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RESEARCH PAPER

Differences in C metabolism of ash species and provenances as a consequence of root oxygen deprivation by waterlogging

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Abstract

The waterlogging tolerance and the physiological responses to this stress were tested in seedlings of *Fraxinus angustifolia*, an ash tree inhabiting riparian forests, and two provenances of the closely related *Fraxinus excelsior*, one derived from a riparian forest (FER) and one from a mountainous region (FEM). Besides visible damage, physiological parameters reflecting adaptations of plants to waterlogging such as net CO_2 assimilation, alcoholic fermentation, and the concentrations of metabolites related to flooding responses were studied. Consistent with the higher flooding tolerance of *F. angustifolia* and FER compared with FEM, net assimilation remained unaffected in *F. angustifolia*, was slightly reduced in FER, but was strongly affected in FEM. Altered carbohydrate concentrations in the roots of the seedlings suggest differences in the ability to supply alcoholic fermentation with substrate during prolonged periods of soil anoxia. Another difference between the seedlings was connected to the γ-aminobutyric acid (GABA) shunt which resulted in alanine accumulation in the flooding-tolerant trees, but strong GABA accumulation in the more sensitive FEM seedlings. This finding indicates differences in GABA conversion into alanine which might result in an accumulation of phytotoxic levels of intermediates. Such provenance-specific differences in Common ash suggest that the selection of appropriate provenances is essential for forest management in flood-prone areas.

Key words: Ash provenances, assimilation, carbon metabolism, flooding, oxygen deficiency, waterlogging.

Introduction

Higher plants are aerobic organisms depending on a steady supply of O_2 (Vartapetian and Jackson, 1997). Plant adaptations to waterlogging and flooding, which are major environmental causes of O_2 deprivation in the soil, include avoidance strategies at the morphological level (e.g. lenticels and aerenchyma, pneumatophores) and plasmatic tolerance mechanisms at the physiological level. One of the major pathways affected by a lack of O_2 is mitochondrial respiration. In order to maintain energy generation under conditions of suppressed O_2 supply, plants switch from respiration to fermentative metabolism. The ability to run alcoholic fermentation under conditions of O_2 deprivation is regarded as an essential requirement of anoxia tolerance of plants since energy metabolism is maintained and NAD^+ is regenerated despite lacking mitochondrial respiration (Drew, 1997). The considerably lower efficiency of fermentative processes compared with respiration to produce energy equivalents may cause a strongly increased demand for carbohydrates in submerged roots of plants. This assumption is supported by the finding of improved survival of flood-sensitive species by exogenous supply of sugars (Saglio and Pradet 1980). Although C and energy metabolism have been identified to play a crucial role for the physiological adaptation of plants to hypoxia, it has so far not been clarified which mechanisms ensure the steady supply of carbohydrates to hypoxic tissues in tolerant plant species (Braendle, 1997).

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As one of the main representatives of the European hardwood alluvial forests, Common ash (*Fraxinus excelsior*) is able to cope with regular flooding. Flood height, duration, and frequency in this habitat vary for different rivers and for each given stand. At the river Rhine, for example, flooding events last for 1–4 d in average years during the vegetation period (April–September), and for 10–35 d in exceptional years (Michiels and Aldinger, 2002). Späth (1988) reported a critical threshold of 35 d for the occurrence of damage in *F. excelsior* seedlings, whereas Siebel and Bouwma (1998) even propose a capability of Common ash to survive flooding for at least 3 months. Surprisingly, Common ash also inhabits rather dry, mountainous sites with calcareous soils and regular drought episodes (Carlier *et al.*, 1992). This observation has led to the hypothesis that specifically adapted ash ecotypes exist, which have been classified as the apparent flood-tolerant 'water ash' and the drought-adapted, apparent flood-sensitive 'limestone ash' (Münch and Dieterich, 1925). Although a series of studies deal with both ash ecotypes (Münch and Dieterich, 1925; Leibundgut, 1956; Weiser, 1995; Dacasa Rüdinger and Dounavi, 2008) it has not been clarified until now if they differ in their capacity to acclimatize to flooding.

The present study therefore aimed at characterizing the degree of flooding tolerance of different seed provenances of *F. excelsior*. In addition, seedlings of *Fraxinus angustifolia*, an ash species well known for its high flooding tolerance (Jelem, 1974; Gerard *et al.*, 2006), were included in the experiments. Moreover, the hypothesis was tested that differences in flooding tolerance are connected to differences in carbon and energy metabolism during periods of O_2 deprivation. To characterize the carbon metabolism of the trees, net CO_2 assimilation, alcoholic fermentation, and the carbohydrate status were determined under conditions of waterlogging. In order to obtain hints for further metabolic processes contributing to physiological adaptation of plants to O_2 deprivation, the concentrations of metabolites involved in pathways affected by flooding (Kreuzwieser *et al.*, 2009) were determined.

Materials and methods

Plant material

Fraxinus excelsior seeds were collected from two natural stands in the federal state of Baden-Württemberg (South Germany) in August 2001. Seeds of provenance 'FER' (*F. excelsior*, riparian site) were derived from 15 mother trees of a mixed ash stand in a natural riparian forest along the river Rhine (48°51′35″N; 8°7′48″E). This site is characterized by regular and intensive flooding events. Seeds of provenance 'FEM' (*F. excelsior*, mountainous site) were randomly collected (from at least 20 mother trees) from a mixed stand in the mountainous regions (470 m a.s.l.) of the Swabian Jura, South Germany (48°29′24″N; 9°24′0″E). Prevailing climatic conditions at these sites were 10.48 °C and 8.61 °C average air temperatures and 857 mm and 964 mm annual precipitation for the FER and FEM stands, respectively. After collection, the seeds were immediately transferred to a soil–turf mixture (7.0 l pots) and kept in a garden, protected from frost by plastic foil. As fertilizer, 'Nitrophoska perfekt' (Compo, Münster, Germany) was used according to the manufacturer's instructions for deciduous tree seedlings.

