in flood tolerant species only, specifically in bottomland woody species (Kludze *et al.* 1994; Pezeshki 1996). The increase in porosity may enhance venting toward the shoot and the atmosphere of phytotoxic compounds, produced in the roots (i.e., ethanol, methane) (Visser *et al.* 1997; Visser and Pierik 2007) and/or enhance the longitudinal diffusion of gases in the roots, thus increasing their aeration (Laan *et al.* 1991; Evans 2004). In fact, the proportion of aerenchyma is generally considered as a key discriminating factor between wetland and non-wetland plants (Vasellati *et al.* 2001).

The development of aerenchyma or lacunae tissues is not unique to roots. They are also observed in the leaf sheath following submergence, forming an interconnecting system of shoot-root ventilation (Jackson and Armstrong 1999; Fabbri *et al.* 2005). Aerenchyma increases tissue porosity which itself can be initiated as a result of osmotic dependant

changes in cell shape (**Fig. 3**) (Justin and Armstrong 1987; Folzer *et al.* 2006). The changes in cell shape and assemblage in the root cortex are most likely linked to enhanced cell wall loosening enzyme activity and with suberin deposition in the exodermis (Colmer 2003; De Simone *et al.* 2003; Armstrong and Armstrong 2005; Enstone and Peterson 2005).

The development of a suberized exodermis correlates with the development of aerenchyma in maize (Enstone and Peterson 2005) and is associated with a decline in radial loss of root O₂ (Visser *et al.* 2000; Armstrong and Armstrong 2005). Such a barrier on the periphery of the cortex may not only reduce the loss of O₂ to the rhizosphere but could also protect the plant from phytotoxins produced by microorganisms in the environment surrounding the roots (Soukup *et al.* 2002; Armstrong and Armstrong 2005; Soukup *et al.* 2007).

The development of aerenchyma has been investigated for many years and it is now clear that at least two types of developmental processes are involved. The first is the constitutive development of aerenchyma as it occurs whether or not the plant is under waterlogged conditions. It forms by cells separating during tissue development. The cell death type taking place through cell separating is termed schizogeny (formed by cell separation) and is developmentally regulated and independent of any external stimuli. It is the outcome of highly regulated tissue specific patterns of cell separation. The other type of cell death process is termed lysogeny (formed by partial breakdown of the cortex), resembles programmed cell death, typically observed during the hypersensitive response of plant pathogen interactions (Mittler *et al.* 1997; Parent *et al.* 2008b) and more recently identified during other abiotic stresses (Pellinen *et al.* 1999; Dat *et al.* 2001; Dat *et al.* 2003; Van Breusegem and Dat 2006). The active cell death process which takes place during aerenchyma development is genetically controlled and shows many similarities with apoptosis, though there is increasing evidence that it generally lacks several features of apoptotic cell death (Buckner *et al.* 2000). In *Sagittaria lancifolia* for example, nuclear changes (clumping of chromatin, fragmentation, disruption of the nuclear membrane), are the earliest events observed following flooding. These nuclear changes are followed by plasma membrane becoming crenulated, tonoplast disintegration, organelle swelling and disruption, loss of cytoplasmic contents and collapse of the cell (Schussler and Longstreth 2000). This sequence of events seems common to most species studied, though the timing of tonoplast disruption varies (Schussler and Longstreth 2000).

CONCLUSION

This short update reviews our current understanding of plant biochemical, physiological and morphological responses to soil waterlogging. The changes taking place in the root zone and their perception by the plant are clearly essential for the establishment of an appropriate response. The alteration in gas diffusion, the soil chemical environment (pH, Eh) and, the accumulation of toxic by-products of anaerobic processes coupled to the decline in O₂ are clearly keys to the capacity of a plant to set up the right response. These adaptive features include changes in metabolism which may help preserve the plant cell integrity. Although less efficient than aerobic processes, the fermentative pathway can help maintain the cell pH but also ATP homeostasis. In addition to the glycolytic pathway to lactate and to ethanol, nitrate reduction could be used as an alternative respiratory pathway to help maintain redox and energy homeostasis under hypoxic and anoxic conditions. Other features such as higher carbohydrate reserves and/or their efficient use, maintenance of photosynthesis and plant water status through shoot elongation or aquaporin gating may greatly improve plant

survival to submergence. Finally, morphological changes such as lenticels formation, aerenchyma development, adventitious roots initiation and/or root suberization can not only ameliorate the rate of O₂ diffusion to the submerged growing parts but also help alleviate water and nutrient deficiencies. Most of these adaptive features have been well characterised in model species adapted to flooding conditions such as maize, rice and carex, however the exact role of lenticels as well as the molecular processes involved in aerenchyma formation still need further scrutiny. In addition, our understanding of the adaptive response of woody species making up forest ecosystems is still in its infancy.

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Les plantes sont capables de s'acclimater à des conditions environnementales défavorables et de mettre en place des adaptations métaboliques et morphologiques leur permettant de survivre aux effets néfastes du stress. Afin de mettre en place une réponse appropriée, le stress doit être détecté par la plante et permettre l'initiation d'une cascade de signalisation spécifique.

La partie bibliographique suivante se penche sur la perception et les voies de signalisation du stress par envoie. Cette revue de la littérature discute notamment des modifications du milieu qui pourraient être détectées par la plante et propose différentes voies de signalisation possibles qui permettraient la mise en place d'une réponse adaptée.

*C. Stress hypoxique, signalisation et
hémoglobine non-symbiotique*

An overview of plant sensing and signaling during hypoxia

Cette partie fait l'objet d'une publication en préparation.

Résumé:

Conséquence du réchauffement global de la planète, l'intensité et la fréquence des précipitations vont augmenter et favoriser les inondations. L'impact de ces événements climatiques sur la croissance et le rendement des cultures agricoles risque d'être important. Ces modifications environnementales vont également exercer une forte pression de sélection sur les écosystèmes naturels. La biodiversité pourrait être menacée et la diversité génétique intra et interspécifique des espèces sera un caractère essentiel pour l'adaptation et la survie aux changements climatiques majeurs prédits. Une exposition plus fréquente à l'ennoyage va entraîner des conséquences sur la croissance, le développement et la survie des végétaux. Depuis une dizaine d'années, les connaissances sur la réponse des plantes à l'hypoxie se sont élargies grâce aux nouvelles techniques génomiques et protéomiques associées à des approches physiologiques et morphologiques. Ces recherches ont permis de caractériser les modifications cellulaires et moléculaires clés impliquées dans la réponse à l'hypoxie. La perception et la signalisation du stress ainsi que la rapidité de la mise en place de ces deux mécanismes sont essentielles pour initier une réponse efficace permettant la survie de la plante. Parmi les nouveaux candidats identifiés, l'hémoglobine non-symbiotique, le monoxyde d'azote et les espèces réactives de l'oxygène semblent occuper une place prépondérante dans la réponse à l'hypoxie. Cependant le calcium, l'éthylène et d'autres molécules plus « classiques » de la réponse au stress sont aussi fortement impliquées dans l'établissement d'une réponse adaptée. Cette *review* fait le point sur les connaissances actuelles des voies de perception et de signalisation impliquées dans la réponse adaptative au stress hypoxique.

An overview of plant sensing and signaling during hypoxia

ABSTRACT

The predicted changes in the regional distribution and frequency of precipitations as a result of climate fluctuations will increase the occurrence of flash floods in the near future. The effect of such events on crop growth and yield will be substantial. Furthermore, these environmental changes will also exert an increased selective pressure on natural ecosystems. Biodiversity will be at risk and species intra and interspecific genetic diversity will be essential in helping species adapt and survive to these global changes. Overall, vegetation will become more frequently exposed to soil waterlogging thus altering growth, development and survival. Over the last decade, our understanding of the response of plants to hypoxia has greatly benefited from the use of large scale genomic and proteomic approaches coupled to physiological and morphological studies. These studies have helped characterize key cellular and molecular changes taking place during hypoxia. The importance of sensing and signaling mechanisms has also been put forward, as the fast and efficient establishment of an appropriate response seems essential for plant survival. Among the new identified players, non-symbiotic hemoglobins, nitric oxide and reactive oxygen species seem to have gained considerable importance in the flooding response, although calcium, ethylene and other more “classical” molecules are still evidently crucial for an adequate response. This update reviews our current understanding of the sensing and signaling networks involved in the adaptive response to hypoxia.

Key words: Adaptation, flooding, hypoxia, nitric oxide; non-symbiotic hemoglobin; plant response, plant signaling

List of abbreviations: ABA, Abscissic acid; ACC, 1-aminocyclopropane 1-carboxylate; ADH, Alcohol dehydrogenase; ANP, Anaerobic protein; CK, Cytokinin; Eh, Redox potential; GA, Gibberellin; GAPC, Glyceraldehyde 3-phosphate dehydrogenase; Hb, Hemoglobin; HIF, Hypoxia-Inducible heterodimeric transcription Factor; IAA, Auxin; LDH, Lactate dehydrogenase; Lp, Hydraulic conductance; PDC, Pyruvate decarboxylase; ROS, Reactive oxygen species; SA, Salicylic acid; XET, Xyloglucan endo-transglycosylase;

INTRODUCTION

There is increasing evidence that plants grown both in natural and agricultural ecosystems will become increasingly exposed to periods of drought and soil waterlogging. Indeed, climate change models predict substantial changes in regional precipitation distribution patterns. Thus the occurrence of periods of drought followed by periods of intensive rain will undoubtedly mark the climate of the coming decades. The impact of drought on plant growth and development has long been studied, however, less is known of the impact of soil waterlogging. In fact, little is known of the intra and interspecific variability of functional traits essential for the establishment of an appropriate adaptive response to hypoxia. Thus, soil waterlogging will become a major abiotic constraint and the strain it imposes on roots will have marked effects on germination and seedling establishment. Furthermore, hypoxia affects the growth, development and survival of numerous plant species, not only in natural ecosystems but also in agricultural and horticultural systems (Dat *et al*, 2006).

One of the best characterized plant adaptations to hypoxia/anoxia includes a switch in biochemical and metabolic processes commonly observed when O₂ availability becomes limiting. The selective synthesis of a set of about 20 anaerobic proteins (ANPs) will enable an oxygen-independent energy-generating metabolic process under conditions unfavourable for aerobic energy production (Subbaiah and Sachs 2003). Other observed adaptations include morphological and anatomical changes which comprise the formation of hypertrophied lenticels, the initiation of adventitious roots and the development of aerenchyma (Vartapetian and Jackson 1997, Jackson and Colmer 2005, Folzer *et al*, 2006).

There is increasing evidence that one of the crucial steps in the response to any stress condition is the kinetic of induction of the sensing and signaling cascades involved. Similarly, during the hypoxia/anoxia, the sensing and signaling mechanisms involved during the early stages of the response are vital. Although, such mechanisms have been extensively studied in animals, they have been more rarely described in plants. This review details the potential sensing mechanisms described during the hypoxia response and examines the signaling network involved in the establishment of adaptive features.

SENSING CHANGES IN THE ROOT ENVIRONMENT?

A slight change in environmental conditions, may affect many physico-chemical parameters. Any of these may in fact be sensed by the plant and it is clear today that whichever the stress or the species considered, the response is dependent on the kinetic of induction with which plant “receptors” will adequately identify the stressful parameter(s) and transmit this information for an appropriate genome switch to take place.

One of the first physical parameters affected during the early stage of soil flooding will be a change in moisture content, as water saturates the soil. This change may be directly sensed by the roots as evidenced by hydrotropic experiments. In a recent series of elegant experiments, results obtained with an *Arabidopsis* mutant (*miz1*) impaired in hydrotropism confirmed that hydrotropism was extremely important to explain root architecture and development (Kobayashi *et al*, 2007). In addition, it was established that the early perception phase of hydrotropism takes place in the root cap (Jaffe *et al*, 1985, Kiss 2007, Kobayashi *et al*, 2007). However, in addition to direct hydro-sensing, soil water saturation will affect gravity. These changes may in turn be sensed through gravi-sensing mechanisms in the root. Gravi-sensing is essential for proper plant growth and development and it ensures that roots will grow downwards into the soil to take up water and nutrients. However, the high water content in the soil during soil flooding will alter the pressure component, thus potentially triggering a gravity response from cells in the roots. In fact, in nature, plant roots generally grow upwards when they come into contact with the water table thus supporting the idea that gravity sensing may be involved in redirecting root growth. These changes in root growth orientation are also sensed in the root cap and are believed to be regulated by lateral auxin gradients (Morita and Tasaka 2004, Perrin *et al*, 2005). Numerous studies have investigated the gravity response in various systems, however to date, the molecular identity of the mechano-sensing mechanism remains unknown (Morita and Tasaka 2004, Perrin *et al*, 2005).

In addition to changes in soil moisture and gravity, as water saturates soil interstices, various gases will be displaced. The displacement of this gaseous environment will lead to the modification of soil physico-chemical characteristics, including an increase in soil compaction.

One of the most important events taking place during soil waterlogging is the depletion of oxygen in the rhizosphere. This will lead to hypoxic/anoxic conditions and a decrease in root metabolism as a result of a reduction in ATP production. The decline in available energy will

have dramatic consequences on cellular processes, thus leading to water and nutrient imbalances and/or deficiency. These environmental changes may also make the plant more prone to other stresses, more particularly to pathogen infection (Munkvold and Yang 1995, Yanar *et al.*, 1997, Balerdi *et al.*, 2003).

In addition, the water saturation of the soil not only reduces the O₂ concentration in the soil but also increases the level of other volatiles such as CO₂, methane, volatile fatty acids and gaseous hormones (i.e. ethylene) which are produced during fermentation.

Under most soil waterlogging conditions, soil pH will approach neutrality. However the pH changes will very much depend on the presence of organic matter and reducible-Fe contents in the soil. pH itself may not directly affect plant growth, although it will lead to the release of various compounds; phytotoxic and/or ions such as manganese and aluminium, and a reduction in organic matter turnover and mineralization.

Soil redox potential (Eh) will generally decline during hypoxia as a result of alterations in soil pH (Pezeshki and DeLaune 1998, Pezeshki 2001). This reduction in Eh may also be compounded by the release in the rhizosphere of by-products of anaerobic metabolism (i.e. CO₂, N₂, H⁺, methane). The presence of these compounds will alter not only the chemiostatic equilibrium but also existing symbiotic relationships between soil microorganisms and the plant.

SENSING AND SIGNALLING NETWORKS DURING HYPOXIA/ANOXIA

In contrast to the wealth of data available on the molecular and cellular mechanisms of hypoxia sensing and signalling in animals, such mechanisms are still poorly understood in plants. In mammals, the Hypoxia-Inducible heterodimeric transcription Factor (HIF) is a key regulatory element in the response to hypoxia (Giaccia *et al.*, 2004, Semenza 2004). To date, no such sensor has been identified in plants (Bailey-Serres and Chang 2005, Agarwal and Grover 2006) even though, recent broad range approaches (i.e. DNA chip technology or proteome analysis) have helped identify novel genes and proteins involved in plant responses to soil waterlogging and anaerobiosis (Chang *et al.*, 2000, Klok *et al.*, 2002, Branco-price *et al.*, 2005, Liu *et al.*, 2005, Loreti *et al.*, 2005, Agarwal and Grover 2006). As a result, novel components of the signal transduction pathway leading to hypoxia-induced gene expression have been documented (Lasanthi-Kudahettige *et al.*, 2007, Miyashita *et al.*, 2007).

CALCIUM AND CALCIUM BINDING PROTEINS

Plants respond to changes in environmental conditions by rapid intracellular signalling. In fact, there is a wealth of data which shows that under most if not all stress conditions, levels of cytosolic calcium are modified (Trewavas and Knight 1994). These changes in cytosolic calcium (Ca^{2+}) are able to bring about responses via a change in the activity of Ca^{2+} -sensor proteins (Lee J. and Rudd 2002). Plants use Ca^{2+} as a cytosolic secondary messenger molecule involved in numerous cell signalling cascades during both abiotic and biotic stress responses (Evans N.H. *et al*, 2001, Snedden and Fromm 2001, Lee J. and Rudd 2002). Several studies on the early events taking place during plant submergence point out to a signalling role for Ca^{2+} (Subbaiah *et al*, 1998, 2000, Luan *et al*, 2002, Liu *et al*, 2005, Rhoads and Subbaiah 2007). For instance, a transient rise in cytosolic Ca^{2+} levels is observed within a few minutes following flooding of maize roots (Subbaiah *et al*, 1994a, 1994b) and *Arabidopsis* (Sedbrook *et al*, 1996). In fact, Ca^{2+} has been shown as an essential component of submergence signalling in *Arabidopsis*, rice, and barley (Sedbrook *et al*, 1996, Chung and Ferl 1999, Tsuji *et al*, 2000, Nie *et al*, 2006). A rise in calcium occurs well before any change in gene expression during anoxia but it is required for alcohol dehydrogenase (*adh1*) and sucrose synthase (*sh1*) induction in both maize and *Arabidopsis* (Subbaiah *et al*, 1994a, Chung and Ferl 1999) and regulates *Adh* and *Hb* transcript levels in barley (Liu *et al*, 2005). Calcium fluxes have also been identified as critical in the signal transduction pathway leading to ethylene biosynthesis (He *et al*, 1996) and subsequent aerenchyma formation in maize roots (He *et al*, 1996). Overall, results suggest that blocking the release of calcium from an internal source disrupts the signalling pathway and there is convincing evidence today that the release of mitochondrial Ca^{2+} is essential for the anoxia response (Subbaiah *et al*, 1998, Klok *et al*, 2002, Rhoads and Subbaiah 2007).

The Ca^{2+} signal modulates cellular processes via high-affinity Ca^{2+} -binding proteins (Reddy 2001). These include calmodulin (CaM) and its isoforms, CaM-related proteins, Ca^{2+} -dependent and CaM-independent protein kinases and Ca^{2+} -binding proteins without an EF hand motif (Zielinski 1998, Harmon *et al*, 2000, Reddy 2001). CaM is, however, the most highly expressed and broadly distributed Ca^{2+} -binding sensor protein present in all kingdoms and the active Ca^{2+} -CaM complex can regulate the activity of many target molecules directly associated with plant stress responses (Snedden and Fromm 2001). Recently, the expression profiles of two CaM families in *Quercus petraea* (*QpCaM1* and *QpCaM2*) were shown to be strongly affected during both early and late events of the hypoxia response (Folzer *et al*, 2005,

2006). There is also evidence that flooding stress initiates a signal transduction pathway in which cytosolic Ca^{2+} stimulates Ca^{2+} /calmodulin-dependent glutamate decarboxylase (GAD) and γ -aminobutyric acid (GABA) synthesis (Baum *et al*, 1993, Aurisano *et al*, 1995, Snedden *et al*, 1996). GABA is synthesised by irreversible α -decarboxylation of L-glutamic acid in a reaction catalysed by GAD (Bouche *et al*, 2004). In fact, GABA increases several-fold during anoxia and is believed to regulate acidosis of the cytoplasm because GAD activity consumes H^+ (Shelp *et al*, 1999, Bouche and Fromm 2004). Interestingly, ethylene production increases when GABA is applied exogenously to sunflower (Kathiresan *et al*, 1997).

Calmodulins are also involved in regulating Ca^{2+} homeostasis by activating plasma and endomembrane Ca^{2+} -ATPase, one of which had its transcript abundance enhanced during anoxia (Subbaiah and Sachs 2000, Snedden and Fromm 2001). Therefore, there is strong evidence for a role for Ca^{2+} and calcium binding proteins in the early and late signalling cascades leading to the hypoxia response.

REACTIVE OXYGEN SPECIES

In addition to Ca^{2+} , reactive oxygen species were also identified as important secondary messengers during the early response to hypoxia (Baxter-Burrell *et al*, 2002). The exact role played by these molecules during hypoxia is still unresolved but it seems that the release of mitochondrial Ca^{2+} , essential for gene regulation during hypoxia (see above), may depend on alterations in cell redox homeostasis. Indeed, inhibition of the mitochondrial electron transport chain can lead to increases in glycolysis and hypoxic genes (Nie and Hill 1997, Rhoads and Subbaiah 2007). Furthermore, there are several reports of a decline in the alternative oxidase (aox) gene expression and enzyme activity during hypoxia (Szal *et al*, 2003, Rhoads and Subbaiah 2007). In addition, a decline in the antioxidant capacity of the cell may arise through a decline in the activity of antioxidant enzymes (i.e. catalase) (Lasanthi-Kudahettige *et al*, 2007) and/or biosynthesis of low molecular weight antioxidants (i.e. glutathione) (Blokhina *et al*, 2003).

An alternative source of ROS during hypoxia may be in the activity of superoxide generating NADPH-oxidase at the plasma membrane, of which several members of the subunit were induced by anoxia treatment (Klok *et al*, 2002, Branco-price *et al*, 2005). This production source is further supported by the production of H_2O_2 co-ordinately with a substantial increase

in ADH activity when *Arabidopsis* is exposed to low oxygen (Baxter-Burrell *et al*, 2002, Fukao and Bailey-Serres 2004). Under such conditions, the production of H₂O₂ is fine-tuned by two proteins, Rop and Rop-GAP4, in which RopGAP4 negatively regulates Rop and Rop has a positive regulatory impact on Rop-GAP4. It is essential that this Rop rheostat controls the levels of H₂O₂ in plants cells precisely, since levels that are too high may trigger formation of ROS that may induce cell death, whereas levels that are too low prevent the expression of adaptive genes (e.g., ADH) that improve survival during low-oxygen stress (Baxter-Burrell *et al*, 2002). In fact, it was speculated that Rop may be responsible for an increase in cytosolic calcium which in turn may activate the NADPH-oxidase thus leading to H₂O₂ accumulation (Bailey-Serres and Chang 2005).

HEMOGLOBINS AND NITRIC OXIDE

Additional candidates involved in sensing and signalling during flooding have recently been identified with the discovery of stress-induced genes that affect plant metabolism and growth under low oxygen tensions (Dordas *et al*, 2003a). Among them, hemoglobins (Hbs) are ubiquitous molecules that have been found in various species from most of the taxonomic kingdoms, including bacteria, yeasts, protists, plants and animals (Wittenberg and Wittenberg 1990, Hardison 1996, Suzuki and Imai 1998). All Hbs contain a heme group carrying an iron ion, which is responsible for the reversible binding to gaseous ligands such as oxygen (O₂) and carbon monoxide (CO) (Weber and Vinogradov 2001). In plants, at least three different Hb families have been identified: symbiotic, non-symbiotic and truncated hemoglobins (Ross *et al*, 2002). Symbiotic Hbs, or leghemoglobins are specifically synthesized in nitrogen-fixing legume root nodules, and their main function is to facilitate oxygen transport and scavenging to protect *Rhizobium* nitrogenase from inactivation (Appleby 1984). Plant truncated Hbs are short versions of the classical globin fold. The function of these proteins, recently detected in organs of angiosperm species such as *Arabidopsis* (Watts *et al*, 2001) and wheat (Larsen 2003), is still unknown. Finally, non-symbiotic Hbs occur at much lower abundance but appear ubiquitous in all plant species examined (Dordas *et al*, 2003a). In vascular plants, two classes occur: class 2 non-symbiotic Hbs present similar O₂-binding properties to that of symbiotic Hb and are inducible by cold stress (Trevaskis *et al*, 1997) or cytokinin treatment (Hunt *et al*, 2001). In contrast, class 1 non-symbiotic Hbs have high O₂ affinity and are induced under hypoxic conditions (Duff *et al*, 1997, Trevaskis *et al*, 1997, Parent *et al*,

2008b). Because of an extremely low O₂-dissociation constant, class 1 non-symbiotic Hbs might participate in the regulation of cellular nitric oxide (NO) levels thus improving the redox and/or energy homeostasis of plant cells during hypoxia (Dordas *et al*, 2003b, Perazzolli *et al*, 2004). NO and Hb are intimately linked during the response to hypoxia in many biological systems (Durner and Klessig 1999, Wendehenne *et al*, 2004). In addition class 1 Hb genes have been shown to affect NO levels in several experimental systems and their main function might be the removal of NO during oxygen deficiency in plants (Dordas *et al*, 2003a, 2004, Perazzolli *et al*, 2004). Recent data on the cellular localisation of an oak ns-Hb (*QbHb1*) indicates that hemoglobin transcripts are most abundant in the protoderm, the outermost layer of living cells in the root directly in contact with the outside environment (Parent *et al*, 2008a, 2008b). Such a localisation could suggest that ns-Hb may be involved in combination with NO in the sensing and signalling of changes in the outside root environment. Additionally, hypoxic root cultures of Hb deficient alfalfa and maize mutants accumulated high levels of NO, 2 to 3 cm behind the root tip during the first 24 h of hypoxia (Dordas *et al*, 2003b, 2004, Igamberdiev *et al*, 2004). Both NO and Hb levels increase in tissues with a similar temporal sequence after exposure to hypoxia or anoxia (Dordas *et al*, 2004), thus suggesting that ns-Hb modulation of NO is closely linked to short term survival to hypoxic or anoxic stress.

There is a growing consensus in plant science that there is considerable cross-talk between signalling cascades during different stress conditions (Gazzarrini and Mccourt 2003, Birnbaum and Benfey 2004, Bostock 2005). Additional complexity arises from the fact that the magnitude, duration and frequency of the signalling cascade influences the cellular response (Mittler *et al*, 2004).

ETHYLENE

The most studied signaling molecule during flooding stress is ethylene. It is involved in regulating such diverse morphological adaptations as root and shoot elongation, radial swelling, adventitious root initiation and aerenchyma development (Gunawardena *et al*, 2001b, Gazzarrini and Mccourt 2003, Visser and Borgemann 2006, Voeselek *et al*, 2006, Pierik *et al*, 2007). The concentration of ethylene increases rapidly during the first hours of submergence in several species (Lorbiecke and Sauter 1999) and this increase is believed to be either a result of physical entrapment (Banga *et al*, 1996) or enhanced biosynthesis as

reported for deepwater rice (Kende *et al*, 1998). For instance, ACC is rapidly synthesized in tomato roots exposed to flooding, and transported to the shoot within 12 h (Shiu *et al*, 1998). Once in the leaves, ACC is converted by ACC oxidase to ethylene, which may cause epinasty. In fact, roots under anaerobic conditions accumulate ACC, as long as enough ATP is available, as the biosynthesis of ethylene is inhibited under anoxic conditions because the conversion of ACC to ethylene by ACC oxidase is an oxygen dependent process (Peng *et al*, 2001).

In non-aquatic plants, ethylene is often associated with root and shoot inhibition, leaf wilting and curling, typical flooding responses (Visser *et al*, 1997, Fiorani *et al*, 2002). In contrast, ethylene is often thought to be one of the primary signals implicated in the promotion of shoot elongation in some flood tolerant species (Benschop *et al*, 2005, Voesenek *et al*, 2006). The dual role played by ethylene as either inhibiting or stimulating growth in plants has recently been attributed to a biphasic response dependent on ethylene concentrations (Pierik *et al*, 2006). Thus, at low concentrations ethylene stimulates growth whereas at high concentrations it inhibits growth.

Ethylene is also considered one of the prime signals controlling apoplast acidification, transcription of expansin (Vreeburg *et al*, 2005), adventitious root growth (Visser *et al*, 1996a, Mergemann and Sauter 2000, Bragina *et al*, 2001, Pan *et al*, 2002) and aerenchyma formation (He *et al*, 1996, Jackson and Armstrong 1999, Gunawardena *et al*, 2001a, Evans D. 2004, Colmer *et al*, 2006). This role is supported by the inhibition of aerenchyma formation when either the ethylene biosynthesis pathway or ethylene receptors are blocked (Kende 1993, Visser and Borgemann 2006). It is also in agreement with the ethylene dependence of many plant programmed cell death events (Overmyer *et al*, 2000, Rao *et al*, 2002, Van Breusegem and Dat 2006). Recently, ethylene response factors (ERFs) have been identified as key regulatory players during submergence in both rice and *Arabidopsis* (Fukao *et al*, 2006, Xu *et al*, 2006, Lasanthi-Kudahettige *et al*, 2007, Perata and Voesenek 2007). In fact, a transcription factor belonging to the B-2 subgroup of the ethylene response factors, located within the Sub1 locus on chromosome 9 in rice, determines submergence tolerance in rice (Fukao *et al*, 2006, Xu *et al*, 2006, Perata and Voesenek 2007).

However, a recent investigation of constitutive formation of aerenchyma in *Juncus effusus* demonstrated that ethylene is not involved in the formation of these structures (Visser and Borgemann 2006). A role for ethylene in plant responses is crucial but how and where it is

positioned in the signaling cascade is still an unknown. Furthermore, how oxygen deprived roots promote ACC oxidase activity in leaves is still unresolved.

OTHER PLANT SIGNALING MOLECULES - PHYTOHORMONES

In addition to the recognized example of ethylene signaling during the response to submergence, other plant growth regulators have also been identified as key players in the adaptive changes commonly observed. Upon accumulation of ethylene, the ABA levels in several submergence-tolerant species (i.e. *Rumex palustris*, deepwater rice, *Scirpus micronatus*) decline through reduced biosynthesis and a promotion of its breakdown (Lee T.-M. *et al*, 1996, Kende *et al*, 1998, Ram *et al*, 2002, Cox *et al*, 2004, Benschop *et al*, 2005, Voeselek *et al*, 2006). The antagonist effect between ethylene and ABA is further supported by the increased expression of a putative ABA 8'-hydroxylase, which catalyses the oxidation of ABA, during ethylene treatment in deepwater rice (Yang and Choi 2006). In *Rumex palustris*, ABA decline is a prerequisite for ethylene induced underwater petiole elongation (Benschop *et al*, 2005). Recently, ABA was also identified as a key negative regulator of ethylene- and GA-induced root emergence in deepwater rice (Steffens *et al*, 2006). However, ABA levels do not always decline upon hypoxia treatment. For instance, submergence did not affect the level of ABA in a flooding-intolerant species; *Rumex acetosa* (Benschop *et al*, 2005), foliar ABA concentrations were increased transiently in tomato (He *et al*, 1996) and, ABA concentrations doubled in xylem sap of de-topped *Phaseolus vulgaris* during flooding (Neuman and Smit 1991). In *Galium aparine*, increased levels of ethylene stimulated ABA biosynthesis (Hansen and Grossmann 2000). In addition, exogenous ABA applications increased anoxia tolerance in maize and *Arabidopsis* (Hwang and Vantoai 1991, Ellis *et al*, 1999), and transcript levels of AtbZIP50, an important transcription factor for ABA signal transduction were transiently increased in anoxia treated *Arabidopsis* root cultures (Klok *et al*, 2002). Finally, a Myb transcription factor (AtMYB2), known to be induced by ABA, was induced by hypoxia (Dennis *et al*, 2000). Thus it seems that the interactive nature of the ethylene and ABA is very much dependent on the species studied.

The importance of ABA levels during the submergence response is also clearly evidenced during root adaptive changes. High levels of exogenously applied ABA reduced root growth (Beaudoin *et al*, 2000) and development of lateral roots in *Arabidopsis* (De Smet *et al*, 2003). Furthermore, ABA is also involved in the regulation of ethylene induced programmed cell

death, a genetically controlled cell death program associated with adventitious root emergence and aerenchyma development (Steffens and Sauter 2005). Because of the contradictory data concerning the ABA effect, it is now believed that ABA action may in fact be linked to its effect on GAs.

It is now well established that ABA and GAs can act as antagonists in various growth responses, and the role of GA in stimulating shoot growth is well established (Hoffmann-Benning and Kende 1992, Rijnders *et al*, 1997, Rademacher 2000). There is also evidence for an increase in GA concentration and sensitivity to ethylene during flooding (Raskin and Kende 1984, Rijnders *et al*, 1997). This is supported by the requirement of GAs for ethylene action in rice leaves during submergence. In fact, the synergism between ethylene and GA is believed to increase the responsiveness of rice internodes to GA. When GA inhibitors are applied to rice seedlings, ethylene and submergence induced growth are inhibited (Raskin and Kende 1984). Finally, enhanced expression of GA-inducible genes coding for cell wall loosening enzymes (i.e. expansins) have been described in the literature. However, as illustrated with *Rumex acetosa*, the response may vary between species, as GA levels remain unchanged although ethylene increased during submergence (He *et al*, 1996).

Other hormonal interplay has also been described during flooding stress. Synergism between IAA and ethylene has been proposed during adventitious root formation. Adventitious root development at the base of the shoot is an important adaptation to flooding and is initiated soon after submergence. Although the endogenous IAA concentration remained unchanged during flooding-induced rooting, a basipetal transport from the shoot to the root zone was essential to keep auxin concentrations stable (Visser *et al*, 1996b). Other commonly observed flooding induced morphological changes such as lateral root formation can also be induced by IAA application. An involvement of IAA in the flooding response is further supported by the transient increase in transcript levels of two auxin-responsive genes during hypoxia treatment of root cultures (IAA2, IAA3) (Klok *et al*, 2002). The Aux/IAA genes are targets of auxin regulation and encode proteins likely to regulate auxin responsive gene expression.

Finally, cytokinin (CK) may also participate in the cross-talk during responses to flooding as sunflower xylem sap CK levels are remarkably reduced following 24 h of flooding (Burrows and Carr 1969). Soil inundation is believed to reduce CK production by lowering O₂ concentrations at the site of CK production, the root apical meristem. However, whether the reduction is due to lower biosynthesis or decreased transport from roots to shoot is still not

demonstrated. Recently, transgenic *Arabidopsis* plants with autoregulated CK production were shown to be more tolerant to flooding. This enhanced tolerance was attributed to the regulation of flooding induced senescence by endogenously produced CK (Chandel *et al*, 1996).

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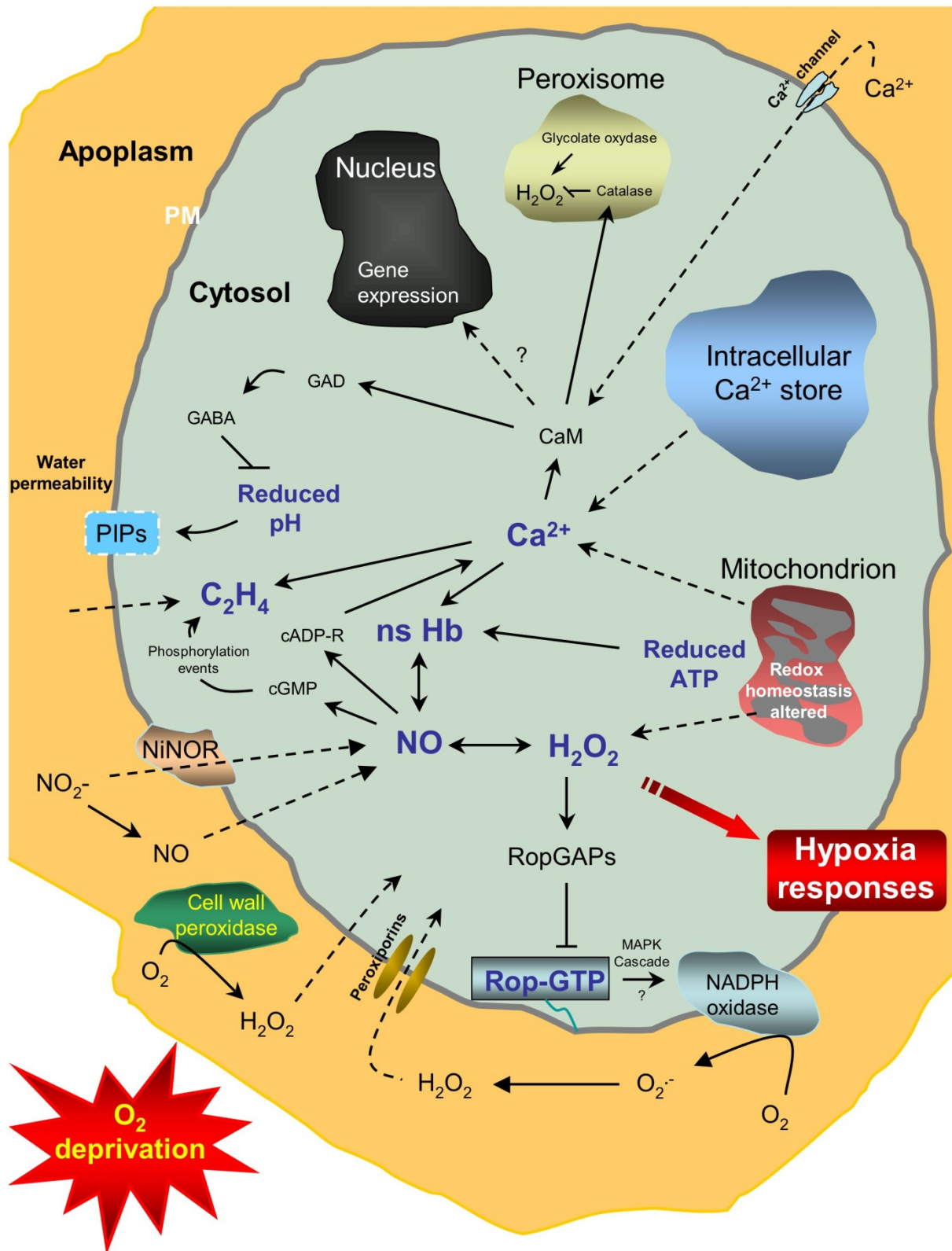


Figure 1. Sensing and signaling pathways in early response to oxygen deficiency in plant cells.

