Rhizosphere dynamics of higher plants in the water fluctuation zone of Yangtze River: Root exudates and mass flow

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Christina Maria Schreiber
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Pflanzenwissenschaften
Forschungszentrum Jülich
Heinrich-Heine Universität Düsseldorf

Referent: PD Uwe Rascher
Koreferent: Prof. Dr. Andreas Weber

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1. Abstract

Rhizosphere dynamics of three flooding-tolerant plant species (*Arundinella anomala* Steud., *Alternanthera philoxeroides* Mart. and *Salix variegata* Franch.), originating from Three Gorges Reservoir (TGR) area (P.R. China), were investigated for reactions at the root-soil interface during flooding periods. This work aims for a better understanding of survival strategies of flooding-resistant plants by observing flooding reactions under close-to-natural and laboratory conditions, adapting a method for high-resolved rhizosphere monitoring to simulate complete submergence. It assesses the ability of the chosen plant species to survive and serve as soil protectors on the banks of TGR.

Flooding was simulated under close-to-natural conditions in open-air pools at Southwest China University Chongqing-Beibei, where plants were waterlogged and then submerged (6 weeks) in their original sediment substrate in pots with access for sterile soil solution sampling via microsuction cups. For comparison of plant and microorganism (MO) contribution in relation to temperature, sediment- and sterile glass bead substrate grown plants were sampled in laboratory during 5 weeks submergence at 10°, 20° and 30°C. Samples were analysed for low molecular weight organic acids (LMWOA) by Capillary Electrophoresis.

The floodable dual-access rhizobox was introduced to gain a high resolution insight to the root-soil interface. It allows non-invasive pH- and oxygen monitoring directly at the root surface as well as simultaneous low-invasive soil solution sampling in high spatial and temporal resolution. The chosen species were treated in short-term (2 day waterlogging, submergence, waterlogging each) and mid-term (2 weeks per phase) in glass bead and sediment substrate at 10° and 20°C and compared to reactions of non-resistant species.

Waterlogged plants showed a relation between microclimatic conditions (mostly PAR) and organic acid (OA) occurrence in soil solution. Fermentation products (acetate, lactate, formate) accumulated slowly during flooding. No bursts of exudation were observed. Patterns of OA almost reached the state of non-submerged control plant sets within one week after de-submergence.

Significant higher fermentative OA appearance occurred in sediment where microorganisms (MO) could interact with roots. In glass, additionally oxalate, malate and citrate were detected, which are seemingly utilized too fast in sediment by MO to appear in samples. Temperature had a significant effect on OA amounts in sediment, which were highest at

30°C. No clear effect was found in glass, implying subjacent temperature dependent MO processes as main source in sediment.

Diurnal rhythms of rhizospheric acidification (>1 pH unit amplitude) compared to bulk soil) were observed during rhizobox treatments, stable during waterlogging and receding, but not ceasing during flooding. With de-submergence these rhythms returned to pre-flood state. All three species exuded oxygen into their rhizosphere, even when submerged, showing that photosynthesis was not completely shut down. No OA exudation bursts as known from non-tolerant species were observed, yet sometimes increased exudation of young active root tips which could be observed in-situ during growth. OA content was highest in sediment at 20°C.

Comparison to non-flooding resistant species (*Zea mays* L., *Hordeum vulgare* L.) in the same treatment (glass bead substrate) showed higher OA occurrence during waterlogging and onset of flooding. Diurnal rhythms ceased during flooding, and the plants died before end of submergence. Oxygen content, which never declined below 30vol% air saturation in the substrate of the tolerant species, was close to zero here after 2 days of complete submergence.

The chosen flooding-resistant species implement several survival strategies. First, turnovers are down-regulated under submergence. No exudative bursts or strong accumulation of fermentation products was observed, minimizing carbon depletion. Initial ethylene production causes rapid shoot elongation, an avoidance strategy, in the first days of flooding in *A. philoxeroides*, followed by down-regulation and quiescence as in the other two. Newly built leaves bear weaker cuticles to facilitate easier gas exchange in water. *S. variegata* produces new adventive roots above soil surface for better exchange of potentially accumulating turnover products. All three tolerant species show radial oxygen loss during waterlogging and even flooding and all survive up to 6 months of submergence. Plant growth accompanied by consistent diurnal rhythms of rhizospheric acidification and oxygenation is considered evidence of good root health status. Therefore could be shown that, after mid-term flooding, the root systems of the three tolerant species are still functioning well, stabilizing the plant and securing growth. Except *A. philoxeroides,* whose roots are too delicate to provide strong mechanical hold, the species are considered well suited for re-vegetation on the TGR banks to mitigate soil runoff.

2. Zusammenfassung

Diese Doktorarbeit befasst sich mit der Dynamik in der Rhizosphäre dreier überflutungstoleranter Pflanzenarten (A. *anomala* Steud., *A. philoxeroides* Mart. and *S. variegata* Franch.) der Ufer des Drei-Schluchten-Reservoirs, VR China. Vorgänge zwischen Wurzeln und Substrat wurden während Flutungsperioden unterschiedlicher Dauer untersucht. Ziel dieser Arbeit war ein besseres Verständnis der Überlebensstrategien dieser flutungsresistenten Pflanzenarten. Dazu wurden die Reaktionen der Pflanzen auf Überflutung unter annähernd natürlichen und Laborbedingungen untersucht. Eine Rhizotronmethode, die die Beobachtung der Vorgänge in der Rhizosphäre hochaufgelöst ermöglicht, wurde angepasst und erweitert, um ihre Anwendung für vollständig überflutete Pflanzen zu ermöglichen. Sie ermöglichte die Beurteilung der Spezies im Hinblick auf ihre Überlebensfähigkeit und ihre Eignung zum Erosionsschutz an den Ufern des Drei-Schluchten-Stausees.

Die naturnahen Überflutungsexperimente wurden in Pools im Freien an der Southwest China University Chongqing-Beibei durchgeführt. Die Pflanzen wurden in Rhizotrontöpfen in ihrem natürlichen Sedimentsubstrat staunass gestellt und für sechs Wochen geflutet. Die Töpfe ermöglichten sterile Beprobung der Bodenlösung mit Mikrosaugkerzen. Die Proben wurden mittels Kapillarelektrophorese auf niedermolekulargewichtige organische Säuren (im folgenden OS) analysiert. Das Vorkommen der OS war abhängig von den mikroklimatischen Bedingungen (hauptsächlich der photosynthetisch aktiven Strahlung, PAR). Fermentationsprodukte (Acetat, Lactat, Format) reicherten sich während der folgenden Flutungsphase nur langsam an, es fanden keine plötzlichen Reaktionen statt. Zusammensetzung und Menge von OS erreichte innerhalb einer Woche nach Ende der sechswöchigen Flutung annähernd den Status der nicht-gefluteten Kontrollsets.

Ein Vergleich von in Sediment- und Glassubstrat gewachsenen Pflanzen während fünf Wochen Flutung bei 10°, 20° und 30°C zeigte signifikant höhere Vorkommen von OS im Sediment, wo Mikroorganismen (MO) mit den Wurzeln interagieren konnten. Im Glas wurden zusätzlich Oxalat, Malat und Citrat festgestellt, die im Sediment anscheinend zu schnell von MO verstoffwechselt werden, um in den Proben zu erscheinen. Im Sediment gab es einen signifikanten Temperatureffekt mit deutlich höheren OS-Mengen. Kein klarer Effekt wurde im Glassubstrat sichtbar, was temperaturabhängige MO-Prozesse als Hauptquelle im Sediment nahelegt.

Die flutbare 'Dual-Access-Rhizobox' wurde vorgestellt, die einen räumlich und zeitlich hochauflösenden Einblick in die Rhizosphäre ermöglicht. Nichtinvasive pH- und Sauerstoffmessungen direkt an der Wurzeloberfläche und wenig invasive Bodenlösungsprobennahmen wurden in kurzen (jeweils 2 Tage Staunässe, Flutung, Staunässe) und mittellangen (jeweils 2 Wochen pro Phase) Überflutungsphasen in Glas und Sedimentsubstrat bei 10° und 20°C an den drei Spezies durchgeführt.

Diurnale Rhythmen einer Ansäuerung der Rhizosphäre (>1 pH-Einheit, verglichen mit dem umgebenden Boden) konnten während des gesamten Experiments beobachtet werden. Sie waren während der Staunässephase stabil und schwächten sich während der Überflutung ab, ohne jedoch zu versiegen. Nach der Flutung kehrten diese Rhythmen innerhalb einer Woche zu der Intensität von Experimentsbeginn zurück. Alle drei Arten exsudierten Sauerstoff in ihre Rhizosphäre, auch während der Flutungsphase, was zeigt, dass die Photosynthese nicht völlig zum Erliegen kam. Junge, aktive Wurzelspitzen zeigten erhöhte Exsudation, was in-situ während des Wachstums beobachtet werden konnten. Das OS-Vorkommen war im natürlichen Sediment bei 20°C erhöht. Ein Vergleich mit nicht flutungsresistenten Arten (*Zea mays* L., *Hordeum vulgare* L.) zeigte bei identischer Behandlung erhöhte OS-Vorkommen während Staunässe und beginnender Flutung. Die anfangs vorhandenen diurnalen Rhythmen versiegten während der Flutungsphase, und die Pflanzen starben nach wenigen Tagen. Der Sauerstoffgehalt im Substrat, bei den resistenten Arten nie unter 30vol% Luftsättigung, war bei den nicht-resistenten Arten bereits nach zwei Tagen vollständiger Flutung nahe Null.

Die resistenten Arten zeigen mehrere Überlebensstrategien. Der Stoffwechsel wurde während der Flutungsphase herabreguliert, was den Kohlenstoffverlust minimiert. Ethylenproduktion bei Flutungsbeginn verursachte rapides Sprosswachstum bei *A. philoxeroides* während der ersten 2-3 Tage. Während der Flutung neu gebildete Blätter hatten eine dünnere Cuticula für erleichterten Gaswechsel unter Wasser. *S. variegata* produzierte neue Adventivwurzeln über der Bodenoberfläche für einen besseren Austausch von potentiell schädlichen Stoffwechselprodukten. Alle drei Arten zeigen radialen Sauerstoffaustritt aus den Wurzeln während Staunässe und auch Flutung. Das stattfindende Wachstum, begleitet von diurnalen Rhythmen rhizosphärischer Ansäuerung, wird als Zeichen eines gesunden Wurzelsystems gedeutet. Es konnte daher gezeigt werden, dass das Wurzelsystem der drei toleranten Arten nach mittellangen Flutungsphasen noch funktionsfähig ist, die Pflanze stabilisiert und Wachstum ermöglicht. Außer *A. philoxeroides,* deren Wurzeln zu delikat sind, um guten mechanischen Halt zu bieten, werden die Arten als geeignet für eine Wiederbepflanzung der TGR-Ufer angesehen.