Fraxinus angustifolia were purchased from a nursery as 3-year old seedlings. The seed origin was Portugal. Trees were delivered with bare roots in February well before bud break, and potted immediately into 7.0 l pots using a mixture of potting soil ('Floraton 2', Floragard, Germany), rough and fine grained silica sand, and Perlite ('Perligran', Perlitdämmstoffe, Dortmund, Germany) as substrate. Additionally, 6 g of slow-releasing fertilizer (Osmocote, Scotts, Nordhorn, Germany) was supplied per pot. Although different types of fertilizers have been used for nutrient supply of the different ash species, effects on reactions to waterlogging treatment are not expected; the fertilizers used caused balanced nutrient supply avoiding development of any nutrient deficiencies or excess.

Five months before the experiment, all trees were transferred to a greenhouse and grown under controlled conditions which were left unchanged until and during the experiments. Temperature was programmed to 25±2 °C during the day and 20±2 °C during the night at a day–night cycle of 16/8 h. Light was supplied at an intensity of ~200 μmol m^{-2} s^{-1} at top canopy level. Irrigation was performed on a daily basis. Only healthy trees with intense root soil penetration were studied.

Waterlogging treatment

For waterlogging experiments, seedlings were placed in 200 l plastic tanks (~100×50×50 cm) and flooded with tap water. The water level was ~2 cm above the soil surface so that the tree shoots were still exposed to ambient air. Each container accommodated five randomly chosen trees of the different provenances and species, respectively. In order to simulate different flooding conditions frequently found in the natural environment, 3 d (short-term) and 10 d (long-term) of waterlogging were applied. After the different exposure times, leaf and fine root samples, phloem exudates, and xylem sap were collected, immediately frozen in liquid N_2, and kept at –80 °C until analysis. Alcohol dehydrogenase (ADH) activities were analysed directly using fresh plant material. Leaf number and development of new leaves were studied in trees flooded twice for 2 weeks each, interrupted by 1 week of normoxia.

Gas exchange measurements

To analyse the CO_2 and H_2O gas exchange of the leaves, a portable gas exchange measuring system (GFS-3000, Walz, Effeltrich, Germany) equipped with a broad-leaf cuvette (4.00 cm^2 leaf area) was used. Light intensities were set to 1000 μmol PPFD m^{-2} s^{-1}, CO_2 concentrations

to 375 ppm, and the air flow through the leaf cuvette was adjusted to 700 μmol s^{-1}. Leaves of five trees per treatment and provenance/species were placed into the cuvette and, after an acclimation time of 15 min, gas exchange parameters were measured six times at 10 s intervals. Means of these values were used for further data analysis.

Extraction of phloem exudates and xylem sap

Phloem exudates were collected from slices of stem bark (~150 mg, 1–2 cm^2) which were separated from the wood, washed in 2 mM EDTA, and allowed to equilibrate in 2 ml of 2 mM EDTA (pH 5.8) for 5 h (Herschbach *et al.*, 2000). Aliquots of these solutions were used for metabolite determination, and concentrations were expressed on a fresh weight basis of the bark slices. Xylem sap of the shoots was collected using the protocol of Rennenberg *et al.* (1996). Shoots of the ash seedlings were cut, and bark and cambium removed at a distance of ~30 mm from the cut end. After rinsing the stripped end with distilled water, the shoots were fitted into the pressure chamber originally described by Scholander *et al.* (1965). The pressure was then raised by adding compressed N_2 at a rate of 0.2 MPa min^{-1} until the first xylem sap appeared. The first protruding solution was discarded to avoid contamination. Then, the pressure was raised by another 0.6 MPa and kept constant for 2 min. The exuding xylem sap was collected in reaction tubes.

Measurement of metabolite concentrations

Leaf pigments were determined photometrically in acetone extracts as described by Lichtenthaler and Wellburn (1983).

For determination of soluble carbohydrates, plant tissues were powdered under liquid N_2 and aliquots of 50 mg were extracted with 2 ml of H_2O by boiling for 5 min. After centrifugation for 5 min at 12 000 *g*, 100 μl of supernatant was injected into an HPLC system (Dionex DX 500; Dionex, Idstein, Germany). The pellet of this centrifugation step was saved for the analysis of starch (see below). Xylem sap and phloem exudates were injected directly into the system after appropriate dilution. Separation of carbohydrates was achieved on a CarboPac PA1 separation column (250×4 mm; Dionex, Idstein, Germany) with 56 mM NaOH as an eluent at a flow rate of 1 ml min^{-1}. Carbohydrates were measured by a pulsed amperometric detector equipped with an Au working electrode (Dionex DX 500, Idstein, Germany). Individual carbohydrates which eluted 8–16 min after injection were identified and quantified by internal and external standards.

For starch determination, the pellets were washed twice with double-distilled H_2O, and starch was completely digested by addition of amyloglucosidase from *Aspergillus niger* as described by Peuke *et al.* (2006). The resulting glucose concentrations were determined by HPLC as described above.

Ethanol contents in tissues were measured according to Kreuzwieser *et al.* (2001), using a commercially available kit (Roche, Basel, Switzerland).

Concentrations of polar low molecular weight metabolites present in the plant samples were analysed by GC-MS using a modified protocol of Fiehn (2006) as described by Kreuzwieser *et al.* (2009). Tissue was homogenized under liquid N_2 and aliquots of 50 mg were added to 87% methanol containing ribitol (0.2 mg ml^{-1}) as an internal standard. After extraction at 70 °C and 1400 rpm for 15 min on a thermoshaker (Eppendorf, Germany), aliquots of 100 μl were dried. For derivatization, 25 μl of methoxyamine hydrochloride (20 mg ml^{-1} pyridine) was added, and samples were incubated at 30 °C/1200 rpm for 90 min (Thermoshaker, Eppendorf, Germany) before addition of 40 μl of MSTFA (Sigma-Aldrich, Germany) and incubation at 37 °C/1200 rpm for 30 min. After addition of 25 μl of alkane standard (Sigma-Aldrich, Germany), 1 μl aliquots were analysed by GC-MS as described by Fiehn (2006).