Legend of figure 1:

Reduction of cellular oxygen levels can result in rapid and transient changes in cytosolic Ca^{2+} concentration and Ca^{2+} -binding proteins, reduction in ATP generation, decline in cytosolic pH, changes in reactive oxygen species homeostasis, including NO and H_2O_2 , the induction of ethylene biosynthesis and Hb gene expression and the accumulation of the active form of the Rop GTPase, Rop-GTP. Diversity in responses may result from interactive networks between the potential signalling cascades.

Abbreviations: ns Hb, nonsymbiotic hemoglobin; NO, nitric oxide; H_2O_2 , hydrogen peroxide; C_2H_4 , ethylene; Rop, Rho-like small G protein; GAPs, GTPase-activating proteins; CaM, calmodulin; GAD, glutamate decarboxylase; GABA, γ -aminobutyric acid; MAPK, mitogen-activated protein kinase; cADP-R, adenosine 3',5'-cyclic diphosphate-ribose; cGMP, cyclic guanosine monophosphate; PIPs, plasma membrane intrinsic proteins; NiNOR, nitrit:NO reductase; PM, plasma membrane.

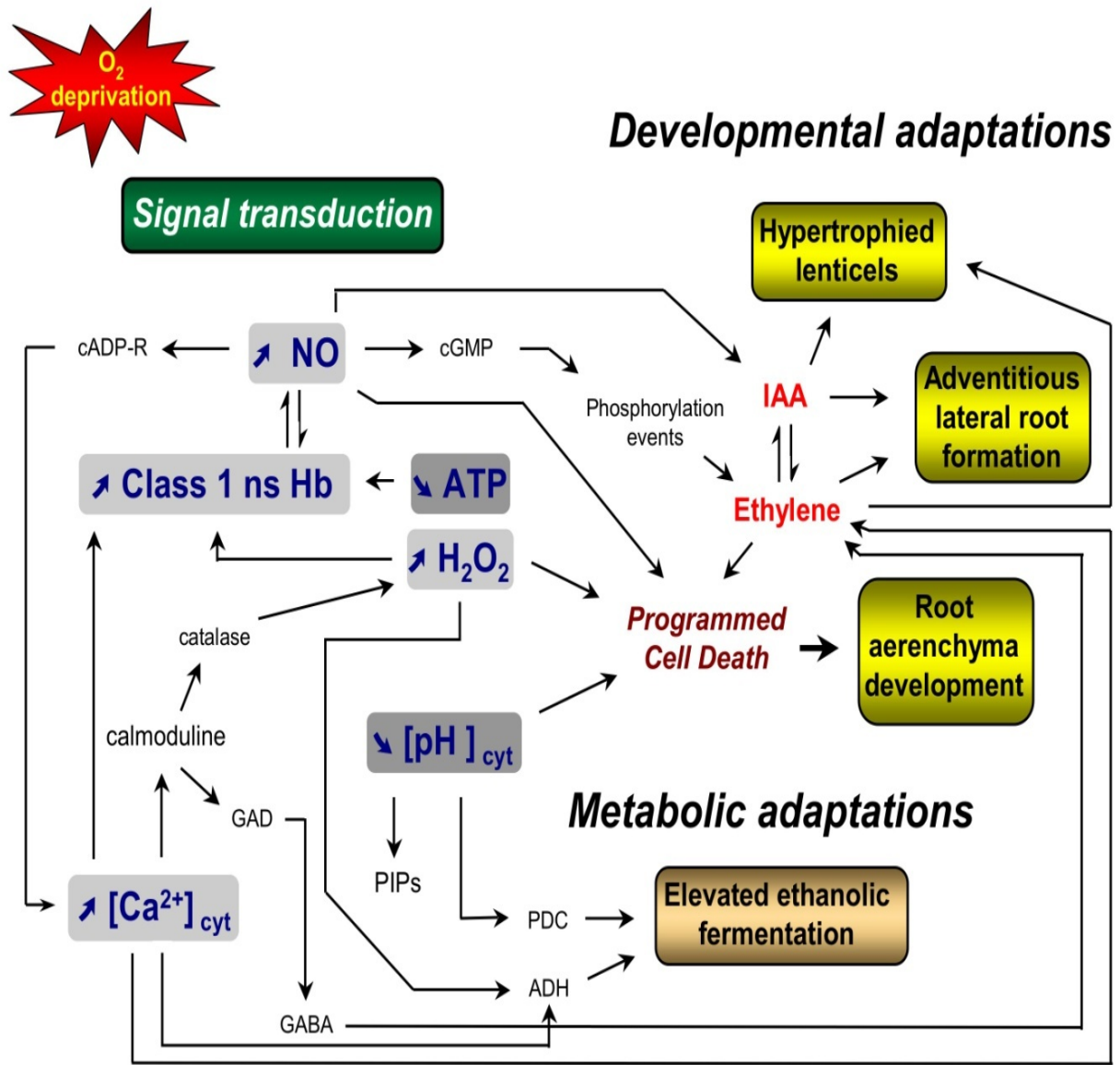


Figure 2. Schematic representation of the potential signalling pathways involved during the response and adaptation of plants to flooding stress.

Legend of figure 2:

Depletion of cellular oxygen levels involves molecules which bind or consume oxygen or that are altered by oxydation state. Short-term metabolic adjustments to hypoxia include modifications of the sucrose degradation, with increased activity of the fermentative metabolism pathway enzymes. Long-term developmental responses in tolerant plants relies on the formation of structures enhancing O₂ diffusion to the root tips, such as hypertrophied lenticels, adventitious roots or aerenchyma.

Abbreviations are as in Fig. 1, with the following additions: IAA, auxin; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase.

Résultats

L'hémoglobine non-symbiotique semble avoir un rôle prépondérant dans l'ennoyage. Cependant les mécanismes par lesquels elle interviendrait sont encore peu connus. Elle pourrait de part son rôle dans la tolérance à l'hypoxie être un des éléments qui expliqueraient la différence de tolérance à l'ennoyage observée entre les deux espèces de chênes étudiées.

*Notre premier objectif a donc été de cloner ce gène chez le chêne et de le caractériser mais aussi de comparer son expression chez les deux espèces en réponse à un ennoyage de courte durée. Ceci dans le but de déterminer si l'hémoglobine était bien impliquée dans la réponse à l'ennoyage mais également de préciser si le chêne sessile et le chêne pédonculé présentaient un profil d'expression différent qui pourrait être à l'origine de leur différence de tolérance à l'ennoyage. L'analyse de *QpHb1* a non seulement montré une expression différentielle entre les deux espèces en réponse à l'hypoxie mais également une répartition dans la racine en conditions témoins qui suggère un rôle constitutif pour l'hémoglobine non-symbiotique. De par sa présence au niveau des cellules du protoderme et du protoxylème, elle pourrait contribuer à la perception et/ou la signalisation du stress.*

*Cette partie est composée de deux articles de recherche, le premier publié dans « *New Phytologist* » présente les principaux résultats, le second paru dans « *Plant Signaling and Behavior* » apporte des résultats complémentaires au précédent et des interprétations plus spéculatives.*

A. Caractérisation du
gène *QpHb1* et expression en
réponse à un stress hypoxique court

1. A novel non-symbiotic hemoglobin from oak: cellular and tissue specificity of gene expression

Cette partie a fait l'objet d'une publication acceptée le 08 août 2007 dans le journal *New Phytologist*. Les auteurs sont C. Parent, A. Berger, F. Folzer, J. Dat, M. Crèvecoeur, P.-M. Badot et N. Capelli

Résumé:

Les résultats présentés dans cette étude portent sur l'isolation et la caractérisation d'un nouveau gène d'hémoglobine non-symbiotique *QpHb1* chez le chêne sessile (*Quercus petraea* Matt. L.). La séquence déduite du clone d'ADNc isolé code pour une protéine de 161 acide aminés qui présente respectivement 71 et 51% d'identité de séquence avec les hémoglobines non-symbiotiques de classe 1 et de classe 2, clonées chez *Arabidopsis thaliana*.

Afin de comprendre le rôle de l'hémoglobine non-symbiotique, nous avons analysé l'expression spatio-temporelle par Northern blotting et hybridation *in situ*. La localisation de *QpHb1* au niveau apical de la racine indique que les transcrits sont plus abondants dans les cellules du protoxylème, dans certaines cellules du cortex ainsi que dans le protoderme. De plus, la comparaison des profils d'expression de *QpHb1* en réponse à un ennoyage de 48h, chez le chêne sessile et le chêne pédonculé, deux espèces présentant une tolérance contrastée à l'hypoxie, montre que le niveau augmente rapidement chez l'espèce la moins sensible en réponse à l'ennoyage.

Les résultats de notre étude suggèrent que *QpHb1* pourrait participer à la perception et aux éléments signalétiques précoces mis en place sous contrainte hypoxique.

A novel nonsymbiotic hemoglobin from oak: cellular and tissue specificity of gene expression

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Summary

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- This study presents the isolation and characterization of a novel nonsymbiotic Hb gene from sessile oak (*Quercus petraea*) seedlings, herein designated *QpHb1*.
- The cellular and tissue expression of *QpHb1* was analysed by Northern blotting and *in situ* hybridization.
- The encoded protein was predicted to consist of 161 amino acid residues, and shares 71 and 51% amino acid sequence identity with the *Arabidopsis* class 1 and 2 nonsymbiotic Hb, respectively. Northern blot analysis revealed that *QpHb1* was strongly expressed in roots. Spatial expression analysis of *QpHb1* in the root apical region of sessile oak by *in situ* hybridization indicated that transcripts were mostly abundant in protoxylem cell initials, some cortical cells and the protoderm. In addition, when comparing the expression profile of *QpHb1* in sessile and pedunculate oak (*Quercus robur*), two species with contrasted hypoxia tolerance, the transcript level of *QpHb1* rose early in the most flood-tolerant species, pedunculate oak, during root submergence.
- The spatial-temporal expression of *QpHb1* suggests that this gene could participate in perception and signaling during hypoxia.

Key words: gene expression, hypoxia, *in situ* hybridization, nonsymbiotic hemoglobin, oak (*Quercus petraea*, *Quercus robur*).

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Introduction

Soil waterlogging has become a major factor affecting the growth, development and survival of many plant species, not only in natural ecosystems, but also in agricultural and horticultural systems (Dat *et al.*, 2006). Transient flooding periods are frequently observed as a result of over-irrigation, inadequate drainage, the removal of vegetation cover and/or global warming. In addition, climate predictions suggest that

the occurrence of this event will increase in frequency in the near future. Some of the best characterized plant adaptations to hypoxia include a switch in biochemical and metabolic processes commonly observed when O₂ availability becomes limiting (Dat *et al.*, 2004). Most plant species synthesize a set of *c.* 20 anaerobic proteins (ANP) that enable an oxygen-independent energy-generating metabolism to proceed when conditions become unfavourable for aerobic energy production (Subbaiah & Sachs, 2003). Other observed adaptations include the formation of hypertrophied lenticels, the development of aerenchyma, root cortical air spaces that enhance the efficiency of gas transfer between aerial and

The nucleotide sequence data reported in this paper will appear in the EMBL/GenBank/DDDBJ databases with accession number EF186909 (QpHb1).

submerged organs, as well as the promotion of adventitious roots (Vartapetian & Jackson, 1997; Jackson & Colmer, 2005; Folzer *et al.*, 2006).

In contrast to the wealth of data available concerning the molecular and cellular mechanisms of hypoxia sensing and signalling in animals, such mechanisms have been more rarely described in plants, even less in woody species. In mammals, the hypoxia-inducible heterodimeric transcription factor (HIF) is a key regulatory element in the response to hypoxia (Giaccia *et al.*, 2004; Semenza, 2004). However, to date no such sensor has been identified in plants (Bailey-Serres & Chang, 2005; Agarwal & Grover, 2006). Recent broad-range approaches (DNA chip technology or proteome analysis) have helped to identify novel genes and proteins involved in plant responses to soil waterlogging and anaerobiosis (Chang *et al.*, 2000; Klok *et al.*, 2002; Agarwal & Grover, 2005; Branco-Price *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005). However, novel components of the signal transduction pathway leading to hypoxia-induced gene expression have been documented exclusively in crop plants (Lasanthi-Kudahettige *et al.*, 2007) and model organisms such as *Arabidopsis thaliana* (Miyashita *et al.*, 2007). These components include rapid changes in cytosolic Ca²⁺ levels (Snedden & Fromm, 1998; Subbaiah *et al.*, 1998, 2000; Luan *et al.*, 2002) and Ca²⁺-binding proteins (Folzer *et al.*, 2005), the induction of ethylene biosynthesis (Drew *et al.*, 2000; Nie *et al.*, 2002), Rop (RHO-related GTPase of plants) G-protein signaling (Baxter-Burrell *et al.*, 2002), as well as a large number of transcription factor families (AtMYB2, ZAT 12, WRKY factors; Bailey-Serres & Chang, 2005). In contrast to the amount of data available for herbaceous species, very little is known about the molecular mechanisms that underlie the sensing and signalling to hypoxia in woody species. This is especially apparent with forest tree species, which are not only of primary interest to the wood industry, but are also critical for the preservation and/or conservation of forest biodiversity.

Recently, additional insight into the response of plants to hypoxia has been provided by the discovery of stress-induced genes that affect plant metabolism and growth under low tensions (Dordas *et al.*, 2003a). Among these, hemoglobins (Hbs) are ubiquitous molecules that have been found in various species from most of the taxonomic kingdoms, including bacteria, yeasts, protists, plants and animals (Wittenberg & Wittenberg, 1990; Hardison, 1996; Suzuki & Imai, 1998). All Hbs contain a heme group carrying an iron ion, which is responsible for the reversible binding to gaseous ligands such as oxygen (O₂) and carbon monoxide (CO) (Weber & Vinogradov, 2001). In plants, at least three different Hb families have been identified: symbiotic, nonsymbiotic and truncated Hbs (Ross *et al.*, 2002). Symbiotic Hbs, or leghemoglobins, are specifically synthesized in nitrogen-fixing legume root nodules, and their main function is to facilitate oxygen transport and scavenging to protect *Rhizobium* nitrogenase from inactivation (Appleby,

1984). Plant truncated Hbs are short versions of the classical globin fold. The function of these proteins, recently detected in organs of angiosperm species such as *Arabidopsis* (Watts *et al.*, 2001) and wheat (Larsen, 2003), is still unknown. Finally, nonsymbiotic Hbs occur at much lower abundance, but appear ubiquitous in all plant species examined (Dordas *et al.*, 2003a). In vascular plants, two classes occur. Class 2 nonsymbiotic Hbs present similar O₂-binding properties to those of symbiotic Hbs and are inducible by cold stress (Trevaskis *et al.*, 1997) or cytokinin treatment (Hunt *et al.*, 2001). In contrast, class 1 nonsymbiotic Hbs have high O₂ affinity and are induced under hypoxic conditions (Duff *et al.*, 1997; Trevaskis *et al.*, 1997). Because of an extremely low O₂-dissociation constant, class 1 nonsymbiotic Hbs might not function as O₂ carriers, as originally thought. In fact, recent studies suggest that their presence could regulate cellular nitric oxide (NO) levels, thus improving the redox and/or energy status of the plant cell during hypoxia (Dordas *et al.*, 2003b; Perazzolli *et al.*, 2004). In an attempt to gain further understanding of the difference in the molecular responses of tree species to hypoxia, the cloning and characterization of a nonsymbiotic Hb gene from sessile oak was undertaken. The analysis was further complemented by comparing the expression profile of the gene in two oak species with a contrasted response to flooding. The genus *Quercus* (oaks), which includes over 300 woody species, is widespread in the northern hemisphere, where it represents the dominant vegetation of temperate forests (Nixon, 1993). We decided to focus on the two predominant European oak species, pedunculate and sessile oak, to investigate the spatial and temporal expression patterns of Hb during the early response to hypoxia. The two species generally cohabit in forest ecosystems; however, sessile oak is found more frequently on well drained soils, whereas pedunculate oak can populate poorly drained sites where temporary waterlogging occurs (Lévy *et al.*, 1992).

Materials and Methods

Plant material and growth conditions

Sessile (*Quercus petraea* (Matt.) Liebl.) and pedunculate (*Quercus robur* L.) oak acorns, harvested in north-eastern France, were provided by the Office National des Forêts (ONF; Preney *et al.*, 1997) and stored in moist vermiculite at 4°C until use. The acorns were sterilized by dipping in a 1% bleach solution and oxygenated overnight in running water to favour germination. Individual acorns were then grown in 1.8l plastic pots containing river sand (Dekoline Carat 4, Aquatic Nature, Belgium). This substrate was chosen to enable harvesting of quality roots for histological and molecular studies. The plants were grown for 5 wk in a controlled growth chamber with environmental conditions set as follows: a 16 h photoperiod, a photosynthetically active radiation (PAR) of 170 μmol m⁻² s⁻¹ at canopy level

(provided by high-pressure sodium lamps), an average temperature of $20 \pm 1.5^\circ\text{C}$ and a relative humidity of 70%. Plants were irrigated four times daily with a fertilizer solution (0.5 ml l^{-1} , NPK 6/5/6; SEM, Compo France, Roche-Lez-Beaupré, France, Germany) using an automated ebb-and-flow system. After 5 wk, which corresponded to the establishment of the first true leaves, each seedling was checked for leaf morphology and growth characteristics to make sure the seedlings grown from acorns provided by the ONF were not misidentified. A clear difference in leaf morphology and growth characteristics could be observed between both species. However, when in doubt, the seedlings were discarded. For molecular analysis, the root system of plants used for shoot water potential measurements were immediately harvested, frozen in liquid nitrogen, and stored at -80°C until use. Other plant samples (shoots and leaves) were treated similarly.

Hypoxia treatment

Low oxygen stress was imposed by immersing 5-wk-old seedlings up to the root collar in the irrigation solution for the desired period (1–48 h). Control plants (not submerged) were harvested in parallel with flooded plants at each time point. In addition, the O_2 level of the solution surrounding the roots was monitored for each stress period by measuring O_2 concentration with a portable O_2 electrode (Cellox325, WTW, Weilheim, Germany) in the rhizospheric solution (see Fig. 7a). The measurements were undertaken on nine individual pots (each containing one plant) from three independent experiments.

Shoot water potential

Shoot water potential measurements were made with a Scholander-type pressure chamber (DPI 700, GE Druck, New Fairfield, CT, USA) on whole shoots of flooded and control 5-wk-old sessile and pedunculate oak seedlings, as described by Folzer *et al.* (2006). The average values for shoot water potential of control and flooded plants were calculated at each time point from 12 seedlings for each species, obtained from four independent experiments. The shoot water potential was then expressed as the difference between the flooded and control values calculated for each species.

5' and 3' rapid amplification of cDNA ends (RACE)

In order to isolate sessile oak nonsymbiotic hemoglobin (Hb) cDNA clones, total RNA from pooled samples of roots of plants exposed to hypoxia for either 1 or 3 h were prepared using the RNeasy Plant Mini Kit (Qiagen S.A., Courtaboeuf, France). cDNA synthesis was carried out using the SMART RACE cDNA amplification kit according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). After treatment with RNase-free DNase, the first-strand

cDNA was synthesized by reverse transcription of $1 \mu\text{g}$ total RNA using the 5'-CDS primer A and the SMART II A oligo (both provided in the kit). The degenerated reverse primer *Hb* ($5' \text{GC[C/T]TC[C/T]TT[A/G/T]AT[A/T/C/G]AT[C/T]T[C/T} [A/TC/G]AG[A/T/C/G]AG[A/T/C/G]G-3'$) for 5'-RACE was designated and synthesized based on the conserved region identified among several plant Hbs (*A. thaliana*, barley, soybean, *Casuarina glauca*, rice) deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>).

Amplification conditions were as follows: pre-amplification denaturation at 94°C for 2 min, 41 cycles of denaturation at 94°C for 15 s, primer annealing at 64°C for 25 s and primer extension at 72°C for 2 min, and a final polymerization and extension step of PCR products at 72°C for 10 min. The resulting 500–600-bp RT-PCR products were purified using the MinElute gel extraction kit (Qiagen), cloned individually using the pGEM-T Easy Vector System (Promega, Madison, WI, USA), and fully sequenced (MilleGen, Biostep, Labege, France). Based on the sequence of the 52-RACE fragment, a gene-specific sense primer *Hb2* ($5' \text{-CGGGTGGTGTTCACCTTCAAAAAGTGGGGTTCG-3'}$) was designated and synthesized to amplify the remaining part of the corresponding Hb gene. RT-PCR was used to isolate the full-length cDNA of *Q. petraea* Hb (designated *QpHb1*).

Northern blot analysis

To obtain a gene-specific probe for *QpHb1*, a 349-bp DNA fragment was amplified by PCR from both the 52-UTR and coding sequence of the cDNA clone. Primers used were *QpHbsp* ($5' \text{-TTTCCAAATCTCTAACTAATTCTTGACC-3'}$) and *QpHbrp* ($5' \text{-AAGTTGCACCGCTGATTCACAAGT CAT-3'}$). Bacteriophage promoters T7 and SP6 were added to primers *QpHbsp* and *QpHbrp*, respectively, generating a PCR product that could be used for *in vitro* transcription (Stoflet *et al.*, 1988). Total RNA ($5 \mu\text{g}$) from each tissue was mixed with sample buffer, separated by formaldehyde-denaturing agarose gel electrophoresis (1.2% w/v) and capillary-blotted with $10 \times$ saline sodium citrate (SSC) onto positively charged nylon membranes (Roche Diagnostics SAS, Meylan, France, Germany). After RNA fixation by baking for 2 h at 80°C , prehybridization (1 h) and hybridization (overnight) were performed in Dig Easy Hyb buffer (Roche Diagnostics) at 68°C . Membranes were washed for 15 min twice with $2 \times$ SSC, 0.1% SDS at room temperature, and for 30 min twice with $0.1 \times$ SSC, 0.1% SDS at 60°C . The hybridized antisense RNA probe was immunodetected with an alkaline phosphataseconjugated antidigoxigenin antibody and visualized with a chemiluminescence system (Roche Diagnostics) as recommended by the manufacturer. The Northern analysis was replicated three times with RNA from three independent experiments and a pool of at least six plants per experiment. Densitometric measurements were performed and a one-way ANOVA was undertaken on the results to identify main effects.

Bio-informatics and phylogenetic analysis

In situ hybridization

Multiple-sequence alignments were carried out using CLUSTALW algorithms from EMBL-EBI (<http://www.ebi.ac.uk/clustalw>). Cluster analysis was performed by the neighbor-joining method (Saitou & Nei, 1987). The GenBank accession numbers for all 56 proteins are given in the legends of Figs 1, 2.

Root segments from *Q. petraea* (7 mm long) were fixed in 0.25% glutaraldehyde (w/v) and 4% formaldehyde (w/v) in 0.1 M phosphate buffer pH 7.2 overnight at 4°C. They were then washed thoroughly in phosphate buffer, dehydrated in a graded-alcohol series and embedded in paraffin. Thin

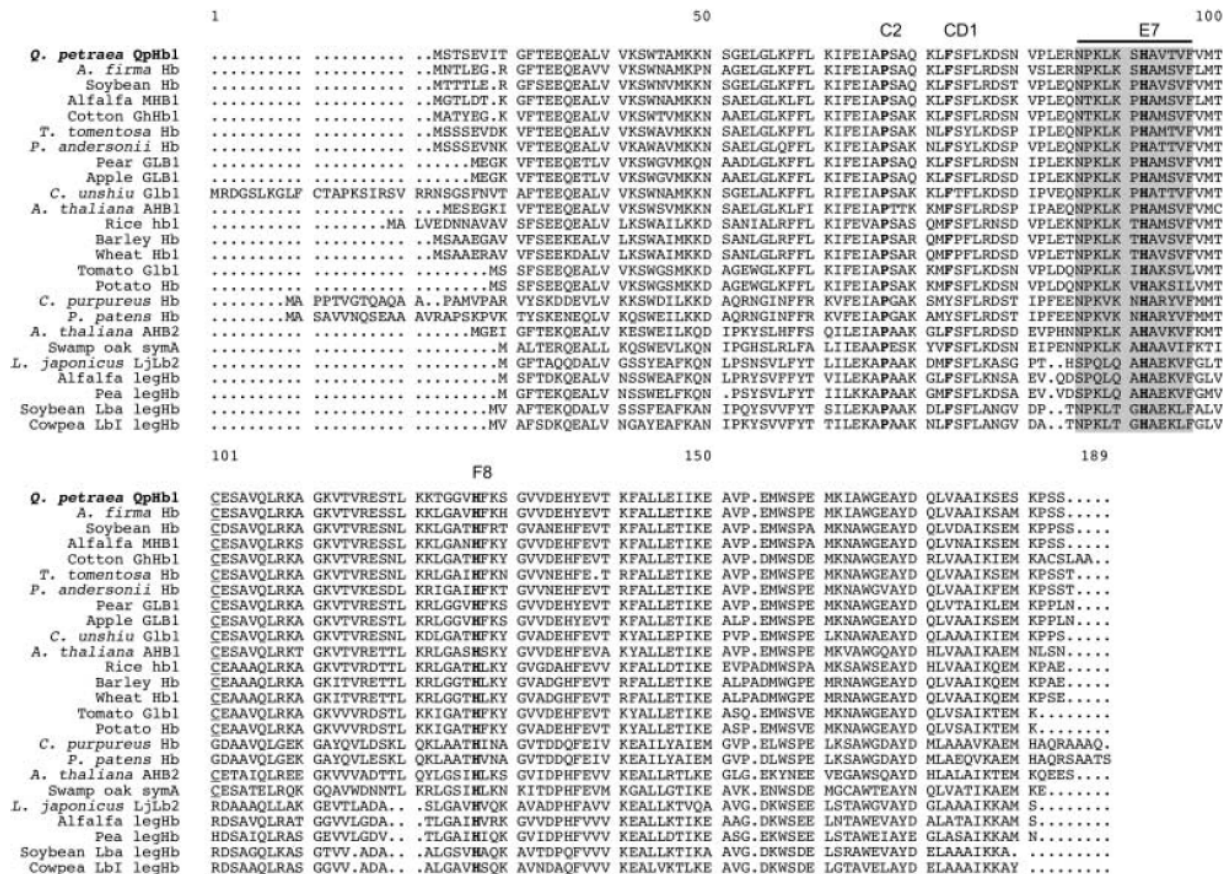


Fig. 1 Comparative alignment of the predicted amino acid sequences of hemoglobin (Hb) from different plant species. The alignment was generated using the CLUSTALW program (<http://www.ebi.ac.uk/clustalw>). Conserved residues of heme- and ligand-binding (distal (E7) and proximal (F8) His residues, Phe (CD1), Pro (C2)) are in bold type; Cys residues are underlined (Ota *et al.*, 1997). The plant globin motif characteristic of plant hemoglobins is indicated in thick gray. Protein sequences were obtained from the GenBank database using the following accession numbers: EF186909 (*Quercus petraea* QpHb1 nonsymbiotic Hb, this study); AB221344 (*Ainus firma* nonsymbiotic Hb, 85% aa identity to QpHb1 protein); U47143 (soybean nonsymbiotic Hb, 80% identity); AF172172 (alfalfa MHB1 nonsymbiotic Hb, 81% identity); AY899302 (cotton GhHb1 nonsymbiotic Hb, 78% identity); Y00296 (*Trema tomentosa* Hb, 82% identity); U27194 (*Parasponia andersonii* Hb, 80% identity); AY224133 (*Pyrus communis* GLB1 nonsymbiotic Hb class 1, 82% identity); AY224132 (*Malus domestica* GLB1 nonsymbiotic Hb class 1, 83% identity); AY026338 (*Citrus unshiu* G1b1 nonsymbiotic Hb class 1, 77% identity); U94998 (*Arabidopsis thaliana* AHB1 nonsymbiotic Hb class 1, 71% identity); AF335504 (rice hb1 nonsymbiotic Hb class 1, 65% identity); U94968 (barley Hb, 70% identity); AY151390 (wheat Hb1, 70% identity); AY026343 (tomato G1b1 nonsymbiotic Hb class 1, 77% identity); AY151389 (potato Hb, 76% identity); AF309562 (*Ceratodon purpureus* Hb, 44% identity); AF218049 (*Physcomitrella patens* nonsymbiotic Hb, 44% identity); U94999 (*Arabidopsis thaliana* AHB2 nonsymbiotic Hb class 2, 51% identity); X77694 (*Casuarina glauca* symA symbiotic Hb, 50% identity); AB238218 (*Lotus japonicus* LjLb2 legHb, 43% identity); X14311 (alfalfa legHb, 46% identity); AB015720 (pea legHb, 47% identity); V00453 (soybean Lba legHb, 43% identity); U33206 (*Vigna unguiculata* Lbl legHb, 40% identity).

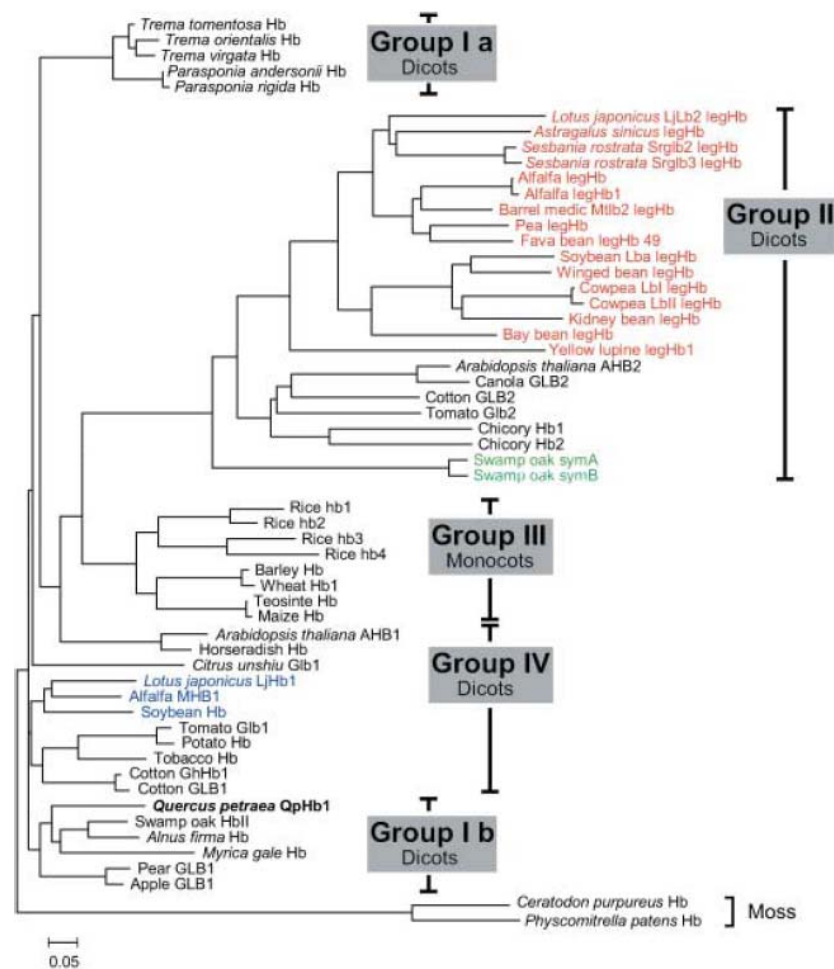


Fig. 2 Phylogenetic relationship of *Quercus petraea* QpHb1 with other hemoglobins (Hb) from various organisms. Complete protein sequences of Hb-encoding genes were aligned and the tree was constructed using the CLUSTALW method (Higgins *et al.*, 1994). The bar indicates a distance of 0.05 substitutions per site. Nonsymbiotic Hbs from leguminous and nonleguminous plants are shown in blue and black, respectively. Symbiotic Hbs from leguminous plants are shown in red; symbiotic Hbs from nonleguminous plants in green. Two dicotyledon plant nonsymbiotic class 1 Hb clusters (groups Ia, Ib; IV), one monocotyledon plant nonsymbiotic class 1 Hb cluster (group III) and a dicotyledon plant symbiotic Hb and class 2 nonsymbiotic Hb cluster (group II) are indicated on the right. Hbs from *Parasponia* and *Myrica gale* were classified as group I, however they are intermediate because they retain both symbiotic and nonsymbiotic specificity. Sequences are as in Fig. 1, with the following additions: *Trema orientalis* Hb (Z99635), *Trema virgata* Hb (AJ131349), *Parasponia rigida* Hb (P68169), *Astragalus sinicus* legHb (DQ199647), *Sesbania rostrata* Srglb2 legHb (X13815), *Sesbania rostrata* Srglb3 legHb (X13814), alfalfa legHb1 (M32883), barrel medic Mtlb2 legHb (X57733), *Vicia faba* legHb 49 (Z54159), *Psophocarpus tetragonolobus* legHb (X65874), *Vigna unguiculata* LblI legHb (U33207), *Phaseolus vulgaris* legHb (P02234), *Canavalia lineata* legHb (U09671), yellow lupin legHb1 (Y00401), *Brassica napus* GLB2 nonsymbiotic Hb class 2 (AY026337), cotton GLB2 nonsymbiotic Hb class 2 (AY026339), *Lycopersicon esculentum* Glb2 nonsymbiotic Hb class 2 (AY026344), *Cichorium intybus* · *Cichorium endivia* nonsymbiotic Hb (AJ007507, AJ277797), *Casuarina glauca* symB symbiotic Hb (X77695), rice Hb (U76031, AF335504), teosinte Hb (AF291052), maize Hb (AF236080), *Raphanus sativus* nonsymbiotic Hb (AY286331), *Lotus japonicus* LjHb1 nonsymbiotic Hb (AB238220), *Nicotiana tabacum* Hb (BQ842804), cotton GLB1 nonsymbiotic Hb class 1 (AF329368), *C. glauca* HbII (X53950), *M. gale* Hb (EF405885).

sections (7 μm) were attached on poly-L-lysine coated slides (Polysine™*) from Menzel-Glaser (Menzel GmbH & Co KG, Braunschweig, Germany) in the presence of DEPC-treated water. The sections were deparaffinized, rehydrated through a graded ethanol series and rinsed in DEPC-treated water. They were then incubated for 30 min at 37°C with 1 $\mu\text{g ml}^{-1}$ proteinase K (Sigma, Buchs, Switzerland) in 100 mM Tris-HCl pH 7.5 containing 5 mM EDTA and washed in 0.2% glycine in PBS. After acetylation with 100 mM triethanolamine pH 8.0

containing 0.25% acetic anhydride, the sections were rinsed successively in 2 \times SSC and in DEPC-treated water. They were then dehydrated in ethanol and dried in a desiccator, and prehybridization was carried out at 50°C for 2 h in the prehybridization solution containing 2 \times SSC, 50% formamide, 1 \times Denhardt's solution, 5% dextran sulfate, 1 $\mu\text{g } \mu\text{l}^{-1}$ salmon sperm DNA and 0.25 $\mu\text{g } \mu\text{l}^{-1}$ yeast tRNA in DEPC-treated water. Slides were then hybridized overnight at 50°C in the prehybridization solution with 4 ng μl^{-1} sense or antisense RNA probe.

After hybridization, the slides were washed at 50°C in decreasing SSC solutions (2-, 1- and 0.5-) and treated with 0.5 g µl⁻¹ RNase A (Roche Diagnostics) in NTE buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA) at 37°C for 30 min. The RNase treatment was followed by 2· 5-min rinse in NTE buffer, 45 min at 50°C in 0.5· SSC and finally in 100 mM Tris-HCl pH 7.5 containing 150 mM NaCl. The immunodetection of probes was performed as described by Carpin *et al.* (1999) and according to the instructions of the manufacturer (Roche Diagnostics). The sections were mounted in PBS-glycerine.

Histological staining

Deparaffinized sections were stained with 1 µg ml⁻¹ 42,6-diamidino-2-phenylindole dihydrochloride (DAPI) in water for 20 min. The sections were rinsed in water and mounted in PBS containing 0.1% *p*-phenylenediamine as antifading reactive and 61% glycerol. For safranin-fast green staining, sections were deparaffinized and rehydrated from alcohol 100 to 70%. They were dipped for 24 h in a solution of 1% safranin T in 50% ethanol, washed in water, stained for a few seconds in 0.2% fast green in 92% ethanol, washed and mounted in Assistent-Histokitt (Assistent-Sondheim/Rhön, Germany).

Photographs

All sections were observed and photographed with a Leica DM IRE 2 microscope equipped with a Leica DC300F CCD color camera. DAPI-stained sections were examined by epifluorescence with the same microscope, with an A filter block (Leica, filters BP 340–380 nm for excitation, LP 425 for emission).

Statistical analysis

For water potential and densitometric data analysis, a one-way ANOVA with the general linear model (GLM) procedure of SAS 6.0 (SAS Institute Inc., Cary, NC, USA) for main effects, species and hypoxia treatment was used. Individual means were compared using Duncan's test.