3. Introduction

3.1 Motivation

The construction of the Three Gorges Dam at Yangtze River, P.R. China, created a reservoir of more than 600km length and 1050m² in area. On 26th October 2010, the maximum water level of 175m was reached for the first time. The management of the reservoir shall mainly provide optimal conditions for energy production (18200MW), enable major ship traffic to Chongqing, and protect the lower river regions from high waters. It also causes a 30m water fluctuation zone 100m above the original water level. This leads to drastic changes in the ecosystem of the riverbanks (Fearnside 1988; Park et al. 2003; Wu et al. 2004), not last because of a changed flood pulse from summer to winter, and the fact that the original vegetation on this level is not used to regular flooding and will suffer. Plants on the higher riverbanks might be able to survive flooding periods (up to 6 months), while in lower areas of the 30m water fluctuation zone only annual species might immigrate between flooding periods. Yet vegetation is needed to mitigate soil runoff, the Yangtze riverbanks being vulnerable to erosion. Flooding resistant species are needed for re-cultivation to provide erosion protection and attractive green banks (Allen 1979; Schiechtl and Stern 1996; Gray 1998; Liu et al. 2004; Wang et al. 2005), and three species (A. *anomala* Steud., *A. philoxeroides* Mart. and *S. variegata* Franch.), which originate from TGR area and are already known to tolerate flooding up to 6 months, have been chosen for a closer look on the reasons for their perseverance.

The plant roots form the key tissue here to investigate, not only because they have to secure plant survival, but because their growth and perseverance is the main factor for soil fixation and stabilization (Angers and Caron 1998; Gyssel et al. 2005). There has been research about soil submergence tolerance and involved rhizosphere reactions (Voesenek et al. 2006) and general knowledge about rhizospheric interactions has been given increased attention in the last decade (Jones 1998, Jones et al. 2003), but studies of rhizosphere dynamics in completely submerged plants are few. Yet this information is needed to assess applicability of tolerant species for the given task and gain more intrinsic knowledge of rhizospheric interactions, especially under hypoxic and anoxic conditions. It is also important to link the laboratory experiments to the field, especially since understanding of plant-microorganism feedback is still scarce (Kozdroj and van Elsas 2000). Comparison to non-resistant species (*Zea mays* L., *Hordeum vulgare* L.) is also crucial to be able to judge the extent and meaning of observed reactions in flooding-adapted species.

3.2 Rhizospheric interactions

Roots have complex architecture and growth patterns, determined by species and soil composition (Uren 2000). Healthy plant roots affect their rhizosphere through water and nutrient uptake, oxygen release, exudation of pH-relevant substances (H^+, O_2, an-/organic ions, organic acids (OA)) and carbohydrates, amino acids, proteins etc.. Thus they influence pH and redox potential and provide energy supply for soil microorganisms (Jones 1998; 2004; Ryan and Delhaize 2001; Hinsinger et al. 2003; Farrar et al. 2003; Walker 2003; Blossfeld and Gansert 2007, Blossfeld et al. 2010). MO species composition is altered by these exudates (Hodge and Millard 1998) and, in turn, alters plant nutrient status through decomposition and mineralization. Exudates are an important carbon- and energy source (Cheng et al. 1996), especially since up to 40% of the photosynthetically fixed carbon may be lost into the soil (Whipps 1990; Bais et al. 2006). Exudates have numerous effects. Carbohydrates mainly serve as nutrition for soil microbiota, while amino acids and amids may inhibit nematodes and produce allelopathic effects on other roots (Willis 2000). Phenolics may serve as *nod*-gene inducer, phytosiderophores and organic or amino acids may chelate poorly soluble mineral nutrients (Philips and Tsai 1992; Dakora et al. 2002). Due to changes in moisture, particles size and absorption potential of soil and the fact that root exudation also depends on species, age of plant and root itself, active growing zones or temperature, the rhizosphere has a high spatial heterogeneity (Vancura 1965; Darrah 1993; Strobel 2001; Bertin et al. 2003; Marschner et al. 2004; Bais et al. 2006). This makes it necessary to approach as low invasively as possible with a high spatial resolution.

Organic acid exudation is of special interest. They present about 10% of total organic carbon exuded and play an important role for availability of metals (desorption, solubilisation, chelation) and MO nutrition (Harter and Naidu 1995; Hinsinger 2001). They may provide information about plant state, since increased OA exudation is known to be induced by nutrient deficiency (P, Fe), toxic cations and anoxia (Smucker and Erickson 1987; Neumann and Römheld 2000). It has further been reported to be positively related to root growth and health (Prikryl and Vancura 1980).

Main OA which have been found in the rhizosphere of the three discussed species are formate, acetate and lactate (**manuscript 2**), fermentation products (aceto- and methanogenesis, lactate/ethanol fermentation) from plant root cells as well as MO's. Further oxalate, malate, succinate and citrate were found, intermediates in the tricarboxylic cycle. They are able to form hydrophosphate chelates in soil solution, which diffuse to root surface, and they may also serve for cation balance (Jones 1998).

The typical exudate release happens via diffusion, which works well for OA's and sugars (passively) due to the gradients between cytoplasma (mM) and soil solution (µM). The membrane permeability determines pass through the lipid bilayer of the plasmalemma, which depends on the polarity of the compounds in question and the physiological state of the root. With normal cytosolic pH of 7.1-7.4 (Marschner 1995), OA exist as anions with low plasmalemma permeability. But a positive charge gradient through cytosolic K+ diffusion potential and proton extrusion (ATPase) promotes cation uptake against anion release (Samuel 1992). It may be enhanced under stress conditions, since membrane potentials change depending of cell energy state which, in turn, are disturbed by stresses. Since OA are involved in many processes concerning plant stress, especially anoxia/fermentation, they pose a most interesting trait for investigation regarding flooding situations like those at TGR.

Exudates also have influence on prevailing pH, which is of utmost importance for plant performance. Availability of nutrients strongly depends on pH, the optimum being between 6.3 and 6.8 (Scheffer and Schachtschabel 2002). Lower pH (<5.5) leads to increased Al, Fe or Mn toxicity (Brady 1990). While OA were found to be relevant for pH sometimes (P-availability in legumes through increased citrate exudation, Gardner et al. 1983; Haynes 1990; Jones and Darrah 1995; Watt and Evans 1999), oxygen in soil (increasing redox potential), excreted protons (H^+ATPase) and also CO_2 (carbonic acid) have greater influence (Mainiero and Kazda 2005). Nutrient uptake means proton excretion in exchange for cation uptake, which acidifies the rhizosphere. Plants can even induce active proton extrusion under Fe-deficiency by increasing H^+-ATPase activity, which also leads to acidification of the rhizosphere (Zocchi and Cocucci 1990). Conversion of NH_4^+ to Glutamine may also contribute to rhizospheric acidification, since uptake of NH_4^+ against protons is a prevailing way of nitrogen uptake in wetland plants (Foyer and Noctor 2002).

Soil acidity in the rhizosphere has been found to be tenfold greater than in the bulk soil (Darrah 1993). PH dynamics in the rhizosphere of some plants (*Juncus effusus*, *Vigna unguicula, Medicago sativa*) follow diurnal rhythms, most likely connected to photosynthesis and internal oxygen diffusion, accompanied by radial oxygen loss due to the gradient of O_2 partial pressure from inside the shoot to the more hypoxic roots (ROL, Jarvis and Hatch 1985; Rao et al. 2002; Blossfeld 2007). ROL can then act as an additional proton source, for example by oxidizing Fe_2^+ and freeing protons (Kirk 2004). Oxygen exudation also means protection from reduced substances in the root vicinity. In combination with hypoxia/anoxia, observation of pH, oxygen and other exudates will lead to conclusions about plant and root state, especially if monitored in situ over longer time periods.

3.3 Plant reactions to flooding: Avoidance, Quiescence, Damage

Plants implement numerous reactions to submergence. Lack of oxygen and the extremely slowed gas diffusion in water (10^4 times lower than in air, Tiedje et al. 1984) present the most impeding factors. Oxygen deprivation leads to avoidance strategies. Ethylene expression, most likely through enhanced ACC synthase as described in rice (Cohen and Kende 1987), promotes reactions like rapid cell elongation or promotion of lysigenous aerenchyma (Blom and Voesenek 1996). Anoxia will lead to fermentation processes inside and outside of plant root cells. Build of adventive roots in upper soil layers helps to dispose of accumulating toxic turnover products like ethanol as described for *Trifolium* (Aschi-Smiti et al. 2003), since ethanol accumulation and its oxidation to acetaldehyde damages membranes (Kato-Noguchi 2002).

Onset of anoxia causes an immediate drop in cytosol pH through inhibition of oxidative phosphorylation and therefore reduction in energy charge (Crawford 2003). Such damage damage is irreversible in *Lettuce* after minutes, *Barley* can suffer irreparable damage after 8h of total anoxia. Other reactions are acceleration of glycolysis due to high CO_2 levels, but this is only effective in short submergence. Surviving longer flooding periods needs down-regulation of metabolism, a quiescence as described in *A. calamus* and *S. tabernaemontani* who survive up to 75 days (Brändle 1991) with a reduced glycolytic rate and a suppressed leaf capacity for photosynthesis and respiration. Additonally, anoxia induces in them a less productive fluorescence-modificated PSII, which, after de-submergence, recovers to full capacity within 7 days (Schlüter and Crawford 2001). *Rumex* species are also capable of photosynthesis under water and subsequently release oxygen into their rhizosphere (Voesenek 1993). Reduced photosynthesis may prevent total oxygen depletion if water is not too turbid. Down-regulation of respiration will also prevent carbon depletion and keep accumulation of toxic substances at bay. Maintaining the energy charge as long as possible to keep membrane integrity is crucial, since its loss means death of the cell.

End of submergence presents another challenge, since rapid aeration means oxidative stress. Reactive oxygen species (ROS) react with lipids and destroy membranes, so-called post-anoxic injury. Resistant plants possess detoxification mechanisms: Superoxiddismutase (SOD), catalase, ascorbate peroxidase, Vitamins C, E etc. (Foyer 1994). It is therefore necessary to survey not only the flooding reaction, but the de-submergence is equally important.

4. Methods and experimental setup

4.1 Measurement at place of origin

To pay respect to the current situation at Three Gorges Reservoir and keep results easily transferable, part of the experiments was kept as close as possible to the natural conditions (**manuscript 2**). A six weeks flooding period was to be observed for changes of OA occurrence in soil solution and general reactions of plants to the treatment. These experiments were conducted at Southwest China University, Chongqing/Beibei (SWU). Sediment soil substrate and plants were collected at Yangtze riverside in Chongqing, N29°28'31.1", E106°31'29.7", 213m NN.

The substrate is a very fine sediment (87% fine sand <20µm diameter) with low nutrient content. Cations were determined by AAS and found to be in a very low range, Ca, Mn, Mg, Fe all being below 10ppm. Nitrate, nitrite and phosphate were also low. All three species are able to grow productively in this nutrient-poor substrate.