ADH activities

ADH activities in plant tissues were determined by an assay adapted from Bouny and Saglio (1996). A sample of 100–150 mg of tissue was homogenized in a chilled mortar with 3 ml of ice-cold extraction buffer [50 mM TRIS-HCl pH 7.5, 10 mM $Na_2B_4O_7$, 15% (v/v) glycerol, 0.02% Triton, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM dithiothreitol (DTT), 5% (w/v) polyvinylpolypyrrolidone (PVPP)]. For each sample, 30 μl of appropriately diluted extract and 140 μl of assay buffer (100 mM tricine, pH 7.5, 0.8 mM NAD^+) were combined. To start the reaction, ethanol was added to a final concentration of 100 μM. The reduction of NAD^+ was followed for 5 min at 340 nm and 25 °C using a microplate reader (Tecan, Germany). ADH activity was expressed as enzyme units (U) per gram of total protein. Total protein in tissue extracts was measured according to Bradford (1976).

Statistics

Statistically significant differences between species/treatments were calculated by applying the Students' *t*-test at a significance level of 5%. For count data, the exact binomial test under the R software package (R Development Core Team, Vienna, Austria) was used. Significant differences are indicated in the figures by asterisks.

Results

FEM and FER differ in flooding tolerance

It was tested if different seed provenances of Common ash differ in their waterlogging tolerance. After waterlogging for 2 weeks twice, interrupted by 1 week of normoxia, seedlings originating from a mountainous population (FEM) exhibited more pronounced leaf loss (Fig. 1a) and less new leaf formation (Fig. 1b) than seedlings deriving

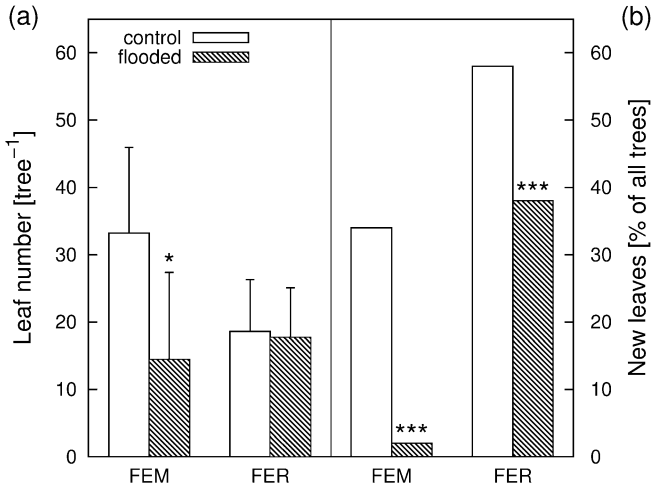

Fig. 1. Effect of flooding on leaf number (a) and development of new leaves (b) of the ash provenances FEM and FER. Trees were flooded twice for 2 weeks each and total leaf number (damaged and undamaged) and development of new leaves were determined. Means (±SD) of 18 (FEM control), 31 (FEM flooded), 52 (FER control), and 77 (FER flooded) plants are given. Statistically significant differences were calculated using Student's *t*-test (a) and the exact binomial test (b), respectively, and are shown by asterisks over the bars.

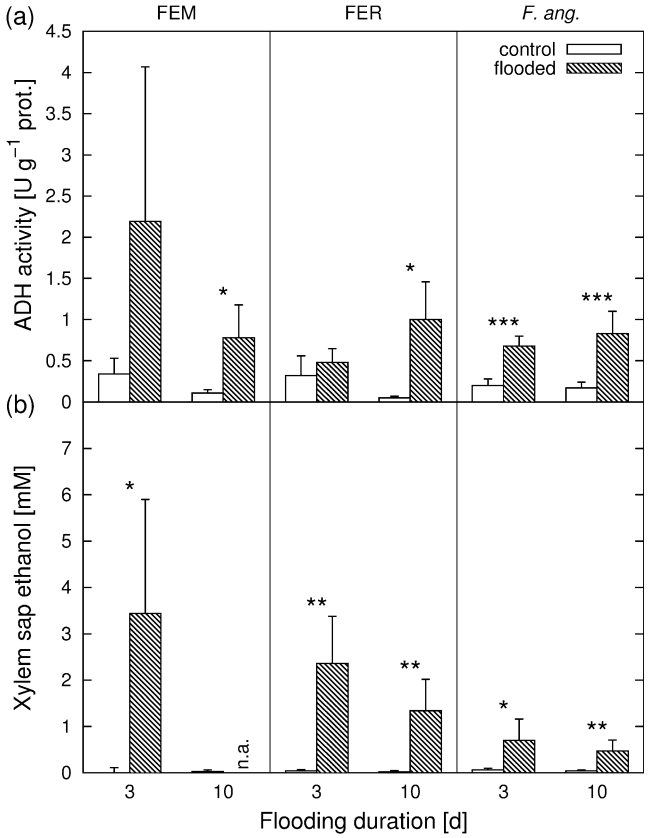

Fig. 2. Effect of flooding on root ADH activity (a) and xylem sap ethanol concentrations (b) in ash seedlings. The root system of the trees was flooded for the times indicated. Means (±SD) of five plants per treatment and provenance/species are shown. Statistically significant differences at $P < 0.05$ between flooded and control plants are indicated by asterisks. n.a., not applicable (due to low sample amount).

from a riparian forest (FER). All flooded trees developed hypertrophied lenticels during the experiment, but provenance-specific differences in shape or number did not occur. Adventitious root formation did not take place in the course of the experiment. As it is well known that *F. angustifolia* is highly flooding tolerant (Jelem, 1974), it was not included in this experiment. Its high flood tolerance became obvious in all of our waterlogging experiments performed with *F. angustifolia* where this species never showed any visible damage or differences from non-flooded controls.