Results

Molecular characteristics of the *QpHb1* gene

The *QpHb1* clone (GenBank accession number EF186909) consists of 729 nucleotides, excluding the poly(A)⁺ tail, with an open reading frame of 486 bp, flanked by a 93-bp 5'-UTR and a 150-bp 3'-UTR. The cDNA sequence encodes a 161-aa predicted polypeptide with a calculated molecular mass of 17 913 Da and an isoelectric point of 8.57, revealed by an analysis with the ExpASY Molecular Biology Server

(<http://us.expasy.org>). Alignment of the predicted *QpHb1* amino acid sequence with selected plant Hb sequences, using the CLUSTALW method (Higgins *et al.*, 1994) is presented in Fig. 1. The *QpHb1* deduced protein contained all conserved amino acid and characteristic peptide motifs of plant Hbs. In an effort to analyse the molecular evolution of the *Q. petraea* nonsymbiotic Hb, 55 full Hb amino acid sequences (incomplete sequences were not included in this study) from other plant species were retrieved from the GenBank database and compared using the CLUSTALW software. Subsequently, a phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei, 1987). The phenogram (Fig. 2) revealed that class 1 nonsymbiotic Hbs diverge into three separate major clusters (groups I, III and IV), while leghemoglobins converge into a single major cluster (group II) with homology to a group of class 2 nonsymbiotic Hbs and symbiotic Hbs from the actinorhizal shrub *C. glauca*. As expected, the monocot sequences form a distinct clade within the class 1 nonsymbiotic Hbs (group III) and *QpHb1* is in group I with other tree species. In addition, the phylogenetic analysis shows that Hbs from the bryophytes *Physcomitrella patens* and *Ceratodon purpureus* constitute a separate clade, which could indicate that Hbs are widespread in land plants.

QpHb1 transcript levels in various organs of sessile oak

The *QpHb1* antisense riboprobe hybridizes with a single specific transcript of approx. 0.75 kb in all vegetative organs examined (root, stem, leaf) in sessile oak seedlings grown under control conditions (Fig. 3a). The mRNA encoding *QpHb1* was detected primarily in root tissues. In contrast, only a very faint signal representing a low level of *QpHb1* mRNA accumulation could be observed in the leaves. The dendrometric analysis of *QpHb1* expression clearly supports the observation that its expression level is generally far superior in roots than in other tissues (Fig. 3c).

In situ expression of *QpHb1* in sessile oak roots

Figure 4 shows cross sections made at three different levels in the primary root tip of *Q. petraea*, in the region where *in situ* hybridization was performed. To identify the different tissues or cell layers in each section, cross serial sections were made from the tip of the root cap (Rc). The position of each section was expressed in micrometres estimated from the extremity of the Rc. The sections allowed us to identify the distance from the Rc at which the first provascular elements can be observed (arrows in Fig. 4c).

Figure 5 shows the *in situ* *QpHb1* expression in cross sections from different regions of a primary root tip. In sections made at 200 µm from the tip of the Rc (Fig. 5a), *QpHb1* expression was associated with the central region of the Rc but was not detected in the outer cell layers. At approx. 400 µm from the tip of the Rc (Fig. 5b), *QpHb1* was expressed mainly in the cell layers corresponding to the outer

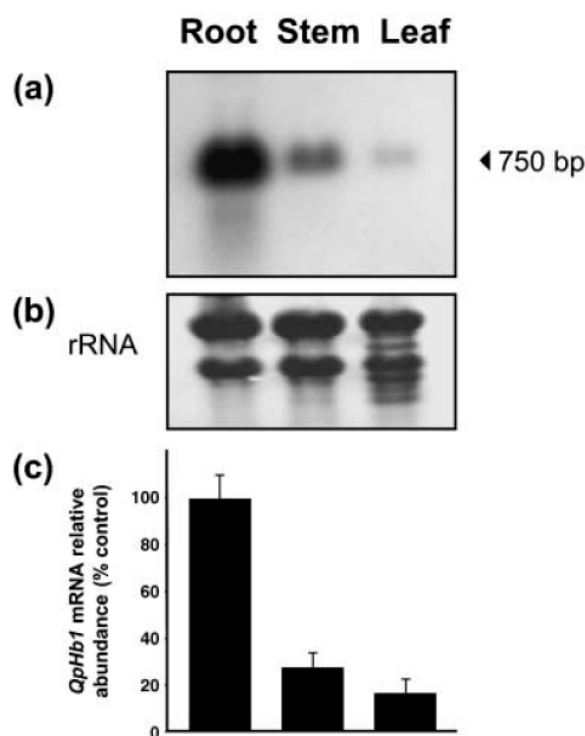


Fig. 3 Transcription pattern of the *QpHb1* gene in vegetative organs of sessile oak (*Quercus petraea*). Total RNAs were extracted from roots, stems and leaves of seedlings grown under control conditions for 5 wk. (a) Hb transcripts (750 bp). (b) Methylene blue staining was used to ensure equal loading in the gel. The low molecular weight zones in the leaf lane correspond to chloroplastic rRNA. (c) Densitometric scanning was used to quantify the signal and the relative mRNA amount was plotted as the percentage of control for each panel. Results are representative of three replicate experiments. Error bars, SEM.

ground meristem. A lower expression was detected in the inner part of the cortex. The outer cell layers, corresponding to the Rc, as well as the central part of the section (future vascular cylinder) did not show any *QpHb1* expression. About 600 μm from the tip of the Rc (Fig. 5c), patterns of *QpHb1* gene expression were as follows: no expression in the outer cell layers corresponding to the Rc, a strong expression in the cell layers located in the outer ground meristem, and finally a lower expression in the cortical parenchyma. *QpHb1* mRNA accumulation was associated with the central region of the vascular cylinder in which the xylem will differentiate. No *QpHb1* expression was detected in the outer region of the vascular cylinder in which protophloem elements are found. Control sections incubated with the sense probe did not show any staining (Fig. 5d). A very similar expression pattern was observed at c. 800 and 1000 μm (Fig. 5f,g). At these levels, *QpHb1* mRNA transcripts were found in cells of the endodermis (Fig. 5g) as well as the epidermis (Fig. 5f,g). DAPI staining of a section at 800 μm from the tip of the Rc

(Fig. 5e) confirms the localization of the epidermal cell layer (densely stained nuclei in the outer region of the section).

Effect of a short hypoxia stress on shoot water potential and *QpHb1* expression level in sessile and pedunculate oaks

We decided to monitor the plant water potential and the expression pattern of *QpHb1* in sessile oak and in another species considered more tolerant to soil waterlogging, pedunculate oak. As the expression pattern of a gene coupled to the plant physiological status may help understand the difference in tolerance between species, we investigated the effect of imposing hypoxia on the roots for 48 h (Figs 6, 7b). The monitoring of O_2 evolution in the rhizosphere of flooded oaks (Fig. 7a) shows that O_2 concentration rapidly decreased within 12 h of the start of treatment. During the next 36 h, the O_2 concentration remained low. The differences in shoot water potential monitored during the same time period in the two oak species indicated a rapid decrease in shoot water potential in both species as early as 3 h after the start of root submergence (Fig. 6). However, the decline was much more pronounced in sessile than in pedunculate oak. An ANOVA of the expression of *QpHb1* during the same stress period in sessile and pedunculate oaks indicated a significant negative effect of hypoxia after 24 and 48 h for both species (Table 1). However, there was also a significant species effect on *QpHb1* expression. Furthermore, the monitoring of *QpHb1* indicated a constant and gradual decline in *QpHb1* expression in sessile oak, whereas there was a significant transient increase in *QpHb1* in pedunculate oak during the first hour of stress (Fig. 7b). This initial rise was followed by a significant drop within 3 h, then the level of expression increased to 60% of its initial level and remained low thereafter, being not significantly different from sessile oak after 24 h of hypoxia.

Discussion

Cloning and characterization of the *QpHb1* gene

The *QpHb1* gene is the first nonsymbiotic Hb gene that has been isolated from oak roots and characterized. The *QpHb1* deduced protein shows characteristic features of other plant

Table 1 ANOVA, effect of species and hypoxia treatment on expression of *QpHb1* in roots

Source	df	F	P
Species	1	8.33	0.007
Hypoxia	6	12.56	0.0001
Species \times hypoxia	6	3.117	0.017
Error	31		

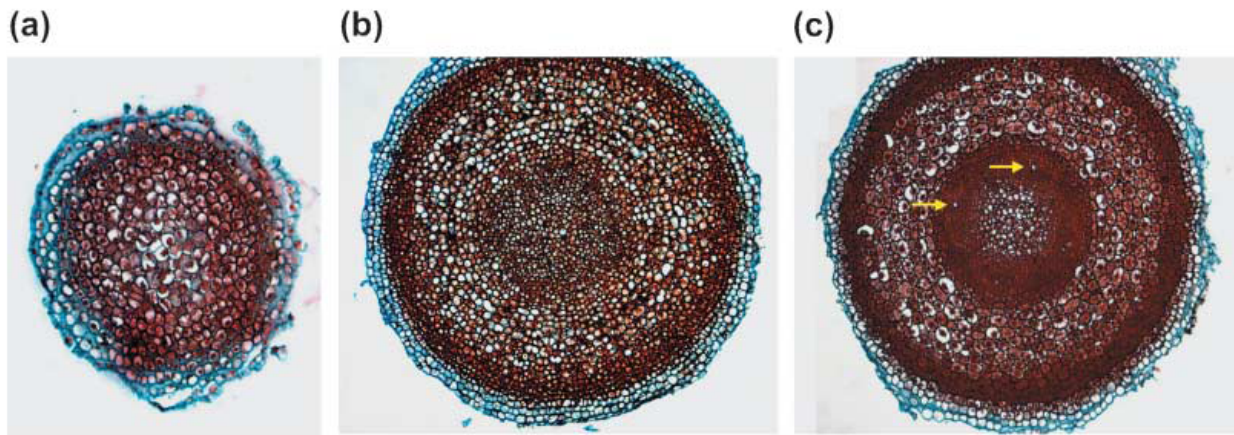


Fig. 4 Safranine-fast green staining of cross sections at different distances from the tip of the root cap: (a) 200; (b) 400; (c) 600 μm . In the sections, cellulose cell walls are stained green; cytoplasm and nuclei, red. Arrows, protophloem. Bar, 100 μm .

Hbs (Fig. 1). These include the CD1 phenylalanine, C2 proline and F8 proximal histidine residues needed for heme binding, and the E7 distal histidine which is involved in ligand binding (Ota *et al.*, 1997). A cysteine residue found in most known plant nonsymbiotic Hbs is also present in QpHb1. Sequence comparison also confirmed the presence of the plant Hbs signature (SN)-P-x-(LV)-x(2)-H-A-x(3)-F (Dickerson & Geis, 1983). Based on these structural features, QpHb1 can be categorized as a class 1 nonsymbiotic Hb. Furthermore, the phylogenetic analysis of QpHb1 with other known Hb sequences available in public resources highlights the fact that QpHb1 forms a cluster together with other woody species (*Trema tomentosa*, *Trema orientalis*, *Parasponia andersonii*, *Alnus firma*, *Malus domestica*), indicating its close primary structural relationship with other lignified plants (Fig. 2). However, as seen in the evolutionary tree constructed with polypeptide sequences, QpHb1 clearly belongs to subgroup Ib, clustering with *A. firma*, pear and apple Hbs, but distant from the *Trema* nonnodulating (Ulmacean) plant Hbs, which are closely related to the nitrogen-fixing tree *P. andersonii* Hb (subgroup Ia).

In plants, three distinct types of Hb have been isolated: symbiotic, nonsymbiotic and truncated. The nonsymbiotic Hbs are widespread in the plant kingdom, suggesting multiple or essential functions (Hebelstrup *et al.*, 2007). They are characterized by a relatively low abundance in plant tissues and are divided into two classes (Dordas *et al.*, 2003a). Class 1 nonsymbiotic Hbs have a high affinity for oxygen (Hill, 1998), are induced during flooding (Taylor *et al.*, 1994; Hunt *et al.*, 2002; Dordas *et al.*, 2003b), and their induction is generally related to cellular ATP levels (Nie & Hill, 1997). In contrast, class 2 nonsymbiotic Hbs have a lower affinity for oxygen and are induced by low temperatures (Trevaskis *et al.*, 1997) and cytokinin treatment (Hunt *et al.*, 2001, 2002). However, the molecular mechanisms behind the distinct

induction characteristics of both classes of nonsymbiotic Hbs have yet to be elucidated.

All plant species studied to date possess nonsymbiotic Hb gene(s), the expression patterns of which vary in different plant tissues and in response to different stress conditions (Hunt *et al.*, 2001). The analysis of the *QpHb1* expression pattern in the various plant organs shows that the gene is strongly expressed in roots as compared with the other plant vegetative tissues, suggesting a more prominent role for *QpHb1* in roots. In most studies in which nonsymbiotic Hb expression has been analysed in the different organs of the same plant, high levels of expression have been found in root tissues (Jacobsen-Lyon *et al.*, 1995; Andersson *et al.*, 1996; Seregelyes *et al.*, 2000; Larsen, 2003; Silva-Cardenas *et al.*, 2003; Qu *et al.*, 2005).

At the tissue level, there is indication that the expression of nonsymbiotic Hbs varies greatly, with generally high levels reported in metabolically active or stressed tissues (Hill, 1998). Using nonradioactive *in situ* hybridization, we describe the distribution under normal conditions of *QpHb1*, in the first millimetre of the primary root tip, in the region including the meristem and the Rc. This is the first report of *in situ* localization of a nonsymbiotic Hb gene in the primary root of a woody plant. In the root meristem, *QpHb1* was mainly expressed in the outer ground meristem and in the differentiation region of the xylem. Differential expression of nonsymbiotic Hb was also reported in soybean, and Northern blot analysis indicated a higher expression in the elongating region than in the root tip (Andersson *et al.*, 1996). Using GUS reporter fusions in *L. corniculatus*, the *C. glauca* nonsymbiotic Hb promoter was detected primarily in meristematic regions of the root tip, the vascular stele and the pith parenchyma (Jacobsen-Lyon *et al.*, 1995). Furthermore, the activity of Hb promoters of *P. andersonii* and *T. tomentosa* was detected in root meristems and in the vascular cylinder of

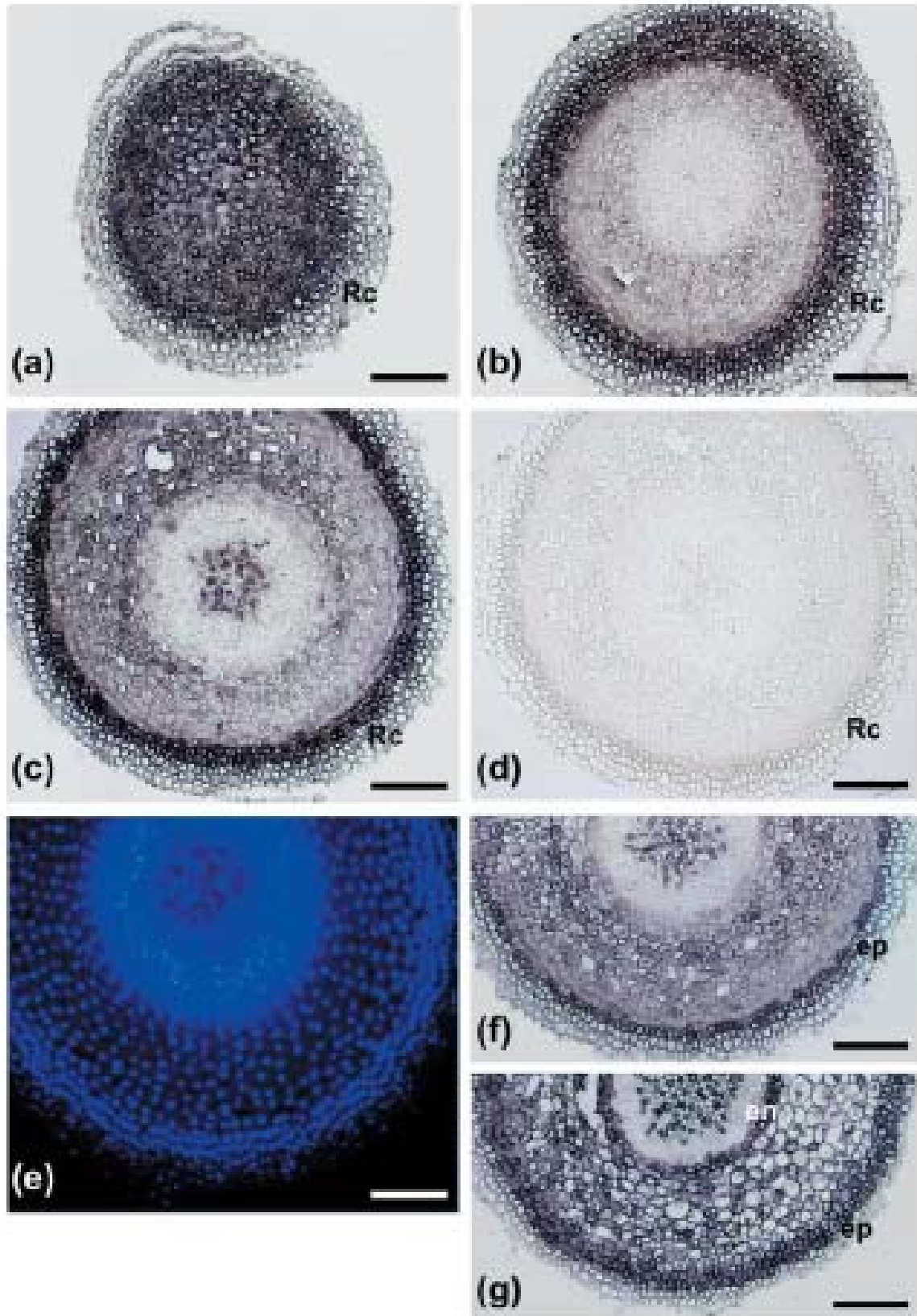


Fig. 5 *In situ* *QpHb1* expression in cross sections at different distances from the tip of the root cap: (a) 200; (b) 400; (c–d) 600; (e–f) 800; (g) 1000 μm . Negative control with sense probe in (d). DAPI-stained section in (e). Rc, root cap; ep, epidermis; en, endodermis. Bar, 100 μm .

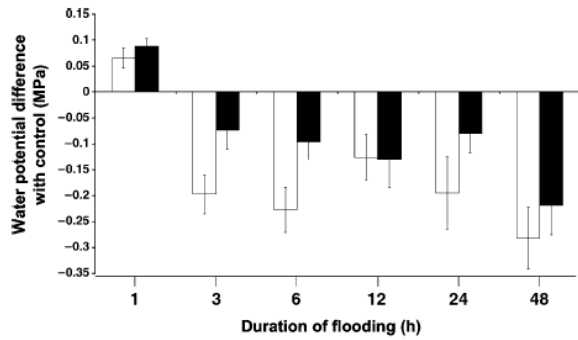


Fig. 6 Changes in the difference in shoot water potential between stressed and control sessile (*Quercus petraea*; open bars) and pedunculate (*Quercus robur*; closed bars) oak seedlings exposed to a short hypoxia treatment. Vertical bars, \pm SEM, $n = 6$.

transgenic tobacco (Bogusz *et al.*, 1990). In monocots, nonsymbiotic Hbs were also immunologically localized in differentiating tissues, mainly the vascular and Rc cells (Arechaga-Ocampo *et al.*, 2001; Lira-Ruan *et al.*,

2001; Ross *et al.*, 2001). In addition to these data, immunolocalization of nonsymbiotic Hb1 in rice has been described in the cytoplasm in primary differentiated and differentiating cell types, including the Rc and the differentiating xylem (Ross *et al.*, 2001). These results suggest that nonsymbiotic Hb proteins are synthesized early during the differentiation of conductive elements, as well as in cells in more advanced stages of xylogenesis. We show here that the expression of *QpHb1* is detected at a very early stage of xylem differentiation, but not in the protofloeum, the earliest vascular tissue to differentiate in *Quercus* and commonly in angiosperm roots. *QpHb1* expression was also found in the Rc cells. A similar localization was reported for a nonsymbiotic Hb protein in 4-d-old rice seedlings, and the authors relate this localization to the formation of new cell types essential for growth of the root (e.g. gravisensing cells; Ross *et al.*, 2001).

However, more recent data on the role of nonsymbiotic Hb in regulating the level of NO in plants may also provide some clues as to the spatial root distribution and function of

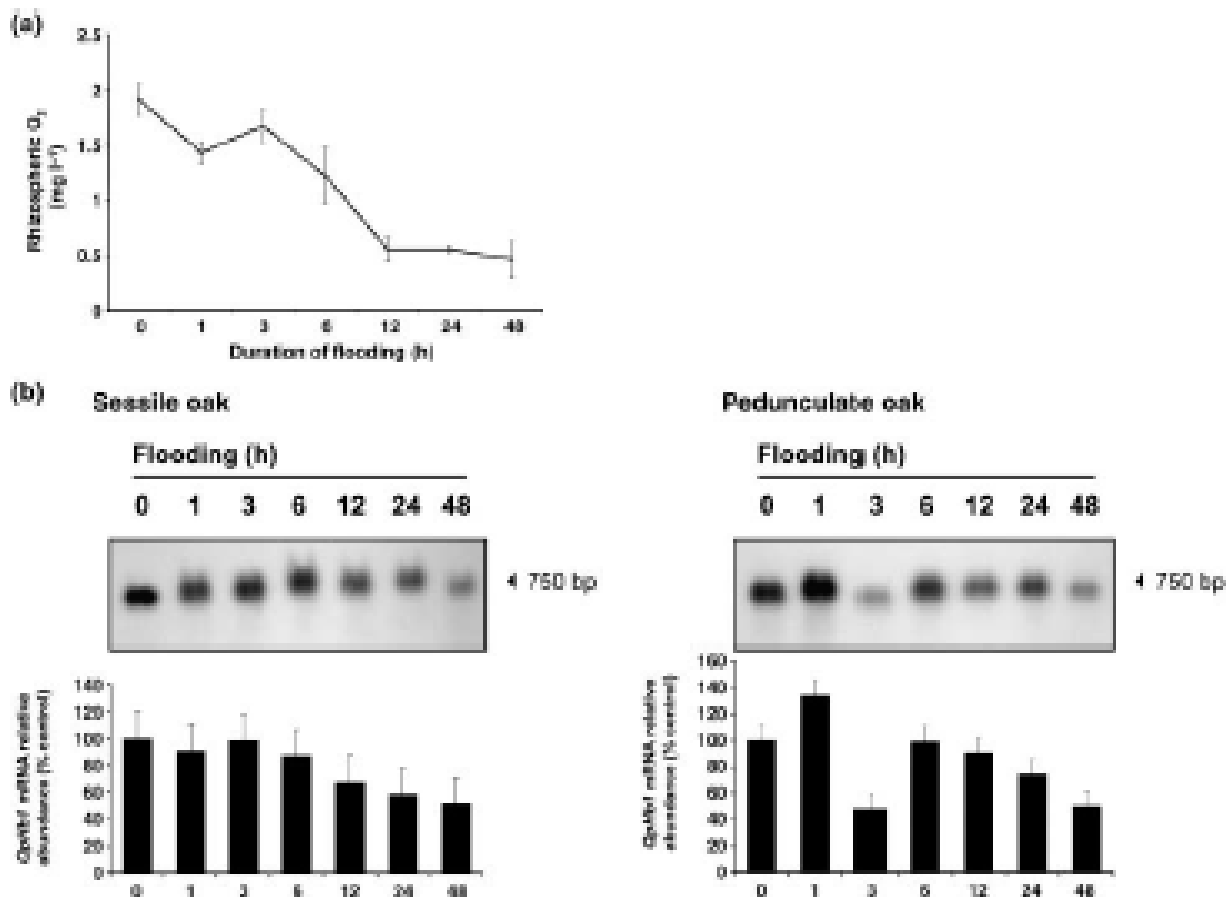


Fig. 7 (a) Evolution in oxygen concentration in the rhizospheric solution during the hypoxia treatment. Vertical bars, \pm SEM, $n = 3$. (b) Timecourse analysis of *QpHb1* gene induction under hypoxia in sessile (*Quercus petraea*) and pedunculate (*Quercus robur*) oak. Five-wk-old oak seedlings were submerged for 0, 1, 3, 6, 12, 24 and 48 h. For each treatment, 5 μ g total RNA was used to determine the transcript level by Northern hybridization. Densitometric scanning was used to quantify the signal as indicated in Fig. 3; the ANOVA for main effects is shown in Table 1

QpHb1 (Ross *et al.*, 2002; Igamberdiev & Hill, 2004; Sakamoto *et al.*, 2004). NO is a stress-related metabolite known to participate in the signalling pathway during plant stress responses, produced in response to a variety of environmental cues (Durner & Klessig, 1999). Interestingly, NO has also been associated with many physiological and developmental processes, including the differentiation of xylem cells (Gabaldon *et al.*, 2005). Thus the spatial expression pattern of *QpHb1* in the protoxylem cells is also in agreement with a role of Hb in controlling NO accumulation in these cells, further supporting a close relationship between both molecules in controlling developmental processes, as proposed previously in *Arabidopsis* (Hebelstrup *et al.*, 2006). On the other hand, the spatial distribution of *QpHb1* in the protoderm cells, the first layer of living cells in the root in contact with the outside environment, strongly suggests that *QpHb1* could play an important role during the perception of changes in the rhizosphere, such as oxygen depletion. In order to address this possibility, we compared the expression of *QpHb1* during a short hypoxia stress in two sympatric oak species: sessile and pedunculate oak.

Possible role of *QpHb1* in the response to hypoxia

Monitoring shoot water potential during hypoxia in both pedunculate and sessile oak seedlings indicated a contrasting response for both species (Fig. 6). The difference between stressed and control plants shows that pedunculate oak is physiologically less affected than sessile oak early during hypoxia. These results suggest an enhanced tolerance to short hypoxia in pedunculate oak. Parelle *et al.* (2006, 2007) have previously shown that pedunculate oak is more tolerant than sessile oak to long-term hypoxia, and the enhanced tolerance could be attributed to a higher capacity to develop aerenchyma tissue as well as adventitious roots. However, no data are available on differences in the early response to hypoxia in both species. The expression analysis of *QpHb1* during hypoxia in both oak species showed that the gene is induced transiently in pedunculate oak (Fig. 7b). However, the decreased expression in sessile oak was more unexpected. Class 1 Hb genes have been shown to affect NO levels in several experimental systems, and their main function may be in the removal of NO during oxygen deficiency in plants (Dordas *et al.*, 2003a, 2004; Perazzolli *et al.*, 2004). Hypoxic root cultures of Hb-deficient alfalfa and maize mutants accumulated high levels of NO, 2–3 cm behind the root tip, during the first 24 h of hypoxia (Dordas *et al.*, 2003b, 2004; Igamberdiev & Hill, 2004). NO and Hb are intimately linked during the response to hypoxia in many biological systems (Durner & Klessig, 1999; Dordas *et al.*, 2003b; Wendehenne *et al.*, 2004). Both NO and Hb levels increase in tissues with a similar temporal sequence after exposure to hypoxia or anoxia (Dordas *et al.*, 2004). It was thus concluded by the authors that Hb modulation of NO is closely linked to short-

term survival under hypoxic or anoxic stress. The data presented here could support the role of nonsymbiotic Hb1 in the spatial and temporal control of NO accumulation.

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2. *A novel non-symbiotic hemoglobin from oak: roles in root signaling and development?*

Cette partie a fait l'objet d'une publication acceptée le 13 mars 2008 dans le journal *Plant signaling and Behavior*. Les auteurs sont C. Parent, A. Berger, N. Capelli, M. Crèvecoeur et J. Dat

Résumé:

Les adaptations moléculaires et cellulaires des espèces ligneuses aux changements environnementaux sont encore mal connues. Dans le cadre d'une précédente étude, nous avons cloné et caractérisé un nouveau gène d'hémoglobine (*QpHb1*) chez le chêne qui présente une distribution cellulaire spécifique au niveau des racines. Le gène *QpHb1* est fortement exprimé dans les cellules du protoderme et du protoxylème chez les deux espèces de chênes (*Q. petraea* and *Q. robur*) lesquelles présentent une adaptation contrastée à l'ennoyage. Cette expression se localiserait au niveau des mêmes zones que le monoxyde d'azote et de façon constitutive. L'hémoglobine non-symbiotique pourrait via la S-nitrosylation jouer un rôle dans la signalisation des changements au niveau rhizosphérique et/ou dans le contrôle de certains aspects du développement racinaire.

Article Addendum

A novel non-symbiotic hemoglobin from oak

Roles in root signaling and development?

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The cellular and molecular adaptations of non-model woody species to environmental changes are still poorly understood. We have cloned and characterized a novel non-symbiotic hemoglobin from oak roots (*QpHb1*) which exhibits a specific cellular distribution in the root. The *QpHb1* gene is strongly expressed in the protoderm and the protoxylem cells in two *Quercus* species (*Q. petraea* and *Q. robur*) with contrasting adaptive potential to drought and flooding. The constitutive expression of *QpHb1* in both oak species in specific root tissues combined with the reported presence of nitric oxide in the same tissues and its potential for protein S-nitrosylation could support a role for non-symbiotic hemoglobins in signaling changes in the root environment and/or in controlling some aspects of root development.

Introduction

The genus *Quercus* (oak) includes over 300 woody species, widespread in the northern hemisphere, where it represents the dominant vegetation of temperate forests.¹ Among these, two sympatric predominant European oak species, pedunculate and sessile oak, are known to display different ecological requirements. The two species generally cohabit in forest ecosystems; however, sessile oak is found more frequently on well drained soils, whereas pedunculate oak can populate poorly drained sites.² This differential spatial distribution is due to the higher tolerance of pedunculate oak for soil waterlogging.

In an attempt to identify pertinent markers to discriminate between the two species, we have cloned and characterized a class I non-symbiotic hemoglobin gene. This class of Hbs has a high O₂ affinity and is induced under hypoxic conditions.^{3,4} However, because of an extremely low O₂-dissociation constant, class I non-symbiotic Hbs may in fact participate in the regulation of cellular

(NO) levels thus improving the redox and/or energy homeostasis of plant cells during hypoxia.^{5,6}

Non Symbiotic Hb Shows Differential Distribution in Oak

We have recently showed that *QpHb1* expression exhibits organ specificity in sessile oak.⁷ We also found that in pedunculate oak (Fig. 1) there is a similar spatial distribution of expression, with a decreasing gradient from roots to leaves. However overall, transcripts are more abundant in pedunculate oak and more specifically in the roots. The fact that ns-Hb is strongly expressed in both species under normal growth conditions, suggests a constitutive role for this protein.

The cellular localization of *QpHb1* in sessile oak roots, as evidenced by in situ hybridization, indicates that hemoglobin transcripts are most abundant in the protoderm and the protoxylem cells. A very similar pattern of expression was also found in pedunculate oak under control conditions (Fig. 2). What could be the significance of such a localization? Could the constitutive expression of *QpHb1* play a role in root development?

A Role for ns-Hb in Root Development and Signaling?

The localization of ns-Hb in cells undergoing differentiation towards xylem elements but not in protophloem cells, suggests a specific role for ns-Hb in the development of root xylem tissues. Interestingly, plant cells which are just predetermined to irreversibly *trans*-differentiate in xylem elements, show a burst in NO production.⁸ This burst is observed when the cells reach a “point of no return” and undergo programmed cell death (PCD), essential to xylem development. The NO/Hb couple is well known to detoxify NO to nitrate via a NAD(P)H dependant mechanism, and thus ns-Hb could play a predominant role for root cell survival by regulating NO levels.⁹⁻¹¹ Other authors have also shown a co-localization of ns-Hb with xylem cells during xylem differentiation in rice.¹² It would be extremely interesting to confirm a responsible for protoxylem differentiation.

NO has also been associated with cell signaling through S-nitrosylation of proteins. This post-translational process permits a conformational change of target proteins which can modify their activity.^{13,14} Recent data suggest that this process is widespread in plants and can be used in regulating various cell processes. Our data

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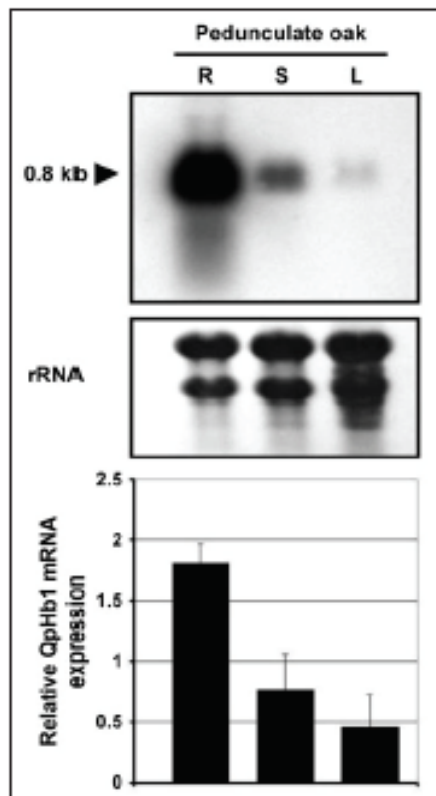


Figure 1. Transcript pattern of the *QpHb1* gene in vegetative organs of pedunculate oak (*Quercus robur*). Total RNAs were extracted from roots, stems and leaves of seedlings grown under control conditions for 5 weeks. R, roots; S, shoot; L, leaves.

showing that ns-Hb is strongly expressed in the root protoxylem could also indicate that the regulation of S-nitrosylation via NO detoxification by ns-Hb or the direct S-nitrosylation of ns-Hb could be key features of root-to-shoot signalling. Indeed, the xylem serves as a direct route between the root and the shoot for various signaling molecules. The constitutive presence of ns-Hb in this tissue may thus serve as a “standby surveillance system” for rapid signalling of changes in the root environment.

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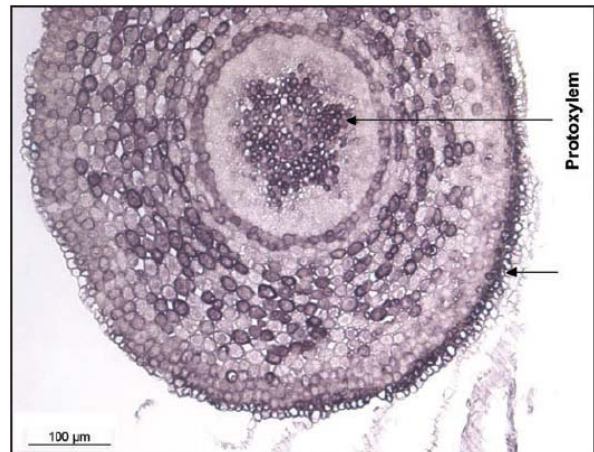


Figure 2. In situ *QpHb1* expression in a cross section realized at 800 µm from the tip of the root cap of *Quercus robur* grown for 5 weeks under control conditions.

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Les deux espèces de chêne présentent une adaptation contrastée à l'ennoyage. Pour caractériser plus précisément de quelle manière se manifeste cette différence, nous avons étudié la croissance ainsi que plusieurs paramètres physiologiques pendant un ennoyage long de 28 jours. Nous avons également entrepris de suivre l'évolution de la porosité du cortex racinaire. Les résultats obtenus confirment la tolérance accrue du chêne pédonculé et notamment sa capacité à former des lacunes dans les racines, pouvant s'apparenter à des aérenchymes. Les précédents résultats ont montré que l'hémoglobine non-symbiotique semblait participer à la signalisation et la réponse rapide à l'hypoxie. Nous nous sommes demandé si elle pourrait également jouer un rôle quand le stress se prolonge et que les adaptations anatomiques et morphologiques se mettent en place. Pour répondre à cette question, nous avons étudié l'expression de QpHb1 dans le système racinaire initial lors de l'ennoyage chez les deux espèces mais également dans les racines d'adaptations ou adventives.