4.1.1 Pot rhizotrons

Plants were submerged in pot rhizotrons which allow close to natural conditions (1.2dm^3), since rhizotron plantings in low-volume boxes may influence root growth and architecture. Pot rhizotrons were built from plant pots with a 35° tilted plexiglass plate on one side to allow access via microsuction cups (MSC) and observation of growing roots. 15 holes were drilled into the plate which would host the MSC (Fig. 1). They were light-proof sealed between measurements. These pots could be waterlogged and completely submerged in 2m deep pools at SWU. The Open-Air character was taken into account by logging microclimate conditions.

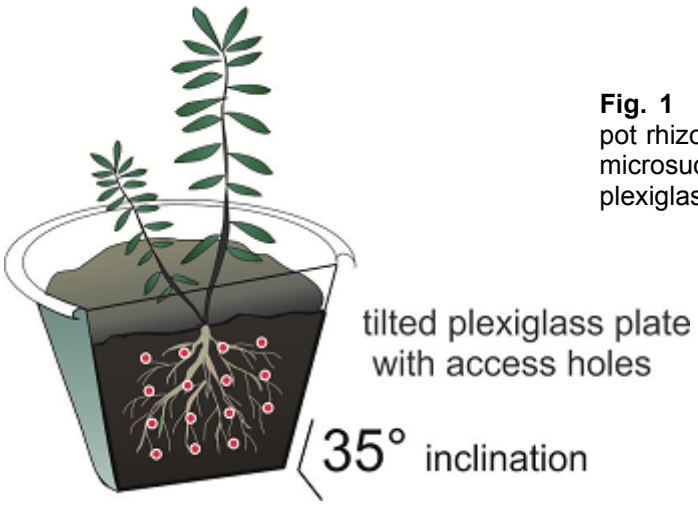

Fig. 1 *S. varieagata*, scheme drawing of pot rhizotron for soil solution sampling with microsuction cups. 15 acces holes in plexiglass plate.

4.1.2 Microsuction cups

Rhizosphere studies have to meet the temporal and spatial variations which are associated with exudation dynamics. Several approaches are known to gain samples for analysis. Extraction techniques like percolation of the plant-containing soil column with water (Johnson et al., 1996; Shen et al., 2004), soil extraction after plant removal (Gerke et al. 1994; Li et al.1997) or exudate collection in hydroponic culture (Dechassa and Schenk 2004) can only determine the total amount of exudation. Collection rings around parts of roots or root parts between filter paper (Hocking and Jeffery 2004; Keerthisinghe et al. 1998; Shane et al. 2003; Watt and Evans 1999; Liang and Li 2003; Neumann and Römheld 1999) offer more spatial resolution. But for use also under field conditions, a method is needed which can take a solid phase and MO existence into account, protecting samples from rapid biodegradation. Microsuction cups solve that problem (Göttlein et al., 1996) in combination with rhizotrons (Dieffenbach et al., 1997). It is a low-invasive, non-destructive method and was tested during this PhD-work for its applicability for OA detection in combination with capillary electrophoresis (**manuscript 1**). MSC's are inserted into substrate and can be connected to a vacuum chamber to sample directly into Eppendorf tubes. Several types of MSC's were tested during this PhD-work for absorption and MO permeability by repeated sampling of standard solution, determining absorption with increasing volumes. MO permeability was tested by sucking a stock solution of 109 MOs/ml of the bacterial strain *Pseudomonas fluorescens* DSMZ no. 50108 (GBF, Braunschweig, Germany) through the MSC by vacuum application. The eluates were examined by fluorescence microscopy total counts (Klauth et al., 2004), plate counting on agar dishes and cell size determination.

The only model impermable to MO was the PES-1. It consists of a 10mm porous polyethersulfone (PES) hollow fibre, containing a polyetheretherketone (PEEK) tube with a hole near the tip ,which was sealed into the fibre with glue. The pore size of the fibre was < 2µm. It was successfully used for a test of OA sampling in different depths of pot rhizotrons during flooding, and therefore utilized in all experiments with pot rhizotrons (**manuscript 1+2**).

4.1.3 Soil solution analysis via Capillary Electrophoresis

Capillary Electrophoresis was chosen as analytical method because it is an effective tool for OA analysis in small sample volumes (10µl), used with a salicylate electrolyte (Bazzanella et

al. 1997). Since OA carry charge, they are easily separated and identified here. HPLC may provide better results in terms of quantification, especially if combined with Mass Spectrometry, but the method would have been more intensive and slower. Since our samples were small snapshots taken from the rhizosphere which were related to each other, and no complete quantitative analysis, this method was considered sufficient for this work, especially regarding the very large amount of samples analyzed.

4.2 Application for simulated flooding in a controlled environment

4.2.1 Dual-Access floodable rhizobox

The pot rhizotron experiment had shown interesting results of plant strategies to cope with submergence. Since plants did not seem inhibited at all by waterlogging and survived a 6 weeks flooding period without greater impediments, it was decided to take a closer look into the rhizosphere under more controlled conditions, and also take the factors pH and oxygen into account. A continuous measuring leads away from snap-shots during a flooding period and provides an even better base to assess plant abilities. Being able to monitor rhizospheric reactions in high temporal and spatial resolution, especially under flooded conditions given the abovementioned challenge, is therefore highly desirable.

To provide this resolution for rhizosphere monitoring, the rhizobox approach was developed further to accommodate a flooded plant. Blossfeld et al. (2007) have developed a rhizobox which allows soil solution sampling via continuously installed and therefore waterproof steel capillaries. The rhizobox has three tubes, in which raster plates can be inserted. Two of these raster plates form a unit, with a filter on the inner side directly contacting the rhizosphere (pore size <0.2µm to prevent microorganism (MO) contamination of samples) and divided by a septum to provide watertightness. The holes in the inner plate narrow to 0.4mm diameter to prevent capillaries from penetrating the filter. It is now possible to install capillaries at a defined point (few mm distances) and sample MO-free soil solution without water loss. This still works with a water column of 30cm after the tank has been mounted on top of the rhizobox to enable submergence (**manuscript 3**).

4.2.2. planar optodes

PH- and oxygen monitoring was done using planar optodes, whose measurement principle is based on the fluorescence decay time of pH- or O_2-sensitive indicator dyes (Blossfeld and

Gansert 2007, Blossfeld et al., 2010, Huber et al., 2001, Klimant et al., 2001, Jensen et al., 2005, Frederiksen and Glud 2008). Foils carry an analyte-insensitive luminophore as reference and an analyte-sensitive fluorophore with similar excitation spectra, but different decay times. The phase angle of excitating light changes depending on the ratio of intensity of reference luminophore and indicator fluorophore and directly reflects analyte concentration. A glass fiber, connected to a light source and measuring device (pH-1 mini, PRESENS, Regensburg, Germany) conveys the excitation light pulse (470nm). The glass fiber is moved by step motors (compare Blossfeld and Gansert 2007) over the foils in 2mm steps (5 seconds per step, 1 measurement per second, resulting in 44 measuring points on an area of 10x30mm). Measuring cyles took 2 hours and could be run 24/7. The average of each 5 measurements per measuring point was processed with SigmaPlot (Systat Software, Inc., San Jose, CA, USA) to create contour plots of pH-distribution along the optode foils. The setup is shown in Fig. 2. These setup allowed assessment of pH- and oxygen dynamics in the mm range, combined with simultaneous soil solution sampling (~2-4mm range) from the corresponding opposite site of the rhizobox. It significantly improves conventional rhizoboxes since it allows non- or low invasive investigations while allowing excellent control of all environmental parameters (**manuscript 3**).

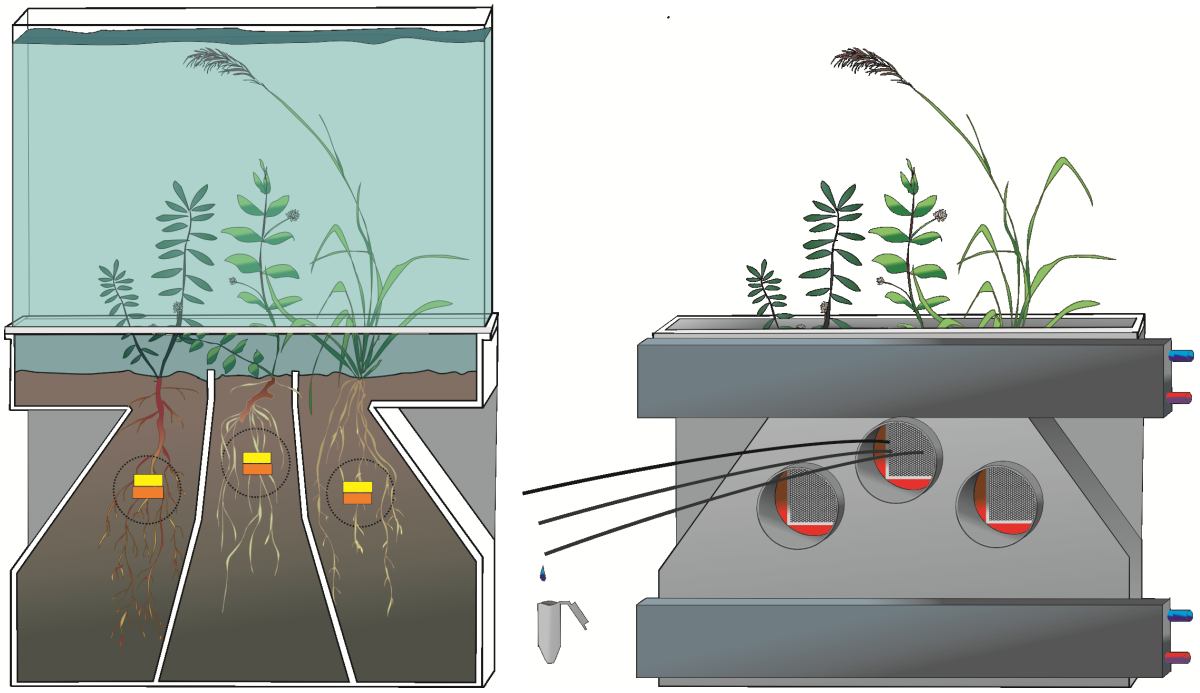

Fig. 2: Dual-Access floodable rhizobox. Left side: view from behind, plexiglass cover with pH-(yellow) and O_2-(orange) sensitive foils which are read by excitation light pulse (pH-mini). Circles imply corresponding tubes on the front (picture on the right side). Capillaries, inserted in raster plate units containing septum and filter, sample soil solution into Eppendorf tubes. The rhizoboxes are tilted 35° to allow roots growth along the plexiglass and, subsequently, the optodes.

5. Results and discussion - assessment of observations

5.1 Three flooding resistant species from TGR area: close-up

It was the first time that rhizosphere reactions have been observed on these flooding resistant species. Aim of this work was a better understanding of how these perseverant plant species manage survival during months of complete submergence. Several points of interest are to be discussed and compared to literature and non-tolerant species with regards to the innovative methods used.