Alcoholic fermentation and ethanol formation and metabolism

ADH activities in roots of all ash trees were strongly induced by waterlogging, reaching average activities between 0.5 U g^{-1} and 2.2 U g^{-1} total protein compared with 0.1–0.3 U g^{-1} total protein in controls (Fig. 2a). While after 3 d waterlogging, FEM tended to exhibit higher activities than FER and *F. angustifolia*, activities after 10 d of submergence were similar among both *F. excelsior* provenances and *F. angustifolia*. Although alcoholic fermentation appeared to be stimulated, as indicated by increased ADH activities in all flooded ash trees, a statistically significant accumulation of ethanol in the roots was only observed in FER (compare Fig. 8). However, waterlogging caused increased ethanol concentrations in the xylem sap of all ash provenances, with the highest concentrations in the xylem sap of FEM (~3.5 mM) and the lowest concentrations (~0.8 mM) in *F. angustifolia* (Fig. 2b). In addition to alcoholic fermentation, lactic acid fermentation was stimulated in the trees studied. Surprisingly, elevated lactic acid concentrations were observed even after 10 d of flooding, indicating prolonged rather than transient lactic acid production in flooded ash (Fig. 8).

To test if ethanol accumulates in the leaves of the trees due to enhanced transport in the xylem, leaf ethanol contents were determined (data not shown). Although ethanol concentrations in leaves were considerably higher than in roots, differences between controls and flooded trees were not observed. This was most probably due to oxidation of ethanol in the leaves as suggested from constitutive leaf ADH activities of 0.2–1.0 U g^{-1} FW in all ash provenances studied (data not shown).

Influence of waterlogging on CO_2 and H_2O gas exchange

Net CO_2 assimilation showed considerable differences between ash seedlings even in non-flooded controls. Probably due to differences in leaf structure, leaves of *F. angustifolia* exhibited higher assimilation rates (4.9±2.8 μmol m^{-2} s^{-1}) than leaves of FEM (1.8±1.2 μmol m^{-2} s^{-1}) and FER (0.3±0.1 μmol m^{-2} s^{-1}) (Fig. 3a). Waterlogging

affected assimilation rates in a species-specific manner. While no effects were observed for *F. angustifolia* (Fig. 3a), 10 d of waterlogging caused >5-fold reduced assimilation rates in FEM seedlings. FER seedlings were also affected by waterlogging, but to a lesser degree than FEM seedlings. Several studies have suggested that reduced net CO_2 assimilation is caused by closure of stomata (Kreuzwieser *et al.*, 2004). In accordance with this assumption, no significant effect of waterlogging on stomatal aperture was detected in *F. angustifolia* (Fig. 3b), whereas the reduced assimilation rates in FEM and FER correlated well with lower stomatal conductance of the leaves. In accordance with unaffected assimilation rates, the leaf pigment content was generally not affected by short-term waterlogging. However, as seen for net assimilation, 10 d of flooding resulted in ~30% lower chlorophyll *a* and *b* concentrations in leaves of FEM seedlings as compared with non-flooded controls (Fig. 4). In contrast, chlorophyll loss in FER and *F. angustifolia* was less pronounced, with decreases of ~15% and ~20%, respectively.

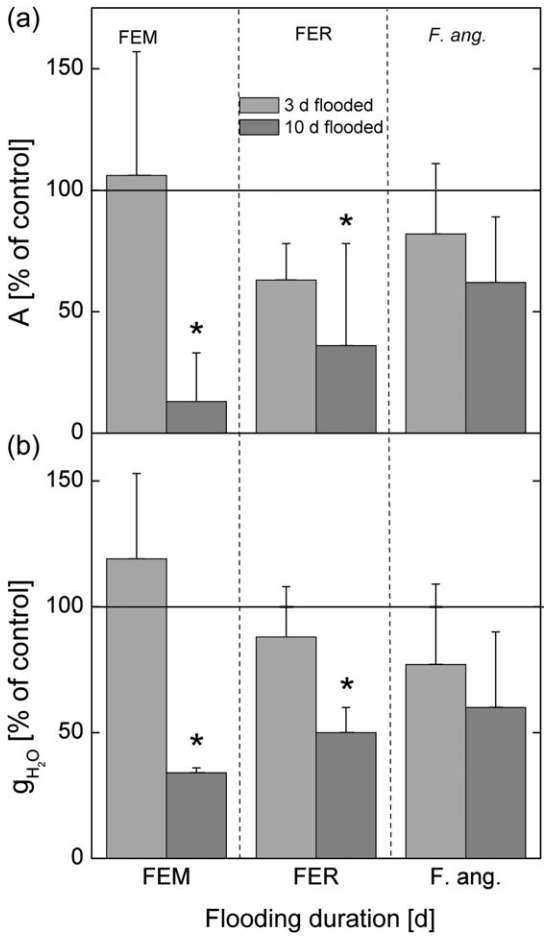

Fig. 3. Net assimilation rate (a) and stomatal conductance (b) of ash seedlings exposed to flooding. The root system of ash trees was flooded for the times indicated and gas exchange was analysed. Gas exchange parameters of flooded trees are expressed as a percentage of controls. Means (±SD) of at least five trees per treatment and provenance/species are shown. Statistically significant differences between flooded trees and controls are calculated by Student's *t*-test and are indicated by asterisks over the bars.

Effects on carbohydrate content

As net CO_2 assimilation was affected by waterlogging in the two *F. excelsior* provenances, a general effect on C metabolism had to be assumed. Indeed, in leaves, carbohydrate concentrations generally increased due to oxygen deprivation (Fig. 5a). However, whereas in the flooding-sensitive FEM elevated carbohydrate levels were observed only during long-term waterlogging, in *F. angustifolia* the increase in carbohydrate concentrations was only transient and concentrations reached the levels of controls during prolonged periods of waterlogging. The changes in total carbohydrate concentrations in the leaves were clearly an effect of changed concentrations of mannitol, which was the main soluble carbohydrate in the leaves of all ash trees studied. The amounts of other sugars, however, remained relatively constant.

The starch content in leaves of the non-flooded controls ranged between 24 and 49 μmol glucose equivalents g^{-1} FW, with the exception of FEM, day 10, where the content was considerably higher (142 μmol glucose equivalents g^{-1} FW) (Fig. 7a). Similar to the pattern in soluble carbohydrates, waterlogging caused starch accumulation in FEM.