*B. Adaptation contrastée des deux
espèces de chênes et expression de
QpHb1 en réponse à un stress long*

Response of two oak species to flooding stress: involvement of nonsymbiotic hemoglobin

Cette partie fait l'objet d'une publication en préparation. Les auteurs sont C. Parent *et al.*

Résumé :

Le chêne sessile et le chêne pédonculé sont deux espèces génétiquement proches mais qui présentent une différence de tolérance à l'ennoyage. L'hémoglobine non-symbiotique améliore la survie des plantes en condition d'hypoxie et son expression pourrait être impliquée dans la tolérance à l'ennoyage. La croissance ainsi que certains changements morphologiques, anatomiques et physiologiques ont été étudiés chez les deux espèces en réponse à un ennoyage de 28 jours. L'expression de l'hémoglobine non-symbiotique *QpHb1* a été analysée par *Northern blotting* et par hybridation *in situ*. La croissance est fortement inhibée chez les deux espèces après 28 jours d'ennoyage. Cependant, l'impact de l'ennoyage est clairement plus marqué chez le chêne sessile. De même, la photosynthèse et le potentiel hydrique diminuent même si le chêne pédonculé en situation d'ennoyage réussit à maintenir son statut hydrique au même niveau que celui de plantes en conditions contrôlées pendant les deux premières semaines de traitement. L'analyse par *Northern* montre que l'expression de *QpHb1* chute dès le début du stress chez le chêne sessile alors que pour le chêne pédonculé, l'expression augmente initialement et diminue seulement après 21 jours d'ennoyage. L'hybridation *in situ* révèle une accumulation de *QpHb1* au niveau des cellules du protoxylème sauf chez l'espèce sensible en conditions d'ennoyage. L'analyse cytologique montre que le chêne pédonculé a tendance à développer des aérénchymes. Les racines adventives, autre modification adaptative, présentent une forte accumulation de transcrits d'hémoglobine non-symbiotique chez le chêne pédonculé, environ 2 fois plus que celles du chêne sessile en conditions contrôles. Cette expression semble se localiser au niveau du protoderme et de certaines cellules du cortex. Les résultats de notre étude suggèrent que la régulation spatio-temporelle de *QpHb1* pourrait être impliquée dans la capacité de tolérance ainsi que dans la cascade de signalisation menant au développement de ces adaptations.

Title:**Response of two oak species to flooding stress: involvement of nonsymbiotic hemoglobin****Summary**

- Sessile (*Quercus petraea* Matt. L.) and pedunculate (*Quercus robur* L.) are two closely related oak species that display a strong differential tolerance to soil flooding. Non-symbiotic hemoglobin has been shown to enhanced survival of plants during hypoxic conditions and its expression could be related to flooding tolerance.
- Physiological, morphological and cellular responses of the two oaks were monitored. In addition, *QpHb1* expression in roots was analyzed by *Northern blotting* and *in situ* hybridization.
- Flooding strongly reduced growth of both oak species but this inhibitory effect was more pronounced in sessile oak. In the same way, photosynthesis and xylem water potential were inhibited in both species even if pedunculate oak succeeded in maintaining its water status at control levels during the first two weeks of flooding stress. *Northern* analysis revealed that *QpHb1* expression fell after 7 days of flooding in sessile oak whereas in pedunculate oak *QpHb1* expression was slightly enhanced and declined only after 21 days of soil waterlogging. *In situ* hybridization indicated that *QpHb1* accumulated in protoxylem cells except in the sensitive species exposed to 14 days of flooding. Anatomical analyses showed that pedunculate oak tends to develop more important aerenchyma type lacunae in its roots whereas this was much less pronounced in sessile oak. Another adaptive response included the formation of adventitious roots which were more frequently present in pedunculate oak and exhibited a strong accumulation of *QpHb1*, about 2 fold that in control sessile oak. Furthermore, the expression seems to localize in the protoderm and in some cortical cells.
- The spatio-temporal expression of *QpHb1* could be directly involved in the tolerance of pedunculate oak to flooding and/or in a signaling cascade leading to the establishment of adaptive features.
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Keywords: adaptation, adventitious roots, aerenchyma, flooding, hypoxia, nonsymbiotic hemoglobin, *in situ* hybridization, oak.

Abbreviations: ANPs, Anaerobic proteins; DW, Dry weight; Fw, fresh weight; NO, nitric oxide; ns-Hb, non-symbiotic hemoglobin; ROL, Radial oxygen loss; SLA, Specific leaf area

Introduction

In temperate forests, two oak species predominate, sessile oak (*Quercus petraea* Matt. Liebl.) and pedunculate oak (*Quercus robur* L.). The two species cohabit in most of Europe, but their local distribution is not the same. Indeed, they have different requirements for various environmental factors such as light, soil, nutrition and humidity and consequently they do not occupy the same ecological niche (Epron & Dreyer, 1990). Sessile oak grows on dense, well-aerated and quite acidic soils while pedunculate oak prefers compact, calcareous and hydromorphic ground (Levy *et al.*, 1992). In fact, the two species exhibit differential tolerance to flooding, with sessile oak considered more sensitive than pedunculate oak (Parelle *et al.*, 2006). Many phenotypic differences distinguish the two species, but they are genetically close and, even if molecular differences exist, they are difficult to identify. As a result, recent research has targeted markers differentially expressed in both species, such as genes implicated in the response to flooding (Folzer *et al.*, 2006; Parelle *et al.*, 2007; Parent *et al.*, 2008b).

Climate change, such as global warming, is likely to have important ecological ramifications through direct and indirect modification of local precipitation regimes. The Intergovernmental Panel on Climate Changes (IPCC, 2007) reports that heavy precipitation events will become more frequent over most areas of the globe. In addition, anthropic activities such as the removal of vegetation, soil proofing due to building, the absence of storm drains and crop over-irrigation will all increase the occurrence of flooding in natural ecosystems (Bailey-Serres & Voeselek, 2008). Furthermore, hydromorphic soils are often inappropriate for agriculture or construction and are thus most often left with forest cover. As a result, forest species, and more specifically trees, will increasingly be exposed to soil waterlogging.

Temporary submergence generally leads to growth inhibition and may ultimately cause plant senescence and death. Moreover, flooding occurs principally in the spring, affecting species regeneration and thus modifying species abundance and distribution (Kozłowski, 1997). Common signs of flooding stress include reductions in root and shoot growth, as well as a decrease in specific leaf area (Dat *et al.*, 2004). However, the first signs of flooding stress will more generally be observed on the plant physiological processes. Indeed, soil inundation will induce multiple plant physiological dysfunctions which will have drastic effects on normal cellular processes, potentially leading to water and nutrient

imbalance and/or deficiency. For instance, flooding will not only reduce stomatal conductance, it may also limit water uptake as a result of a decline in hydraulic conductivity consequent to a decrease in root permeability (Dat *et al.*, 2006; Parent *et al.*, 2008c). Furthermore, stomatal closure, as well as other factors, such as leaf chlorophyll degradation or early leaf senescence, will contribute to photosynthesis inhibition (Pezeshki, 1993). The consequent decline in photoassimilate production will be an important factor limiting metabolic activity. Thus, maintenance of photosynthetic activity is considered a key factor affecting survival to flooding stress (Chen *et al.*, 2005).

However, the adverse effect of flooding is largely due to the hypoxic conditions that develop in the rhizosphere as a result of water saturation of the soil. Thus, the principal consequence of soil waterlogging is a decline in O₂ availability. As a consequence, aerobic ATP production cannot proceed, and a switch to anaerobic fermentation will take place (Agarwal & Grover, 2006). This metabolic change will be accompanied by a general decrease in protein synthesis, except for about 20 newly synthesized anaerobic proteins (ANPs) including a class-1 non-symbiotic hemoglobin (ns-Hb). Indeed, ns-Hb is induced under hypoxic conditions in a number of different species (Duff *et al.*, 1997; Trevaskis *et al.*, 1997; Parent *et al.*, 2008a). However, its role during the hypoxia response is still not clearly established but its potential link with nitric oxide (NO) is particularly interesting. Indeed, class 1 ns-Hb can modulate NO levels and thus act as a regulator of the NO response (Perazolli *et al.*, 2004). In fact, an Hb/NO cycle has been proposed in which the reaction between Hb and NO consumes NADH and maintains ATP levels in an, as yet unknown, mechanism (Igamberdiev *et al.*, 2005). Furthermore, we have also recently shown that transcripts of an oak class 1 non-symbiotic Hb1 (*QpHb1*) were not only strongly expressed in protoxylem cells, some cortical cells and most notably the root protoderm layer (the outermost layer of living cells in the root) but also accumulated during early flooding stress, thus suggesting a potential role in flooding stress signaling (Parent *et al.* 2008a; 2008b). However, the localization of *QpHb1* could also suggest other roles during the flooding response, most notably through NO regulation. Indeed, a regulatory function of NO in, aerenchyma formation and adventitious root initiation has been demonstrated (Pagnussat *et al.*, 2003; Igamberdiev *et al.*, 2005; Lanteri *et al.*, 2006). Consequently, ns-Hb1 may also participate in these morphological changes by regulating NO phytotoxicity and/or signaling function.

The development of aerenchyma in roots is considered as a key adaptation to flooding and can greatly enhance plant survival (Evans, 2003). In fact, root cortex porosity increases in most plants exposed to flooding, even if it does not necessarily lead to functional aerenchyma (Drew *et al.*, 2000; Colmer, 2003; Evans, 2004). However the signaling pathways leading to these morphotypes are still unclear. Other morphological changes are also believed to help alleviate hypoxic stress. For instance, hypertrophied lenticels can develop at the stem base. These are believed to facilitate downward diffusion of O₂ and/or help vent phytotoxic byproducts of anaerobic metabolism (Kozłowski & Pallardy, 2002; Mielke *et al.*, 2003; Folzer *et al.*, 2006; Parelle *et al.*, 2006). Another important morphological adaptation to flooding is the development of adventitious roots, which replace the original root system when the latter becomes necrotic and incapable of supplying the shoot with the required water and minerals (Gibberd *et al.*, 2001; Malik *et al.*, 2001; Bacanamwo and Purcell, 1999). Interestingly, the development of some of these adaptive processes has been related to NO function.

With the aim of better understanding the mechanisms involved in the susceptibility/tolerance of oak to flooding, we have undertaken a comparison of physiological parameters, root morphological and cellular changes and, hemoglobin expression, in two oak species (sessile and pedunculate) with contrasting tolerance to flooding.

Materials and methods

Plant material

Sessile (*Quercus petraea* (Matt.) Liebl.) and pedunculate (*Q. robur* L.) oak acorns, harvested in northeastern France, were provided by the Office National des Forêts (ONF) (Preney *et al.*, 1997) and stored in moist vermiculite at 4°C until use. The pericarps were removed from acorns, which were sterilized by dipping in a 1% bleach solution and oxygenated overnight in running water to favor germination. Individual acorns were then grown in 1.8 l plastic pots containing river sand (Dekoline Carat 4, Aquatic Nature, Belgium). This substrate was chosen to enable harvesting of quality roots for histological and molecular studies. The plants were grown for 5 weeks in a controlled growth chamber with environmental conditions set as follows: a 16 h photoperiod, a photosynthetically active radiation (PAR) of 170 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy level (provided by High Pressure Sodium lamps), an average temperature of 20°C \pm 1.5°C and a relative humidity of 70%. Plants were irrigated 4 times daily with a fertilizer

solution (0.5 ml l⁻¹, NPK 6/5/6, SEM, Germany) using an automated *Ebb-and-Flow* system. After five weeks, which corresponded to the establishment of the first true leaves, each seedling was checked for leaf morphology and growth characteristics to make sure the seedlings grown from the acorns provided by the ONF were not misidentified. A clear difference in leaf morphology and growth characteristic can be observed between both species. However, when in doubt, the seedlings were discarded. Low oxygen stress was imposed by immersing 5 week-old seedlings up to the root collar in the irrigation solution for the desired period (from 7 to 28 days). Control plants (unflooded) were harvested in parallel to flooded plants at each time point.

Physiological and biomass measurements

For stem length and biomass determination, leaves, stem and roots were harvested from control and flooded plants, they were weighed immediately and stem height was measured. Tissues were then dried at 70°C for a minimum of 72h and weighed. Leaves were then scanned for determination of foliar surface using the free image analysis ImageJ 1.40 software (<http://rsbweb.nih.gov/ij/index.html>; Bethesda, Maryland, USA). Specific Leaf Area (SLA) was expressed as the ratio of leaf area to leaf dry mass (cm² g⁻¹). These measurements were obtained from 3 plants for each sampling point, for each treatment (unflooded/flooded) and were repeated three independent times.

At each point of experience, net photosynthesis and stomatal conductance of single, attached, mature leaves (one leaf of three different plants for control and flooding treatment) was measured using a Li-6400 Portable System (Li-Cor® Inc., Lincoln, NE, USA). Measurements were made at growth temperature, the flow velocity was set as 200m⁻²s⁻¹, the photosynthetic photon flux density was fixed at 400µmol m⁻² s⁻¹ and the CO₂ concentration injected in the reference chamber was set at 400 µmol mol⁻¹. These measurements are repeated three independent times.

Shoot water potential measurements were made with a Scholander type pressure chamber (DPI 700, Druck, France) on whole shoots of flooded and unflooded sessile and pedunculate oak seedlings, as described in Folzer *et al.* (2006). The average values for shoot water potential of unflooded and flooded plants were calculated at each time point from 12 seedlings for each species, obtained from 4 independent experiments.

Molecular analysis

For northern blot analysis, immediately after harvest, the samples were frozen in liquid nitrogen and stored at -80 °C until used. The RNA was then extracted and processed as described by Parent *et al.* (2008b). To obtain a gene-specific probe for *QpHb1*, a 349 bp DNA fragment was amplified and labelled by PCR from both the 5'-UTR and coding sequence of the cDNA clone according to PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Germany). Primers used were *QpHbsp* (5'-TTTCCAAATCTCTAACTAATTCTTGACC-3') and *QpHbrp* (5'- AAGTTGCACCGCTGATTCACAAGTCAT-3'). The membranes and the hybridization conditions were also prepared as described by Parent *et al.* (2008b). The northern analysis was replicated three times with RNA from three independent experiments and a pool of at least 6 plants per experiment. Densitometric measurements were performed using ImageJ software.

In situ Hybridization

Root segments from sessile and pedunculate oak (7 mm long) were fixed in 0.25% glutaraldehyde (w: v) and 4% formaldehyde (w: v) in phosphate buffer. After washing, they were embedded in paraffin. Thin sections (7 µm) were hybridized overnight at 50°C in the hybridization solution with 10 ng µl⁻¹ of sense or antisense RNA probe. All protocols details were described in Parent *et al.* (2008b).

Histological study

For all histological studies, root systems of sessile and pedunculate seedlings at each time point of experience were gently washed in water and the root apex segment (about 5mm in a zone situated 2cm from the tip of the root) was cut with a razor blade. The segments were fixed in 3% formaldehyde (w/v) and 0.3% glutaraldehyde (w/v) in 0.1 M phosphate pH7.2 buffer during 4h, then were washed four times in PBS buffer and dehydrated in an graded ethanol series (50, 70, 90, 100% ethanol) and finally embedded in Technovit® 7100 (Kulzer, Germany). Semi-thin sections (5–8 µm) were stained with a 2,5% toluidine blue solution (Sigma-Aldrich, Germany) for 10 min, rinsed in water and observed with light microscope (Eclipse TE300, Nikon, Japan). Changes in root cell morphological characteristics (root porosity and roundness) were assessed in the digitized images with the image analysis ImageJ. Porosity was calculated from the digitized images as the percentage area occupied by intercellular spaces per total cortex area. Results are the combined analysis of at least 600 individual cells from three sections of three independent seedlings per treatment at each sampling time, in sessile and pedunculate oak obtained from three independent experiments.

For adventitious roots, segments were embedded in paraffin as described in Parent *et al.* (2008b) and stained in Alcian blue (1%) for 30 min and then in Safranin O (1%) for 10 min. About 8mm sections were photographed with a Leica DMIRE 2 microscope equipped with a digital DC300F CCD color camera.

Statistical analysis

The experiment consisted of three factors, two categorical (species and flooding treatment) and one metric (treatment duration). Data on stem height, mass, photosynthesis, stomatal conductance and xylem water potential were analyzed with a one-way analysis of variance (ANOVA) with the General Linear Model (GLM) procedure of SAS 6.0 (SAS Institute, Cary, NC). Individual means between unflooded and flooded for a same species at a point of experience were compared by the Student's *t* test.

Results

Differential growth responses of sessile and pedunculate oak during flooding

The shoot growth development of both oak species was found to be very different, irrespective of flooding stress (Fig. 1). In fact, control pedunculate oak stem length was about 200 mm while that of control sessile oak reached only 130 mm at the beginning of the experiment (Fig. 2) and, this difference in height remained during the entire experiment. A difference in stem increment between both species was also observed during flooding as height growth was inhibited as early as 7 days after the start of the treatment for sessile oak whereas it was affected only after 14 days in pedunculate oak.

Specific leaf area (SLA) of control sessile and pedunculate oak was nearly the same for the duration of the experiment (Fig. 3). However, SLA was significantly lower in flooded than in unflooded sessile oak after 14 days whereas it was only clearly reduced after 21 days in pedunculate oak.

Other shoot characteristics were differentially affected by flooding in both species. An analysis of variance indicated that species (S), experiment duration (D) and flooding treatment (F) significantly influenced leaf number per plant, total leaf area as well as specific leaf area (Table 1). However, leaf number per plant was the only shoot growth characteristic affected by an interaction between species and experiment duration (S x D), whereas total leaf area

and specific leaf area were affected by a combination of species, experiment duration and flooding treatment (S x D x F).

At the root level, both fresh (FW) and dry weights (DW) were affected by flooding in sessile oak (Table 2). In fact, fresh root growth ceased after 21 days. In contrast, the reduced root growth was much less pronounced for pedunculate oak, and roots still grew after 28 days of submergence. An analysis of variance indicated a significant effect of flooding and treatment duration on root FW and DW. Furthermore, a significant interaction of species, flooding and treatment duration on root FW was observed (Table 3).

Leaf FW was significantly different between flooded and unflooded seedlings of both species after 21 days. The analysis of variance further supported this observation as there was a significant interaction of species, flooding as well as between species, flooding and treatment duration on leaf FW (Table 3). In contrast, a significant effect of flooding on leaf DW was noted only in sessile oak after 28 days (Table 2) and the analysis of variance indicated a significant interaction between species and flooding only.

The fresh shoot/root ratio was only significantly increased by flooding treatment after 28 days in sessile oak and after 14 days in pedunculate oak (Table 1). In contrast, the dry shoot/root ratio was not significantly altered between flooded and unflooded sessile oak seedlings but it was significantly increased in pedunculate oak after 7 and 14 days of submergence (Table 1). Although, a comparison of root/shoot ratio between control and flooded seedlings, at each sampling point, does not show a significant trend, the analysis of variance clearly indicates a significant alteration of the ratio by flooding (Table 3).

Finally, the total FW and DW of both oak species was inhibited by flooding (Table 2). The reduction in growth was more pronounced for sessile oak with a significant difference between control and flooded seedlings for total FW and DW after 21 days. Similarly, the analysis of variance indicated that there was a significant effect of species and flooding on total DW and a significant interaction effect between species, treatment duration and flooding on total FW (Table 3).

Effect of flooding on physiological parameters and *QpHb1* expression

Stomatal conductance and photosynthesis declined rapidly in response to the flooding treatment (Fig. 4a). After 7 days, photosynthesis was about 60 and 54% of that of controls for pedunculate and sessile oak, respectively. Overall, it continuously declined in the flooded

plants irrespective of the species. However the decrease was generally more pronounced in sessile oak. After 28 days of flooding, the photosynthesis level of the flooded plants was about 20% that of the unflooded controls. The stomatal conductance didn't show the same continuous decrease, indeed since 7 days of flooding, in pedunculate oak, it was stabilized.

In contrast to photosynthesis, the effect of flooding on shoot xylem water potential was different for both species. Although, the xylem water potential declined in flooded plants during the course of the experiment, the effect was much more pronounced during early submergence in sessile oak (Fig 4b). In contrast, the xylem water potential of pedunculate oak declined gradually to reach a minimum value after 21 days.

In order to gain some insight into the molecular mechanisms involved in the differential flooding response of both oak species, we monitored the long term expression profile of *QpHb1*, a ns-Hb we had previously cloned and characterized in sessile oak (Parent *et al.*, 2008b). The expression profile of the gene shows a clear difference between both species (Fig 5). Indeed, *QpHb1* expression drops to 40% of its initial level within 7 days of submergence in sessile oak and reaches a minimum of 20% after 21 days. In contrast, *QpHb1* expression remains above control levels for 14 days and only drops to 60% of control level after 21 days in pedunculate oak.

In an attempt to localize *QpHb1* expression in the roots, *in situ* hybridization was performed (Fig 6). Based on the physiological analysis and the *QpHb1* expression profile obtained, we decided to compare *QpHb1* expression in control roots with that in plants flooded for 14 days. Indeed, after 14 days of flooding pedunculate oak seemed to be at a hinge point whereas sessile oak was already clearly affected. In sessile oak, *QpHb1* was expressed in the cells of the outer ground meristem in both control and flooded plants, but in flooded roots, the expression was more pronounced (Fig 6a and 6b). In both species, *QpHb1* mRNA was already found to accumulate in protoxylem in normoxic conditions (Fig 6a and 6c) (Parent *et al.*, 2008a; 2008b), but under hypoxic conditions, only pedunculate oak seedlings accumulated hemoglobin in their protoxylem cells (Fig 6b and 6d). Interestingly, contrary to sessile oak, *QpHb1* was found in the endoderm layer in pedunculate oak. In the four sections observed, *QpHb1* expression was observed in the cytoplasm of some disseminated cortical cells (Fig 6, arrows).

Differential root anatomical changes and adaptations during flooding in both oak species

One of the best characterized responses to flooding includes the development of lacunae spaces in the root cortex which are believed to either help alleviate oxygen deficiency (aerenchyma) or evacuate toxic by products of anaerobic metabolism (Dat *et al.*, 2006; Parent *et al.*, 2008c). In order to assess whether the development of lacunae was different in both oak species, we decided to undertake a detailed assessment of cellular changes taking place in the root cortex under flooding. As can be observed in Fig. 7, there is a clear increase in intercellular spaces in the cortex of both oak species after 28 days of flooding (Fig. 7c and 7f). In fact, we observed a clear disorganization of root cortical cells after 14 days of flooding (Fig 7e), which was more pronounced in pedunculate oak. This was confirmed by a significant decline in cell roundness after 14 days of flooding in pedunculate oak and after 21 days in sessile oak (Table 4). In addition, determination of cell cortex porosity indicated that there was a significant increase in cortex porosity with flooding (Fig 7) and the porosity was significantly higher in pedunculate than sessile oak.

In addition to root anatomical changes, we also observed significant differences in the initiation of adventitious roots and hypertrophied lenticels in both species. Figure 9 shows the typical adaptations found in pedunculate oak: (a) adventitious roots developed horizontally at the soil/air interface whereas the original root system was necrotic (b) hypertrophied lenticels on the stem base corresponding to an aggregation of loose cells.

Rather unexpectedly, both sessile and pedunculate oak present adaptive roots but they appeared more abundantly and earlier in pedunculate oak (after only 7 days of flooding) whereas their presence was found only after 28 days in sessile oak (data not shown). In order to investigate a possible role for ns-Hb in the tolerance and adaptation to flooding in oak, we analyzed the expression of *QpHb1* in adventitious roots, harvested after 28 days of flooding, in both oak species (Fig 10). The expression of *QpHb1*, relative to its level in control sessile oak roots, shows a clear difference between the two species. Indeed, the expression of ns-Hb in pedunculate oak roots was induced by more than two-fold whereas in sessile oak, it was inhibited by roughly 32% of its expression in controls.

Adventitious root anatomy clearly exhibited cortex lacunae with a radial development (Fig 11a). Safranin-O stained in magenta the lignified cell walls and the cytoplasm of the cells comprising the outer ground meristem, the cortex, the endodermis and the xylem vessels. In order to localize the strongly induced mRNA of *QpHb1* in adventitious roots of pedunculate oak (Fig 10b), *in situ* hybridization was performed at the same level as in Fig

11a. Results showed a high presence of transcripts in the protoderm, specifically in the cytoplasm (Fig 11b). Some of the cortical cells showed a strong expression of ns-Hb, but contrary to that observed in control roots and in 14 days-flooded roots, no *QpHb1* expression was noted in neither the xylem cells nor the endoderm.

Discussion

Contrasting physiological responses between sessile and pedunculate oak

Our integrated study not only confirms that sessile and pedunculate oak exhibit a contrasting response to flooding stress (Schmull & Thomas, 2000; Parelle *et al.*, 2006) but also demonstrates that these differences can be related to adaptive mechanisms which are more developed in pedunculate oak. An inhibition of stem elongation, leaf area as well as whole plant biomass was observed in both species as a result of flooding (Fig 2 and 3; Tables 1, 2 and 3). This growth effect is similar to that reported for poplar (Gong *et al.*, 2007), and in other tree species (Gravatt & Kirby, 1998), in field bean (Pociecha *et al.*, 2008), in maize (Lizaso *et al.*, 2001) and in rice (Suralta & Yamauchi, 2008). The global decrease in biomass production may be related to a reduced metabolic activity of hypoxic roots as indicated by the decline in several important physiological parameters i.e. photosynthesis, water deficit (Table 2). Furthermore, the negative impact of flooding on root functioning is further confirmed by the higher increase in shoot/root dry weight ratio in flooded plants during the first weeks of flooding than in unflooded ones (Table 2). With prolonged submergence, the root/shoot ratio of flooded plants reached that of unflooded ones, a probable sign of leaf water deficit leading to senescence coupled to the apparition of adventitious roots. In contrast, the decrease in SLA observed during flooding (Fig 3) may be an avoidance mechanism, reducing transpirational area, thus maintaining the water balance (Sanchez-Blanco *et al.*, 2002).

Overall however, the decrease in plant biomass production may be directly related to the effect of reduced stomatal conductance on net photosynthesis thus reducing carbon assimilation (Mielke *et al.*, 2003). Moreover, it is well known that oxygen deficiency caused by flooding leads to reduced root permeability and hydraulic conductivity causing reductions in xylem water potential and stomatal conductance (Else *et al.*, 2001). In our study, photosynthesis of both oak species was inhibited after 7 days of flooding (Fig 4a) whereas at the same time, pedunculate oak maintained its shoot xylem water potential (Fig 4b). Naumann

et al. (2008) showed a similar disruption in *Myrica Cerifera*, a shrub growing in frequently flooded barrier islands, with a small decrease in water potential even after 12 days of flooding whereas photosynthesis fell at 50% of control within 8 days. In fact, photosynthesis was probably inhibited by a restriction in CO₂ diffusion due to stomatal closure. At the same time, this process limited water loss through transpiration and thus allowed water potential to be partially maintained. The plant capacity to maintain xylem water potential, photosynthesis and photosynthetic products translocation from leaves to roots can be considered discriminating factors for flooding tolerance (Gravatt & Kirby, 1998). Thus, our results would tend to favour pedunculate oak as being more tolerant to soil waterlogging as this species is able to better maintain its water homeostasis and photosynthetic activity during flooding. Interestingly, the slight reestablishment of xylem water potential after 14 days of flooding in sessile oak is associated with the first apparition of hypertrophied lenticels (data not shown). These outgrowths are permeable to water and could allow water-uptake when original roots become incapable to do so (Parelle *et al.*, 2007). In pedunculate oak, hypertrophied lenticels also develop, however it seems that their presence is less crucial as the plant water balance is less affected by soil waterlogging and other adaptive features (i.e. aerenchyma, adventitious roots) develop simultaneously.

A role for non-symbiotic hemoglobin?

We have previously characterized the spatio-temporal expression of *QpHb1* in both oak species in response to short-term flooding (Parent *et al.*, 2008a; 2008b). In addition, its differential expression was hypothesised to be related to a role in stress perception and signaling during hypoxia through its effect on nitric oxide (NO) levels. Here, we investigated the possible implication of *QpHb1* in response to long-term flooding and its role as a discriminating marker during flooding tolerance. *QpHb1* belongs to class 1 ns-Hbs which have a high O₂ affinity and low oxygen dissociation rate constant. Their expression is directly associated with protection against hypoxia stress, and it appears to depend on mitochondrial ATP level rather than oxygen levels (Dordas *et al.*, 2003; Hoy *et al.*, 2007). Over the last few years, the function of ns-Hb has been intensively investigated (see for review: Igamberdiev *et al.*, 2005; Garrocho-Villegas *et al.*, 2007; Hoy & Hargrove, 2008) and it is now clearly established that NO function is directly linked to ns-Hb during many plant processes (Borisjuk *et al.*, 2007; Borisjuk & Rolletschek, 2008). Ns-Hb possesses NO dioxygenase activity, which enables it to convert NO to nitrate accompanied by an NADH oxidation (Hebelstrup *et al.*, 2007). This function avoids toxic NO accumulation but also helps maintain

glycolytic ATP synthesis under hypoxic conditions (Perazzolli *et al.*, 2004), thus providing protection during oxygen deficiency. In sessile oak, the most sensitive species studied, *QpHb1* expression declined strongly soon after the start of the treatment and remained very low for the entire duration of the flooding treatment (Fig 5a). In contrast, in pedunculate oak, *QpHb1* expression initially increased and its level decreased only after 14 days of flooding (Fig 5b). As a result, the higher flooding tolerance of pedunculate oak could be related to an enhanced protection against NO accumulation provided by *QpHb1*, at least during the first week of treatment. The subsequent inhibition of *QpHb1*, after 14 days, could be the result of the increasing anoxia conditions developing in the roots thus disabling transcription and/or to a NO production exceeding the nsHb detoxifying capacity (Borisjuk & Rolletschek, 2008).

It is now generally agreed that nsHb is mainly expressed in roots, but there are still very few reports on the precise cellular location of nsHb. The nsHb have generally been observed in parenchyma cells and in vascular bundles, more specifically in xylem parenchyma (Bogusz *et al.*, 1990; Jacobsen-Lyon *et al.*, 1995; Heckmann *et al.*, 2006). When we compared the distribution of *QpHb1* expression in 14 days flooded roots with that of control plants for both species (Fig 6), we found that nsHb localized in the protoderm, the first layer of living cells in the root in contact with the flooded rhizosphere, as previously observed under normal growth conditions (Parent *et al.*, 2008a; 2008b). This localization does not only support the hypothesis of a signaling role for nsHb during the flooding response but it could indicate a potential participation in detoxifying high NO levels produced by anaerobic activity of microorganisms in the rhizosphere.

QpHb1 was also generally expressed in the endoderm and in the protoxylem of oak plants except in flooded sessile oak. The presence of *QpHb1* in protoxylem vessels could be linked to the necessity of communication between organs and tissues (i.e root to shoot), to signal hypoxic stress and initiate the appropriate response. In addition, the presence of hemoglobin in cortical cells could act as a protective measure against NO toxicity which can lead to membrane and protein damage (Borisjuk & Rolletschek, 2008). This possibility is supported by the observation that *QpHb1* seems to be more present in cortical cells of sessile oak than in pedunculate oak after 14 days of flooding (Fig 6). In fact, the presence of *QpHb1* correlates with cortical cells being better preserved in sessile than pedunculate oak thus further supporting the latter hypothesis (Fig 7).

Alternatively, a promoting effect of NO on ethylene biosynthesis has also been proposed by Igamberdiev *et al.* (2005) and this effect could thus promote aerenchyma formation. In pedunculate oak, root cortex porosity was strongly enhanced by flooding (Figs 7 and 8) and the development of these large gas spaces (lacunae) may favor internal gas exchange between hypoxic roots and oxygenated shoots. Thus, modulation of *QpHb1* expression and its effect on ethylene biosynthesis in cortical cells could be involved in aerenchyma formation.

Adventitious roots

Another typical adaptation to flooding stress is the formation of adventitious roots. Flood tolerant species commonly develop these particular roots which emerge during waterlogging thus replacing the degenerating original root system (Li *et al.*, 2006). We thus decided to investigate *QpHb1* expression in adventitious roots of both sessile and pedunculate oak and found a very contrasting response (Fig 10). Indeed, transcripts levels of *QpHb1* were highly abundant in pedunculate oak adventitious roots as compared to sessile oak. A role for NO in the signaling cascade leading to adventitious root development having been shown in cucumber (Pagnussat *et al.*, 2003; 2004), the abundance of hemoglobin transcripts in adventitious roots of pedunculate oak could be related to a protective role against toxic NO accumulation which is produced to maintain glycolytic ATP synthesis. To further investigate the over-expression of *QpHb1* in pedunculate adventitious roots, we used *in situ* hybridization. Interestingly, hemoglobin transcripts accumulated in the protoderm layer but not in the protoxylem vessels (Fig 11b) as previously evidenced on the original root system. As a result, this localization could be linked to a role against radial oxygen loss (ROL) as ns-Hbs have a high oxygen dissociation rate. A role against NO produced by anaerobical microorganisms may not be excluded either. Overall however it was surprising to find a low level of *QpHb1* expression in sessile oak adventitious roots whereas the development of this tissue is considered as an adaptive response. As a result, the level and spatial distribution of ns-Hb inside root tissues may be a discriminating factor between tolerant and sensitive species. In conclusion, the low levels of *QpHb1* expression in root cortical cells of sessile oak may correspond to an avoidance strategy, whereas the stronger expression in pedunculate oak may help maintain the redox potential and energy status and the localization in the xylem vessels could participate in signal transduction and correspond to an adaptive strategy.

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Fig 1: Picture of control pedunculate (a) and sessile (b) oak.



Fig 2: Effect of soil flooding on stem length of sessile and pedunculate oak. Bars represents \pm S.E. (n= 9). Control P = pedunculate control plants; Hypoxia P = pedunculate flooded plants; Control S = sessile control plants and Hypoxia S = sessile flooded plants.

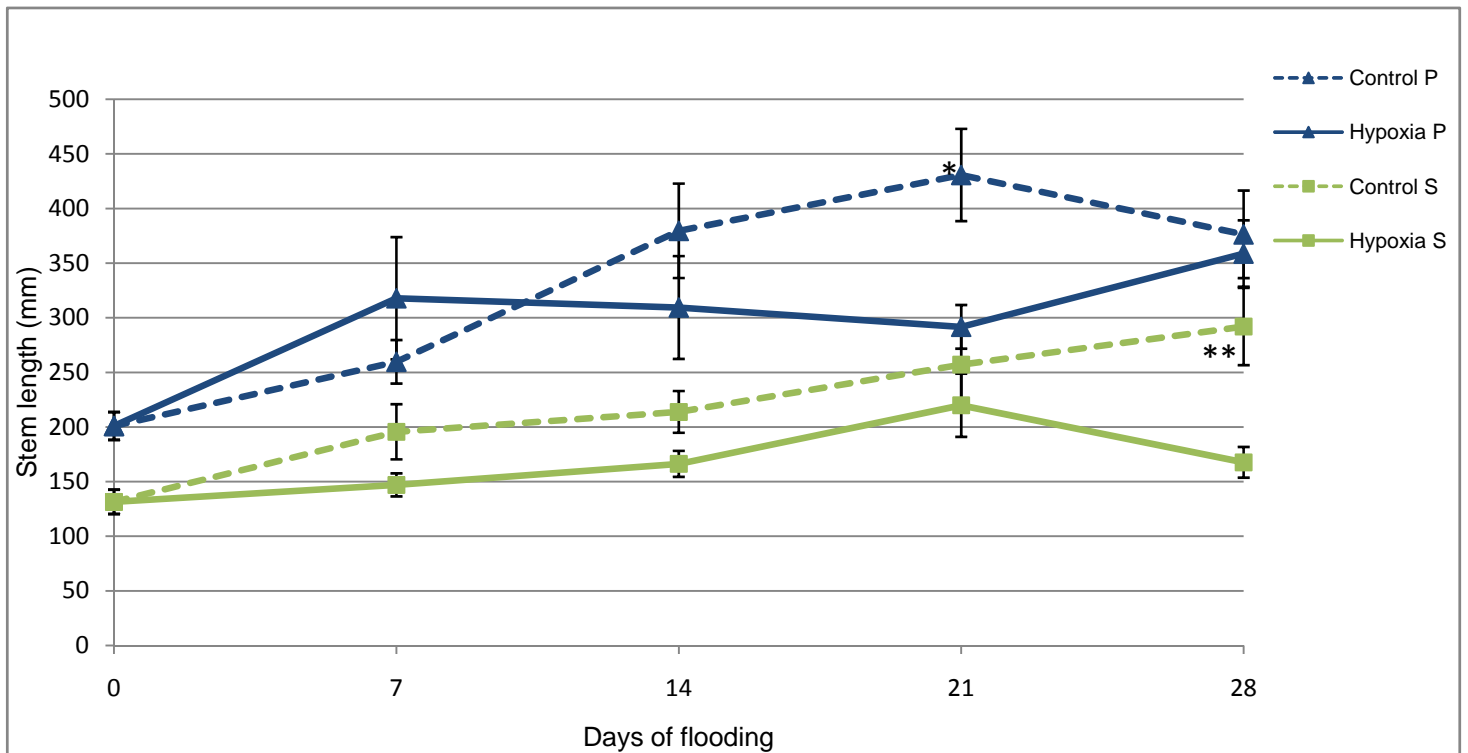


Fig 3: Effect of soil flooding on specific leaf area (SLA) of sessile and pedunculate oak. Bars represent \pm S.E. ($n \geq 6$). Significant differences between flooded and control for each species are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

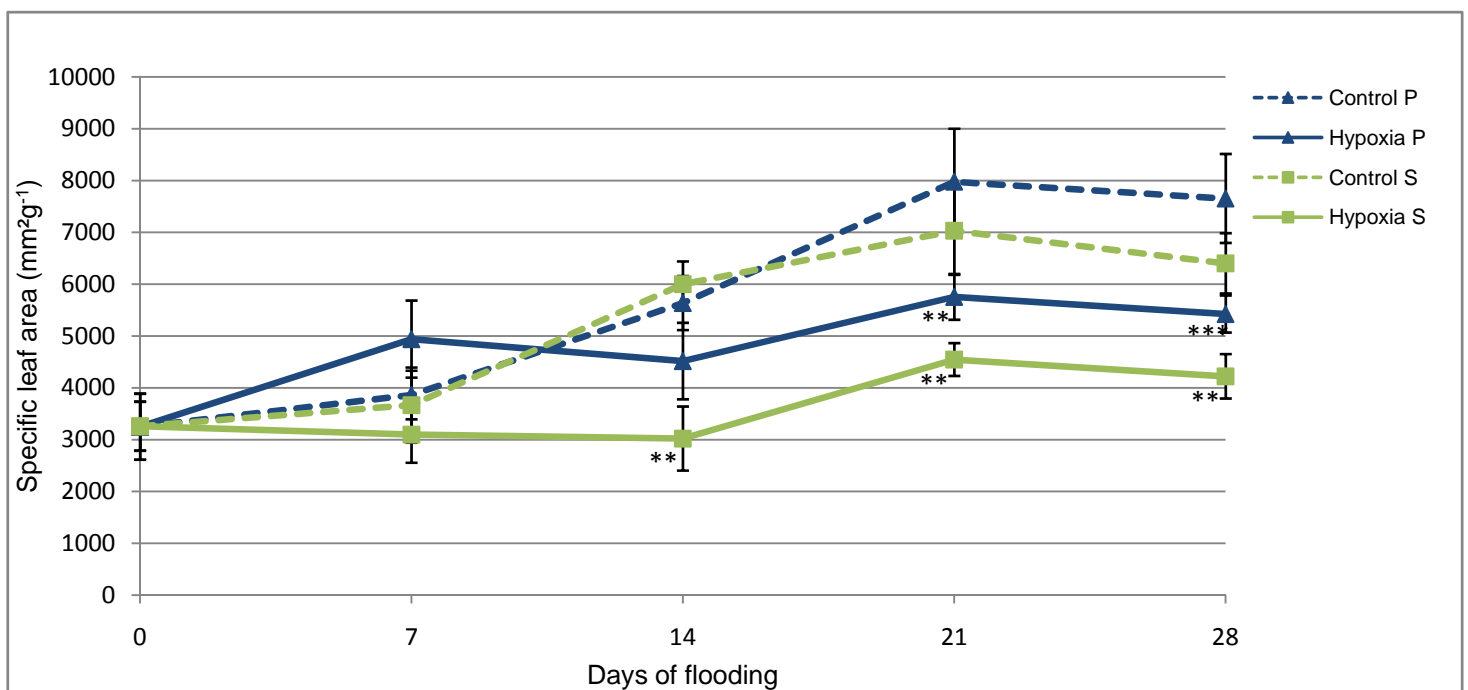


Table 1: Analysis of variance (ANOVA) of different leaf growth parameters of sessile and pedunculate oak. The Degrees of freedom for Species (S), Flooding (F) and their interaction (S x F) are 1, for Duration (D) and its interaction with Species (S x D), 4 and for interaction between the third 6.