→ Plant performance: waterlogging is no hindrance

When the pot rhizotrons were exposed to three weeks waterlogging, their behaviour did not differ significantly from that of moderate moist control plant sets. Most plants react stressed to prolonged waterlogging, which means hypoxic, if not anoxic conditions in the rhizosphere, and ethylene as well as anaerobic metabolites may accumulate (Voesenek 2003; McKee and McKevlin 1993). This was not the case here. Patterns of OA occurrence were quite similar between the species as well as their controls, showing an increase of OA values after one week of waterlogging (**manuscript 2**). These patterns correlated with microclimate data, namely a high PAR regime during that time. Stress through waterlogging cannot have been very impeding, since reaction to "normal" microclimatic conditions as well as shoot growth rates were identical to control plants. Since Pikryl and Vancura (1985) describe root exudation and plant health as positively linked, it can be deduced that increased photosynthetic rates and higher turnovers lead to higher carbon assimilation and therefore also carbon loss into the rhizosphere. Since mainly fermentation products were detected in soil solution, it can be assumed that we did not only record plant root exudation, but also MO contribution, which exist in a close feedback relation (Jones 1998). Nevertheless, increased carbon loss/exudation means increased nutrition for fermenters in a hypoxic waterlogged soil. The MO communities will, with their products, reflect the input by the plant root. Therefore it is possible to carefully conclude on root activity. It can further be assumed that the well-developed aerenchyma in all three species helped to avoid anoxia in the roots themselves, which means that energy charge in root cells was always ensured.

→ Initial flooding causes no exudation bursts and no accumulation of fermentation products

Pot rhizotrons were sampled for OA after 1, 3 and 7 days of complete submergence (**manuscript 2**). There was no "burst" of exudation visible as reported from more flooding-sensitive plants like *Medicago sativa* and *Lotus corniculatus* (Barta 1986). Instead, a slow rise of fermentation products occurred. After prolonged flooding (6 weeks), this level had not risen much further (probing inbetween was not possible because of soil disturbance and oxygen entry through water movement). Schreiber et al. showed that the three species follow a quiescent strategy during prolonged submergence, even if rapid shoot elongation of *A. philoxeroides* strongly implies high ethylene production within the first three days. The fact that there is no further accumulation of lactate, formate or acetate shows that further carbon rhizodeposition had receded along with photosynthesis as described by Brändle (1999) to a lower level, providing not much nutrition for fermentative processes. Yet it could be shown that turnovers did not cease completely. After de-submergence, all three species were sporting fresh buds and even some new leaves, which had thinner cuticles, which are useful for better gas exchange in water.

→ After flooding: return to productivity within one week

De-submergence produced plants with delicate new buds and leaves (most old leaves had been discarded). Fermentation-related OA had slightly accumulated. The following de- and latter increase of overall OA amounts can be explained through re-aeration of soil, which helped aerobic degradation of fermentation products. Schreiber et al. could show that especially *A. anomala* releases much oxygen into its rhizosphere after de-submergence. After 7 days, also OA like oxalate, malate and citrate were detectable, and the pattern of submerged plants started to reach control plant levels. Parallel to this, renewed growth and leaf development was observed. This showed that plants did not take much damage from prolonged flooding (**manuscript 2**).

→ Survival strategies: diverse reactions to submergence

Observations during experiments revealed some traits in the plants which are not clearly quiescent, yet may be effective. As mentioned above, *A. philoxeroides* was found to be capable of rapid shoot elongation (**manuscript 2**). Up to 50cm and more can be bridged within 2-3 days. This combinative strategy is very effective during shorter or shallow flood events. If the plant reaches the surface, oxygen supply to roots is secured, because it consists

almost only of aerenchyma (compare Voesenek 1993,2003). Additionally it can re-grow from just one node and two leaves. If the shallow shoot should break in the current, that node can build a swimming carpet on the water surface and distribute easily, which is one reason why *A. philoxeroides* is such an invasive weed.

Another trait could be shown in *S. variegata*, which produced new adventive roots during prolonged flooding directly above soil surface in water (**manuscript 2**). Quiescent plants like these three keep up very low metabolism, but something must remain (Schlüter and Crawford 2001). This energy charge must be supplied from carbon storage, if no light or CO_2 is available (if the water is turbid or water pH too high, so that only CO_3^{2-} remains, which is not usable for plants. Ye (2010) showed that after 60 days the water soluble sugar storages of *A. anomala* in 20°C cold water were used up, in a slow process. (This would be a crucial point since *A. anomala* does not seem to be able to tap starch reserves while submerged. It needs further investigation.) In *S. variegata*, these adventive roots show need for better exchange with the surrounding matrix. Low diffusion coefficient of gases are one thing, exuding toxic substances like ethanol the other. Exuded by roots in anoxic soil, ethanol would accumulate fast because it is not processed further (Kato and Naguchi 2002) and cause injury to cells. But from roots in the surrounding water body it will easily be deposited.

→ MO interact with plants: Sediment vs. Glass beads connecting field to lab

The accompanying experiment, analyzing and quantifying the OA production of whole root systems of the three species in glass bead substrate and glass sediment at 10°, 20° and 30°C, should provide a first connection between field and lab applications, comparing an MO-colonized treatment to an almost sterile treatment to assess MO contribution **(manuscript 2)**. We decided against estimating exudation rates instead of assessing total production, since OA exudation does not occur over the whole root surface and is therefore underestimated when related to the whole root system (Ryan and Delhaize 2001). Comparison between complete root system analysis and sampling with higher spatial resolution is therefore needed. It could be shown that temperature had no effect in glass bead setup, and that formate, lactate and acetate were still dominating OA, which hints that in field they do indeed not only originate from fermenters, but also from roots. A temperature dependence of OA accumulation could be shown in the sediment setup for fermentation related OA. Overall OA levels in glass were much lower than in sediment, implying the lack of feedback between soil organisms and roots. It is not possible yet in field experiments to distinguish

quantitatively between plant and MO exudation since they are closely linked (Jones 1998). But laboratory culture alone will also not show real plant behaviour (Grayston et al. 1997; Jones 2003). We suggest that comparison between field and lab are, at the current time, a well suited method to gain more information, especially when related to temperature, which is suspected to have strong influence on microbial turnovers in soil and has to be kept in mind.

5.2 High resolved insight into the rhizosphere of completely submerged plants

The Dual-Access floodable rhizobox as an approach for high resolved rhizosphere monitoring. Schreiber et al. showed a first application of investigation in the rhizosphere of completely submerged plants (**manuscript 3 + 4**).

→ Root growth: observed in-situ

We confirmed earlier findings of Marschner (1995) and Jones (1998) who found the root tip and root elongation zone the areas of strongest rhizospheric acidification. As shown by Hinsinger et al., (2003) and Kopittke and Menzies (2004), the root tip could acidify its surrounding by 1-2 units (observed for *A. philoxeroides* and *S. variegata*). Acid-growth mechanism and the, also here observed, ROL add to the acidifying effect (Peters 2004; Versel and Pilet 1986). Considering the amount of oxygen exuded and the values of accompanying OA, we postulate that OA exudation is hardly pH-relevant in our three study species. This part is taken by protons and oxygen. The root could be observed parallel to growing and sampled for OA at corresponding sampling spots (**manuscript 3**). After much adaptation and programming, the rhizobox and motor system was developed to a stable running system which was easily to handle. We tested this in the 2-weeks-cycles: 2 weeks waterlogging, 2 weeks flooding, 2 weeks waterlogging at 10° and 20°C (**manuscript 3**).

→ MO interact with plants: Sediment vs. Glass beads, temperature and OA

The former results regarding low temperature influence in glass bead setup were confirmed in the rhizobox setup (**manuscript 4**). In contrast, the 20°C sediment setup appeared to show a generally much higher pH (>1unit) and also the highest OA levels of all treatments (fermentation!). An explanation could be that the warm solution can not solve as much O_2 than the colder variation, yet the 20°C glass setup did not have an increased pH compared to

the 10°C glass setup. The sediment setup does in any case experience more respiration in soil by roots and MO's, so oxygen may be utilized fast before oxidizing rhizospheric components and releasing protons. Assessing these questions is quite important, since the original habitat is prone to some temperature regime changes because of the change of flood pulse from summer to winter.

→ Rhythmic roots: Diurnal acidification and spatio-temporal effects

We found diurnal rhythms of acidification in all rhizobox experiments. Acidification follows photosynthesis with a shift, having its peak at 4-5pm. The spread within root and bulk soil was normally around 0.4-0.5, the highest values in bulk soil reaching 7.2-7.4 in glass bead substrate (**manuscript 4**). Diurnal rhythms were less in spread in sediment substrate, the sediment having slight buffering effects. Such rhythmics were already described for *Vigna unguicula, Medicago sativa* and *Juncus effusus* (Jarvis and Hatch 1985; Rao et al. 2002; Blossfeld 2007). Yet we did not find nightly alkalinization as reported from *Medicago* and *Vigna.* Rhythms receded during submergence and re-improved in the following waterlogging back to initial levels. It can be concluded that plants reached a state again that was similar to the initial state, and that 2 weeks flooding did not have negative consequences on plant performance.

→ Radial oxygen loss: spatio-temporal dynamics

Dynamic oxygen occurrence was detected for all three species. Interestingly, not all roots which were in measuring windows exuded oxygen (**manuscript 3 + 4**). Those which did not, did also only have very weak acidification. Oxygen release is not homogeneous over the root system and may have varying intensities. Barriers to oxygen loss in basal root regions through cell suberization, as shown in *Typha* and *Cladium* with methylene blue in agar, secures in combination with aerenchyma oxygen transport into root tips (Chabbi 2002). ROL can act as an additional proton source, oxidizing cations and freeing protons (Kirk 2004). Since it occurred parallel to the observed acidifications, it is very likely to have happened here. ROL followed the same temporal dynamics as pH, dependent on photosynthesis dynamics. That was still the case during complete submergence, even if weaker. Oxygen in soil never dropped below 30vol% air saturation during 2 weeks complete submergence (**manuscript 4**). Compared to *Maize* and *B* which had almost zero oxygen in their rhizosphere in the same experiment setup, that is a huge advantage, and proves

photosynthesis was still active, if on a lower level (oxygenation being about half as strong as during waterlogging, when photosynthesis was not impeded). *A. anomala, A. philoxeroides* and *S. variegata* survived all treatments, as did *P. arundinacea*. *Maize* and *Hordeum* died during submergence (**manuscript 4**).

→ Non-tolerant species vs. floodable rhizobox: oxygen for everyone?

Maize, *Hordeum* and *Phalaris* have been treated in the rhizobox to serve as comparisons and control plants for the three study species, since Maize and Hordeum are known as being not very resistant to anoxia (Wenkert et al. 1981, Mayne et Lea 1984) and could therefore serve well to help value the reactions of the adapted species. Most obvious result was that *Maize* and *Hordeum* did not survive flooding. Despite *Maize* being able to form aerenchyma, both plants were not tolerant enough to endure this task. Several studies have been performed on them, showing a fast anoxia response in *Maize* which leads to aerenchyma forming (Lenochová et al. 2009, Drew et al. 2000). They also show diurnal rhythms, but during flooding, these ceased as the plants deceased. Schreiber et al. (**manuscript 4**) could also show that almost no oxygen was released into the rhizosphere by those species despite this aerenchyma. While *Phalaris* could keep up 30vol% and the study species 45-70vol%, *Maize* and *Hordeum* reached five to zero. Anoxia must have been accompanied by loss of energy charge, which is supported by the fact that in the beginning of flooding, OA levels were very high here. They were in fact highest of all treatments, receding after the plants had died. So overexeeding fermentation as well as carbon loss through a non-quiescent working metabolism as reaction to flooding, and subsequently failing energy balance, caused the non-adapted plants to die.