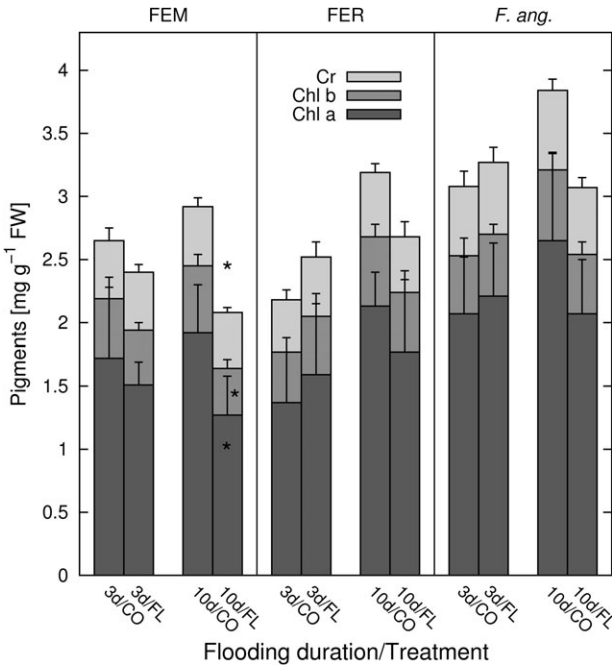

Fig. 4. Leaf pigment contents in ash seedlings exposed to flooding. Trees were flooded for the times indicated and contents of leaf pigments were determined. Means (±SD) of five trees per treatment and provenance/species are shown. Asterisks above the bars designate significant differences in total carbohydrate concentrations between controls and flooded trees. Asterisks next to the bar segments indicate differences of individual carbohydrate concentrations between flooded trees and controls. Chl a, chlorophyll *a*; Chl b, chlorophyll *b*; Cr, carotinoids.

In contrast, in FER and *F. angustifolia* prolonged waterlogging either caused depletion in starch or did not affect starch contents, respectively.

As in leaves, mannitol was quantitatively the most important sugar in roots, making up 33–79% of total soluble carbohydrates, followed by glucose (19–43% of the total) and sucrose (6–23%). Also in roots an accumulation of carbohydrates was observed in response to waterlogging (Fig. 5b). In both species, this increase was almost exclusively due to increased mannitol concentrations. In FEM, root carbohydrate concentrations strongly increased after 3 d of waterlogging, but decreased with prolonged oxygen deprivation to control levels. In contrast, long-term waterlogging in *F. angustifolia* caused up to 2.5-fold increased root sugar concentrations. This effect was mainly caused by increased mannitol levels. Another species-specific difference was seen in the concentrations of glucose and to a minor extent in fructose. The concentrations of both carbohydrates clearly decreased in flooded roots of FEM and FER but they were unaffected in *F. angustifolia*.

Sugar accumulation in response to flooding was also observed along the transport paths. In the phloem of FEM, long-term waterlogging resulted in >3-fold higher carbohydrate concentrations, mainly due to increased mannitol and sucrose abundance (Fig. 6a). A similar, 2-fold increase was found in the phloem of *F. angustifolia* and FER. The carbohydrate concentrations in the xylem sap amounted to up to ~15 mM in FER and *F. angustifolia* (Fig. 6b). Significantly higher concentrations were observed as a consequence of long-term flooding. Compared with FER and *F. angustifolia*, the xylem sap of FEM contained much higher sugar concentrations. While in controls

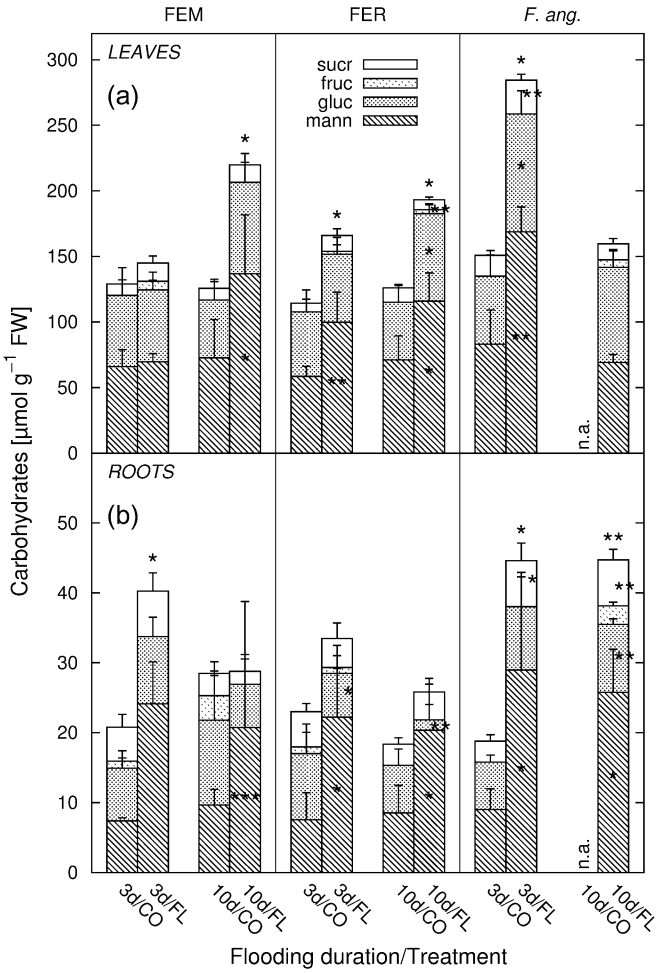

Fig. 5. Soluble carbohydrate concentrations in leaves (a) and roots (b) of ash seedlings exposed to flooding. Trees were flooded for the times indicated and carbohydrates were analysed. Means (±SD) of five trees per treatment and provenance/species are shown. Asterisks above the bars designate significant differences in total carbohydrate concentrations between controls and flooded trees. Asterisks next to the bar segments indicate differences of individual carbohydrate concentrations between flooded trees and controls. In the case of *F. angustifolia*, day 10, statistics were calculated against the control of day 3. n.a., not applicable (due to sample loss).