Variables	Sources	F-values	P-values
Leaf number per plant	Species (S)	8.21	0.0046
	Duration (D)	12.10	0.0001
	Flooding (F)	16.54	0.0001
	S x D	0.27	0.9001
	S x F	0.14	0.7059
	S x D x F	1.81	0.0994
Total leaf area	Species (S)	5.26	0.0229
	Duration (D)	16.25	0.0001
	Flooding (F)	20.76	0.0001
	S x D	0.23	0.9188
	S x F	1.37	0.2440
	S x D x F	2.49	0.0244
Specific leaf area	Species (S)	9.17	0.0047
	Duration (D)	17.34	0.0001
	Flooding (F)	23.43	0.0001
	S x D	0.20	0.9394
	S x F	2.01	0.1579
	S x D x F	2.3	0.0363

Table 2: Fresh (FW) and dry weights (DW) of roots and leaves, shoot/root ratio and whole plant weight (root, stem and leaf weight) of the sessile and pedunculate oak in response to flooding (mean \pm S.E, n \geq 9). Difference in weight between flooded and unflooded plants was calculated by Student *t*-test (*P<0.05; **P<0.01; ***P<0.001).

Organ	Duration (days)	Fresh weight (g)				Dry weight (g)			
		Sessile		Pedunculate		Sessile		Pedunculate	
		Unflooded	Flooded	Unflooded	Flooded	Unflooded	Flooded	Unflooded	Flooded
root	0	5.40 \pm 0.29		5.62 \pm 0.26		3.75 \pm 0.17		3.73 \pm 0.12	
	7	7.20 \pm 0.40	5.79 \pm 0.36*	5.41 \pm 0.24	6.13 \pm 0.45	3.83 \pm 0.07	3.62 \pm 0.07	3.57 \pm 0.04	3.70 \pm 0.07
	14	6.00 \pm 0.43	5.40 \pm 0.38	6.53 \pm 0.55	5.33 \pm 0.23	3.73 \pm 0.04	3.65 \pm 0.05	4.02 \pm 0.14	3.69 \pm 0.08*
	21	8.39 \pm 1.02	5.95 \pm 0.27***	8.02 \pm 0.74	6.29 \pm 0.30**	4.09 \pm 0.16	3.76 \pm 0.06*	4.07 \pm 0.12	3.77 \pm 0.05*
	28	8.72 \pm 0.41	5.93 \pm 0.27***	7.46 \pm 0.56	6.93 \pm 0.32	4.03 \pm 0.06	3.74 \pm 0.05*	4.00 \pm 0.09	3.96 \pm 0.08
leaf	0	5.41 \pm 0.31		4.79 \pm 0.27		4.09 \pm 0.21		3.75 \pm 0.13	
	7	6.40 \pm 0.43	5.23 \pm 0.39	4.96 \pm 0.25	6.12 \pm 0.53*	4.18 \pm 0.09	3.96 \pm 0.14	3.74 \pm 0.08	4.17 \pm 0.15*
	14	6.50 \pm 0.31	5.37 \pm 0.25	5.97 \pm 0.26	5.71 \pm 0.31	4.25 \pm 0.13	4.11 \pm 0.08	4.14 \pm 0.12	4.09 \pm 0.12
	21	7.66 \pm 0.60	5.86 \pm 0.24**	7.78 \pm 0.71	6.18 \pm 0.24**	4.51 \pm 0.17	4.18 \pm 0.09	4.59 \pm 0.12	4.26 \pm 0.08
	28	7.41 \pm 0.43	5.77 \pm 0.29**	7.50 \pm 0.59	6.32 \pm 0.30*	4.63 \pm 0.13	4.26 \pm 0.12*	4.59 \pm 0.18	4.38 \pm 0.12
shoot/root ratio	0	1.81 \pm 0.04		1.64 \pm 0.03		2.07 \pm 0.03		1.99 \pm 0.02*	
	7	1.54 \pm 0.06	1.61 \pm 0.06	1.79 \pm 0.08	1.81 \pm 0.06	2.03 \pm 0.02	2.07 \pm 0.02	2.05 \pm 0.02	2.19 \pm 0.04**
	14	1.90 \pm 0.11	1.86 \pm 0.10	1.78 \pm 0.10	2.04 \pm 0.13*	2.11 \pm 0.05	2.12 \pm 0.02	2.01 \pm 0.03	2.17 \pm 0.04**
	21	1.61 \pm 0.11	1.78 \pm 0.06	1.76 \pm 0.06	1.85 \pm 0.07	2.03 \pm 0.04	2.12 \pm 0.04	2.13 \pm 0.04	2.20 \pm 0.03
	28	1.46 \pm 0.05	1.74 \pm 0.04**	1.88 \pm 0.11	1.73 \pm 0.06	2.10 \pm 0.02	2.13 \pm 0.02	2.20 \pm 0.05	2.18 \pm 0.04
whole plant	0	16.21 \pm 0.87		15.20 \pm 0.75		11.93 \pm 0.58		11.23 \pm 0.37	
	7	19.99 \pm 0.16	16.25 \pm 1.10*	15.32 \pm 0.68	18.36 \pm 1.45	12.18 \pm 0.24	11.53 \pm 0.36	11.05 \pm 0.18	12.04 \pm 0.37
	14	19.01 \pm 0.85	16.14 \pm 0.69	18.47 \pm 1.03	16.74 \pm 0.69	12.23 \pm 0.26	11.86 \pm 0.21	12.29 \pm 0.37	11.86 \pm 0.31
	21	23.70 \pm 1.96	17.67 \pm 0.70***	23.58 \pm 2.10	18.66 \pm 0.72**	13.11 \pm 0.49	12.11 \pm 0.21*	13.24 \pm 0.50	12.29 \pm 0.18*
	28	23.53 \pm 1.18	17.47 \pm 0.82***	22.46 \pm 1.60	19.58 \pm 0.85	13.29 \pm 0.32	12.25 \pm 0.29*	13.17 \pm 0.44	12.71 \pm 0.28

Table 3: Analysis of variance (ANOVA) of different growth parameters between sessile and pedunculate oak during flooding. The Degrees of freedom for Species (S), Flooding (F) and their interaction (S x F) are 1, for Duration (D) and its interaction with Species (S x D) 4 and for interaction between the third 6.

Variables	Sources	F-values	P-values
Root fresh weight	Species (S)	0.03	0.8670
	Duration (D)	15.29	0.0001
	Flooding (F)	31.17	0.0001
	S x D	1.12	0.3500
	S x F	6.35	0.0125
	S x D x F	2.66	0.0168
	Root dry weight	Species (S)	0.65
Duration (D)		6.47	0.0001
Flooding (F)		13.67	0.0003
S x D		0.90	0.4660
S x F		0.89	0.3454
S x D x F		1.57	0.1570
Leaf fresh weight		Species (S)	0.02
	Duration (D)	11.38	0.0001
	Flooding (F)	24.84	0.0001
	S x D	0.35	0.8422
	S x F	7.05	0.0086
	S x D x F	2.62	0.0184
	Leaf dry weight	Species (S)	0.82
Duration (D)		10.14	0.0001
Flooding (F)		5.61	0.0188
S x D		0.55	0.7028
S x F		4.84	0.0289
S x D x F		1.58	0.1549
Shoot/Root dry weight ratio		Species (S)	4.26
	Duration (D)	7.30	0.0001
	Flooding (F)	12.49	0.0005
	S x D	3.02	0.0191
	S x F	4.84	0.0290
	S x D x F	1.44	0.2028
	Total fresh weight	Species (S)	0.06
Duration (D)		12.34	0.0001
Flooding (F)		32.61	0.0001
S x D		0.40	0.8065
S x F		7.65	0.0062
S x D x F		2.73	0.0144
Total dry weight		Species (S)	0.25
	Duration (D)	9.24	0.0001
	Flooding (F)	8.07	0.0050
	S x D	0.44	0.7792
	S x F	4.14	0.0431
	S x D x F	1.55	0.1630

Fig. 4 Stomatal conductance (a), photosynthesis (b) and xylem water potential (c) of sessile and pedunculate oak in response to soil flooding. Bars represents \pm S.E. (n = 12). Significant differences between flooded and control for each species are represented by * p<0.05, ** p<0.01 and *** p<0.001.

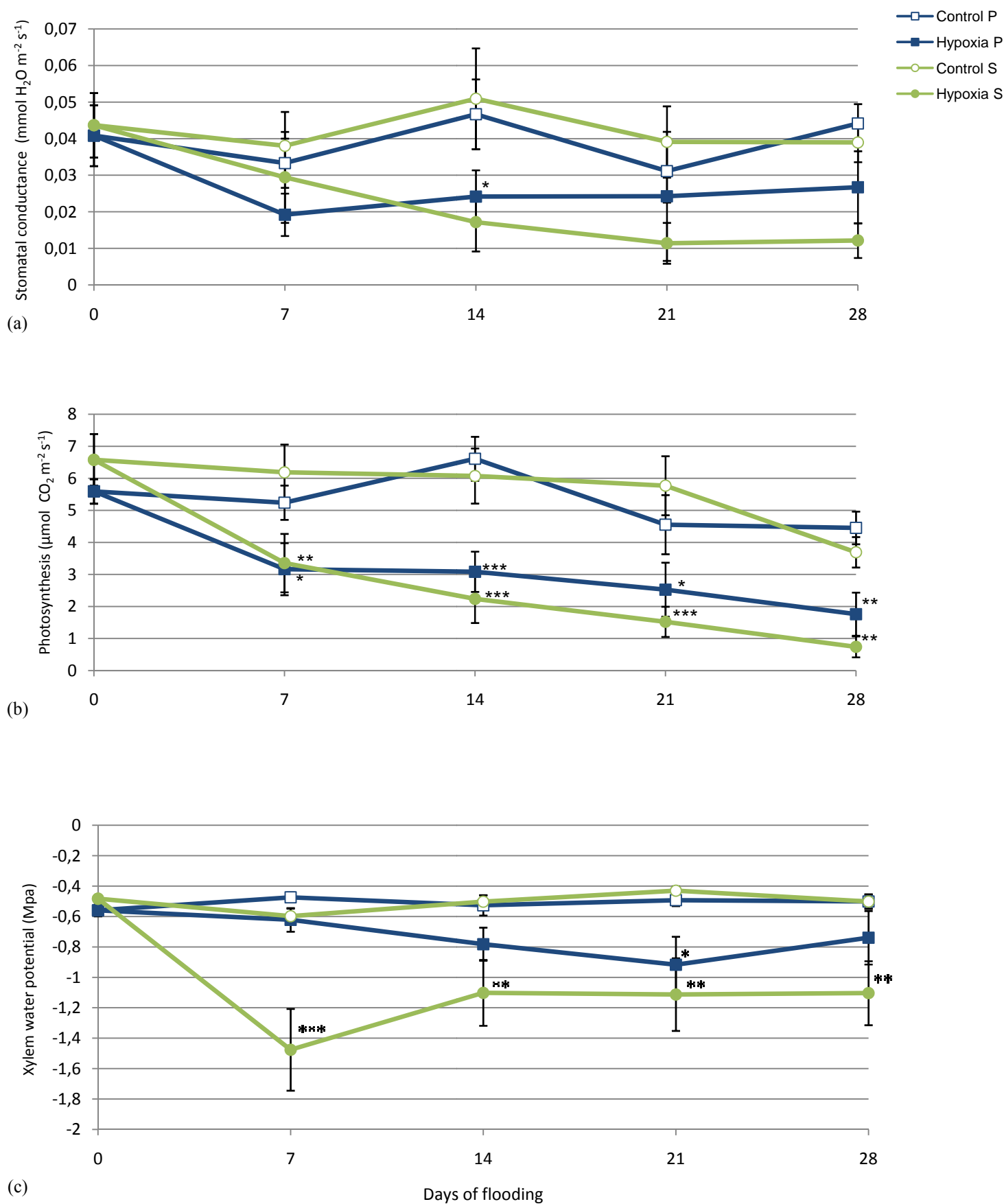
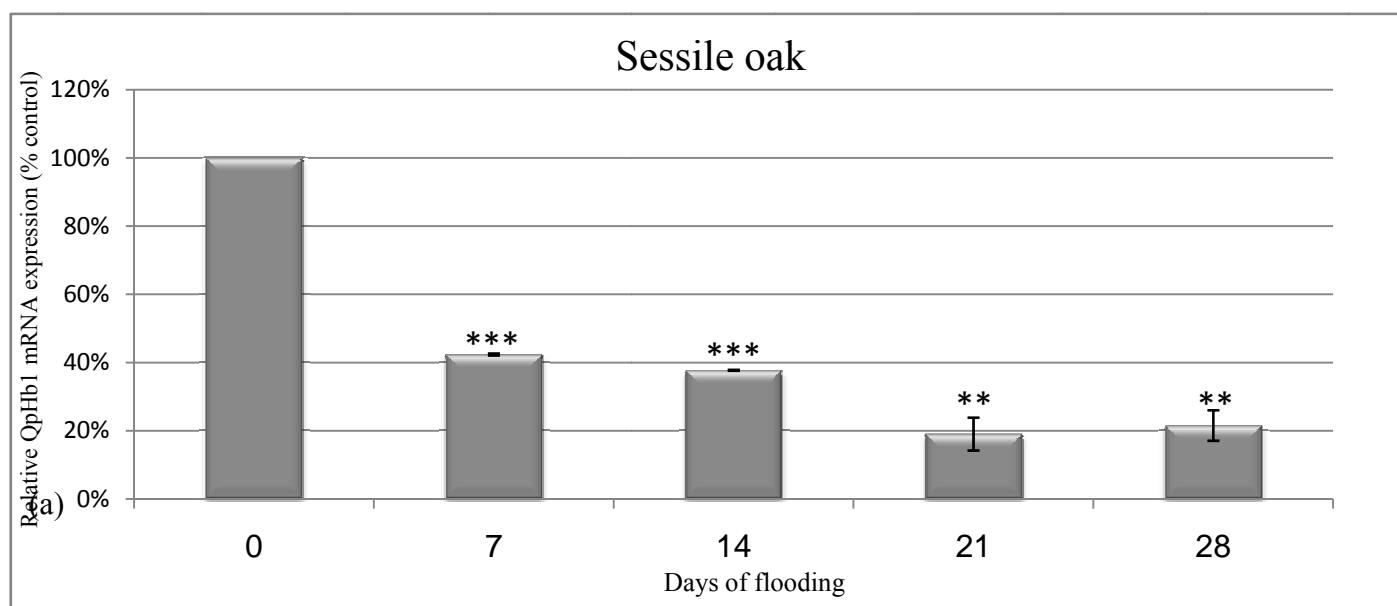


Fig. 5 Effects of flooding on the expression of *QpHb1* on sessile (a) and pedunculate (b) oak. Vertical bars represents \pm S.E. (n = 3). Significant differences between flooded and control for each species are represented by * p<0.05, ** p<0.01 and *** p<0.001.



(b)

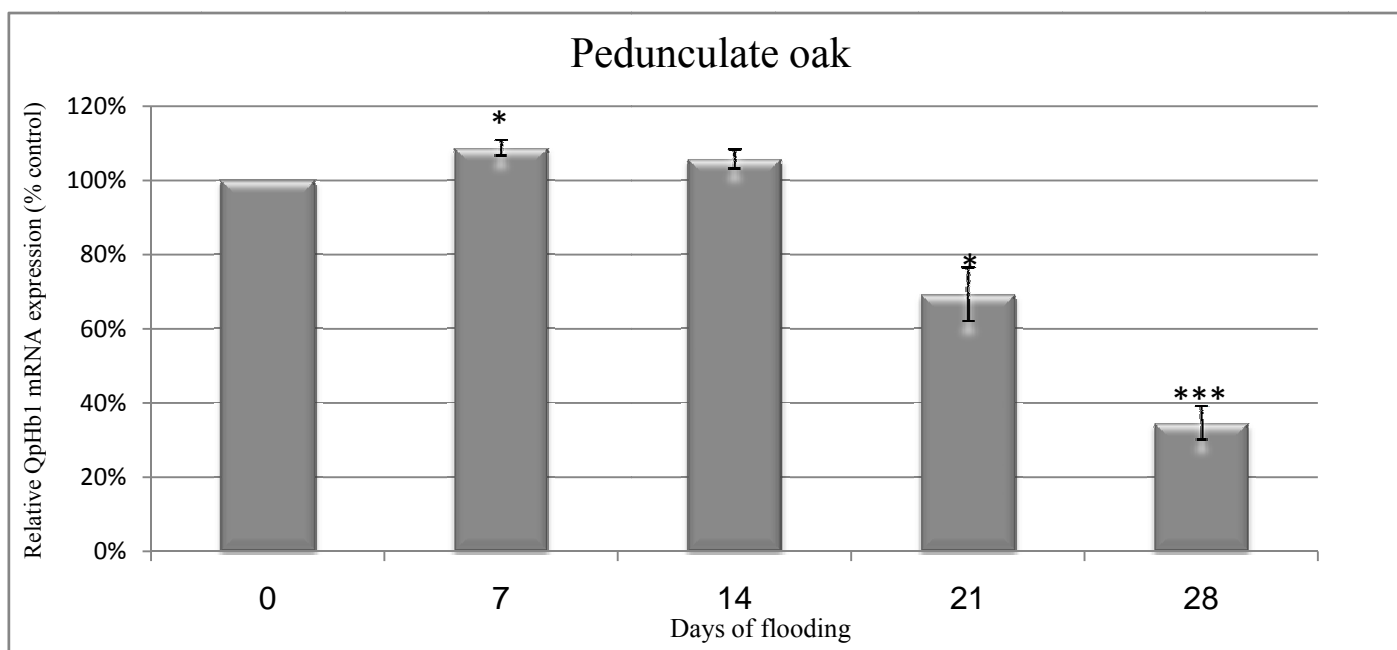


Fig 6: *QpHb1* expression by *in situ* hybridization in a cross section in sessile (a) control (b) 14 days flooded and pedunculate oak seedlings (c) control (d) 14 days flooded.

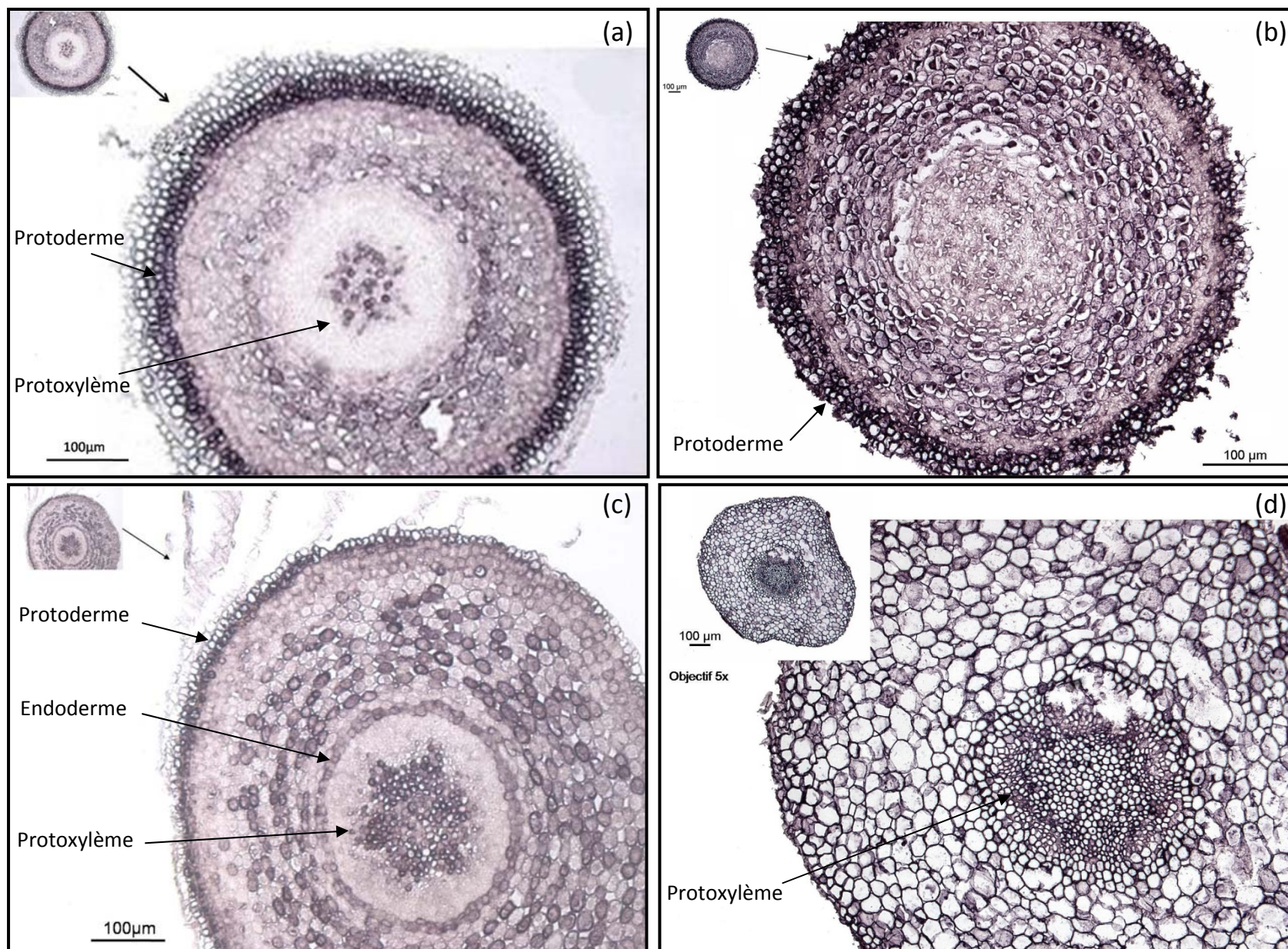


Fig 7: Root cross section realized between 2200 μm and 2800 μm from the root tip stained with toluidine blue after different duration of flooding treatment in sessile (a) control (b) 14 days (c) 28 days and pedunculate oak seedlings (d) control (e) 14 days (f) 28 days. Scale bar represents 100 μm .

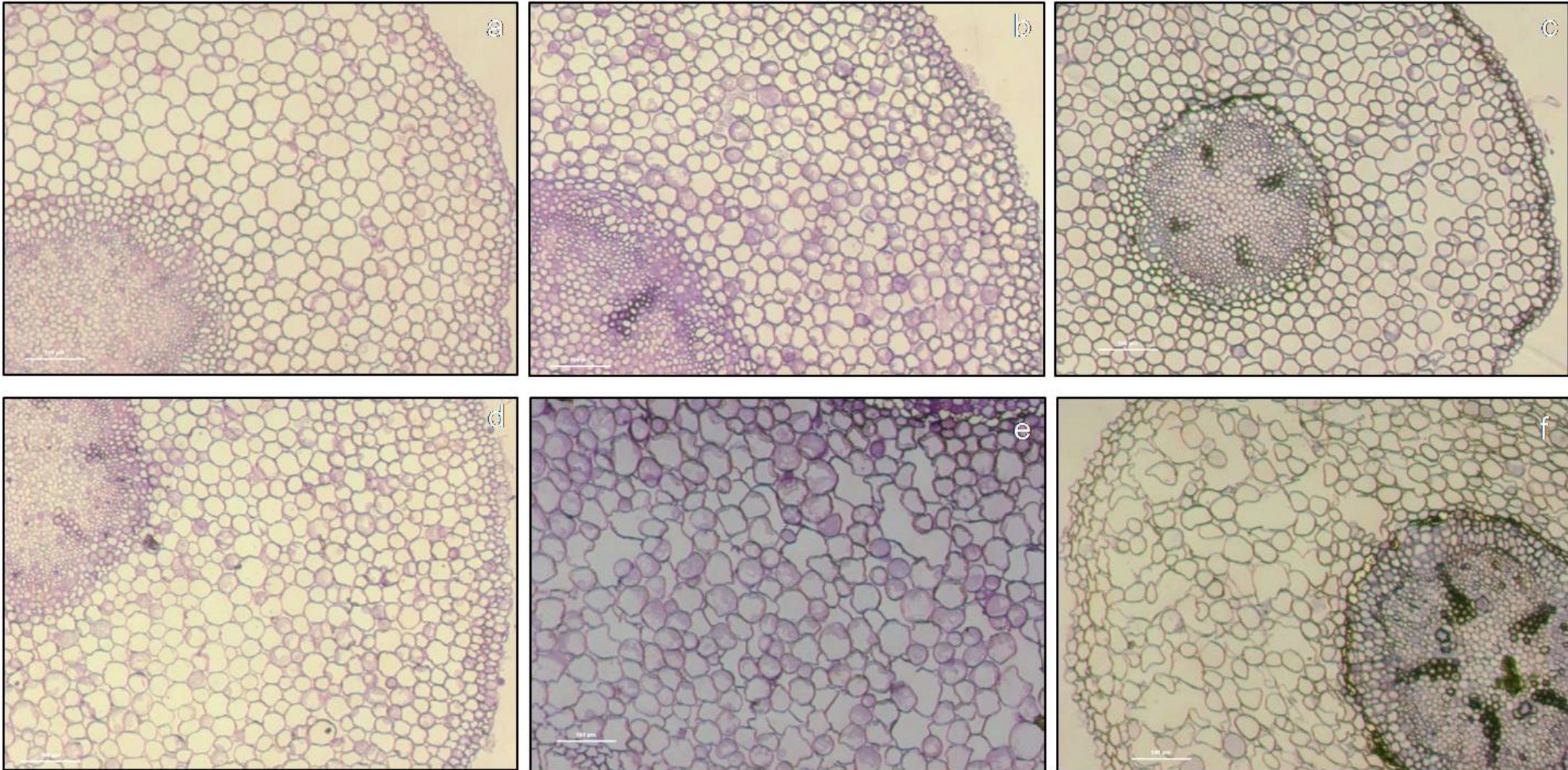


Table 4: Changes in cell circularity (roundness) in cortex of sessile and pedunculate oak in response to soil flooding (mean \pm S.E. $n \geq 248$). Significant differences between flooded and control for each species are represented by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Duration (days)	Roundness	
	Sessile	Pedunculate
0	0.80 \pm 0.004	0.82 \pm 0.005
7	0.81 \pm 0.006*	0.80 \pm 0.008*
14	0.80 \pm 0.005	0.72 \pm 0.017*
21	0.76 \pm 0.009*	0.76 \pm 0.012*
28	0.77 \pm 0.008*	0.76 \pm 0.014*

Fig 8: Effect of soil flooding on root cortex porosity expressed as percentage of intercellular spaces in the sessile and pedunculate oak. Bars represents \pm S.E. ($n = 3$). Significant differences between sessile and pedunculate oaks are represented by * $p < 0.05$ and ** $p < 0.01$.

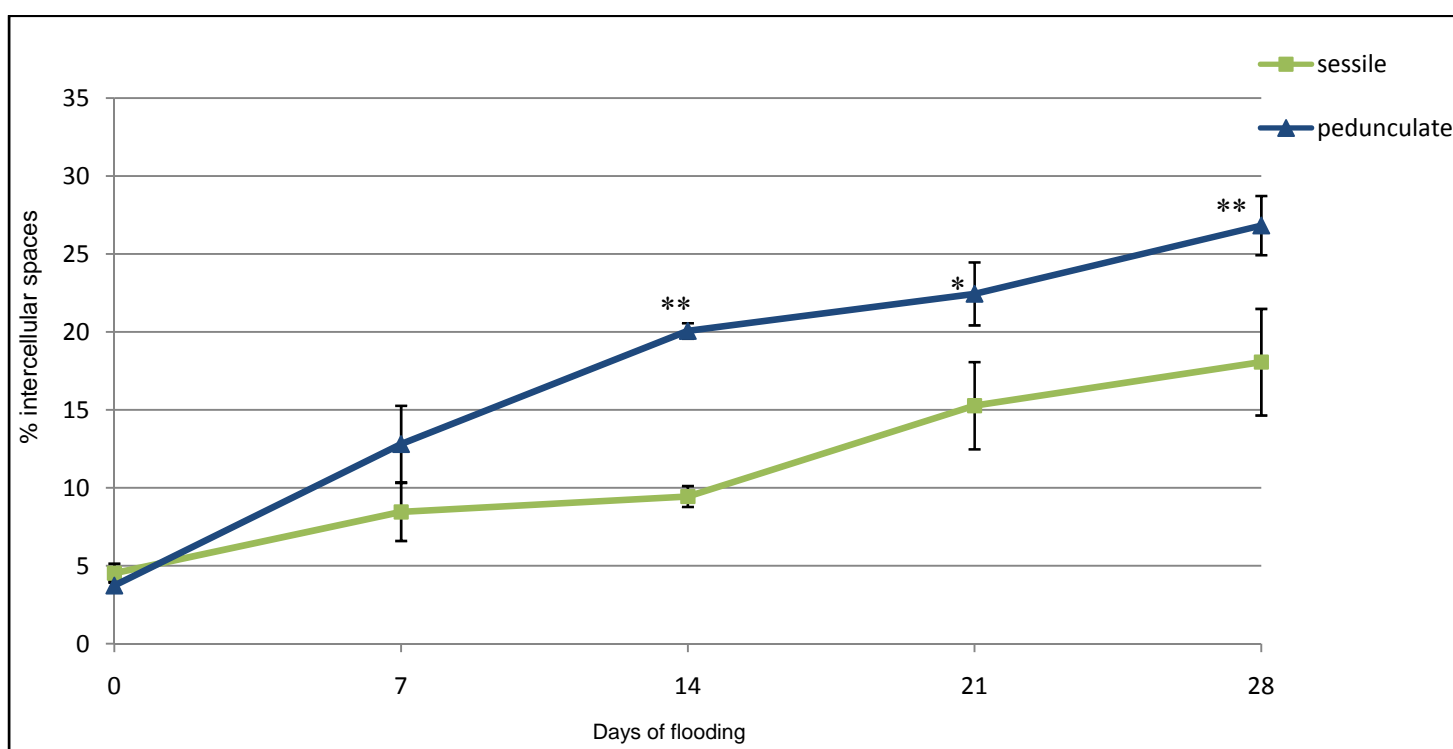


Fig 9: Observations of adaptations in pedunculate oak after 28 days of flooding. (a) adventitious roots (b) hypertrophied lenticels (see arrows).

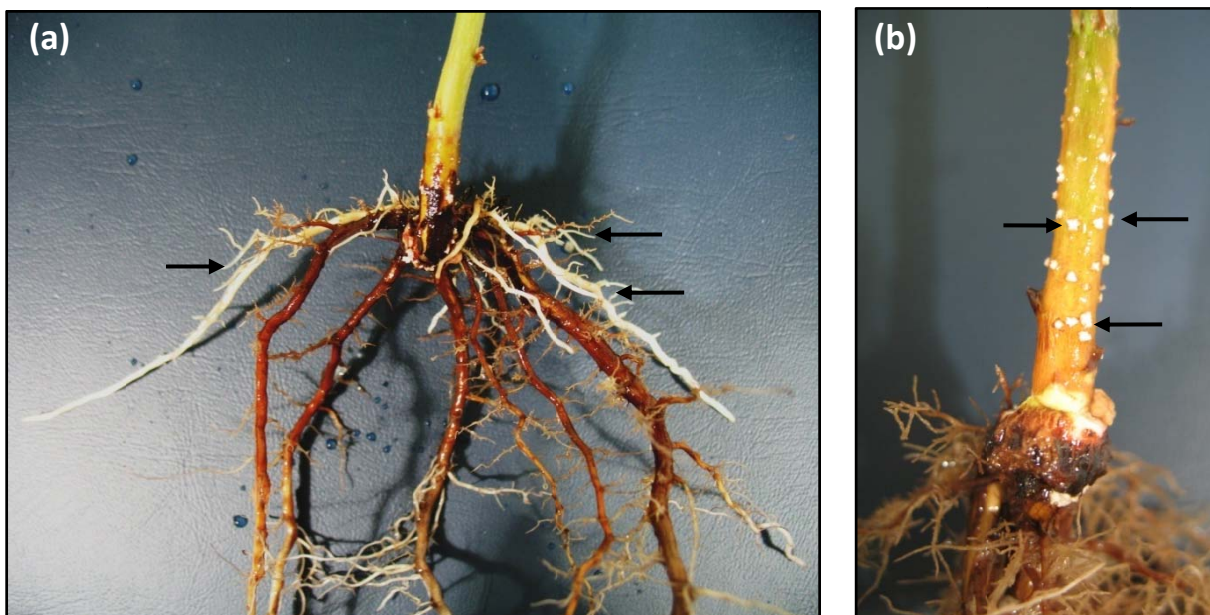


Fig 10: Effects of flooding on the expression of *QpHb1* in sessile and pedunculate adventitious roots. Densitometric scanning (n = 3) was expressed relatively to *QpHb1* expression in sessile control.