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6. Synopsis and outlook

The Dual-Access rhizobox is considered a valuable tool for high-resolved rhizosphere monitoring and simulating complete submergence. Its uses could be further stressed, since there are still many questions to assess. The role of MO in the rhizosphere is still widely unaddressed, as is carbon fate after exudation by plant roots. The rhizobox and the involved techniques, especially the planar optodes, can, in combination with isotopes or imaging techniques, make a contribution to clear much more in relation to processes in the rhizosphere.

However, based on these results and the fact that dying plants loose their periodicity, Schreiber et al. suggest that regular steady diurnal rhythms of acidification and ROL can be seen as an indicator for a functioning root system**.** This can be transferred to other studies and species.

Aim of this work was to understand what makes the study species so persistent during stressful flooding events, and their strategies have been apprehended. They are considered highly suitable for surviving longer flooding events. However, for the task of soil protection, only two of the three are considered applicable since the roots of *A. philoxeroides* are too delicate to provide good hold on sedimental soil.

Temperature had faster and stronger effect on the complete soil community (including roots) than on roots alone. This should not be neglected in further studies to estimate effects of temperature correctly. An interesting question in that regard could be the ramifications of the new temperature regime in TGR. Before the Dam construction, flooding took place in summer (25°C water temperature). Now it will happen in winter, at far lower temperatures (5-10°C). Of course that means lower respiration, less carbon loss and overall less pressure. But competition does not sleep. If now in winter survival conditions are better, more and different species may survive the flooding period. When the vegetation period begins, competition might be stronger than before. We do not know yet if this really is the case and which role rhizosphere reactions will have in this, but this problem has to be discussed in future. The TGR presents a great intrusion and damage to existing ecosystems. Yet we have the possibility to watch as the ecosystems react, and learn.

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7.1 Publications of dissertation

C. M. Schreiber, Bo Zeng, U. Rascher, Marian Kazda, U. Schurr and A. J. Kuhn: Surviving Submergence: Rhizosphere dynamics of flood-stressed plant species, observed by non- and low invasive methods.(submitted)

Christina M. Schreiber, Bo Zeng, Stephan Blossfeld, Uwe Rascher, Marian Kazda, Ulrich Schurr, Agnes Höltkemeier, Arnd J. Kuhn (submitted): Rhizosphere dynamics of 2 short-time flooded plants species from the water fluctuation zone of Three Gorges Reservoir, observed by means of high-resolution minimal-invasive rhizobox techniques. (submitted)

Christina M. Schreiber, Björn Thiele, Edelgard Schoelgens, Peter Klauth, and Arnd J. Kuhn (submitted): Low-invasive sterile sampling of soil solution for low molecular weight organic acid monitoring in the rhizosphere with different types of micro suction cups. First results from an application in flooded soil. (submitted)

Christina M. Schreiber, Bo Zeng, Vicky M. Temperton, Uwe Rascher, Marian Kazda, Ulrich Schurr, Agnes Höltkemeier, Arnd J. Kuhn (submitted): Dynamics of organic acid occurrence under flooding stress in the rhizosphere of three plant species from the water fluctuation zone of the Three Gorges Reservoir, P.R. China. (submitted)

7.2 Other publications (including talks and posters)

Schreiber C. M., Zeng B., Schurr U., Kuhn A. J. (2010): Wurzelexsudation in der Wasserfluktuationszone des Drei-Schluchten-Reservoirs, VR China. Ein Ansatz für Einblicke in eine überflutungsgestreßte Rhizosphäre am Beispiel von *Salix variegata* Franch. In: Steuerungsfaktoren von Rhizosphärenprozessen, 19. Borkheider Seminar zur Ökophysiologie des Wurzelraumes 2:45-53, ISBN 978-3-86168-556-8- Verlag Ulrich E. Grauer, Stuttgart.

talks

Schreiber C. M., Zeng B., Schurr U., Höltkemeier A., Kuhn A. J.: Rhizosphere Dynamics of 3 riparian plant species from TGR water fluctuation zone: A floodable rhizobox as an approach to observe O_2, pH and soil solution properties in high resolution. oral presentation at GfÖ Anniversary meeting, Giessen 2010.

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8. Manuscripts

8.1 List of manuscripts and declaration of own contribution

This chapter describes the process from initialisation of each study until completion with special regard to my contribution. Single sections compromise:

concept:	idea for study and development of experimental design
data acquisition:	executing actual experiments in lab and field
data analysis:	translation of raw data into actual results, programming of processing applications
literature research:	acquisition of background information
writing:	the actual process of banning results and thoughts into a manuscript
editing:	rewriting after discussion and implementation of comments of co-authors for final versions of manuscripts

Manuscript 1

Title: Low-invasive sterile sampling of soil solution for low molecular weight organic acid monitoring in the rhizosphere with different types of micro suction cups. First results from an application in flooded soil

Authors: Christina M. Schreiber, Björn Thiele, Edelgard Schoelgens, Peter Klauth, and Arnd J. Kuhn.

Corresponding author:	C. M. Schreiber
Status:	submitted at Journal of Plant Nutrition and Soil Science
Own contribution:	concept (10%), data aquisition (15%), data analysis (20%), literature research (35%), writing (35%), editing (80%).

Manuscript 2

Title: Dynamics of organic acid occurrence under flooding stress in the rhizosphere of three plant species from the water fluctuation zone of the Three Gorges Reservoir, P.R. China.

Authors: Christina M. Schreiber, Bo Zeng, Vicky M. Temperton, Uwe Rascher, Marian Kazda, Ulrich Schurr, Agnes Höltkemeier, Arnd J. Kuhn

Corresponding author: C. M. Schreiber

Status: submitted at Journal of Plant Nutrition and Soil Science

Own contribution: concept (75%), data aquisition (80%), data analysis (75%), literature research (80%), writing (100%), editing (70%).

Manuscript 3

Title: Rhizosphere dynamics of 2 short-time flooded plants species from the water fluctuation zone of Three Gorges Reservoir, observed by means of high-resolution minimal-invasive rhizobox techniques

Authors: Christina M. Schreiber, Bo Zeng, Stephan Blossfeld, Uwe Rascher, Marian Kazda, Ulrich Schurr, Agnes Höltkemeier, Arnd J. Kuhn

Corresponding author: C. M. Schreiber

Status: submitted at Journal of Plant Nutrition and Soil Science

Own contribution: concept (60%), data aquisition (100%), data analysis (75%), literature research (80%), writing (80%), editing (65%).

Manuscript 4

Title: Surviving Submergence: Rhizosphere dynamics of flood-stressed plant species, observed by non- and low invasive methods

Authors: C. M. Schreiber, Bo Zeng, U. Rascher, Marian Kazda, U. Schurr and A. J. Kuhn

Corresponding author: C. M. Schreiber

Status: submitted at Plant, Cell and Environment

Own contribution: concept (80%), data aquisition (100%), data analysis (60%), literature research (80%), writing (80%), editing (70%).

8.2 Manuscript 1

Cover page

Number of text pages: 10
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Running title:

Low-invasive sterile sampling of soil solution for low molecular weight organic acid monitoring in the rhizosphere with different types of micro suction cups. First results from an application in flooded soil.

Corresponding author information:
Christina Schreiber
Institute of Bio- and Geosciences (IBG-2): Plant Sciences, Research Center Jülich GmbH,
Leo-Brandt-Str., D-52425 Jülich, Germany
Fax: +49 2461 612492
Phone: +49 2461 615493
email: c.schreiber@fz-juelich.de

Low-invasive sterile soil solution sampling: different micro-suction-cups in comparison and application

Christina M. Schreiber[1], Björn Thiele[2,3], Edelgard Schoelgens[1], Peter Klauth[3], and Arnd J. Kuhn[1]

[1] Institute of Bio- and Geosciences (IBG-2): Plant Sciences, Research Center Jülich GmbH, Leo-Brandt-Str., D-52425 Jülich, Germany

[2]Institute of Bio- and Geosciences (IBG-2): Plant Sciences/Phytospec, Research Center Jülich GmbH, Leo-Brandt-Str., D-52425 Jülich, Germany

[3]Institute of Bio- and Geosciences (IBG-3): Agrosphere, Research Center Jülich GmbH, Leo-Brandt-Str., D-52425 Jülich, Germany

Key words: Arundinella anomala Steud., micro suction cup, organic acids, root exudation, anoxia

1. Abstract

A new type of micro suction cups, made of polyethersulfone hollow fibres for minimal-invasive monitoring of soil solution chemistry, has been tested to validate the method and also test applicability for monitoring rhizospheric reactions under non-laboratory conditions. Starting point of the study was a number of different materials for micro suction cups including borosilicate, aluminiumoxide, nylon membranes at the top of teflon tubes and polyethersulfone hollow fibres. Tests on their impermeabilities for microorganisms (*Pseudomonas fluorescens*) indicated that only the polyethersulfone hollow fibre of all 5 tested materials was impermeable for MOs. Adsorption tests revealed that the recoveries of organic acids were slightly lower with plastic materials than with ceramic materials. Both tests indicated the micro suction cups made of polyethersulfone hollow fibre (PES-1) with 1 mm diameter as the best. With these, samples were taken and examined for organic acid composition in the rhizosphere of the flooding-resistant grass *A. anomala* Steud. on three days during a seven day flooding event. Analysis of samples was performed by capillary electrophoresis. No exudation bursts were observed, but a slow rise of fermentation-related organic acids. PES-1 microsuction cups proved to be an effective tool to collect non-MO-affected soil solution samples, and to enable insights into a flooding-stressed rhizosphere.