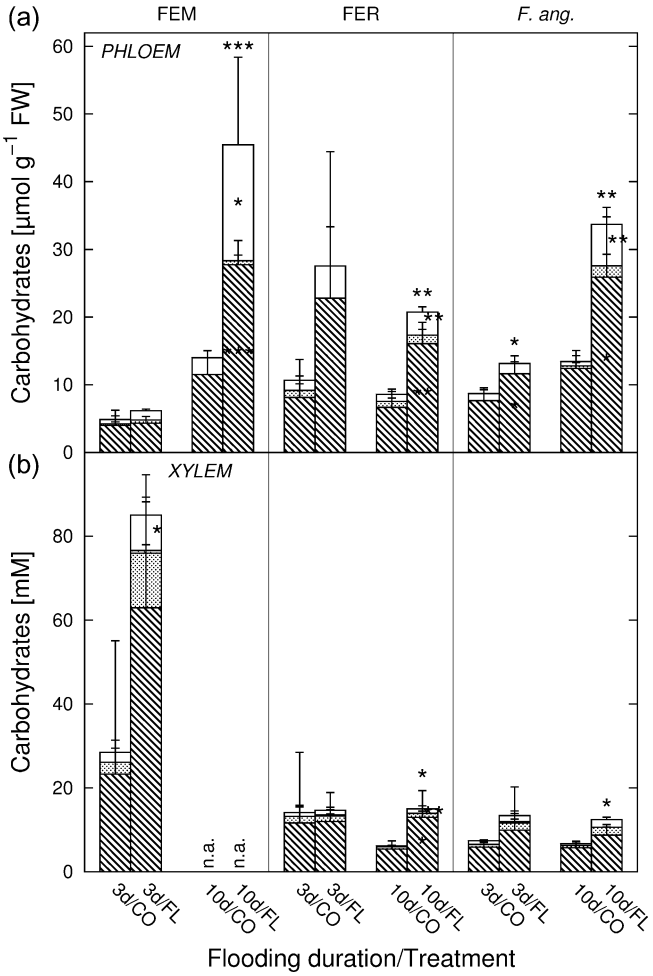

Fig. 6. Soluble carbohydrate concentrations in phloem exudates (a) and xylem sap (b) of ash seedlings exposed to flooding. Trees were flooded for the times indicated and carbohydrates were analysed. Concentrations for phloem exudates (a) are expressed on a fresh weight basis of the bark slice exudated. Concentrations in (b) are that of pure xylem sap. See legend of Fig. 5 for further explanations. n.a., not applicable (due to low sample amount).

concentrations of ~30 mM were observed, flooded trees accumulated up to 90 mM carbohydrates. In all species, the increased concentrations observed were mainly due to mannitol.

Starch concentrations in roots of FEM and *F. angustifolia* decreased ~3-fold in response to waterlogging. In contrast, an accumulation of starch was observed in roots of FER seedlings (Fig. 7b).

Effects on intermediates of the TCA cycle and the γ-aminobutyric acid (GABA) shunt

The most obvious difference between the ash species was found in concentrations of GABA shunt metabolites (Fig. 8). In roots of FEM flooded for 10 d, GABA accumulated to 25 times higher concentrations than in non-flooded control trees. In contrast, highly flooding-tolerant *F. angustifolia* did not accumulate GABA but showed strongly increased concentrations of the amino acid alanine, an intermediate of GABA metabolism. The provenance FER showed both GABA and alanine accumulation in flooded roots. Compounds upstream and downstream of GABA also showed changed concentrations in the roots of flooded ash species. Generally, metabolite concentrations in flooding-sensitive FEM seedlings tended to decline (e.g. citrate, glutamate, glutamine, succinic acid, and malic acid) whereas no clear pattern was obtained for FER and *F. angustifolia* (Fig. 8).

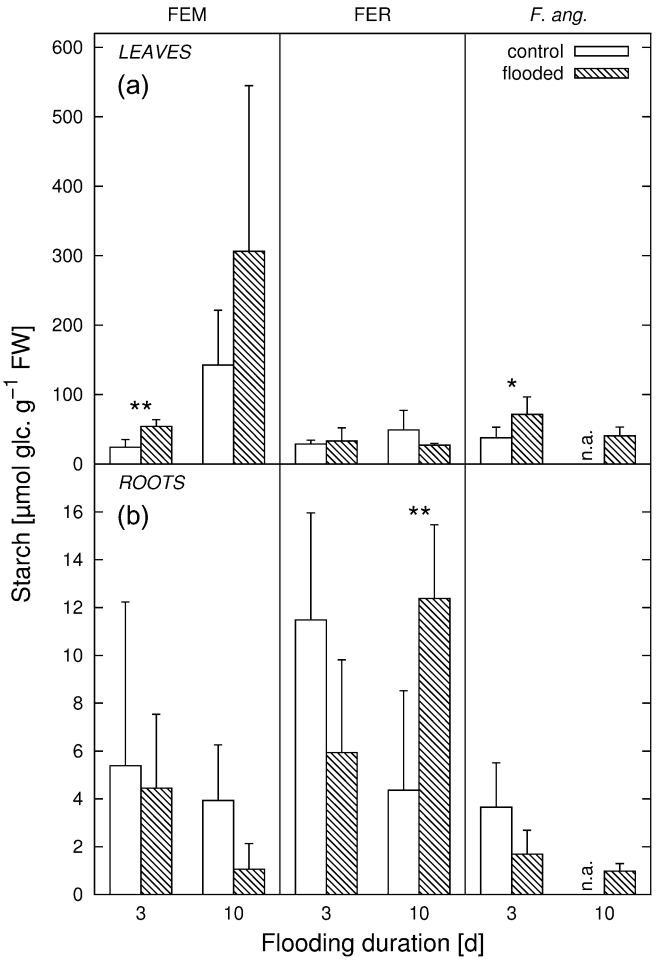

Fig. 7. Starch content in leaves and roots of ash seedlings as affected by flooding. Trees were flooded for the times indicated and starch contents determined in leaves and roots. Means (±SD) of five plants per treatment and provenance/species are shown. Asterisks above the bars indicate significant differences between flooded and control trees as calculated by Student's *t*-test at $P \geqslant 0.05$. n.a., not applicable (due to sample loss).