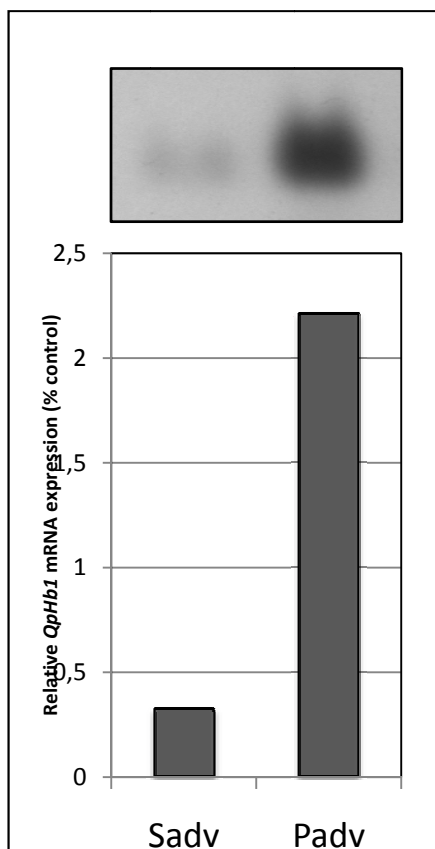
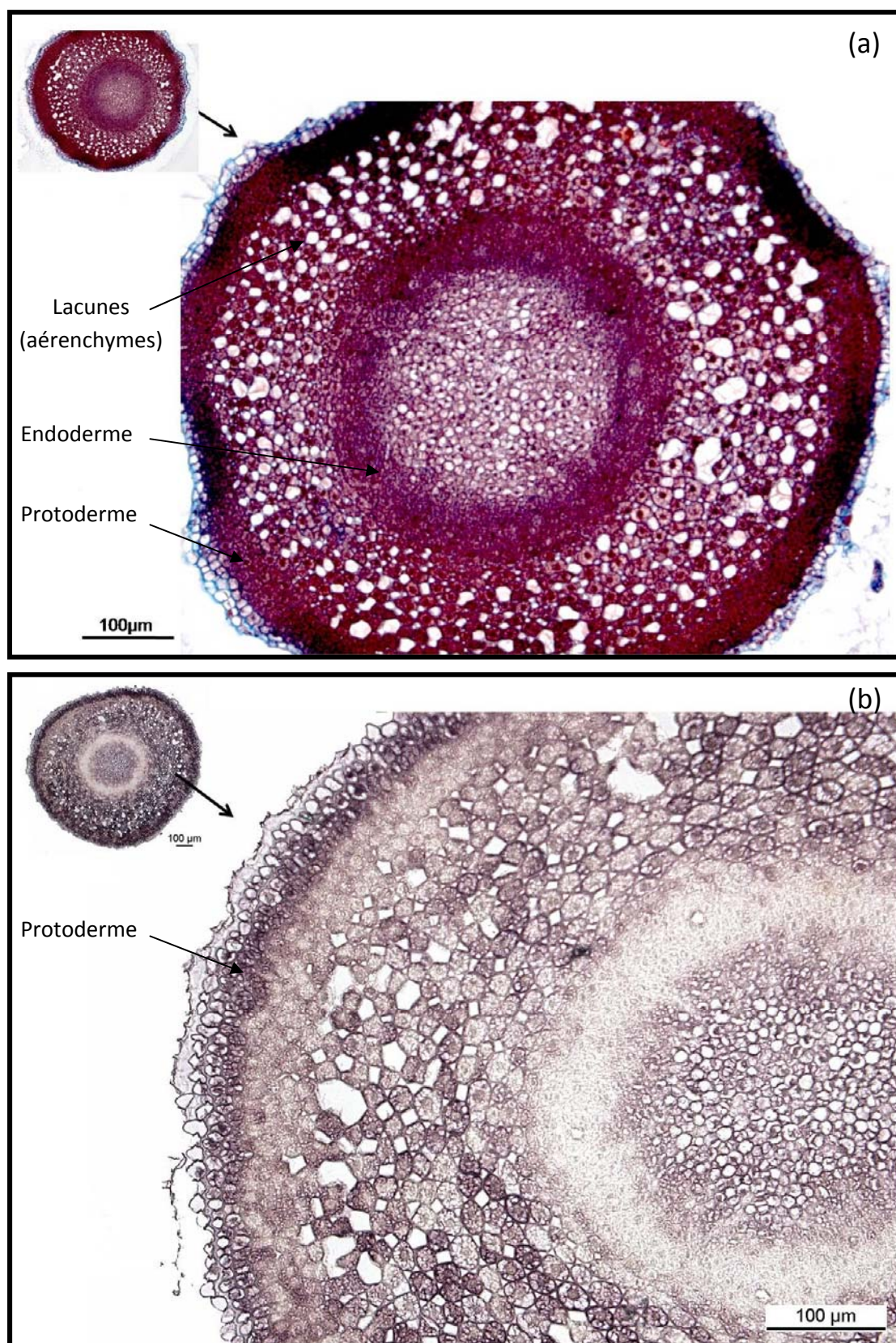


Fig 11: (a) Adventitious root cross section at approximately 600 μ m from the tip stained with Alcian blue/Safranin O in pedunculate oak; (b) In situ *QpHb1* expression in a cross section realized at 600 μ m from the tip of pedunculate adventitious root.



Discussion générale et Perspectives

Cette partie reprendra les points les plus importants des discussions fournies dans les précédents articles et traitera de la complémentarité et de l'originalité des résultats obtenus dans un contexte plus spéculatif. Un premier thème abordera les réponses morphologiques, physiologiques et cellulaires du chêne à l'ennoyage et plus particulièrement les différences observées chez les deux espèces. Le second thème sera consacré à l'hémoglobine non-symbiotique et aux différents mécanismes cellulaires dans lesquels elle pourrait être impliquée.

L'ennoyage retarde ou inhibe la germination des graines, limite la survie des semis et réduit la croissance des végétaux. La régénération naturelle, de même que la répartition des différentes espèces en est ainsi bouleversée. Le stress hypoxique résultant des conditions d'ennoyage a des effets directs non seulement sur le système racinaire mais également sur la plante entière. La croissance globale de la plante est inhibée (Table 2 p.86). Chez le chêne, ces conséquences sur la croissance ont bien été observées. Cependant, même si on a pu constater un début de sénescence foliaire en fin d'expérience (après 28j d'ennoyage) sur de rares plants de chêne sessile, aucune mortalité n'a été relevée contrairement à l'étude de Parelle *et al.* (2006). Ces différences peuvent s'expliquer par des conditions expérimentales différentes (milieu de culture, âge des plantules, application du stress...). Dans nos conditions, l'élongation de la tige est ralentie voire stoppée quand l'ennoyage se prolonge (Fig. 2 p.83) et la croissance foliaire est inhibée aussi bien en termes de surface foliaire que de biomasse (Tables 1&2 p.85-86). L'apparition d'un déficit hydrique mais également une diminution de la photosynthèse pourraient être à l'origine de ces bouleversements.

En effet, l'excès d'eau dans le sol n'est pas synonyme d'un « excès d'alimentation en eau » mais au contraire on observe les mêmes symptômes que lors d'un stress hydrique. La chute du potentiel hydrique dès les premières heures d'ennoyage traduit ce dysfonctionnement d'absorption de l'eau par les racines (Fig.6 p.56). Elle est principalement due à la diminution de la conductivité hydraulique elle-même associée à la fermeture des stomates (Else *et al.* 2001). Pour le stress à plus long terme, la réduction du potentiel hydrique (Fig 4.c p.88) peut également être mise en relation avec la surface foliaire, car plus la surface est importante, plus les pertes d'eau par transpiration seront grandes. La diminution de la SLA (specific leaf area, Fig.3 p.84) pourrait donc être considérée comme un moyen d'éviter un stress hydrique interne trop important. D'autres événements antérieurs et/ou associés à la fermeture des stomates pourraient également être à l'origine de la chute du potentiel hydrique. Dans cette hypothèse, la fermeture d'aquaporines pourrait être un des événements précoces impliqués dans la chute

de la conductance racinaire (Tournaire-Roux *et al.* 2003). L'étude de ces canaux et de leur régulation représente donc une perspective de recherche intéressante qui permettrait de mieux comprendre les phénomènes impliqués dans la réponse à l'ennoyage. Cependant, on ne peut évidemment pas négliger la probable participation d'autres mécanismes de signalisation issus des racines comme l'acide abscissique ou le pH dans la régulation de la conductance hydraulique de la plante (Parent *et al.* en préparation p21). La participation du système racinaire dans la régulation du potentiel hydrique est aussi en accord avec nos résultats et ceux de Schnull et Thomas (2000), qui montrent que la mise en place de racines adventives entraîne une augmentation de la conductance hydraulique racinaire (Fig.4c p.88). L'apparition de ces adaptations ainsi que l'hypertrophie des lenticelles permettraient donc une légère amélioration du potentiel hydrique de l'appareil aérien.

L'inhibition de la croissance de la plante n'est probablement pas seulement due au déficit hydrique mais également à la diminution de l'activité photosynthétique (Fig. 4b p.88). Par ailleurs, cette diminution de la photosynthèse serait elle aussi liée à la fermeture précoce des stomates, limitant l'entrée de CO₂. Le maintien de la capacité photosynthétique pendant l'ennoyage est souvent considéré comme un facteur déterminant de la tolérance à l'ennoyage (Pezeshki 2001). Cependant les précédents travaux portant sur l'assimilation du CO₂ chez les chênes sessile et pédonculé n'ont pas montré de différence entre les deux espèces (Dreyer *et al.* 1991, Wagner & Dreyer 1997, Schnull & Thomas 2000). Dans le cas de notre étude, le chêne sessile semble davantage affecté que le pédonculé, car même si les valeurs d'assimilation du CO₂ des plants stressés ne sont pas significativement différentes, il faut tenir compte du fait que l'activité photosynthétique en conditions normoxiques du chêne sessile est globalement supérieure à celle du pédonculé (Fig. 4b p.88). En effet, on observe une différence significative entre chêne sessile et pédonculé à 7 et 21 jours d'ennoyage si on considère la diminution de la photosynthèse relativement à celle des témoins. La diminution continue de la photosynthèse n'est pas uniquement due à la réduction des échanges gazeux par la fermeture des stomates. En effet la conductance stomatique mesurée parallèlement à la photosynthèse ne suit pas la même tendance (Fig. 4a p.88). Après une nette diminution suivant l'application du stress, elle se stabilise dès 7 jours chez le chêne pédonculé et à partir de 21 jours chez le chêne sessile. La poursuite de l'inhibition photosynthétique pourrait être alors attribuée à une diminution de l'activité de la Rubisco et du photosystème II (Pocieta *et al.* 2008) et à plus long terme, à la dégradation de la chlorophylle (Mielke *et al.* 2003, Gerard *et al.* in press) et à la diminution de la surface foliaire.

L'appareil aérien n'est pas le seul à subir les conséquences du stress et c'est d'ailleurs au niveau racinaire que les conséquences sont les plus importantes. De manière générale, les deux espèces voient leur croissance racinaire rapidement inhibée par l'ennoyage, et leur système racinaire se nécrose de plus en plus avec le prolongement du stress (Tables 2&3 p86-87). Cette conséquence peut être considérée comme un phénomène d'acclimatation visant à diminuer le nombre de racines métaboliquement actives et ainsi limiter les dépenses énergétiques. Cette nécrose se traduit dans un premier temps par la désorganisation du cortex et conduit dans un deuxième temps, à l'apparition de lacunes aérifères (Fig.7 p91). Ces espaces, dus à la mort de certaines cellules du cortex, forment des aerenchymes comme on peut l'observer chez le chêne pédonculé après plusieurs semaines d'ennoyage. Ce réseau gazeux favorise la diffusion interne d'oxygène des parties aériennes (appareil aérien) vers les parties racinaires ennoyées, afin de maintenir la croissance et la survie de la plante (Evans 2003). Ce transport permet également, en diffusant les gaz à l'extérieur des racines hypoxiées, d'évacuer certaines substances réduites et/ou issues du métabolisme fermentaire, pouvant être toxiques pour les racines (acide lactique, éthanol, ...). Il existe deux voies de formation des aerenchymes, une appelée schizogénie, caractérisée par la séparation des cellules corticales qui se divisent et grandissent de façon à former des espaces entre elles. L'autre, appelée lysogénie, correspond à une mort cellulaire programmée (PCD) probablement initiée par la voie de l'éthylène. Certaines caractéristiques observées dans nos racines tendent à montrer que la création des aerenchymes chez le chêne pédonculé se ferait par PCD. En effet, on a pu observer une condensation de la chromatine dans les noyaux de cellules corticales au même niveau que les lacunes (données non publiées, Master A. Berger, 2007 ; voir annexe). Afin de valider la formation des aerenchymes chez le chêne par lysogénie, on se propose de vérifier si le clivage de l'ADN (DNA laddering), caractéristique de la PCD, est bien observé dans les racines soumises à un ennoyage de longue durée. Une étude par microscopie électronique des modifications des organites des cellules du cortex racinaire chez le chêne en hypoxie est aussi envisagée pour essayer d'identifier d'autres signes de la PCD (condensation de la chromatine, altération de la membrane plasmique, vacuolisation..).

Le développement des aerenchymes s'accompagne d'une hypertrophie des lenticelles à la base de la tige et du pivot au niveau de la zone faisant interface entre l'eau et l'air (Fig.9b p.93). Ces excroissances sont issues d'une réponse à l'ennoyage fréquemment observées chez les ligneux (Colin-Belgrand *et al.* 1991, Kozłowski 1997, Dat *et al.* 2006, Parelle *et al.* 2006, 2007a). Les lenticelles hypertrophiées permettent les échanges entre les tissus de la tige et le milieu extérieur et pourraient former un continuum gazeux avec les aerenchymes (Mancuso &

Marras 2003). Elles sont aussi perméables à l'eau (Groh *et al.* 2002) et pourraient donc permettre à la plante de s'alimenter en eau et ainsi réduire son déficit hydrique (Chen *et al.* 2002). Leur apparition chez le chêne sessile coïncide d'ailleurs avec une légère remontée du potentiel hydrique à 7 jours (Fig.4c p.88). L'absorption d'eau est également favorisée par la mise en place de racines d'adaptation, appelées adventives (Fig. 9a p.93). Ces racines nouvellement émises colonisent la partie supérieure de la nappe d'eau et participent ainsi au transport de l'oxygène. Si la présence de racines adventives a souvent été rapportée chez les deux espèces de chêne, c'est la première fois qu'elles sont analysées. L'étude de leur structure cellulaire montre comme chez d'autres espèces, la formation de lacunes dans le cortex (Drew *et al.* 1979, Colmer *et al.* 2006, Fig.11a p.94). En effet, elles présentent un tissu cortical très poreux et les aerenchymes, qui semblent s'y former, faciliteraient aussi la diffusion de l'oxygène présent en plus forte concentration dans cette zone plus proche de l'atmosphère. C'est également la première fois qu'une étude est réalisée sur l'expression d'un gène au niveau de ce tissu (Fig.10&11b p.93-94). Il est intéressant de noter que d'un point de vue chronologique, l'apparition des racines adventives semble aussi être associée à la réouverture des stomates, suggérant un rôle important dans le rétablissement des échanges gazeux en conditions d'ennoyage (Fig.4a p.88).

Ces trois types d'adaptations analysées au cours de cette étude (aerenchyme, lenticelles hypertrophiées et racines adventives) aident toutes à maintenir un certain niveau d'oxygène nécessaire au métabolisme et donc à la survie de la plante lorsque le stress se prolonge. Chacune d'entre elles semble apparaître plus rapidement et/ou de façon plus importante chez l'espèce la plus tolérante, le chêne pédonculé.

C'est au niveau des racines, premier tissu à percevoir le stress, que l'initiation d'une réponse d'adaptation à l'ennoyage doit s'effectuer. Afin d'établir une réponse le plus rapidement possible, les racines doivent disposer d'un système de veille, prêt à « détecter » les modifications environnementales et à initier une cascade signalétique relative au stress imposé. En conditions témoins, l'hémoglobine non-symbiotique (Hb-ns) est généralement davantage exprimée dans les racines que dans les autres tissus (Fig.3 p.53 & Fig.1 p.62 ; Larsen *et al.* 2003, Wang *et al.* 2003, Parent *et al.* 2008a, 2008b). Cette localisation suggère un rôle prépondérant pour l'hémoglobine au niveau du système racinaire. Son expression n'est d'ailleurs pas répartie uniformément dans la racine et nos expériences d'hybridation *in situ* effectuées sur la pointe de la racine ont montré une localisation au niveau de certains tissus ou groupements cellulaires spécifiques (Fig.5 p.55 & Fig.2 p.62 ; Parent *et al.* 2008a,

2008b). Aussi bien chez le chêne pédonculé que chez le chêne sessile, les transcrits de *QpHb1* abondent au niveau des cellules vivantes les plus externes de la racine. En effet, en amont de l'apex, on trouve la « zone externe » de la coiffe, sous une couche de cellules mortes, qui se différenciera en protoderme puis en épiderme. Ces trois zones présentent une forte expression de l'Hb. Cette localisation pourrait être associée à un rôle de senseur de perturbations rhizosphériques. La présence d'Hb pourrait également être importante comme barrière protectrice contre les effets toxiques du monoxyde d'azote (NO) produit durant les conditions d'ennoyage par les microorganismes de la rhizosphère. L'Hb-ns semble être également impliquée dans la régulation de processus indépendants de la réponse aux stress. Elle pourrait ainsi protéger les cellules vivantes de l'endoderme contre la mort cellulaire. En effet, le signal de mort cellulaire impliqué dans la dégradation ou la mort de cellules composant une partie de la coiffe, ne doit pas s'étendre aux couches de cellules sous-jacentes. Ce signal pourrait être transmis par le NO et la présence d'Hb à ce niveau permettrait de le détoxifier et de protéger ces cellules contre l'initiation d'une mort cellulaire.

L'expression de *QpHb1* au niveau du cylindre central dans les cellules du protoxylème mais pas au niveau du protophloème (Fig.5 p.55 & Fig.2 p.62 ; Ross *et al.* 2001, Parent *et al.* 2008a, 2008b) pourrait être synonyme d'un autre rôle pour les ns-Hbs. Cette localisation pourrait être associée à la nécessité de communication entre les différents tissus, notamment en conditions d'hypoxie. Les vaisseaux conducteurs du xylème représentent en effet une voie de communication privilégiée entre appareil racinaire et aérien (Duff *et al.* 1998, Smaggle *et al.* 2007). Le NO est produit en réponse à l'hypoxie et il a été démontré que l'hémoglobine permet d'éviter une trop forte accumulation de NO, pouvant s'avérer toxique pour les cellules (Dordas *et al.* 2003a). Cependant le NO est une molécule signal du stress et la réaction de l'Hb avec le NO pour former du nitrate empêcherait cette fonction de signalisation. L'Hb peut interagir d'une autre façon avec le NO en se fixant sur le résidu cystéine par S-nitrosylation, formant la S-nitrosoHb (Perazolli *et al.* 2004). Cette modification post-traductionnelle pourrait aussi permettre l'activation d'une fonction signalétique, tout en évitant un stress nitrique pour les cellules. De plus, la S-nitrosylation d'autres protéines pourrait être responsable de l'activation ou l'inhibition de protéines clés de la réponse à l'hypoxie et ce mécanisme pourrait aussi être régulé par une modulation du niveau intracellulaire de NO par l'Hb. Il a été montré notamment que le NO était impliqué dans la fermeture des stomates (Neill *et al.* 2008). Ainsi, on peut supposer que la régulation de la conductance stomatale en conditions d'hypoxie pourrait être liée à l'Hb-ns. La localisation de l'Hb au niveau du xylème pourrait donc permettre cette signalisation rapide vers l'appareil aérien. De plus, la formation

des vaisseaux du xylème requiert un « burst » de NO correspondant à un point de non-retour pour la cellule, puisque ce signal initie la PCD nécessaire à la formation du xylème (Gabaldon *et al.* 2005). Cette co-localisation pourrait donc être nécessaire à la différenciation des cellules du xylème. Ces deux formations cellulaires (cellules mortes de la coiffe et vaisseaux du xylème) font intervenir une mort cellulaire dépendante du NO. Le rôle prépondérant du NO dans la mort cellulaire est depuis longtemps connu mais la modulation de l'activité du NO dans cette fonction par l'Hb-ns pourrait être primordiale.

Comme discuté précédemment, le monoxyde d'azote peut jouer de nombreux rôles chez les plantes, aussi bien en réponse au stress que dans des processus constitutifs comme dans la régulation de la croissance et du développement des plantes (Wendehenne *et al.* 2004). Ainsi le NO a récemment été identifié comme modulateur du gravitropisme (Hu *et al.* 2005) et nos résultats d'hybridation *in situ* (Fig.5a p.55), de même que ceux de Ross *et al.* (2001) montrent une accumulation de transcrits de *QpHb1* au niveau de la partie de la coiffe correspondant à la columelle. La columelle est impliquée dans la perception de la gravité et une accumulation asymétrique de NO induite par l'auxine dans la racine permettrait d'orienter la croissance (Hu *et al.* 2005). La présence d'Hb dans ces cellules pourrait indiquer sa participation directe dans la régulation du niveau intracellulaire du NO et donc du gravitropisme ou bien afin d'éviter les effets potentiellement toxiques dus à une trop grande concentration en NO. Il est intéressant de constater que le NO est également impliqué dans la mise en place des racines adventives chez le concombre (Pagnussat *et al.* 2002, 2003). Ces racines adaptatives se développent latéralement et parallèlement au niveau de la nappe et la régulation du gravitropisme est donc modifiée pour permettre cette particularité. Le couple Hb/NO pourrait, encore une fois, être impliqué dans ce mécanisme. Cependant, l'expression de *QpHb1* dans les racines adventives (Fig.10 p.93) pourrait également être en relation avec la demande énergétique liée à la mise en place de ces nouvelles racines. En effet, malgré les conditions d'hypoxie limitant fortement la production d'énergie, des racines adventives sont initiées. La réaction d'oxydation du NO par l'oxy-hémoglobine et produisant du nitrate, pourrait permettre la production d'ATP par la réduction du nitrate en NO. Cette séquence de réactions dépendante du NAD(P)H, proposée par Igamberdiev *et al.* (2005), pourrait fournir de l'ATP de façon plus efficace que la glycolyse. Cette production alternative d'énergie expliquerait comment la plante serait capable de produire de nouvelles racines en situation de stress hypoxique. La localisation de *QpHb1* par hybridation *in situ* dans les différentes couches cellulaires de la racine, montre une accumulation de transcrits au niveau du protoderme (Fig.11b p.94) qui pourrait comme dans le système racinaire originel en

conditions normoxiques, permettre une protection contre le milieu extérieur. Cette localisation pourrait également servir de barrière contre la perte radiale d'oxygène (ROL : radial oxygen loss). En effet, les racines adventives présentent un cortex lacuneux d'aérenchymes (III.B. Fig11a p.94) favorisant la diffusion de l'oxygène des parties aériennes vers les racines et cette enceinte protectrice d'Hb éviterait la fuite du peu d'oxygène disponible vers la rhizosphère. L'Hb se localise également dans certaines cellules du cortex, et d'après les résultats de Northern, y est fortement exprimée. Elle pourrait donc permettre le maintien du potentiel redox ainsi que du statut énergétique.

QpHb1 s'exprime et se localise différemment dans les deux espèces de chêne. Le niveau d'expression, de même que le lieu d'expression de l'ns-Hb pourraient être des éléments clés de discrimination de la tolérance à l'ennoyage. Dans une certaine mesure, l'hémoglobine pourrait assurer un rôle de protection et d'évitement des conditions critiques dues à l'hypoxie. D'autre part, elle pourrait entraîner une réponse appropriée afin de mettre en place les adaptations nécessaires à la survie dans ces conditions.

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Annexes

Liste des abréviations

ABA	acide abscissique
ACC	1-aminocyclopropane 1-carboxylate
ADH	alcool déshydrogénase
ADN	acide désoxyribonucléique
ADP	adénosine diphosphate
ANOVA	analyse de variance
ANPs	anaerobic proteins
ARNm	acide ribonucléique messenger
ATP	adénosine triphosphate
CaM	calmoduline
CK	cytokinine
CMT	cortical microtubule
CO	monoxyde de carbone
CO₂	dioxyde de carbone
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DEPC	diéthylpyrocarbonate
DW	dry weight
EDTA	ethylene diamine tetra-acetic acid
Eh	potentiel rédox
En	endoderme
ENO	enolase
Ep	epiderme
ERF	ethylene response factor
FAO	Food and Agriculture Organization of the United Nations
FW	fresh weight
GA	gibbérelline
GAD	glutamate décarboxylase
GABA	acide γ amino-butérique
GAPC	glycéraldéhyde 3-phosphate déshydrogénase
GIEC	Groupe d'experts Intergouvernemental sur l'Evolution du Climat
Hb	hémoglobine

HIF	hypoxia-Inducible heterodimeric transcription Factor
H₂O₂	peroxide d'hydrogène
IAA	auxine
IDF	Institut pour le Développement Forestier
IFN	Inventaire Forestier National
ISH	<i>In situ</i> hybridization
LDH	lactate déshydrogénase
Lp	conductance hydraulique
MetHb-R	methemoglobin reductase
NAD(P)⁺	nicotinamide adénine dinucléotide (phosphate), forme oxydée
NAD(P)H	nicotinamide adénine dinucléotide (phosphate), forme réduite
NO	nitric oxide ou monoxyde d'azote
ns-Hb	hémoglobine non-symbiotique
NTP	nucléoside triphosphate
O₂	oxygène
ONF	Office National des Forêts
PAR	photosynthetically active radiation
PBS	phosphate buffered saline
PCD	programmed cell death (mort cellulaire programmée)
PDC	pyruvate décarboxylase
PIPs	plasma membrane intrinsic proteins
<i>QpHb1</i>	<i>Quercus petraea</i> hemoglobin gene 1
Rc	root cap
ROL	radial oxygen loss
Rop	RHO-related GTPase of plants
ROS	reactive oxygen species (espèces réactives de l'oxygène)
SA	salicylic acid
SDS	sodium dodecyl sulfate
S.E.	standard error
SLA	specific leaf area
SSC	saline sodium citrate
TCA cycle	tricarboxylic acid cycle ou cycle de Krebs
XET	xyloglucan endotransglycosylase

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I. Introduction

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II. Synthèse bibliographique

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Liste des publications et communications

Publications dans des revues à comité de lecture

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Parent C., Capelli N., Dat J. (2008b) Reactive oxygen species, stress and cell death in plants. *C. R. Biologies* 331, 255–261

Parent C., Berger A., Folzer H., Dat J., Crèvecoeur M., Badot P.-M., Capelli N. (2008a) A novel non-symbiotic hemoglobin from oak: Cellular and tissue specificity of gene expression. *New Phytologist* 177, 142–154

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Parent C., Berger A., Capelli N., Crèvecoeur M., Badot P.M., Dat J.F. Analyse de l'expression d'un gène d'hémoglobine non-symbiotique chez deux espèces de chênes *Quercus petraea L.* et *Q. robur L.* en réponse rapide à l'ennoyage. (2008) 10ème Rencontre du groupe de Biologie Moléculaire des Ligneux, 5-7 mai, Nancy, France.

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Figures annexes

Résultats Master A. Berger

3.4.2. Dégradation des noyaux

Au niveau du 4^{ème} centimètre, lors d'un stress de 7 jours, on peut observer des altérations des noyaux du parenchyme cortical. Certains noyaux de formes rondes présentent des amas denses de chromatine (figure 15.A). D' autres prennent une forme plus allongée et on voit, là encore des amas denses de chromatine (figure 15.B) à la périphérie de l'enveloppe nucléaire. Enfin certains noyaux paraissent s'être complètement désagrégés et on peut observer la chromatine à l'état libre se dispersant dans la cellule (figure 15.C).

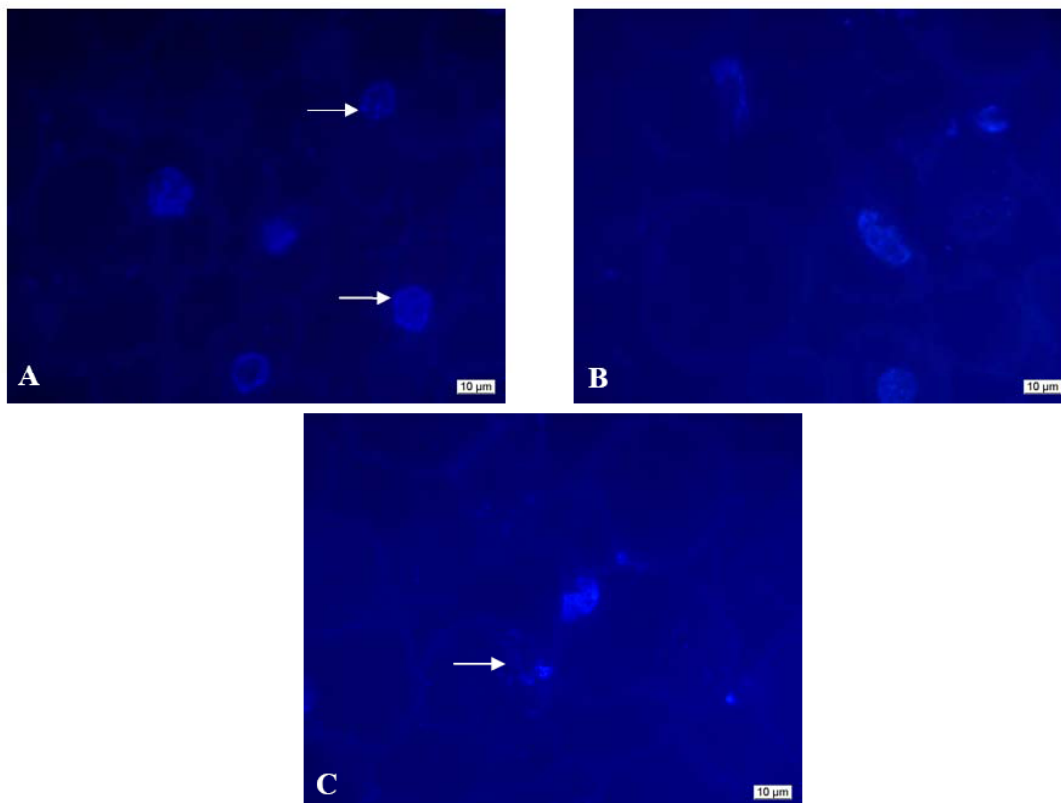


Figure 15: altérations nucléaires observées dans le parenchyme cortical après un stress hypoxique

Coupes paraffine au niveau du 4^{ème} cm dans une racine de chêne sessile à 7 jours d'envoyage. Coloration DAPI.

Hypoxia Stress: Current Understanding and Perspectives

Cette annexe correspond à un chapitre d'ouvrage intitulé Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues (1st Edition). Les auteurs sont J.F. Dat, H. Folzer, C. Parent, P.-M. Badot, N. Capelli.

Résumé:

Aussi bien dans leur milieu naturel que lorsqu'elles sont cultivées, les plantes sont fréquemment exposées de façon temporaire ou permanente à un déficit en oxygène au niveau de la rhizosphère suite à l'engorgement du sol. Ainsi, l'hypoxie et/ou l'anoxie sont parmi les contraintes environnementales couramment subies par le système racinaire. Le manque d'oxygène se répercute sur la croissance, le développement et la survie de la plante.

Depuis une dizaine d'années, le développement des approches génomiques et protéomiques associées aux études physiologiques et morphologiques a permis d'améliorer considérablement nos connaissances sur les réponses des plantes à l'engorgement. Cependant même si, les mécanismes de réponse impliqués dans la tolérance à l'engorgement sont désormais mieux compris, il paraît indispensable de mieux appréhender les mécanismes adaptatifs afin d'opérer une meilleure sélection des espèces candidates.

Ce chapitre a pour objectif premier de faire l'inventaire des réponses métaboliques, physiologiques et morphologiques ainsi que des stratégies d'adaptation adoptées par les plantes en conditions d'engorgement. Il donne également certains éléments clés pour évaluer les perspectives d'amélioration de la tolérance des plantes à l'engorgement par croisement, biotransformation et sélection grâce aux gènes marqueurs de tolérance.

X

Hypoxia Stress: Current Understanding and Perspectives

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Keywords: adaptation, flooding, plant response

ABSTRACT

Under both natural and agricultural culture conditions, plants are frequently exposed to transient or permanent low O₂ levels in the soil atmosphere or soil solution as a consequence of flooding. Thus hypoxia and/or anoxia are environmental stresses commonly encountered by plant root systems. These O₂ restriction conditions will have drastic effects on plant growth, development and survival. Over the last decade, the introduction of large scale genomic and proteomic approaches coupled to physiological and morphological studies have allowed a great leap forward in our understanding of plant responses to flooding. As a result, the response and adaptation mechanisms involved in flooding tolerance are sufficiently understood allowing the selecting and breeding of stress tolerant plant varieties. However, it is crucial to understand the basic adaptive mechanisms that allow for the efficient selection of candidate species. This chapter reviews the metabolic, physiological and morphological responses and adaptation strategies of plants to flooding and assesses the prospect of improving plant tolerance to flooding through breeding, biotransformation and marker aided selection.

1. INTRODUCTION

Hypoxia and/or anoxia conditions around the root system are a major issue for plant growth and development, not only in natural ecosystems but also in agriculture, horticulture or silviculture. In natural or semi-natural habitats, the expected rise in sea level, the building of dams or water channels, or the removal of vegetation cover make flooding one of the most important stress factors limiting growth and development of numerous plant species (Kovlowski 1997). In horticulture and agriculture, under field or greenhouse growth conditions, hypoxia is frequently observed as a result of either poor drainage and/or root aeration. Furthermore, flood-irrigation techniques are still commonly used in many parts of the world to control undesired vegetation and/or plant diseases. Although this technique is convenient and rather inexpensive, from an agricultural perspective, it may dramatically alter the soil chemical and physical properties which will subsequently affect plant growth, development and regeneration. Finally, the use of aero- or hydroponic growth techniques for many crop and horticultural species may also lead to conditions of hypoxia and/or anoxia. Thus, an understanding of growth and development under oxygen limiting conditions is critical to the improvement of successful regeneration and culture techniques as well as increasing crop yield.

There has been great progress in the understanding of the response of plants to flooding since the introduction of large scale genomic and proteomic approaches. Furthermore, current knowledge of the response at the whole plant level has greatly benefited from integrated studies of the adaptive mechanisms at the physiological, cellular and molecular levels.

This chapter investigates some of the basic molecular, cellular and morphological characteristics that make some plant species more tolerant to hypoxia/anoxia conditions than others. It also addresses research issues and perspectives for improving plant regeneration and growth under hypoxia/anoxia conditions.

2. SYMPTOMS OF HYPOXIA/ANOXIA

The effect of oxygen deprivation on plant growth and development has long been recognised. There are numerous historic examples of dramatic flood events destroying annual crop production and reducing harvest yield. On the other hand, the occurrence of flash floods, although common in many areas on a yearly basis, often does not attract as much attention. However, transient floods, caused by a seasonal rise in the water table (spring or fall), will have marked effects on plant growth and development. When these events occur in the spring there will often reduce seed germination and seedling establishment. In general, root hypoxia will only be detected after the plant has been experiencing the stress for some time and, only once the aerial part of the plant shows symptoms of stress such as leaf wilting, bleaching, and senescence.

Several factors will determine how plants are affected by flooding. Timing (period of the year) and duration of the flooding event, air/soil

Abbreviations: ABA, abscissic acid; ACC, 1-aminocyclopropane 1-carboxylate; ADH, aldehyde dehydrogenase; Eh, redox potential; GA, gibberellin; GAPC, glyceraldehyde 3-phosphate dehydrogenase; IAA, auxin; LDH, lactate dehydrogenase; Lp, hydraulic conductance; PDC, pyruvate decarboxylase; SA, salicylic acid; XET, xyloglucan endo-transglycosylase

temperatures as well as plant developmental stage will impact plant growth. Overall however, early plant symptoms resemble those typically observed during drought stress. Both stresses, although considered physically opposite, often lead to similar alterations in plant growth and development. For instance, the occurrence of hypoxia in the root zone will inevitably lead to a reduction in water absorption (Jackson 1997, Kozłowski 1997, Pezeshki 2001), thus creating physiological conditions very similar to drought stress. As a result, sensitive plants exposed to hypoxia often wilt in a similar way as plants exposed to drought. The most commonly observed early symptoms on the aerial part of a plant include: leaf curling (epinasty) and stem twisting, leaf chlorosis and wilting, marginal browning of the leaf and shedding/defoliation, as well as fruit drop (Fig. 1). Other signs of stress include reductions in specific leaf area and weight, a decline in shoot growth and sprouting, dieback of the stem and limb and eventually death of the plant. All these symptoms occur as a consequence of root waterlogging, which itself will generally lead to a reduction in root growth and alteration in root to shoot ratios. However, one must keep in mind that symptoms of root hypoxia/anoxia are very general signs of stress and it may be difficult to differentiate them from other stress symptoms.