2. Abbreviations

ac	acetate
ANOVA	analysis of variance
CE	capillary electrophoresis
cit	citrate
form	formate
GLM	General Linear Model
gl	glyoxylate
IDL	instrument detection limit
lac	lactate
LMWOA	low molecular weight organic acids
mal	malate
MO	mircroorganisms
MSC	micro suction cups
ox	oxalate
PAR	photosynthetically active radiaton
PEEK	polyetheretherketone
PES	polyethersulfone
suc	succinate

Introduction

Organic acids as exudates from living plant roots have been hypothesized to be involved in the solubilization of nutrients for the roots, the detoxification of metals and nutritional input for soil microbiota (*Jones*, 1998; *Ryan* et al., 2001). Therefore, soil solution chemistry in the immediate vicinity of roots strongly differs from that in bulk soil (*Marschner* et al., 1986). OA exudation may provide information about plant root condition, since for example nutrient deficiency, toxic cations and anoxia are beyond the strongest stimuli for increased OA exudation (*Smucker* and *Erickson,* 1987; *Neumann* and *Römheld*, 2000). Studies on rhizosphere chemistry have to meet the high spatial and temporal variations which are associated with the dynamics of root exudation. Extraction techniques like percolation of the soil column containing the plant with water (*Johnson* et al., 1996; *Shen* et al., 2004), soil extraction after removal of the plant (*Gerke* et al., 1994; *Li* et al., 1997) and collection of root exudates from plants in hydroponic culture (*Dechassa* and *Schenk*, 2004; *Sas* et al., 2001) only provide the total amount of exudates. Higher spatial resolution offers the embedding of small parts of the roots from plants grown in hydroponic systems in collection rings (*Hocking* and *Jeffery*, 2004; *Keerthisinghe* et al., 1998; *Shane* et al., 2003; *Watt* and *Evans*, 1999) or between moist filter paper (*Liang* and *Li*, 2003; *Neumann* and *Römheld*, 1999). However, exudates from roots in hydroponic systems are not affected by interaction with microbes or the solid phase as under natural conditions in soil (*Jones* et al., 2003). For better understanding of the impact of organic acids on soil processes experiments in soil are necessary which require micro-scale extraction systems for sampling soil solution at defined distances from the roots.

The development of micro suction cups consisting of ceramic aluminiumoxide tips with 1 mm diameter (*Göttlein* et al., 1996) in combination with rhizotrones (*Dieffenbach* et al., 1997) has enabled the non-destructive investigation of soil solution chemistry with high spatial and temporal resolution for the first time. Changes in the concentrations of nutrient cations and Al^{3+} in the vicinity of roots were determined (*Dieffenbach* et al., 1997; *Göttlein* et al., 1999; *Wang* et al., 2001; *Gao* et al., 2009). The rhizotrone/micro suction cup system was also used for in-situ application of stable isotope tracers to the rhizosphere soil. Thus, ion uptake of roots was investigated under natural conditions (*Göttlein* et al., 2005). Analysis of organic acids, however, often failed due to their concentrations below detection limit (*Göttlein* and *Blasek*, 1996). Recently developed micro suction cups constructed with nylon membranes showed a distinct adsorption of organic acids implying their minor utility for studies on root exudation (*Puschenreiter* et al., 2005).

Present micro suction cup methodologies for studying rhizosphere chemistry still show deficiencies regarding sampling of soil solution containing organic acids. Collected soil solutions were not protected from microbial biodegradation and losses of organic acids occurred due to adsorption. In this study, micro suction cups constructed of different materials have been tested for their permeabilities of microorganisms (MOs) and their adsorption of organic acids. The favoured micro suction cup was used in flooded rhizotrons containing *A. anomala* to examine rhizospheric reaction to flooding. *A. anomala* is a very flooding resistant *Poaceae* (*Luo* et al., 2008, *Ye* et al., 2010), native to the water fluctuation zone of Three Gorges Reservoir, P.R. China, and subject to several studies for its reaction to submergence and its capability to prevent erosion on the regularly flooded banks of Three Gorges Reservoir region (TGR). Understanding rhizosphere processes like organic acid exudation during submergence helps estimate the state of the root system, since exudation is positively related to root growth and health (*Prikryl* and *Vancura*, 1980). The interaction of plant roots with their direct surrounding (Water and nutrient uptake, oxygen loss, organic carbon release and more) influences pH and redox potential and provides nutrition for soil microbiota (*Jones* 1998, 2004; *Ryan* and *Delhaize*, 2001; *Hinsinger* et al., 2003; *Farrar* et al., 2003; *Blossfeld* and *Gansert,* 2007) which may or may not be beneficial under stressful conditions like anoxia. A species which is able to maintain healthy roots during longer submergence is suitable for re-vegetating riverbanks, and thus helping prevent erosion (*Allen*, 1979; *Schiechtel* and *Stern*, 1996; *Liu* et al., 2004; *Wang* et al., 2005). Being able to sample low-invasively under close-to-natural conditions and yet without MO-contamination will bring more information about rhizospheric reactions, since sterile laboratory cultures will not show real exudation patterns due to lack of MO feedback or different root development (*Grayston* et al., 1997; *Jones*, 2003; *Schulz* and *Vetterlein*, 2007). Furthermore, capillary electrophoresis allows analysis of very small sample volumes (<10µl), which makes sampling even less invasive (concentration gradients, influence of neighbouring sampling spots) and is therefore an effective tool to complete this technique (*Dabek-Zlotorzynska* and *Keppel-Jones*, 2000; *Göttlein*, 2005).

Materials and methods

Micro suction cups

The micro suction cups tested for their abilities to sample organic acid containing soil solution are shown in table 1.

((Table 1))

The most suitable micro suction cup with a polyethersulfone (PES) hollow fibre was further improved and miniaturized in cooperation with "Rhizosphere Research Products" (Wageningen, The Netherlands). The final design of the micro suction cup PES-1 consisted of a 10 mm long PES hollow fibre as the porous part (compare *Gao* et al., 2009, *Göttlein* et al., 1999). A polyetheretherketone (PEEK) tube (0.6 mm O.D.) with a hole near its end was inserted into the hollow fibre until the fibre's end. The tip was sealed with glue. For better handling and higher stability the open side of the hollow fibre was inserted into a polystyrene tube (2.0 mm O.D., 1.0 mm I.D.) and fixed with glue resulting in a porous tip length of the micro suction cup of 8 mm. The void volume of PES-1 (46 ± 2 µl) was determined by weighing several micro suction cups before and after filling with water. All micro suction cups were tested for leaks. Before use the micro suction cups were sterilized with 3 % hydrogen peroxide solution by suction, and subsequently rinsed with autoclaved deionised water.

Tests on microorganisms permeability

The bacterial strain *Pseudomonas fluorescens* DSMZ no. 50108 (GBF, Braunschweig, Germany) was used for the tests. The organisms were grown in medium 461 according to DSMZ protocol (DSMZ, 2004) which contained 0.5 g/l glucose as sole energy and carbon source. The cells were harvested at the end of the logarithmic phase. Concentrations of stock solutions of MOs were measured with a Coulter Counter (Beckman Coulter Multisizer 3, Krefeld, Germany). A stock solution of 10^9 MOs/ml was sucked through the micro suction cups by application of a vacuum of 400 mbar. The vacuum corresponded to that one applied by *Dieffenbach* et al., (1997) in their rhizotrone experiments. After collection of 1.5 – 2 ml eluate per micro suction cup the eluates were both examined by fluorescence microscopy total counts (*Klauth* et al., 2004) and by plate counting on agar dishes (R2A Agar, Oxoid, Lenexa, USA). For plate counting, the eluates were progressively diluted by factors of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} and incubated at 25°C for 24 h. The frequency distribution of cell sizes of *Pseudomonas* bacteria was determined by use of image processing according to (*Klauth* et al., 2004).

Tests on adsorption affinities for organic acids

The tests of the micro suction cups on their adsorption of organic acids were conducted with a test solution containing 10 µM of oxalate, formate, succinate, malate, acetate, glyoxylate, lactate and citrate. The micro suction cups were connected to a syringe to collect the eluate of each micro suction cup into individual sterile Eppendorf vials. With each of 5 micro suction cups first a fraction of 100, 150 and then 3 times 250µl was collected, weighing the eluate.

Validation of capillary electrophoresis analysis for organic acids in nutrient solution

Standard solutions (5, 10, 20 and 50 µM) of 7 organic acids (oxalate, formate, succinate, malate, acetate, glyoxylate, lactate and citrate) in water and 10% Hoagland solution were analyzed 3 times a day on 3 consecutive days by capillary electrophoresis.

Plant growth and culture

11 individuals of *A. anomala* were collected from Yangtze riverside and transferred into pot rhizotrons filled with Yangtze sedimental soil (collected at N29°28'31.1", E106°31'29.7", 213m NN) after washing the roots carefully with tap water. The pots had a total volume of 1.2dm³, closed on one side with a 35° tilted plexiglass plate with a 15 hole grid, which would host the MSC during sampling. The distance to visible roots was documented with photographs. In-between samplings, the holes were sealed by cling wrap and black rubber foil. The plants were not fertilized since they were growing in one of their habitual substrates. The control plants were watered every second day with tap water. The treatment plants were kept waterlogged after 1 week acclimatization.

3 weeks after planting, 9 pots were submerged (2m depth), 2 were kept as control sets under natural conditions in the experimental garden at Southwest University Chongqing-Beibei. The microclimatic conditions (PAR (Sykes quantum sensor), air, water and soil temperature, precipitation) were monitored (logger Minicube Mini 32, Jiří Kučera - Environmental Measuring Systems).

Sampling of rhizosphere soil solution

Soil solution sampling in the flooded *A. anomala* sets took place after 1, 3 and 7 days of submergence. 3 pots and the control sets were collected on each date and immediately sampled by PES-1 micro suction cups to avoid reactions to aeration in the rhizosphere, applying a vacuum of 200-300mbar. Sampling time was 5-10 minutes to collect 50-70µl of

soil solution. Before each sampling, the MSC were dried by air-pressure to avoid dead volume. Each day the MSC were sterilized with ~65% ethanol and thoroughly rinsed with deionized water before sampling. Control samples from a sediment-filled rhizotrone without plant were obtained in the same manner.

Analysis of organic acids

All samples were analyzed for organic acids (oxalate, formate, succinate, malate, acetate, lactate, citrate) using capillary electrophoresis with a salicylate electrolyte (*Bazzanella* et al., 1997). A capillary electrophoresis system G1600A (Agilent, Böblingen, Germany) was used, equipped with a built-in diode-array detector. Fused silica capillaries (Polymicro, Phoenix, USA) of 75 µm I.D. x 64.5 cm total length (56 cm to detector) were used. The electrolyte solution contained 7.5 mM salicylic acid, 15 mM TRIS, 0.5 mM dodecyltrimethylammonium hydroxide and 0.3 mM $Ca(OH)_2$. A voltage of 30 kV was applied during all separations, with temperature maintained at 25°C. Injections were carried out hydrodynamically with a pressure of 50 mbar for 30 s. The separated compounds were detected by indirect UV-detection at a wavelength of 232 nm. Quantification was performed using external calibration with aqueous standard solutions (5, 10, 20 µM) and internal standardization using phenylacetic acid as internal standard. The rhizosphere samples (5 µl) were diluted with 40 µl deionized water and 5 µl 100 µM internal standard solution prior to analysis.

Statistical analysis of A. anomala rhizotron experiment

In the rhizotron experiment we tested mean values for organic acid concentrations using general linear model (GLM, Type 4 sum of squares) Repeated Measurements ANOVA for time series data with statistical significance defined at $P < 0.05$ unless otherwise stated. Here we tested for overall effects of different experimental treatments (Between Subjects Effects, i.e. irrespective of time: depth of measurement (2, 5, 7cm)) as well as effects of time (within subjects) on the effects on the organic acid concentration. Interaction of factors time*depth was tested. Time intervals between measures were not identical. The Greenhouse-Geisser correction was used to adjust significance values if assumption of sphericity was not met. [Games-Howell post hoc test was used since not all sets showed homogeneity of variance after Levene's test ($P < .05$).]