Discussion

Common ash is a tree species with a wide ecological amplitude, growing in habitats as diverse as frequently inundated riparian forests and quickly drying chalk sites. Plants from these habitats differ in growth performance (Münch and Dieterich, 1925; Weiser, 1995), wood properties (see references in Weiser, 1964), and water uptake (Carlier *et al.*, 1992), but it has been unclear if they also differ in their waterlogging tolerance. The present study indicates that Common ash provenance FER, originating from a frequently inundated riparian forest of the river

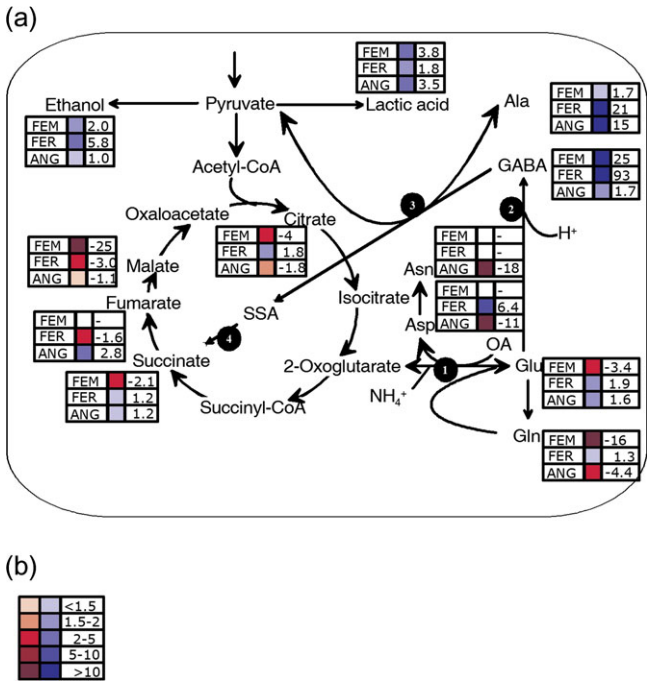

Fig. 8. Effect of long-term flooding on TCA cycle and GABA shunt metabolites in roots of ash seedlings. Trees were flooded for 10 d and metabolites were determined in root tissue as described in Materials and methods. (a) Fold changes (flooded versus non-flooded) were calculated from means of five plants per treatment and provenance/species. (b) The colour code indicating the extent of changes caused by flooding is shown; fold changes are given in addition to the colour code. Upper square, FEM; middle, FER; lower, *F. angustifolia*. Enzymes involved in the GABA shunt: (1) glutamate dehydrogenase, GDH; (2) glutamate decarboxylase, GDC; (3) GABA transaminase, GABA-T; (4) succinic semialdehyde dehydrogenase, SSADH.

Rhine, is much less sensitive to waterlogging than provenance FEM. Due to waterlogging, FEM lost more leaves and developed fewer new leaves than FER seedlings (Fig. 1). In contrast, long-term differences in growth between 'water' and 'limestone' ash provenances were not observed in response to occasional flooding episodes (Weiser, 1995). Apart from the fact that seed provenances were not identical in the two studies, flooding conditions in the present study were possibly harsher than in Weiser's experiment, exposing tolerance differences between the provenances more clearly. Interestingly, a recent study dealing with the genetic structure of the *ADH-B* locus, an essential gene for anaerobic metabolism, also did not find differences between Common ash provenances from limestone and riparian sites (Dacasa Rüdinger *et al.*, 2008). This, however, does not exclude provenance-specific differences in other genes involved in the response to oxygen deficiency, and thus does not contradict the present results.

The occurrence of considerably different flooding tolerance of FEM and FER provided the opportunity to analyse physiological features potentially explaining these observations. In good agreement with the flooding tolerance assessed, net CO_2 assimilation of FEM was more affected by flooding than that of FER and *F. angustifolia* (Fig. 3a). Similar correlations between flooding tolerance and disturbance of net assimilation have been observed for other tree species (Wagner and Dreyer, 1997; Graves *et al.*, 2002). However, assimilation rates also differed between untreated plants of FEM, FER, and *F. angustifolia*. This was possibly due to different growth rates, since growth requires continuous carbon fixation. Ash is known for its rhythmic growth (Kerr and Cahalan, 2004), and *F. angustifolia* seedlings were possibly in a phase of intense growth, while seedlings of *F. excelsior* were not. In addition, the 15–30% higher leaf chlorophyll contents of *F. angustifolia* as compared with *F. excelsior* probably also contributed to the higher control assimilation rates of this species (Fig. 4).

It is generally assumed that stomatal closure determines decreased assimilation rates under conditions of O_2 deficiency (Gravatt and Kirkby, 1998; Mielke *et al.*, 2003). This is also supported by the present study, where the response of stomatal conductance to waterlogging resembled that of photosynthesis (Fig. 3b). However, non-stomatal factors probably also contributed to the reduction in assimilation, as suggested by the observation of significant chlorophyll loss in FEM flooded for 10 d (Fig. 4) and carbohydrate accumulation in leaves of FEM and FER (Fig. 5a). Chlorophyll degradation, which may indicate increased N mobilization as a result of impaired N nutrition during flooding (Kreuzwieser *et al.*, 2002), was strongest in FEM, coinciding with particularly low assimilation rates. Increased photoassimilate levels in leaves may repress assimilation by feedback inhibition (Goldschmidt and Huber, 1992).