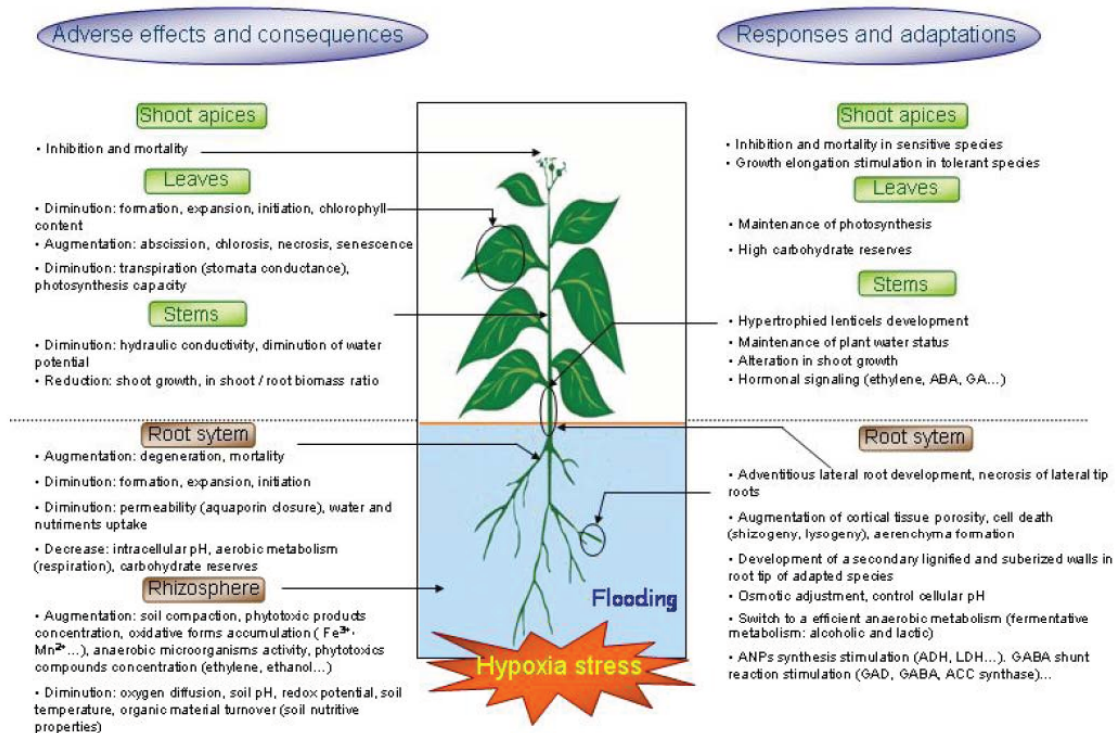


Fig. 1 Schematic representation of the responses and adaptations of the various plant organs to hypoxia/anoxia.

3. CHANGES IN THE RHIZOSPHERE DURING HYPOXIA/ANOXIA CONDITIONS

To best understand the impact of hypoxia/anoxia on plants, it is critical to know how stress affects the root system and its surrounding environment. Indeed, soil flooding not only creates conditions of hypoxia, it affects many other soil characteristics. As water enters the soil, gases are displaced. This water saturation of the soil interstices changes the physico-chemical characteristics of the root environment. First, the presence of water will reduce the gas exchange capacity of the soil by displacing the gaseous environment surrounding the roots. Gases being much less diffusible in water than air (gases diffuse 10^4 faster in air than in water; Armstrong 1979), a decline in the concentration of O_2 will rapidly take place (reviewed below) whereas levels of CO_2 , methane, volatile fatty acids and various gaseous hormones (i.e. ethylene) produced as by-products of fermentative metabolism, will rise. As gases become entrapped in and around the root they will cause some alteration in the composition of dissolved gases in the rhizosphere. Some of these changes may also apply to hydroponic-type culture systems.

A second string of changes taking place following soil flooding consists of changes in pH and redox potential (Eh). Both the pH and Eh changes are derived from the alterations to the gaseous environment described above. Following soil flooding, regardless of their original pH, soil pH will approach neutrality as the pH of acid soils increases whereas that of alkaline soil will tend to decrease. These changes are however very much dependent on the presence of organic matter and reducible-Fe contents of soils. As the soil becomes reduced, iron and iron oxides will also become reduced leading to a change in the proton and cation balance. This process will be amplified in the presence of CO_2 as the partial pressure of CO_2 will buffer carbonate thus lowering the pH. Although pH itself may not directly affect plant growth, it will lead to the release of i) phytotoxic compounds or ions such as manganese and aluminium through solubilisation, and a reduction in ii) the turnover of organic matter, iii) mineralization and, iv) calcium availability. In aero- and hydroponics systems changes in solution pH will directly affect the availability of certain nutrients. Micronutrients will be excessively uptaken under low pH ($pH < 7$) whereas under high pH ($pH > 7$) precipitation will reduce their availability. In addition, numerous studies have demonstrated a strong correlation between reductions in plant growth and changes in root zone aeration under soil less culture conditions (Morad and Silvester 1996, Morard *et al.* 2000, Tesi *et al.* 2003, Urrestarazu and Mazuela 2005).

Changes in soil redox potential (Eh) will be directly linked to alterations in soil pH, as the pH of the soil will tend to increase with chemical reduction and to decrease with oxidation. As a result, soil Eh will generally decline during hypoxia (Pezeski and DeLaune 1998, Pezeshki 2001). The changes in Eh will affect the charge conditions of certain clay minerals and their cation exchange capacity and thus the mobility and availability of many nutrients. The decline in Eh will favour the release of cations through adsorption of ferrous iron on exchange complexes and phosphorus by dissolution of oxides. Upon reduction, NO_3^- will turn into NH_4^+ which will then be fixed on the soil's cation exchange complexes. These changes in Eh followed by reductions in uptake will often lead to a decline in plant nitrogen and phosphorus concentrations. In contrast, oxides of manganese (Mn^{4+}) and iron (Fe^{3+}) will be reduced thus forming more soluble forms, Mn^{2+} and Fe^{2+} (Laanbroek 1990) and leading to increased accumulation of both in plant tissues (Gries *et al.* 1990). When O_2 limiting conditions are prolonged anaerobic bacteria may further convert sulfate (SO_4^{2-}) to sulfite (H_2S). This may be compounded by the release in the root environment of CO_2 , N_2 , H^+ , ethylene and methane thus altering the chemiostatic equilibrium and affecting not only soil pH but also the symbiotic relationship between the soil microorganisms and the host plant. In aero- and hydroponic systems, root development and plant growth will also be affected by the decline in O_2 levels as a result of reduced diffusion combined with the potential accumulation of phytotoxic plant derivatives in the nutrient medium.

Hypoxia/anoxia conditions will also affect soil structure and texture over the long term. These changes will tend to increase soil impedance especially following recession of the water table, creating a physical constraint to root development, thus altering plant growth. Finally, the reduction in biomass production and C mineralization will also alter the soil over the long term.

One of the most important events during flooding is the depletion of oxygen. The reduction in gas diffusion to the root environment as a result of the presence of excessive water in soil or poor aeration in soil less cultures (gases diffuse better in air than in water), accompanied by depletion of available oxygen by aerobic processes (i.e. root and microbial respiration), will deprive the rhizosphere of available O_2 . This will lead to anoxic conditions and a decrease in root metabolism as a result of a reduction in ATP production. The reduced availability in energy will have dramatic consequences on cellular processes leading to water and nutrient imbalance and/or deficiency. These stress conditions will also make crop species more prone to other stresses, more particularly to pathogen infection (Yanar *et al.* 1997). There exist a wide range of "secondary" opportunistic fungi and insects (phloem and wood borers) that selectively invade their host when they are weakened by stress (Munkvold and Yang 1995). In addition, certain root rot diseases of the *Phytophthora* and *Phyium* species are known to prevail under oxygen deficient conditions (Balerdi *et al.* 2003).

4. PLANT RESPONSES TO HYPOXIA/ANOXIA AND TOLERANCE STRATEGIES

As indicated in the paragraph above, numerous culture systems are prone to inducing hypoxia/anoxia stress. As a result it is important to clearly understand the various plant responses and adaptive strategies. As with any stress response, a change in the surrounding environment will alter the capacity of the plant to grow and develop normally.

4.1. Physiological responses and consequences on plant growth

4.1.1. Stomatal regulation and plant water relations

One of the earliest physiological consequences of hypoxia is a reduction in stomatal conductance (Sena Gomes and Kowlowski 1980, Pezeski and Chambers 1985, Folzer *et al.* 2006). In fact, changes in plant water homeostasis during flooding stress share similarity with those commonly observed during drought stress. Indeed, soil water saturation may not only reduce stomatal conductance but also limit water uptake, thus leading to internal water deficit (Jackson and Hall 1987, Smith and Huslig 1990, Ismail and Noor 1996, Pezeski *et al.* 1996a 1996b, Else *et al.* 2001). However, this is not always the case and the initial decline in stomatal opening can occur without significant changes in the plant water status (Tang and Kozlowski 1982, Pezeski and Chambers 1985). When flooding stress is prolonged, changes in stomatal conductance are generally coupled with increased water stress and leaf dehydration (Pezeshki 2001). In many woody fruit trees for instance, flooding adversely altered stomatal conductance and plant water status (Zaerr 1983, Schaffer and Ploetz 1989, Nicolas *et al.* 2004). Interestingly, an enhanced ability to keep stomates open during flooding was suggested as a key parameter of enhanced tolerance among selected flood-tolerant *Populus deltoides* clones (Cao and Conner 1999). Similarly, after 34 days of flooding, stomatal conductance of the more flood tolerant tamarack (*Larix laricina*) was higher than that of the more flood sensitive black spruce (*Picea mariana*; Islam and McDonald 2004). It is thus probable that part of the tolerance to flooding resides in the ability to keep a favourable plant water status by enabling an adequate supply of water to reach the shoot.

The development of water stress during flooding can result from a change in hydraulic conductivity (L_p) consequent to a decrease in root permeability (Clarkson *et al.* 2000, Else *et al.* 2001). In fact, the decrease in L_p has recently been linked to aquaporin gating by cytosolic pH (Toumaire-Roux *et al.* 2003). Prompt stomatal closure will temper the adverse effect of reduced root L_p , thus limiting water loss through transpiration and maintaining an adequate plant water status. Consequently, decreased transpiration rates are a common feature of the response to flooding (Cao and Conner 1999, Folzer *et al.* 2006) and rapid stomatal closure is probably an adaptive feature of flooded plants (Jackson *et al.* 2003). An alternative adaptation feature is the maintenance of high root L_p thus allowing adequate water uptake as observed in tamarack (Islam *et al.* 2003). Water stress responses under hypoxia are, however, clearly species and age specific and, dependent on the duration and intensity of the applied stress (Koslowski 1997).

Features of stomatal control are not fully understood, though several mechanisms have been proposed (Else *et al.* 1995 2001, Jackson *et al.* 2003, Dat *et al.* 2004). The delivery rates from root to shoot being strongly reduced during flooding, root ABA levels are probably not involved in stomatal closure (Else *et al.* 2001). Leaf ABA levels probably serve as relay to other root derived signals (Jackson 1990 2002). Other signals including negative messages in the form of pH and decreased solute delivery are thus good candidates (Jackson *et al.* 2003, Dat *et al.* 2004).

4.2. Photosynthesis and carbohydrate reserves

Following the hypoxia-induced initial decline in stomatal conductance, a rapid reduction in the rate of photosynthesis is commonly observed in flood-intolerant plants (Ismail and Noor 1996, Huang *et al.* 1997, Kozlowski 1997, Gravatt and Kirby 1998, Pezeski and DeLaune 1998, Ashraf and Habib-ur-Rehman 1999, Malik *et al.* 2001). Indeed, stomatal aperture is often considered a limiting factor for CO_2 exchange rates (Liao and

Lin 1998 2001). Other factors such as a decrease in leaf chlorophyll content, early leaf senescence and reduction in leaf area may also contribute to inhibition of photosynthesis (Sena Gomes and Kozlowski 1980). For instance, submerged *Populus deltoides* exhibited a strong decrease in fluorescence thus reducing photosynthetic activity (Cao and Conner 1999). When flooding is prolonged the reduction in photosynthesis may involve inhibitory effects on carboxylation enzymes (Pezeshki 1993), chlorophyll loss (Cao and Conner 1999), reduction in metabolic activity and translocation of photoassimilates, and/or a decline in photosynthetic activity of the mesophyll (Vu and Yelenosky 1991, Huang *et al.* 1994, Liao and Lin 1994, Pezeshki *et al.* 1996a). Indeed, numerous studies have indicated that following soil waterlogging the rate of photosynthesis rapidly declines within a few hours to a few days (Sena Gomes and Kozlowski 1980, Pezeski and Chambers 1985). In addition, a reduced leaf water potential will reduce Rubisco activity as well as disrupt photoassimilate transport (Drew 1990, Pezeshki 1994 2001). The outcome of a decline in photosynthesis on plant growth and development may be dramatic, leading to concurrent physiological dysfunctions such as the inhibition of carbohydrate and water transport and changes in hormone relations (Kozlowski 1997, Vuylsteker *et al.* 1998, Kato-Noguchi 2000, Else *et al.* 2001, Gunawardena *et al.* 2001). In fact maintenance and/or recovery of photosynthetic capacity have been suggested as an adaptive response in some flood tolerant species (Pezeshki 1993, Liao and Lin 1994 2001, Pezeshki 2001).

A direct consequence of a decline in photosynthetic capacity will be the effect on carbohydrate reserves. Carbohydrate supply is correlated with the level of tolerance to hypoxia in many species, presumably through its involvement in providing energy during anaerobic conditions (Setter *et al.* 1997, Ram *et al.* 2002). An increased capacity to utilise sugars through the glycolytic pathway enables rice seedlings to survive longer periods under anoxia (Ito *et al.* 1999). Another illustration of the importance of carbohydrates lies in the fact that older seedlings, which contain higher reserves, are generally more flood tolerant than younger ones (Atkins *et al.* 1990). Protective effects of sugars have also been demonstrated by the enhanced survival rates when sugars are fed to plants during hypoxia (Vartapetian and Jackson 1997). In addition, treatments such as shading which tend to depress starch levels, will generally reduce tolerance to hypoxia (Jackson and Ram 2003). This however may not always be the case as a limited interaction was found between long-term waterlogging and irradiance on photosynthetic performance of three oak species (Wagner and Dreyer 1997). On the other hand, damage was more pronounced in field grown alfalfa exposed to waterlogging under cloudy weather (Barta 1988). Pre-flooding reserves are also often associated with enhanced survival (Setter *et al.* 1997, Sarkar 1998, Das *et al.* 2005). Overall, there is a strong correlation between flooding tolerance and carbohydrate reserves, although these are not necessarily sufficient for higher flood tolerance. Disruption of translocation of photoassimilate may in part explain this discrepancy.

Even though a plant may have high sugar reserves they must be available and be converted readily through an efficient glycolytic pathway. Furthermore, photoassimilates must be available to the organ (i.e. roots) subjected to anaerobiosis. This in fact has been proposed as one of the limiting steps in waterlogged plants (Pezeshki 2001). Flooding will tend to reduce the translocation of photosynthetic products from "source" leaves to "sink" roots, thus reducing availability for glycolysis during root anoxia (Kozlowski 1997). In fact, there is strong evidence that carbohydrate allocation patterns to the various plant organs may be one of the critical tolerance factors to hypoxia (Yamamoto *et al.* 1995, Liao and Lin 2001, Alaoui-Sossé *et al.* 2005). A decreased translocation from leaves to roots during waterlogging has been reported for many species and may be responsible for decreased tolerance (Barta and Sulc 2002). It is common to observe an accumulation of leaf starch coupled to a decline in photosynthetic rate during waterlogging (Topa and Cheeseman 1992, Liao and Lin 2001). As a result, the maintenance of photosynthetic activity and accumulation of soluble sugars to roots is clearly an important adaptation for species under flooding (Chen *et al.* 2005).

4.3. Plant growth and biomass accumulation

Decrease biomass allocation is a common feature of many wetland and non-wetland species during waterlogging (Blanch *et al.* 1999, Cooling *et al.* 2001, Sarkar *et al.* 2001). Indeed, growth processes will not continue without an adequate supply of oxygen (Drew 1990). Consequently, roots will be the first organ to sustain growth reduction. A rapid consequence of the decline in root respiration during flooding is the drastic reduction in root growth in both flood tolerant and intolerant plants. In fact, the capacity of seminal roots to maintain relatively high respiration rates is associated with flood tolerance in some species (Su and Lin 1996). This suggests that although anaerobic conditions will reduce root respiratory capacity, re-establishment and maintenance of respiratory metabolism is a crucial factor for flooding tolerance.

One of the first morphological evidence of hypoxia stress in a variety of plants is the necrosis of secondary root apices (Webb and Armstrong 1983, Barrett-Lennard *et al.* 1988, Folzer *et al.* 2006). Sacrificing a part of the root system through rapid anoxic root tip death may be an alternative to allow sufficient oxygen to reach the root apical meristem. This hypothesis is further supported by the enhanced survival of maize seedlings during hypoxia following removal of root tips (Subbaiah and Sachs 2003). Indeed, rapid root tip death is one of the first observed consequences of anoxia conditions generally followed by the initiation of adventitious roots in flood tolerant species. Under soil water saturation conditions, adaptive roots will tend to develop at the surface of the water table where oxygen concentration is still sufficient for aerobic processes. In fact, adventitious roots (roots initiated by tolerant species) will develop horizontally at the stem base, at the interface between the saturated soil and the atmosphere where oxygen is more readily available. Stimulation of adventitious root formation often corroborates with a reduction in new root initiation (Pezeshki and Delaune 1990, Rogers and West 1993, Blom *et al.* 1994, Pezeshki *et al.* 1996b, Watkin *et al.* 1998, Bacanamwo and Purcell 1999, Gibberd *et al.* 2001, Malik *et al.* 2001). As a result, root growth is inhibited through reductions in new root initiation (except adventitious roots) and branching and enhanced root decay. Overall however, the decline in root growth may be attributed to a decrease in root carbon allocation, reduction in metabolic activity as a result of reduced nutrient supply, phytotoxic accumulation of metabolites, and blockage of oxidative phosphorylation as a result of decreased ATP production as anaerobic conditions prevail (Drew 1997).

As a consequence of the decline in root growth, shoot growth is also generally inhibited. This decline in shoot growth is characterised by suppression of leaf formation and expansion, increased abscission, necrosis and leaf senescence as well as shoot die back (Hurng *et al.* 1994, Ismail and Noor 1996, Vann and Magonigal 2002). Reductions in shoot growth are attributed to water and nutrient deficiencies and generally take place during long term flooding stress. However, shoot growth reductions may be considered an adaptive strategy during flash floods as it helps preserve the energy reserves required for further growth. In contrast, shoot elongation of wetland and amphibious plants is generally promoted under flooding conditions and during long term flooding events (Jackson 1990, Ito *et al.* 1999). This feature is clearly an escape strategy to promote growth under more favourable conditions. It is common for many aquatic/marsh plants (e.g. rice) to be regulated by at least

two phytohormones: ethylene and gibberellins (Setter and Laureles 1996, Kende *et al.* 1998). In rice for instance, two main adaptive strategies have been identified: i) to elongate and escape or ii) not to elongate and preserve resources (Ram *et al.* 2002). The first strategy is therefore clearly an adaptive response to submergence, allowing continuous development of the shoot above the water level (Cooling *et al.* 2001).

5. METABOLIC RESPONSES AND ADAPTATIONS TO HYPOXIA/ANOXIA

5.1. Maintenance of cellular ATP homeostasis

The immediate consequence of soil waterlogging will be a period of hypoxia as long as enough oxygen is still available for aerobic processes. Thereafter, anoxia conditions will develop and a dramatic reduction in ATP generation will occur as a result of decreased O₂ availability (Blom and Voeselek 1996). As respiration declines, the electron flow through the respiratory pathway is reduced, thus diminishing ATP production. Consequently, chemical oxidising power (i.e. nicotinamide adenine dinucleotide, NAD⁺) must be generated via alternative pathways that do not use O₂ as a reductant acceptor (Roberts *et al.* 1984, Drew *et al.* 1994, Drew 1997, Summers *et al.* 2000). As limitation of adenosine diphosphate (ADP) oxidative phosphorylation occurs, plants shift their metabolism. This switch from aerobic respiration to anaerobic fermentation involves the induction of glycolytic and fermentative genes (Peng *et al.* 2001) and occurs in both tolerant and intolerant species (Fukao and Bailey-Serres 2004). However, the kinetics of induction of the various enzymes involved in glycolysis and fermentation is very much dependent on the tolerance level (Umeda and Uchimiya 1994).

Following the initial sensing of a drop in O₂, total protein synthesis is drastically altered, resulting in the selective synthesis of approximately 20 proteins (anaerobic proteins, ANPs; Sachs *et al.* 1980, Chang *et al.* 2000). Most genes and proteins identified during anoxia in acclimated maize root tips are soluble metabolic enzymes involved in glycolysis, fermentation and primary carbohydrate metabolism (Chang *et al.* 2000). These include alcohol dehydrogenase 1 (ADH1; Sachs *et al.* 1980), enolase 1 (ENO1; Lal *et al.* 1991 1998), glyceraldehyde-3-phosphate dehydrogenase (GAPC; Russell and Sachs 1991) and a pyruvate decarboxylase (PDC; Kelley *et al.* 1991). Additional proteins whose synthesis is altered during maize root tip hypoxia treatment have recently been identified (Chang *et al.* 2000). Genes whose expression is also modified include transcription factors, signal transduction components, non-symbiotic haemoglobin as well as those involved in ethylene biosynthesis, nitrogen metabolism and cell wall loosening (Klok *et al.* 2002, Nie *et al.* 2002, Dordas *et al.* 2003 2004, Paul *et al.* 2004, Liu *et al.* 2005). Thus, as O₂ levels decline, the fermentative pathway serves as a metabolic safe route. This is performed through two steps: carboxylation of pyruvate to acetaldehyde (catalysed by PDC) and the subsequent reduction of acetaldehyde to ethanol with concomitant oxidation of NADPH to NADP⁺, catalysed by alcohol dehydrogenase (ADH; Vartapetian and Jackson 1997, Kingston-Smith and Theodorou 2000, Nakasono *et al.* 2000). In flooded plants, three main metabolic fermentative pathways are active: an ethanolic pathway, a lactic acid one and one involving alanine aminotransferase (Dennis *et al.* 2000; Fig. 2).

Glycolysis, linked principally to ethanol and to a lesser extent lactate fermentation is the principal means of recycling the NADH necessary to maintain glycolysis and ATP production. However, anaerobic fermentation is very inefficient, releasing a fraction of the ATP normally generated under aerobic conditions (Summers *et al.* 2000). To compensate for this loss, the glycolysis flux can increase (Pasteur effect), thus restoring in part the nucleoside triphosphate (NTP) pool (Gout *et al.* 2001). Consequently, glycolysis can still take place as long as the NADPH generated can be oxidised into NAD(P)⁺. However, the "Pasteur effect" *per se* is not correlated to flooding tolerance.

Anoxia tolerant species often share the following characteristics: i) a capacity to sustain ethanol fermentation, ii) release of ethanol and other by-products of anaerobic metabolism to the surrounding environment, iii) carbohydrate reserves allowing glycolysis to be maintained and iv) an ability to generate ATP through the adaptation of the TCA cycle (Fox *et al.* 1994). Although ethanolic and lactate fermentation do occur under limiting O₂ conditions, in most cases they are not sufficient for long term flooding tolerance (Laan and Blom 1990).

5.2. Cytoplasmic pH homeostasis

Low-oxygen stress also triggers other basic cellular responses. A fall in cytoplasmic pH is a common phenomenon observed in most living organisms during hypoxia and is considered an important factor for survival (Xia and Saglio 1992, Wilkinson 1999, Felle 2005). In plants, acidification of the cytoplasm is considered a common cause of cell death (Roberts *et al.* 1984). Thus, it is crucial that cytoplasmic pH is kept close to neutrality. Several parameters may be involved in the observed decline in cytoplasmic pH: increased influx of H⁺, hydrolysis of Mg²⁺ pools, NTP and sugar phosphates, accumulation of non-processed glycolytic intermediates and synthesis of lactate. Initially, the parallel decline in pH and the increased synthesis of lactic acid suggested that this compound was involved in the decline in pH of the cytoplasm. Lactate accumulation coupled to a restriction of tonoplast proton pumps as ATP levels decline, results in a rapid pH decline (Drew 1997). However, cytoplasmic acidosis was not correlated with increased lactate synthesis in rice (Menegus *et al.* 1991). More recently, release of H⁺ ions with the Pi-liberating hydrolysis of NPT was shown to be the principal cause of the initial drop in cytoplasmic pH in *Acer* cells exposed to anoxia (Gout *et al.* 2001). Interestingly, when cytosolic pH falls below 7, the optimum for lactate dehydrogenase (LDH) but within the range of PDC, pyruvate is converted to acetaldehyde which can then be converted to ethanol by ADH (Kingston-Smith and Theodorou 2000). In fact, ethanol is most probably the major product of fermentation whether the plant is tolerant or not to O₂ deficiency (Smith and Ap Rees 1979).

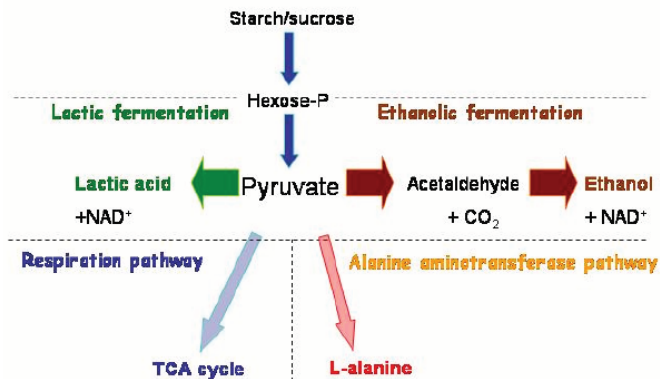


Fig. 1 Schematic diagram of the three main metabolic fermentative pathways in flooded plants.

6. MORPHOLOGICAL ADAPTATIONS TO HYPOXIA/ANOXIA

6.1. Aerenchyma development

One of the most important response of wetland and some non-wetland species to waterlogging is the development, in the root cortex, of gaseous spaces that could help provide O₂ to active cells in the roots (Fig. 3). The development of lacunae gas spaces in the root cortex is one of the most important adaptive response to soil flooding in many flood tolerant species (Vartapetian and Jackson 1997, Schussler and Longstreth 2000, Chen *et al.* 2002, Evans 2003), more specifically, bottomland woody species (Kludze and DeLaune 1994, Pezeshki and Anderson 1997). The increase in porosity may enhance venting of phytotoxic compounds being produced in the roots during waterlogging (i.e: ethanol, methane; Visser *et al.* 1997 2000) and/or reduce longitudinal diffusion of gases in the roots, thus increasing its aeration (Laan *et al.* 1991, Evans 2003). The proportion of aerenchyma generally distinguishes wetland from non-wetland plants (Vasellati *et al.* 2001). Ethylene dependent cell death and lysis occurs behind the root apex in the region of cell expansion and forms continuous gas-filled channels which help oxygen diffusion from shoot to roots (Drew 1997). The development of aerenchyma or lacunae tissues is not unique to roots. They are also observed in the leaf sheath following soil flooding, forming an interconnecting system of shoot-root ventilation (Jackson and Armstrong 1999, Fabbri *et al.* 2005). Their development increases root porosity which itself can be initiated as a result of osmotic dependant changes in cell shape (Justin and Armstrong 1987, Folzer *et al.* 2006; Fig. 3).

Injury and cell death of roots have in part been attributed to the accumulation of toxic end products of anaerobic metabolism and could thereby be qualified as necrosis. This hypothesis is supported by the observation that when glucose is supplied to roots under anoxia, thereby inhibiting the accumulation of anaerobic by-products, survival is increased (Drew 1997). However, death of root cells by phytotoxic accumulation of anaerobic metabolic derivatives is not the only type of cell death observed during aerenchyma development. The development of aerenchyma has been categorised into two types of developmental processes. The first is the constitutive development of aerenchyma as it occurs whether or not the plant is under waterlogged conditions. It forms by cells separating during tissue development. This type of cell death process is termed schizogeny (formed by cell separation) and is developmentally regulated and independent of any external stimuli. It is the outcome of highly regulated tissue specific patterns of cell separation. The other type of cell death process is termed lysogeny (formed by partial breakdown of the cortex) resembles programmed cell death (PCD) typical of plant pathogen interactions (Mittler *et al.* 1997) and during other abiotic stresses (Pellinen *et al.* 1999, Dat *et al.* 2001 2003). This active cell death process is genetically controlled and shows many similarities with apoptosis, though there is increasing evidence that it generally lacks several features of apoptotic cell death (Buckner *et al.* 2000). In aerenchyma development, cells of the cortex are specifically targeted, thus supporting PCD (Kawai *et al.* 1998). In *Sagittaria lancifolia* for example, nuclear changes (clumping of chromatin, fragmentation, disruption of the nuclear membrane), are the earliest events observed following flooding. These nuclear changes are followed by plasma membrane becoming crenulated, tonoplast disintegration, organelle swelling and disruption, loss of cytoplasmic contents and collapse of the cell (Schussler and Longstreth 2000). This sequence of events seems common to most species studied, though the timing of tonoplast disruption varies.

Development of aerenchyma is often associated with the enlargement of cortical cells and aerenchyma formation correlates with increased cellulase and/or xyloglucan endo-transglycosylase (XET) abundance (Drew *et al.* 1979, Saab and Sachs 1996, Gunawardena *et al.* 2001, Peng *et al.* 2001). These transglycosylase enzymes play a role in cell wall metabolism during cell expansion. They modify xyloglucans by cleavage and rejoining of the $\beta(1-4)$ -xyloglucan backbone, thus potentially altering cell size and form through cell wall loosening or tightening (Cosgrove 1999, Burstin 2000). Additional cellular changes take place during aerenchyma development, among them, orientation of cortical microtubule arrays (CMT). CMT arrays become diagonal rather than perpendicular and, the randomisation of CMT arrays correlates with aerenchyma development and is induced by ethylene treatment (Baluska and Barlow 1993, Schussler and Longstreth 2000).

In addition to schizogenic and lysigenic aerenchyma development a third type has been described in the aquatic plant, *Pontederia cordata*. This unique type of aerenchyma development originates from differential expansion of cells along walls lining intercellular spaces (Seago *et al.* 2000). Wetland species often have roots with lignified and suberized secondary walls that develop within a few centimetres of the root tip, thus limiting the radial diffusion and loss of O₂ to the surrounding environment. This reduction in root permeability is presumably sufficient to reduce the loss of O₂ from the inner root cortex towards the surrounding environment thus improving the efficiency of aerenchyma in providing O₂ to root cells.

6.2. Hypertrophied lenticels

The presence of hypertrophied lenticels is a common anatomical change observed in many woody species during flooding (Yamamoto *et al.* 1995, Kozlowski 1997; Fig. 3). Hypertrophic growth appears as swelling of tissues at the stem base and is believed to result from radial cell

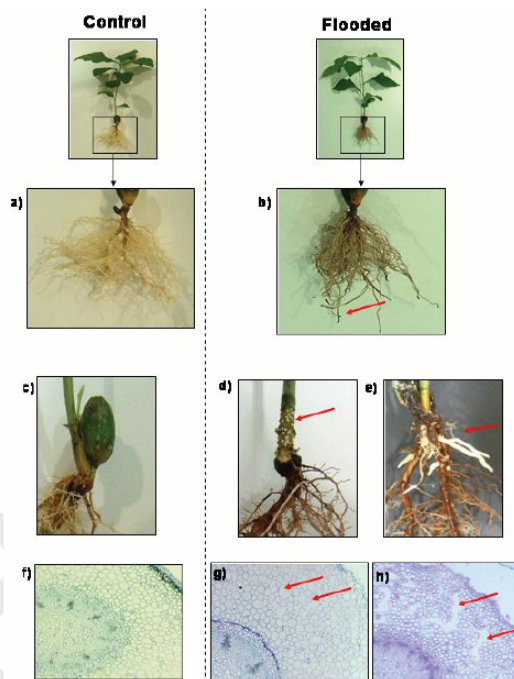


Fig. 1 Figure showing some of the adaptive features observed in oak seedlings during hypoxia: root system of a) control and b) flooded seedlings (arrow indicates necrosis at root tips); shoot base of c) control and flooded seedlings d) hypertrophied lenticels (arrows), e) adventitious roots (arrows). Cortical main root tissue of, f) control and flooded seedlings showing g) disorganised cortical tissue (arrows) and h) aerenchyma (arrows).

division and expansion. It is associated with auxin and ethylene production (Blake and Reid 1981, Kozłowski 1997) and the development of large root cortical intercellular air spaces in flood-tolerant species (Topa and McLeod 1986, Mielke *et al.* 2003). The development of hypertrophied lenticels at the stem base is believed to facilitate the downward diffusion of O₂ as well as potential venting structures to release phytotoxic compounds produced as by-products of anaerobic metabolism (ethanol, ethylene, CO₂). Interestingly, the tendency for stomatal conductance to return towards control levels after a transient decrease, has generally been associated with the development of hypertrophied lenticels, lateral roots and flood tolerance (Pezeshki *et al.* 1996, Gravatt and Kirby 1998, Folzer *et al.* 2006).

6.3. Adventitious roots

Another important morphological adaptation to flooding is the growth of adventitious roots, which functionally replace basal roots (Bacabamwo and Purcell 1999, Gibberd *et al.* 2001, Malik *et al.* 2001). The formation of these specialised roots takes place when the original root system becomes incapable of supplying the shoot with the required water and minerals, because the energy required for active transport is insufficient (Mergermann and Sauter 2000). The formation of adventitious roots takes place at the base of the stem of flooded plants and their growth is horizontal rather than vertical like normal roots. Their presence at the interface between the water saturated soil and atmosphere reflects their importance in replacing the normal root system. The ability to produce adventitious roots is commonly associated with enhanced tolerance to flooding and their development is mediated by ethylene, as evidenced by experiments using ethephon (Mergermann and Sauter 2000). The higher tolerance to flooding of pedunculate oak (*Quercus robur*) as compared to sessile oak (*Quercus petraea*), is clearly based on the development of functional aerenchyma (Folzer *et al.* 2006). A similar analysis was previously used to explain the greater tolerance of *Eucalyptus camaldulensis* compared with *E. globulus* (Blake and Reid 1981).

7. SIGNALLING AND HORMONAL CONTROL OF PLANT RESPONSES TO HYPOXIA/ANOXIA

Flooding is characterised by changes in the levels of several plant growth regulators. The most studied, ethylene, is induced by a variety of biotic and abiotic stresses. The enzyme ACC synthase (S-adenosyl-L-methionine ethylthioadenosine-lyase, EC 4.4.1.14) catalyses the rate limiting step in ethylene biosynthesis, the conversion of S-adenosyl-L-methionine to 1-aminocyclopropane 1-carboxylate (ACC; Olson *et al.* 1995). In higher plants the immediate precursor of ethylene, ACC is produced as a result of the Yang cycle (Grichko and Glick 2001). Increased ethylene production occurs rapidly (within 4 h) following hypoxia treatment of *Arabidopsis* (Peng *et al.* 2001) or rice (Lorbiecke and Sauter 1999). In addition, ethylene is required for aerenchyma and adventitious root formation in maize (Kende 1993, He *et al.* 1996, Lorbiecke and Sauter 1999, Drew *et al.* 2000). Ethylene production rate in leaves of flooded plants closely correlates with the induction of ACC synthase and the accumulation of ACC in the roots (Kende 1993, Olson *et al.* 1995). In tomato plants exposed to flooding, ACC is rapidly synthesised in the roots, and transported to the shoot within 6 to 12 h (Shiu *et al.* 1998).

In addition to the convincing example of root-to-shoot ethylene signalling events during flooding, there is increasing interest in the possible role played by abscisic acid (ABA), gibberelin (GA) and auxin (IAA; Jackson 1997, Dat *et al.* 2004). In tomato, foliar ABA concentrations are increased transiently following soil flooding (He *et al.* 1996) and exogenous ABA application increased anoxia tolerance in maize and *Arabidopsis* (Hwang and Van Toai 1991, Ellis *et al.* 1999, Kato-Noguchi 2000). Furthermore, synergism between IAA and ethylene has been proposed during adventitious root formation (Visser *et al.* 1998), as IAA supplied to chicory and two *Rumex* species induced the formation of lateral and adventitious roots (Visser *et al.* 1995, Vuylsteker *et al.* 1998). In contrast, in flooded *Rumex palustris*, auxin delivered to the shoot base is a prerequisite for rooting but no accumulation of auxin takes place (Visser *et al.* 1995). Finally, the ethylene increase following flooding causes an increase in GA concentration and sensitivity to that hormone (Raskin and Kende 1984, Rijnders *et al.* 1997). In contrast, in *R. acetosa* although ethylene increases, GA levels remain unchanged (He *et al.* 1996). Recent results obtained using transgenic *Arabidopsis* plants producing high levels of cytokinin, showed that the increased tolerance of the plants during and after flooding stress coincided with an increase in cytokinin level (Zhang *et al.* 2000, Huynh *et al.* 2005).

Other signalling molecules such as salicylic acid (SA) may also be involved in the responses and adaptation to hypoxia. Indeed, a role for SA during both biotic and abiotic stress responses is becoming increasingly evident (Dat *et al.* 1998, Scott *et al.* 1999). In addition, SA has been implicated with ABA in the regulation of stomatal opening during drought stress (Larqu e-Saavedra 1978), stimulating root growth and adventitious root formation (Singh 1995, Gutierrez-Coronado *et al.* 1998), although, to date no direct evidence is available on a potential role for SA during hypoxia.

8. PERSPECTIVES FOR IMPROVEMENT OF HYPOXIA/ANOXIA TOLERANCE

8.1. Limiting hypoxia/anoxia conditions

Of course the first and foremost alternative to flooding damage is to limit the occurrence of this stress on agricultural and natural ecosystems. This can be done by applying various techniques to both the field and greenhouse grown cultures. Numerous technical examples of field drainage systems, modified irrigation techniques, fertilization, enhancing root aeration have been described in more specialized literature and will not be reviewed here. On the other hand, the choice of species and genotypes particularly adapted to flooding is an obvious positive evolution in dealing with the adverse effect of hypoxia.