Statistical analysis was done using PASW Statistics 18, graphical presentation was done using Sigmaplot 10.0 (PASW: SPSS Inc., Chicago, USA. Sigmaplot: Systat Software Inc., London, UK).

Results and Discussion

Microorganisms permeability

Cell densities in *Pseudomonas fluorescens* solutions after suction through different micro suction cups were identical whether determined by total counts with a fluorescent dye (data not shown) or plate counting (Fig. 1). In case of the hollow fibre, the cell density was too low for total counting according to the protocol of *Klauth* et al. (2004).

((Figure 1))

As shown in Fig. 1, the ceramic materials borosilicate (BSiO-6 and BSiO-2.5) and aluminiumoxide (AlO-1) as porous tips of the micro suction cups were almost completely permeable for *Pseudomonas fluorescens*. Micro suction cups made of nylon membranes showed a significant decrease in the number of MOs but only the hollow fibre was totally impermeable for MOs. Total counts revealed nearly null countable cells for hollow fibre, even in undiluted eluates. The pore sizes of the micro suction cups are the decisive factor for their MO permeabilities. As shown in table 1 the MO impermeable PES hollow fibre has the smallest pore size of 0.1 µm. In comparison, the cell sizes of *Pseudomonas fluorescens* bacteria in a stock solution were in the range of 0.2 – 1.2 µm (Fig. 2). A depth filter effect which could have been supposed in the case of the relatively thick borosilicate micro suction cup (BSiO-6) was obviously irrelevant.

((Figure 2))

Adsorption affinities of organic acids

Adsorption of organic acids by the micro suction cups is shown in Fig. 3. Among the ceramic and plastic materials borosilicate (BSiO-2.5) came off best with no adsorption of any of the organic acids. The nylon membrane (Ny-4) caused slight losses of < 10 % for all organic acids whereas BSiO-6 and AlO-1 retained 20 % citric acid from all collected fractions. *Sandnes* et al. (2005) also reported that micro suction cups of the AlO-1 type tended to adsorb different organic acids. Micro suction cups PES-2.5 made of hollow fibres adsorbed more than 20 % of all organic acids at the beginning. Adsorption gradually decreased with increasing elution volume. A miniaturized type of this micro suction cup (PES-1) with a thinner hollow fibre tip and PEEK tube significantly improved the performance resulting in almost no adsorption of the acids. A second test with the PES-1 for 7 different organic acids (ox, form, suc, mal, ac, lac, cit) confirmed this result (Fig. 4).

((Figure 3))((Figure4))

Validation

Organic acid determination with capillary electrophoresis (CE) worked well in a concentration range of 5-20µmol/l. In this range, determination coefficients even over 3 days measuring were close to 1, slightly better in hoagland solution than in water (Tab. 2). Samples have to be diluted to fit into this concentration range, since higher concentrations cause broader peaks and inaccuracy (see standard deviations. Tab. 3, for c=50µmol/l). Instrument Detection Limit (IDL) was 1-1.5µmol/l. Multiple sampling over three consecutive days showed small standard deviations mostly <1 (Tab. 3). CE is therefore considered a suitable tool for analyzing small volume samples.

((Table 2, Table 3))

Rhizotrone experiment with A. anomala

The favoured micro suction cups PES-1 consisting of polyethersulfone hollow fibres with 1 mm diameter were tested in a rhizotron experiment with *A. anomala.* Analysis of soil solution, obtained from completely submerged individuals of *A. anomala* showed no immediate response in the rhizosphere as known from other species exposed to flooding stress like *Medicago sativa* and *Lotus corniculatus* (*Barta*, 1986) or *Zea mays* (*Xia* and *Saglio*, 1992), but a slow yet significant rise (Tab. 4) of mostly fermentation related products (lactate, acetate, formate) towards day 7 (Fig. 5).

((Table 4)) ((Figure 5))

Higher concentrations of lactate and acetate can be a consequence of anaerobic conditions. In this case plants undergo anaerobic respiration which produces potentially phytotoxic metabolic endproducts like lactate. To prevent lethal acidification of the cytoplasm of the root cells, lactate is released in the rhizosphere (*Rivoal* and *Hanson*, 1993; *Xia* and *Roberts*, 1994). Since this experiment took place in a non-sterile environment, also MO's could be the source of these anaerobic turnover products. But a sudden root exudation event would also show in this case since root excretions serves as nutritional input for soil microbiota and increases their turnovers (*Harter* and *Naidu*, 1995; *Hinsinge*r, 2001). Since no immediate strong reaction in the rhizosphere could be observed and all sets reacted quite similarly, this hints towards a quiescent strategy of the concerned species. Additional observations over the experiment time showed that 1 week submergence does not visibly hamper the performance of *A. anomala* (no leaf loss or discolouring, all plants survived). However, organic acid (OA) turnovers in the rhizosphere were not changed significantly directly after beginning of

flooding, and the differentiation between plant root or microbial/fungal contribution to the rise of fermentation products still has to be done. Avoidance of exudation bursts combined with long-lasting root tissues of course helps prevent carbon depletion during prolonged submergence. Already under normal conditions, up to 40% of photosynthetically produced LMW carbon molecules are excreted into the rhizosphere (*Bais*, 2006). Minimizing carbon loss under stress conditions is therefore one part of the ability of these species to survive up to 6 months flooding. The polyethersulfone MSC's proved to be a simple to install and enduring tool for sampling under these conditions.

Concluding remarks

A comparative test of several micro suction cups revealed a polyethersulfone hollow fibre as the most suitable material for in-situ studies of organic acids in rhizosphere samples. The hollow fibre has been shown to be impermeable for microorganisms and to have no tendency to adsorb organic acids. Due to sterile filtration of the soil solution by these micro suction cups conservation of the samples with chemical additives is not necessary. In combination with the rhizotrone system investigation of root exudation at different root zones is possible, as the results of the *A. anomala* experiment have shown.

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Tables and Figures

Tables

Table 1. Tested micro suction cups

	BSiO-6	**BSiO-2.5**	**AlO-1**	**Ny-4**	**PES-2.5**	**PES-1**
Construction	Borosilicate ceramic at the top of a glass tube connected to a teflon tube	Borosilicate ceramic at the top of a V2A tube connected to a teflon tube	Aluminiumoxide ceramic connected to a PEEK tube	Nylon membrane fixed on one end of a teflon tube	Polyethersulfone hollow fibre connected to a teflon tube	Polyethersulfone hollow fibre connected to a PEEK tube (further details see text)
Manufacturer (Order No.)	Ecotech[$] (43141)	Ecotech[$] (prototype)	Self-made (Göttlein et al., 1996)	Ecotech[$] (prototype)	Ecotech[$] (43142/10GF)	Rhizosphere Research Products* (19.21.82)
Outer diameter [mm]	6.0	2.5	1.0	4.0	2.5	1.0
Pore size [µm]	1.0	1.0	1.0	0.45	0.1	0.1

[$] E-Mail: ecoTech@ecoTech-Bonn.de

* E-Mail: Info@Rhizosphere.com

Table 2. Coefficients of determination (linear regression) for the calibration curves (5, 10, 20µmol/l) of the means of 8 OA standards in Hoagland solution and water, measured 3 times on 3 consecutive days

organic acid	hoagland	H2O
ox	0,9996	0,977
form	0,9938	0,9655
suc	0,9984	0,9634
mal	0,9966	0,9532
ac	0,9985	0,9646
gly	0,9994	0,9627
lac	0,9966	0,9526
cit	0,9999	0,9848

Table 3. mean Standard Deviations (SD) of measured standards of 8 OA measured 3 times on 3 consecutive days in Hoagland solution and water

mean SD	Matrix \ c[µmol] of input standard	5µmol	10µmol	20µmol	50µmol
over day1	hoagland	0,36	0,83	2,13	2,33
	h2o	1,45	2,40	4,42	10,41
over day2	hoagland	0,56	0,71	1,25	2,01
	h2o	0,89	2,08	2,94	15,08
over day3	hoagland	0,75	1,08	1,09	1,72
	h2o	2,26	2,94	5,49	10,22

Table 4. 7 day flooding test: P values from Repeated Measures ANOVA on effects of depth and sampling time (day 1, 3, 7) on 5 organic acids in the soil solution from submerged pots of *A. anomala*. If the assumption of sphericity of data was not met, Greenhouse-Geisser correction was used. (* = significant at p< .0.05 level). n = 3 for depth, n = 4 for sampling time

		within subj.		between subj.
		Over time	time*depth	depth
Treatment effects OA found in soil solution of 7 day flooded rhizotrons	ox	.002*	.577	.910
	form	.009*	.603	.888
	ac	.005*	.007*	.229
	gly	.466	.787	.548
	lac	.001*	.218	.725

Figure legends and figures

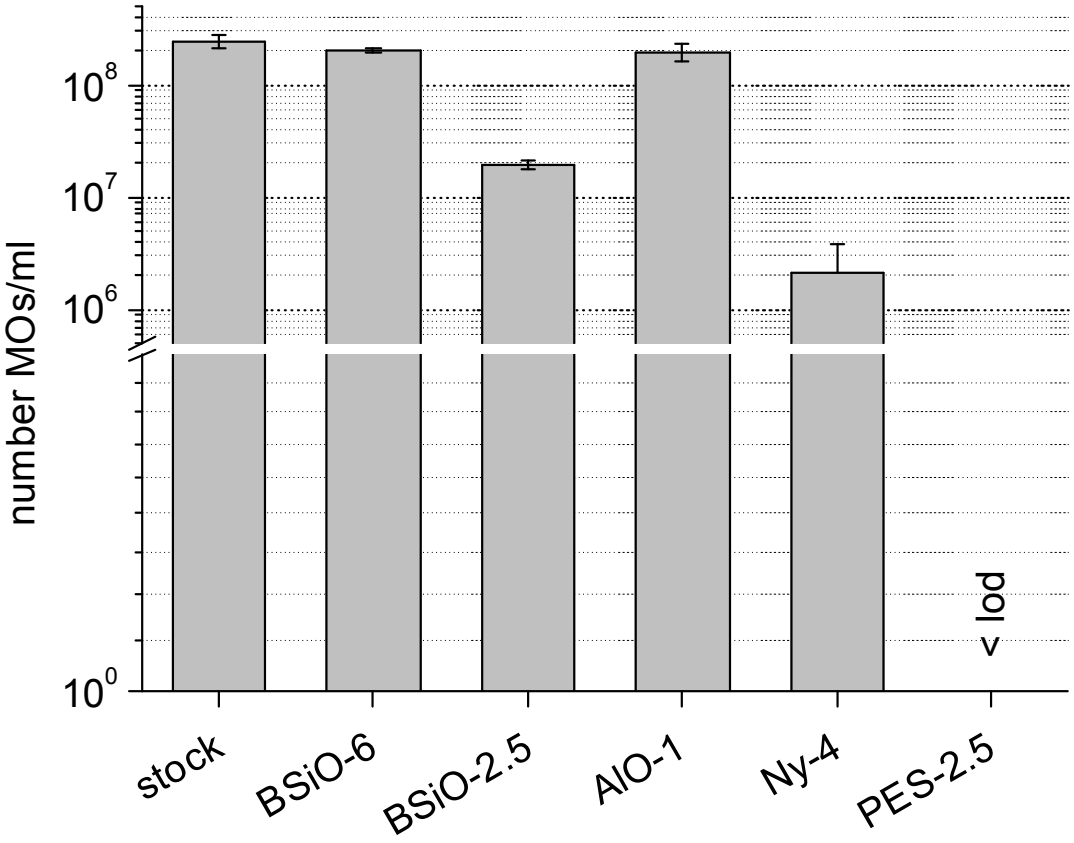
Fig. 1 Change of the cell densities after suction of a bacterial solution of *Pseudomonas fluorescens* through different micro suction cups. Colony forming units (CFU) were counted after plating diluted samples on nutrient agar plates followed by incubation. For abbreviations of the micro suction cups see table 1. Error bars indicate the standard deviation of the mean (n = 2).