Differences in flooding tolerance have been associated with differences in the maintenance of energy metabolism in roots during periods of anoxia (Bouny and Saglio, 1996). Both *F. excelsior* and *F. angustifolia* induced lactic acid and ethanolic fermentation, as indicated by high lactic acid and/or ethanol concentrations (Fig. 8), and increased ADH activities (Fig. 2a) in flooded roots. ADH activity after 10 d waterlogging was similarly increased among both *F. excelsior* provenances and *F. angustifolia*. However, this does not preclude different substrate flux through ethanolic fermentation, since the latter is usually limited by pyruvate decarboxylase (PDC), and not ADH activity (Tadege *et al.*, 1999). Moreover, to allow continued alcoholic fermentation in oxygen-depleted tissues, the availability of carbohydrates as substrate for glycolysis is crucial (Waters *et al.*, 1991; Perata *et al.*, 1992). In the present study, short-term waterlogging caused sugar accumulation in all ash trees investigated independent of flooding tolerance (Fig. 4b). Long-term waterlogging, however, caused increased sugar availability only in roots of FER (+20%) and *F. angustifolia* (+200%), whereas in FEM no differences between controls and flooded trees were observed. Moreover, in FEM the concentrations of glucose and fructose decreased, suggesting that substrate availability in roots of this provenance may become limited as a consequence of prolonged oxygen deficiency.

The accumulation of carbohydrates in roots of FER and *F. angustifolia* was mainly due to increased mannitol levels, which in roots may be a consequence of starch degradation as suggested from low starch contents in *F. angustifolia* seedlings (but not in FER seedlings) (Fig. 7b), or by enhanced phloem transport of mannitol and sucrose from leaves to roots. The latter might be concluded from higher concentrations of these sugars in the phloem of flooded trees (Fig. 6a). However, as it was the case that also in FEM seedlings phloem sucrose concentrations increased considerably during long-term waterlogging, but root sucrose concentrations were unaffected, impaired phloem unloading of this sugar may be assumed in this provenance. This idea is supported by increased leaf carbohydrate concentrations (Fig. 5a).

The effect of flooding on carbohydrate metabolism in plants is well studied but with very heterogeneous results. Several studies demonstrated the occurrence of reduced carbohydrate concentrations in roots of flooded trees (Vu and Yelenosky, 1991; Angelov *et al.*, 1996). However, increased concentrations have also been observed in response to flooding (Schlueter *et al.*, 1996; Hsu *et al.*, 1999). The latter has been explained by a reduced demand for carbon caused by a decline in root growth and nitrogen metabolism or by a high flooding tolerance (Angelov *et al.*, 1996). The results for leaf carbohydrate levels are less contrasting. Flooding generally caused increased carbohydrate contents (Drew and Bazzaz, 1978; Gravatt and Kirby, 1998), which is in good agreement with the present findings.

Differences in carbohydrate metabolism might be one reason for differences in flooding tolerance of the trees studied. Another reason might be related to the GABA shunt. In good agreement with results for other species exposed to hypoxia (Smith and ap Rees, 1979; Kreuzwieser *et al.*, 2002), strongly increased concentrations of the free amino acids GABA and alanine were found in the present

study (Fig. 8). This effect, however, varied between the FEM, FER, and *F. angustifolia*, indicating differences in the performance of this pathway. As the decarboxylation of glutamate consumes protons, GABA biosynthesis might counteract cytosolic acidification in hypoxic roots (Shelp *et al.*, 1999). Lactic acid production, as observed in all ash plants (Fig. 8), may contribute to such cytosolic pH decline (Roberts *et al.*, 1984), though this is not the case in all plant species (Felle, 2005). In addition, both GABA and alanine may serve as temporary C and/or N storage, as well as osmolytes (Bouché and Fromm, 2004). The differences in GABA accumulation between the different provenances may be a consequence (i) of different production rates, e.g. due to a different demand to consume protons; or (ii) of different GABA metabolism. As the activity of succinic semialdehyde dehydrogenase (SSADH) is strongly impaired by high levels of NADH (Busch and Fromm, 1999), GABA catabolism might be inhibited in plants with strongly disturbed redox balance, which could be the case in roots of flood-sensitive FEM. The view of impaired GABA metabolism in FEM but not in FER and *F. angustifolia* is supported by the observed alanine concentrations (Fig. 8). FER and *F. angustifolia* may accumulate alanine due to conversion of GABA. This hypothesis is consistent with observations from *Arabidopsis thaliana* where alanine accumulation was largely attributed to the activity of GABA transaminase (Miyashita and Good, 2008). Further evidence for inhibited GABA breakdown in FEM and, as a consequence, decreased succinate entry into the tricarboxylic acid (TCA) cycle, is provided by concentrations of TCA cycle intermediates. Succinate, malate, and oxaloacetate showed clearly diminished concentrations in FEM, but only small or no changes in FER and *F. angustifolia* (Fig. 8).

In the present study, waterlogging caused stomatal closure, reduced rates of photosynthesis, and accumulation of compatible solutes such as malate, mannitol, and proline, which also constitutes typical symptoms of drought stress in trees (Chaves, 1991). O_2 deprivation and drought stress can produce similar symptoms in plants, such as wilting and epinastic growth of leaves (Bradford and Hsiao, 1982), probably due to the reduced water uptake capacity of flooded roots (Else *et al.*, 1996). Thus, the strong accumulation of mannitol in various ash tissues is probably a response to compensate for decreased tissue water potential, just like under conditions of water shortage. Ash has previously been described as a 'tolerator' (Ludlow, 1989), being able to cope with extreme leaf water potentials as low as –4 MPa (Guichert *et al.*, 1997). The present finding of mannitol accumulation suggests that this physiological strategy also plays a role under conditions of oxygen deprivation.

Conclusions

In the present study, seedlings of common ash from different provenances were tested for differences in their physiological response and overall resistance to waterlogging. The alluvial provenance FER turned out to be more tolerant to water-saturated soil than the provenance FEM from a mountainous environment, indicating the presence of genetic adaptation to a reduction of oxygen in FER. Genotypic differences may also determine the ability of an ecotype to acclimatize to changing environmental conditions, a trait that may influence the development of a tree. This may be essential in forests along rivers which have been cut from the natural hydrology for years and are now restored as a riparian forest or used as artificial flood water retention areas. In order to ensure ecologically and economically valuable ecosystems in such areas, provenance selection seems critical.

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