8.2. Different natural strategies of tolerance to hypoxia/anoxia

As indicated by this review, plants have evolved numerous strategies to cope with hypoxia/anoxia conditions. The diversity of strategies can be attributed to a large extent to the range of ecological conditions under which plants grow. Indeed, plants frequently found in wetland environments will often have more drastic strategies to survive flooding than non wetland plants. Furthermore, marsh or aquatic plants, commonly used to long term flooding or total submergence will have developed strategies which may not be adapted to flash floods. As a result, one must clearly identify the ecological origin of a species to get some insight into its adaptive capacity to flooding.

Generally, adaptive tolerance strategies can be regrouped into three broad categories: i) escape, ii) avoidance and iii) tolerance. The first strategy is used by plants that will improve their tolerance to flooding by alleviating its adverse effects by promoting shoot elongation, formation of adventitious roots and root aerenchyma. This strategy is used by many wetland species. Although adventitious root formation will help the plant by developing a new root system in non-submerged parts of the soil, this is an expensive process as it generally means sacrifice of part of the taproot. The second strategy of avoidance is illustrated by plants that will become dormant during stressful conditions. This strategy is used by many species under various stress conditions. Although, often successful for short exposures to adverse environments, this strategy is inadequate for long term tolerance and/or adaptation. Finally, the third strategy will be used by plants that can accelerate glycolysis and ethanolic fermentation. This metabolic switch has many advantages including released pressure from O₂ dependent processes by a change from aerobic to anaerobic metabolism, while continuously generating the energy required for cell growth and survival. However, by-products derived during this process (i.e. ethanol and lactic acid) can accumulate to toxic levels in the root environment. Consequently, plants can only tolerate the stress for a limited time and need to dispose of these toxic compounds.

9. ENGINEERING TOLERANCE

9.1. Molecular markers and target transformation

There have been numerous attempts to improve tolerance to flooding in various model species. The use of large scale genomic and proteomic approaches has allowed the identification of several candidate genes for improving tolerance to waterlogging. In addition, identification of molecular markers would become an efficient and reliable screen for selecting tolerant genotypes. However, to date, no molecular markers are available for waterlogging tolerance in cereals for instance (Seeter and Waters 2003). As a result much hope is based on large scale genomic and proteomic approaches to identify new candidate genes. Among these, hemoglobin has received much attention in recent times. Indeed, oxygen binding proteins such as hemoglobin may help provide oxygen to cells under anaerobic conditions. This protein is characterised by its conserved structure, high O₂ affinity and reversible combination with O₂ in the ferrous state. It is one of the best characterised heme protein and plays such a role in animal cells. It may thus serve a similar function in plants.

Hemoglobin were only recently identified in plants. Two types of hemoglobins have been identified in plants: one symbiotic (leghemoglobin) the other not (non-symbiotic hemoglobin). The first was originally isolated in leguminous species and is present mainly in nodules where it helps oxygen transport (Hardison 1996). In contrast, the exact function of non-symbiotic hemoglobin is still a matter for debate although three main possible roles have been proposed for non-symbiotic hemoglobin (*Hb*) under hypoxic conditions. Hemoglobin may i) serve as an O₂ carrier as in animal cells (myoglobin) to help preserve mitochondrial respiration under anoxic conditions, ii) serve as an O₂ sensor capable of regulating the gene expression under anoxia (Nie and Hill 1997), or iii) help sustain glycolytic metabolism in stressed tissues (Sowa *et al.* 1998). Hemoglobin proteins of several species have a high avidity for O₂ (Arrendondo-Peter *et al.* 1997, Duff *et al.* 1997), indicating that the free protein will remain oxygenated at O₂ concentrations well below those at which anaerobic processes are activated.

Several transgenic plants have been produced to study the role of *Hb* in plants. Transformed maize cells over- or under-expressing a barley *Hb* gene showed no difference in culture growth rates or consumption of O₂ under normoxic conditions, suggesting that *Hb* may only play a role under conditions of anoxia (Sowa *et al.* 1998). A lower level of ADH activity in the *Hb* overexpressing line suggested that *Hb* may be an alternative to fermentation when the plants were under hypoxia. In addition, another series of experiments indicated that *Hb* maintains the energy status of the cell independent of mitochondrial oxidative phosphorylation. These results in combination with those obtained with barley support the idea that *Hb* utilises the available cellular O₂ to maintain ATP homeostasis, in cells exposed to conditions that reduce cell ATP levels (Taylor *et al.* 1994). Recently, *Hb* has been proposed as a mediator of aerenchyma development through an interaction with nitric oxide (Dordas *et al.* 2003 2004, Dat *et al.* 2004, Perazzoli *et al.* 2004). Thus, although several lines of research have indicated an interesting role of *Hb* in flooding tolerance, it is still unclear what exact function *Hb* may play in tolerance to flooding.

Another target of genetic transformation could involve the manipulation of transcription factors, as successfully used to improve tolerance to chilling (Jaglo-Ottosen *et al.* 1998). The *Arabidopsis* MYB2 transcription factor can regulate the anaerobic response (Hoeren *et al.* 1998) and the GT motif has been identified as the most commonly represented element in promoters of hypoxia-induced genes (Dennis *et al.* 2000, Liu *et al.* 2005). The use of promoters may thus be an interesting approach to overexpress key genes responsible for tolerance.

Alteration of genes involved in anaerobic metabolism has also been undertaken. For instance, modification of the post-transcription of sucrose synthase leads to dramatic changes in tolerance to flooding (Subbaiah and Sachs 2003). Overproduction of pyruvate decarboxylase in rice and *Arabidopsis* enhances submergence tolerance (Qiumio *et al.* 2000, Dolferus *et al.* 2003) and ADH null mutants are more sensitive to flooding than wild type (Ellis *et al.* 1999). Some of these new approaches are promising and the identification of new genes involved in flooding tolerance for the production of transgenics will certainly benefit from the recent advances in genomics and proteomics.

9.2. Genetic approaches and breeding

The use of genetic selection has long been used in many countries to improve tolerance to flooding. As a result, there has been a strong selective pressure on various crop species around the world and this has allowed the identification and selection of hypoxia tolerant cultivars (e.g. rice FR13A, Vaidehi). For instance, two key physiological traits have been described as essential for tolerance for flash flooding in lowland rice: i) minimal underwater elongation and ii) high storage carbohydrate concentration (Setter and Laureles 1996, Setter *et al.* 1997, Ram *et al.* 2002). In contrast, in deepwater conditions, internode elongation is crucial for allowing foliage to develop above the water level and sustain aerobic respiration and photosynthesis. In maize, a fairly simple dominant trait was identified as a key factor in tolerance by genetic analysis (Subbaiah and Sachs 2003). In wheat, there is genetic variability for flooding tolerance and heritability is medium to high for this trait thus giving the opportunity for breeding for flooding tolerance. Other physiological traits include seed and seedling carbohydrate reserves which may be valuable if germination and early growth must take place under water. The efficiency of ethanolic fermentation may also be a key parameter to alleviate the effect of flooding on growth and development. Finally, morphological traits are increasingly put forward as essential for tolerance. These include the capacity to develop aerenchyma and adventitious roots. Waterlogging tolerance among a diverse range of *Trifolium*

accessions was correlated with a high root porosity and extensive lateral rooting (Gibberd *et al.* 1999 2001). Another approach may reside in grafting less tolerant genotypes/species on flooding resistant rootstocks, as demonstrated by the enhanced flooding tolerance of tomato plants grafted on eggplant rootstocks (Lin *et al.* 2004). Thus there is panel of techniques available today to improve tolerance to flooding. However, many of these approaches are still at the experimental stage and it may take a few more years to be able to routinely breed, transform and produce highly flood tolerant species.

10. CONCLUSION

In general, survival to flooding includes one or more of the following responses: control of energy metabolism, availability of abundant energy sources, provision of essential gene products and synthesis of macromolecules, and protection against post-anoxic injury. These strategies have been studied for more than a decade and the cellular and molecular adaptations to tolerate hypoxia are becoming clearer. This understanding is crucial to identify and select plants capable of withstanding increased environmental pressure. In addition, the natural selective pressures are increased in frequency and duration with climate change and species may be unable to evolve in time to survive. It is therefore crucial that plant strategies are analysed and characterised to help better select species and individuals capable of tolerating these stresses.

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Formes réactives de l'oxygène, stress et mort cellulaire chez les plantes

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Résumé:

Les plantes sont constamment soumises à des variations environnementales. Ces changements peuvent engendrer un stress qui modifie l'homéostasie cellulaire par la production de formes réactives de l'oxygène. L'accumulation phytotoxique de ces différents radicaux oxygénés peut entraîner la mort de la plante ; cependant, ils ont récemment été identifiés comme des acteurs essentiels de la réponse au stress et leur rôle comme messenger secondaire est maintenant clairement établi. Leur implication dans la régulation de l'expression génique a aussi permis de démontrer leur rôle d'inducteurs de la mort cellulaire programmée, mort génétiquement contrôlée que l'on retrouve non seulement dans les processus développementaux, mais également typiquement observée dans la réponse au stress. Cette revue présente les récentes avancées dans la caractérisation du rôle des formes réactives de l'oxygène chez les plantes.

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Revue / Review

Formes réactives de l'oxygène, stress et mort cellulaire chez les plantes

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Résumé

Les plantes sont constamment soumises à des variations environnementales. Ces changements peuvent engendrer un stress qui modifie l'homéostasie cellulaire par la production de formes réactives de l'oxygène. L'accumulation phytotoxique de ces différents radicaux oxygénés peut entraîner la mort de la plante ; cependant, ils ont récemment été identifiés comme des acteurs essentiels de la réponse au stress et leur rôle comme messenger secondaire est maintenant clairement établi. Leur implication dans la régulation de l'expression génique a aussi permis de démontrer leur rôle d'inducteurs de la mort cellulaire programmée, mort génétiquement contrôlée que l'on retrouve non seulement dans les processus développementaux, mais également typiquement observée dans la réponse au stress. Cette revue présente les récentes avancées dans la caractérisation du rôle des formes réactives de l'oxygène chez les plantes. *Pour citer cet article : C. Parent et al., C. R. Biologies 331 (2008).*

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Abstract

Reactive oxygen species, stress and cell death in plants. Plants are constantly exposed to changes in environmental conditions. During periods of stress, the cellular redox homeostasis is altered as a result of reactive oxygen species accumulation. The change in redox is responsible for the symptoms commonly observed during periods of stress and reflects the phytotoxic nature of oxygen radical accumulation. However, oxygen radicals have recently been identified as key actors in the response to stress and their role as secondary messengers is now clearly established. The identification of their role in gene regulation has allowed one to identify them as key regulators in the induction and execution of programmed cell death typically observed during developmental processes as well as during stress responses. This review presents recent advances in the characterisation of the role of reactive oxygen species in plants. *To cite this article: C. Parent et al., C. R. Biologies 331 (2008).*

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Mots-clés : Formes réactives de l'oxygène ; Mort cellulaire programmée ; Stress ; Plantes*Keywords:* Reactive oxygen species; Programmed cell death; Stress; Plant

1. Introduction

Les plantes utilisent l'oxygène, via le métabolisme aérobie, pour fournir l'énergie nécessaire à leur crois-

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sance et à leur développement. Cependant, la réduction de l'oxygène par les cytochromes de la chaîne respiratoire s'accompagne inévitablement d'une production de radicaux oxygénés. En effet, lorsque cette réduction est incomplète, des molécules hautement réactives, dérivées de l'oxygène sont produites, parmi lesquelles des radicaux libres comme le radical superoxyde ($O_2^{\bullet-}$), le radical perhydroxyle (HO_2^{\bullet}), le radical hydroxyle ($\bullet OH$), le radical peroxyde (RO_2^{\bullet}) et le radical alkoxyde (RO^{\bullet}), ainsi que des formes non radicales comme le peroxyde d'hydrogène (H_2O_2). Parallèlement, les chaînes de transfert d'électrons au niveau de l'appareil photosynthétique sont capables de produire de grandes quantités de ces formes réactives de l'oxygène (ROS : *Reactive Oxygen Species*).

Même si la majeure partie de l'oxygène cellulaire subit une réduction tétravalente conduisant à la production de l'eau, une partie des électrons peut s'échapper et réduire de manière monoélectronique l'oxygène, conduisant à la formation du radical $O_2^{\bullet-}$. La toxicité de ce radical envers les substrats bioorganiques est directe, mais aussi indirecte, car il peut réagir avec H_2O_2 et ainsi donner naissance à des $\bullet OH$ ou des peroxydites, radicaux du monoxyde d'azote (NO). Le radical $O_2^{\bullet-}$ a une durée de vie de l'ordre de quelques secondes, qui lui permet de diffuser au-delà de son lieu de production pour atteindre ses cibles. Il peut cependant être éliminé par une des superoxydes dismutases (MnSOD; FeSOD; Cu/ZnSOD), métallo-enzymes qui catalysent la dismutation du radical $O_2^{\bullet-}$ en H_2O_2 . Le peroxyde d'hydrogène ainsi formé n'est pas un radical libre, car tous ses électrons sont appariés, mais c'est malgré tout un intermédiaire réduit toxique qui possède une durée de vie relativement longue (quelques minutes). Comme le radical $O_2^{\bullet-}$, il possède la capacité de diffuser loin de son site de production et peut traverser les membranes en utilisant les canaux aqueux (aquaporines), grâce à sa grande similitude chimique avec H_2O [1,2]. Sa concentration est régulée par des enzymes telles que l'ascorbate peroxydase (APX), la catalase (CAT) ou bien la glutathion peroxydase (GPX). H_2O_2 peut également être produit lors de la réduction biélectronique de l'oxygène en présence d'oxydases telles que la glycolate oxydase ou encore l'amine oxydase. La toxicité d' H_2O_2 est principalement liée à sa capacité à produire le radical $\bullet OH$ durant les réactions de Fenton et d'Haber-Weiss. Ce radical, comme les autres ROS, est particulièrement délétère vis-à-vis de tous les constituants cellulaires (ADN, protéines, lipides...), mais sa durée de vie est de l'ordre de la microseconde.

Pour ces raisons, les ROS sont généralement considérées comme des molécules phytotoxiques. Cepen-

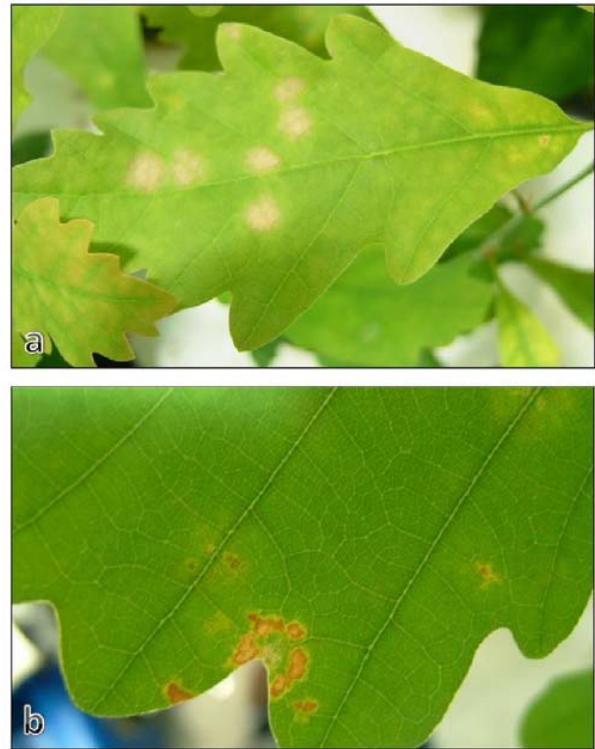


Fig. 1. Feuille de chêne montrant (a) les symptômes d'oxydation de la chlorophylle et (b) les nécroses liées à la mort de cellules.

dant, il est aujourd'hui reconnu que leur synthèse, liée aux métabolismes respiratoire et photosynthétique, joue un rôle essentiel dans la vie et la mort de la cellule végétale. En effet, elles peuvent jouer un rôle opposé à celui de molécules délétères qu'on leur connaît bien, et agir comme molécules de la signalisation cellulaire pour mettre en place des mécanismes de défense vis-à-vis d'un stress, qu'il soit d'origine biotique ou abiotique.

2. Importance des formes réactives de l'oxygène dans la vie d'une plante

Les ROS sont depuis longtemps reconnues pour leur importance dans la réponse des plantes aux contraintes environnementales. Certains symptômes observés en situation de stress d'origine biotique ou abiotique (par exemple, blanchiment des feuilles, nécroses) sont la conséquence d'une forte accumulation de radicaux libres oxygénés et d'une altération de l'homéostasie cellulaire. Ces symptômes traduisent à la fois une oxydation de la chlorophylle au niveau des feuilles, mais aussi la mort par nécrose de cellules isolées ou de groupements de cellules dans les différents tissus végétaux (Fig. 1). Ainsi, même si les ROS sont formées au cours du métabolisme normal de la plante, l'augmentation de

leur concentration intracellulaire est souvent synonyme de stress. La plupart des symptômes liés aux conditions de stress est amplifiée par l'effet des ROS. Par exemple, une forte augmentation du taux de peroxyde d'hydrogène dans les chloroplastes et les peroxyosomes est observée en réponse à une exposition à de fortes intensités lumineuses [3]. Lors d'un stress hydrique ou salin, l'inhibition de la photosynthèse, et plus précisément la fuite d'électrons due à la diminution de la fixation du CO₂, entraîne une forte accumulation de ROS [4]. C'est cette même inhibition de la cascade photosynthétique qui est à l'origine de la production de formes réactives de l'oxygène lorsque la plante subit des fluctuations importantes de température [5]. De nombreuses autres contraintes abiotiques s'accompagnent également d'un stress oxydatif. On observe ainsi, en présence de métaux lourds, une peroxydation lipidique due à l'accumulation des ROS, les UV entraînant la formation de radicaux superoxydes ; il en est de même pour l'ozone ou encore les stress mécaniques [4].

Cependant, malgré leur nature extrêmement réactive, les ROS ne sont pas uniquement impliquées dans des réponses délétères chez les végétaux.

De nombreuses études ont montré que les ROS peuvent aussi intervenir dans les cascades de signalisation responsables de l'induction et de la régulation de nombreux gènes de défenses (protéines chaperonnes, *Heat Shock Proteins* ; enzymes antioxydantes, ascorbate peroxydase (APX), glutathione-S-transferase (GST) ; gènes liés à la pathogenèse (PR) ; [6–9]). Les ROS sont désormais aussi considérées comme agents régulateurs de la mort cellulaire programmée (PCD : *Programmed Cell Death*) chez les plantes [10,11]. Ce processus de mort cellulaire, actif et contrôlé génétiquement, se retrouve tout au long de la vie des plantes. En effet, différents types cellulaires ou organes sont éliminés, au moment approprié, au profit de l'organisme et de la population ; ils constituent des modèles de la PCD végétale. Au cours du développement, la PCD est impliquée dans de nombreux phénomènes comme la germination (couche aleurone), la différenciation des vaisseaux conducteurs de la sève brute et élaborée, la croissance (coiffe racinaire), la reproduction (tube pollinique), ou bien encore la sénescence (feuilles). Par ailleurs, les plantes ont aussi recours à cette mort contrôlée pour s'adapter et résister aux conditions adverses de leur environnement, comme durant des déficiences en alimentation minérale ou hydrique, les extrêmes de température, l'hypoxie ou encore l'attaque pathogène.

Lors de l'établissement de la réponse hypersensible (RH), une des réponses les plus étudiées entre un pathogène et une plante hôte [12–14], le développement

d'une lésion nécrotique localisée autour des sites d'infection du pathogène permet à la plante d'isoler l'agent infectieux. Ce processus est initié par la production rapide et transitoire de ROS autour du site d'infection. Cette forte accumulation se déroule en deux phases distinctes. La première est commune aux interactions compatibles et incompatibles ; la seconde débute environ 6 à 12 h après le début du stress et n'est observée que lorsque l'interaction est incompatible [12]. Ce second *burst* oxydatif est impliqué dans la cascade de signaux nécessaire à l'induction de nombreux gènes de défense (par exemple, protéines de la pathogenèse, PR), dans la fortification des parois cellulaires (par exemple, lignification, subérisation...), et il joue sans doute aussi un rôle antimicrobien.

Après le premier *burst* de ROS, la biosynthèse de nombreuses hormones végétales est stimulée (acide jasmonique, éthylène...) et notamment l'acide salicylique (AS), dont l'accumulation précède la seconde production de ROS lors d'interactions incompatibles [12]. D'ailleurs, dans de nombreux cas étudiés à ce jour, l'accumulation de l'AS et des ROS est nécessaire pour l'induction de la PCD durant la réponse hypersensible.

3. Origine des formes réactives de l'oxygène durant le stress

Comme indiqué précédemment, la production de formes réactives de l'oxygène est une réponse cellulaire commune à de nombreux stress chez les végétaux qui se localise au niveau de différentes sources, selon, notamment, qu'il s'agisse d'un stress d'origine biotique ou abiotique [4,11]. Chez les animaux, les mitochondries constituent la source principale de ROS. Chez les plantes, la production de ROS par les mitochondries a été historiquement minimisée par rapport à celle des chloroplastes. Cependant, avec l'identification de l'alternative oxydase (AOX), la mitochondrie pourrait devenir un acteur important dans la régulation du stress oxydatif chez les plantes [15–17]. En effet, cette enzyme agit comme une « soupape de sécurité », contrôlant la réduction du pool d'ubiquinone, source importante de ROS [18]. Pour cette raison, la mitochondrie a été proposée comme médiateur entre les changements métaboliques, la production de ROS et l'induction de gènes. Cependant, la contribution de la mitochondrie à la production de ROS lors de la réponse au stress reste encore mal définie.

Dans la plupart des situations où les conditions environnementales sont modifiées, une forte augmentation de la production des ROS est observée au niveau des chloroplastes (Fig. 2) [4,19,20]. En effet, de nom-

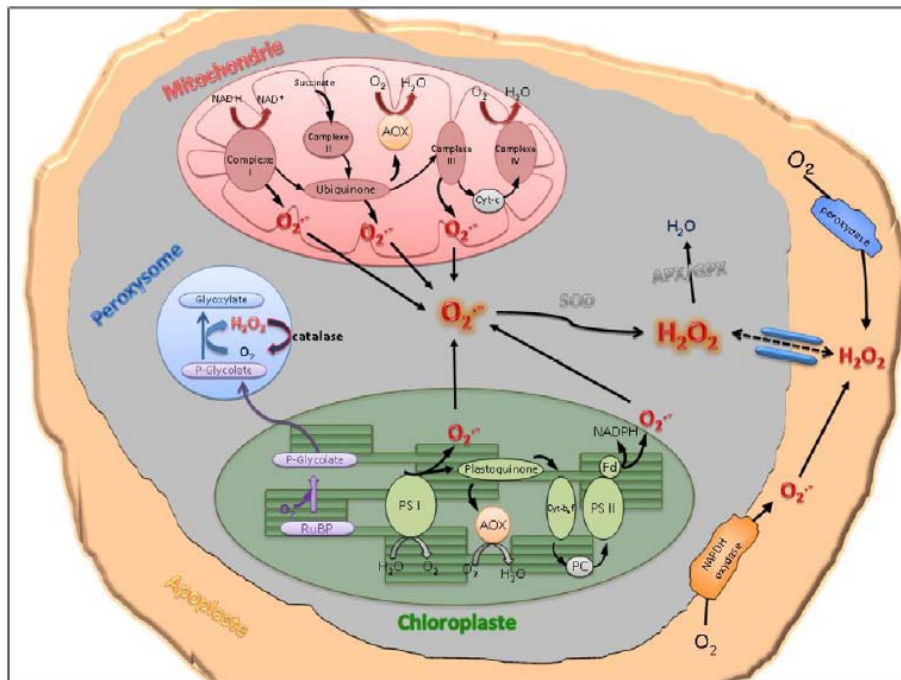


Fig. 2. Sites de production intra-organites des formes réactives de l'oxygène (ROS) dans la cellule végétale.

breuses situations de stress abiotiques entraînent une inhibition de la photosynthèse et les électrons qui ne participent plus à la fixation du CO_2 vont entraîner la production et l'accumulation de ROS. Durant les conditions de photoinhibition, la carboxylation du ribulose 1,5-biphosphate (RuBP) est inhibée, favorisant son oxygénation et entraînant la production de phosphoglycolate. Celui-ci est transporté vers le peroxysome où il est converti en glyoxylate par la glycolate oxydase, produisant ainsi le peroxyde d'hydrogène. Les chloroplastes et les peroxysomes sont ainsi considérés comme « *sensors/capteurs* » des changements environnementaux. Les ROS se comportent donc comme signaux « rédox », dérivés des chloroplastes, et sont susceptibles de réguler l'expression de gènes de la réponse et de l'adaptation au stress [21,22].

Lors de la réponse à un stress d'origine biotique, la production de radicaux oxygénés est généralement attribuée à un complexe NADPH-oxydase associé à la membrane plasmique (Fig. 2) [23]. Cependant, des études récentes indiquent que, lors d'une attaque par un pathogène incompatible, la production de ROS par les chloroplastes participe activement au blocage de la progression de la maladie [21,24]. Ceci est illustré par la réponse du tabac à une agression pathogène, où deux profils différents de peroxydation des lipides sont observés selon la présence ou l'absence de lumière [24]. Toutefois, l'origine de la production de ROS ne se li-

mite pas au chloroplaste, à la NADPH-oxydase et à la mitochondrie. La grande variété des types de stress nécessite la mobilisation de multiples mécanismes de production des ROS, parmi lesquels les amines oxydases ou les peroxydases jouent certainement un rôle important (Fig. 2) [25].

4. Rôle des formes réactives de l'oxygène dans la signalisation et la mort cellulaire programmée

Dans le règne animal, les ROS sont depuis longtemps reconnues comme des molécules de la signalisation, en particulier durant le processus de mort cellulaire [26–29]. Chez les plantes, l'hypothèse selon laquelle les ROS pourraient avoir une fonction autre que celle de simples molécules toxiques dérivées du métabolisme respiratoire et/ou photosynthétique est assez récente. Cependant, de nombreuses études ont récemment démontré que la balance entre production et détoxification des ROS peut être considérée comme essentielle pour de nombreux processus cellulaires [4,19,30–33].

La capacité signalétique des ROS a été mise en évidence lors de premières expériences démontrant la possibilité pour le peroxyde d'hydrogène (H_2O_2) de traverser les membranes biologiques et de modifier l'activité de la glutathion peroxydase (GPX) [34]. En effet, grâce à la présence dans les membranes cellulaires végétales d'aquaporines, capables de laisser diffuser de petites

molécules non spécifiques, H_2O_2 peut oxyder des protéines distantes de son lieu de synthèse [35,36].

En plus de cette action directe, les ROS sont aussi impliquées dans une cascade régulant l'expression génique. En effet, la plante adapte ses réponses selon un mécanisme de régulation génique qui dépend de la concentration cellulaire en ROS [6,7,9,37–41]. Ainsi, la production systématique et régulière de ROS durant de nombreux processus métaboliques, associée à des pics de production durant des conditions environnementales défavorables, aurait permis aux cellules d'exploiter l'effet négatif des radicaux oxygénés en un système de « veille environnementale ». Dès que la concentration en ROS dépasse un certain seuil, celle-ci servirait de signal capable d'induire et de réguler l'expression de gènes de défense.

Les données scientifiques actuelles laissent supposer que cette voie de régulation pourrait jouer un rôle essentiel dans la PCD chez les plantes [9–11,24,42,43]. Ce processus hautement conservé au cours de l'évolution, participe à une multitude d'événements qui illustrent parfaitement son caractère universel au sein des différents phylums du monde vivant. Chez les végétaux, la PCD a été particulièrement bien décrite durant la réponse hypersensible qui caractérise la réponse incompatible d'une plante soumise à l'attaque d'un pathogène [44]. La PCD a aussi été observée au cours de nombreuses phases du développement végétal et dans la réponse à certains stress d'origine abiotique. Elle intervient depuis la germination [45,46] jusqu'à la sénescence [47], en passant par la formation des éléments de vaisseaux conducteurs de la sève brute et élaborée [48]. Elle est aussi rencontrée à chaque épisode de stress d'origine abiotique comme l'ozone [49], les stress thermiques (températures extrêmes) ou anaérobies (hypoxie et anoxie, [50]). Le rôle des ROS dans la PCD n'est pas limité à la phase d'exécution, mais concerne également la phase d'induction [9,11,24].

Comme chez les animaux, dès que la production de ROS dépasse la capacité antioxydante des cellules végétales, plusieurs symptômes sont observés. Cette hyperaccumulation se traduit le plus souvent par un blanchiment des feuilles lié à une oxydation de la chlorophylle [4] ou, à terme, par une mort cellulaire de type nécrotique [51]. L'orientation de la mort cellulaire vers un type nécrotique ou programmée est aussi corrélée à la concentration et à la durée d'exposition aux ROS [11,52–55].

Ainsi, les premiers résultats qui ont mis en avant le rôle essentiel des ROS dans la régulation de la PCD ont été obtenus grâce à des expériences consistant à traiter des cultures de cellules de soja avec du per-

oxyde d'hydrogène [34,43]. La production endogène d' H_2O_2 peut aussi conduire à une PCD, comme cela a été démontré sur des plants de tabac transgéniques, déficients en catalase (Cat1AS, [56]). Lorsque ces plantes, Cat1AS, sont exposées à de fortes intensités lumineuses ($>500 \mu\text{mol m}^{-2} \text{s}^{-1}$), H_2O_2 s'accumule et entraîne la mort des cellules. Ce processus létal peut être bloqué par l'injection de cycloheximide (inhibiteur de la synthèse de protéines), d'inhibiteurs de flux ioniques ou bien encore par celle d'inhibiteurs de la phosphorylation [10]. Ainsi, la production de ROS *in planta* peut entraîner une PCD, et cette cascade signalétique implique non seulement la synthèse *de novo* de protéines mais aussi des flux d'ions [10]. Le rôle d' H_2O_2 dans la PCD a également été observé dans plusieurs autres modèles d'études végétaux [10,37,40,57,58]. Il faut également noter que le peroxyde d'hydrogène participe à l'initiation de la PCD lors d'infections par un pathogène incompatible (*Pseudomonas syringae* *pv* *syringae*) et lors d'une exposition à l'ozone [33,49,59]. L'utilisation de tabacs Cat1AS a d'ailleurs permis de démontrer que la PCD induite par une accumulation importante, mais transitoire, d' H_2O_2 présente la même signature en acides gras hydroxylés que celle obtenue lors de l'induction de la PCD par un éliciteur d'origine pathogène, comme la cryptogéine [24]. En revanche, lorsque les plantes sont exposées à une production continue d' H_2O_2 , la PCD présente les mêmes caractéristiques d'oxydation lipidique que celles observées lors de « nécroses » ou d'infection pathogène en présence d'une forte intensité lumineuse [10,24]. Enfin, remarquons que H_2O_2 n'est pas la seule espèce réactive de l'oxygène capable de réguler l'expression génique (par exemple, O_2^- et 1O_2 ; [60,61]). À ce jour, cependant, peu de données existent sur la spécificité de ces autres formes réactives de l'oxygène.

5. Conclusion

Les ROS sont impliquées non seulement dans le développement de symptômes, mais aussi dans la signalisation lors de la réponse des plantes aux stress. Le rôle prépondérant des ROS dans l'initiation et l'exécution de la mort cellulaire a déjà été démontré pour de nombreux processus du développement végétal, comme la germination ou la sénescence. Cependant, les acteurs clés de l'induction de la PCD restent encore mal connus à ce jour. Le séquençage du génome de plantes modèles ainsi que les approches génomiques et protéomiques globales couplés à l'utilisation des outils de la transgénèse devraient rapidement permettre d'identifier les acteurs de l'initiation et de l'exécution de la PCD, mécanisme es-

sentiel du développement, aussi bien chez les plantes que chez les animaux.

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Résumé de thèse

*L'ennoyage est un phénomène courant qui se répercute sur les végétaux en diminuant leur croissance, leur développement et leur régénération. Il entraîne une forte diminution du taux d'oxygène (hypoxie) dans le compartiment racinaire résultat de l'excès d'eau dans le sol. Face à ce stress, certaines plantes sont capables d'intensifier leur métabolisme anaérobie et développer des adaptations morphologiques (lenticelles hypertrophiées, aérochymes, racines adventives). Le chêne sessile (*Quercus petraea* Matt L.) et le chêne pédonculé (*Quercus robur* L.) présentent une différence de tolérance à l'ennoyage du sol mais sont génétiquement proches. Cependant, il existe peu de connaissances sur les processus physiologiques mis en place par ces deux espèces durant ce stress. La croissance ainsi que certains changements morphologiques, anatomiques et physiologiques étudiés chez les deux espèces en réponse à un ennoyage, ont confirmé que le chêne sessile était davantage sensible à ce stress. Le chêne pédonculé, espèce plus tolérante, parvient à maintenir son statut hydrique de même que son activité photosynthétique plus longtemps et à des niveaux moins critiques que le sessile. L'hémoglobine non-symbiotique améliore la survie des plantes en conditions d'hypoxie en interagissant avec le monoxyde d'azote (NO). Le clonage d'une hémoglobine non-symbiotique chez le chêne sessile a permis d'isoler le gène *QpHb1* codant pour une protéine de 161 acides aminés et présentant toutes les caractéristiques communes aux hémoglobines non-symbiotiques de classe 1. Chez les deux espèces, *QpHb1* est davantage exprimé dans les racines que dans la tige ou les feuilles, suggérant un rôle particulier dans ce tissu. L'expression de *QpHb1* analysée par Northern blotting a montré une chute d'expression dans les racines dès les premières heures de stress chez le chêne sessile qui se poursuit durant toute la durée de l'expérience (28 jours). Alors que chez le chêne pédonculé, l'expression de *QpHb1* suit un schéma plus complexe : on observe un pic d'expression après 1h d'ennoyage suivi d'une forte inhibition après 3h. Cette régulation pourrait être synonyme d'une implication de l'hémoglobine non-symbiotique dans la signalisation rapide du stress et par conséquent dans la mise en place de la tolérance. D'ailleurs sa localisation par hybridation *in situ* au niveau racinaire a montré que *QpHb1* se situait au niveau des cellules du protoderme, pouvant suggérer un rôle dans la détection des modifications de la rhizosphère mais aussi au niveau du protoxylème qui pourrait lui permettre de participer à la signalisation entre l'appareil racinaire et l'appareil aérien via la S-nitrosylation. Dans la réponse à un ennoyage de plusieurs semaines, le chêne pédonculé met en place des adaptations (aérochymes, lenticelles hypertrophiées et racines adventives) et exprime davantage *QpHb1* que le chêne sessile notamment dans les racines adventives. La régulation spatio-temporelle de *QpHb1* pourrait être impliquée dans la capacité de tolérance ainsi que dans la cascade de signalisation menant au développement de ces adaptations.*

Flooding is a common environmental stress inducing a decline in plant growth, development and regeneration. Waterlogging leads to low oxygen levels (hypoxia) in the root environment due to an excess of water in the soil. In response to hypoxia, plants switch their metabolism from aerobic respiration to anaerobic fermentation and can develop morphological adaptations (aerenchyma, hypertrophied lenticels and adventitious roots). Sessile and pedunculate oak are two closely related species that display a strong differential tolerance to soil flooding. However, few reports exist about the physiological processes leading to their response to flooding. Growths, but also morphological, anatomical and physiological changes studied in both species in response to waterlogging, have confirmed that sessile oak is more sensitive to this stress. Pedunculate oak, the more tolerant species, succeeded in maintaining its water status and its photosynthetic activity at a higher level than sessile oak. Non-symbiotic hemoglobin enhances plant survival in hypoxic conditions by interacting with nitric oxide (NO). We cloned and characterized a novel non-symbiotic hemoglobin gene in sessile oak, QpHb1, coding for a 161 amino acid protein and showing all characteristics of class I non symbiotic hemoglobins. In both oak species, QpHb1 is more highly expressed in roots than in stem or leaves, suggesting a particular role in this tissue. QpHb1 expression analyzed by northern blotting showed a strong decline from the first hours of stress in sessile oak which continued until the end of the experiment (28 days). In contrast, QpHb1 expression in pedunculate oak, exhibits a more complex scheme: an early rise in expression after 1h of waterlogging followed by a strong decline after 3h. This regulation could be related to the implication of non-symbiotic hemoglobin in rapid stress signaling and consequently in the induction of tolerance. Indeed, QpHb1 root localization performed by in situ hybridization showed a strong expression in protoderm cells, suggesting a sensing function of rhizospheric modifications. In addition, the protoxylem localization of QpHb1 could also indicate a role in root to shoot signalization by S-nitrosylation. In response to several weeks of flooding stress, pedunculate oak displayed morphological adaptations (aerenchyma, hypertrophied lenticels and adventitious roots) and exhibited a higher level of QpHb1 transcripts than sessile most notably in adventitious roots. The spatio-temporal regulation of QpHb1 could be involved in the tolerance capacity as well as in signaling cascade leading to developmental adaptations.