Fig. 2 Histogram of cell diameters of *Pseudomonas fluorescens* bacteria. The sample was taken from a stock solution at the end of the logarithmic phase.

Fig. 3 Change of the concentration of oxalic acid (-□-), tartaric acid (-●-) and citric acid (-▲-) in sample fractions with increasing elution volume after suction of a 10 µM standard solution through different micro suction cups. For abbreviations of the micro suction cups see table 1. Error bars indicate the standard deviation of the mean (n = 3).

Fig. 4 Change of the concentration of 7 organic acids in sample fractions with increasing elution volume after suction of a 10 µM standard solution through PES-1. Error bars indicate the standard deviation of the mean (n = 3).

Fig. 5 Concentration of main root exudates (5 OAs [µmol/l]) in the soil solution in three different depths of the rhizosphere of *A. anomala* (distance to root <5mm) during a flooding period of 1, 3 and 7 days in pools with 2m water depth. Sampling of soil solution was done by micro suction cups (MSC) in rhizotrons with original sediment. Control plants in rhizotrons with original sediment were kept close to the pools under natural conditions at the original location and were also sampled by MSC. Error bars indicate the standard deviation of the mean.


number MOs/ml
10^8
10^7
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BSiO-6
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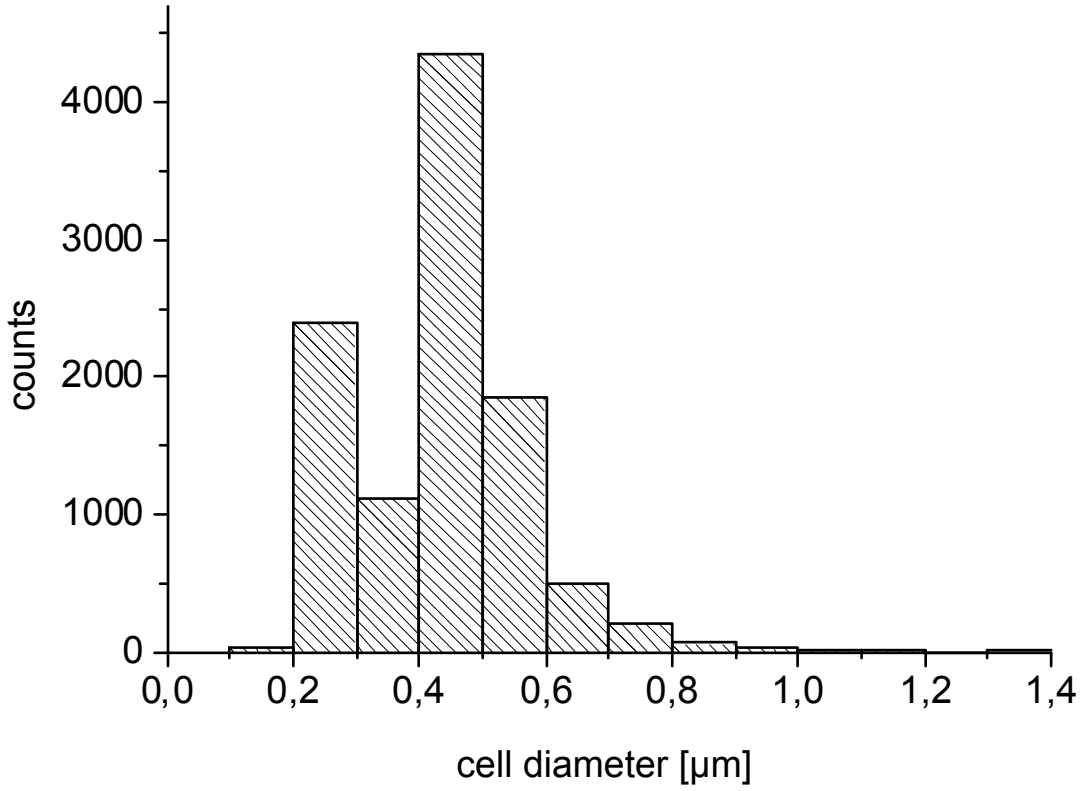
AlO-1
Ny-4
PES-2.5

Fig. 1


4000
3000
2000
1000
0
counts
0,0
0,2
0,4
0,6
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1,0
1,2
1,4
cell diameter [µm]

Fig. 2

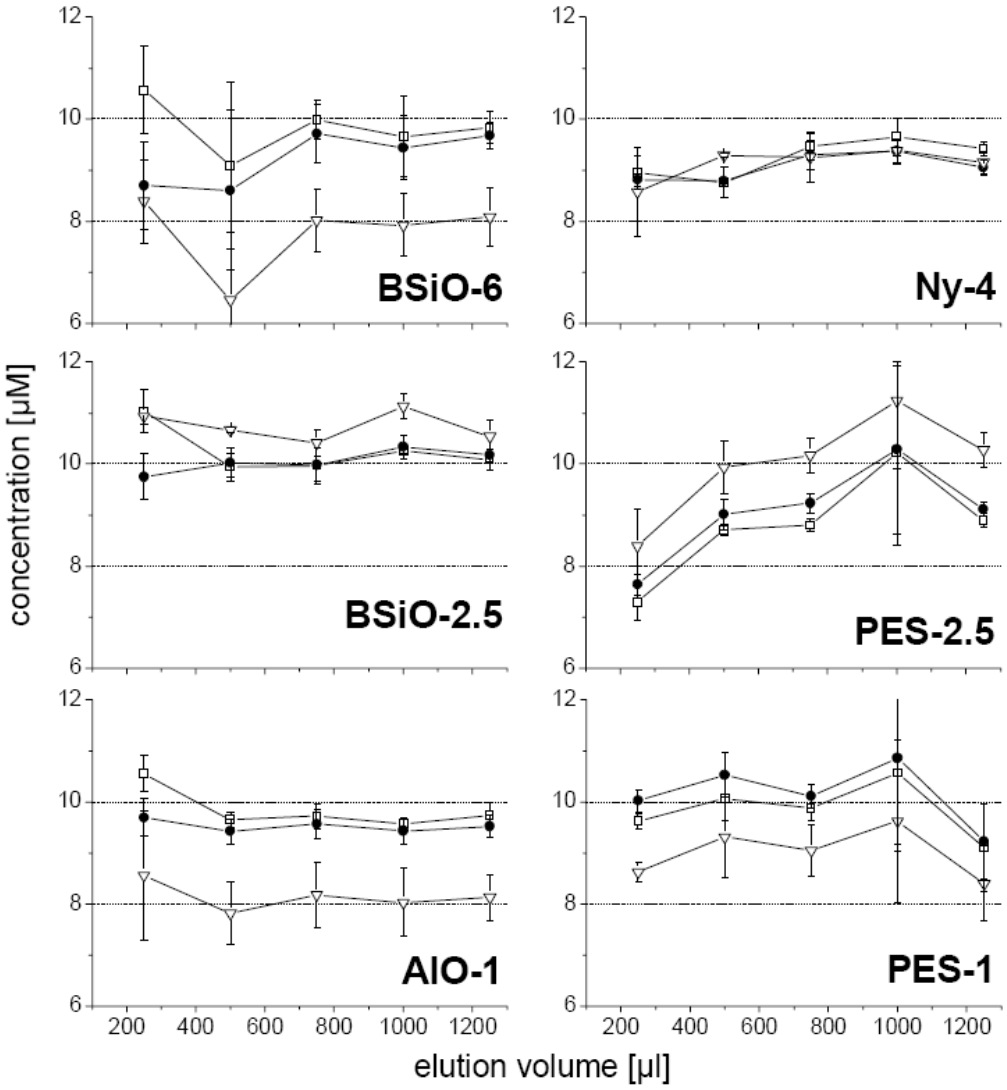
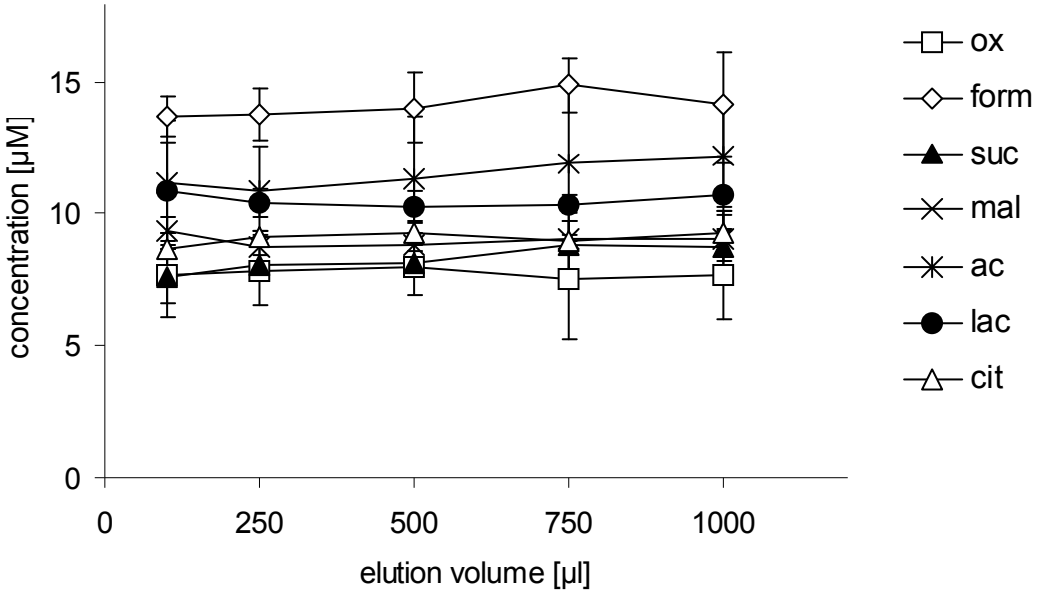

BSiO-6
Ny-4
BSiO-2.5
PES-2.5
AlO-1
PES-1
concentration [µM]
elution volume [µl]
12
10
8
6
200
400
600
800
1000
1200

Fig. 3


concentration [µM]
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10
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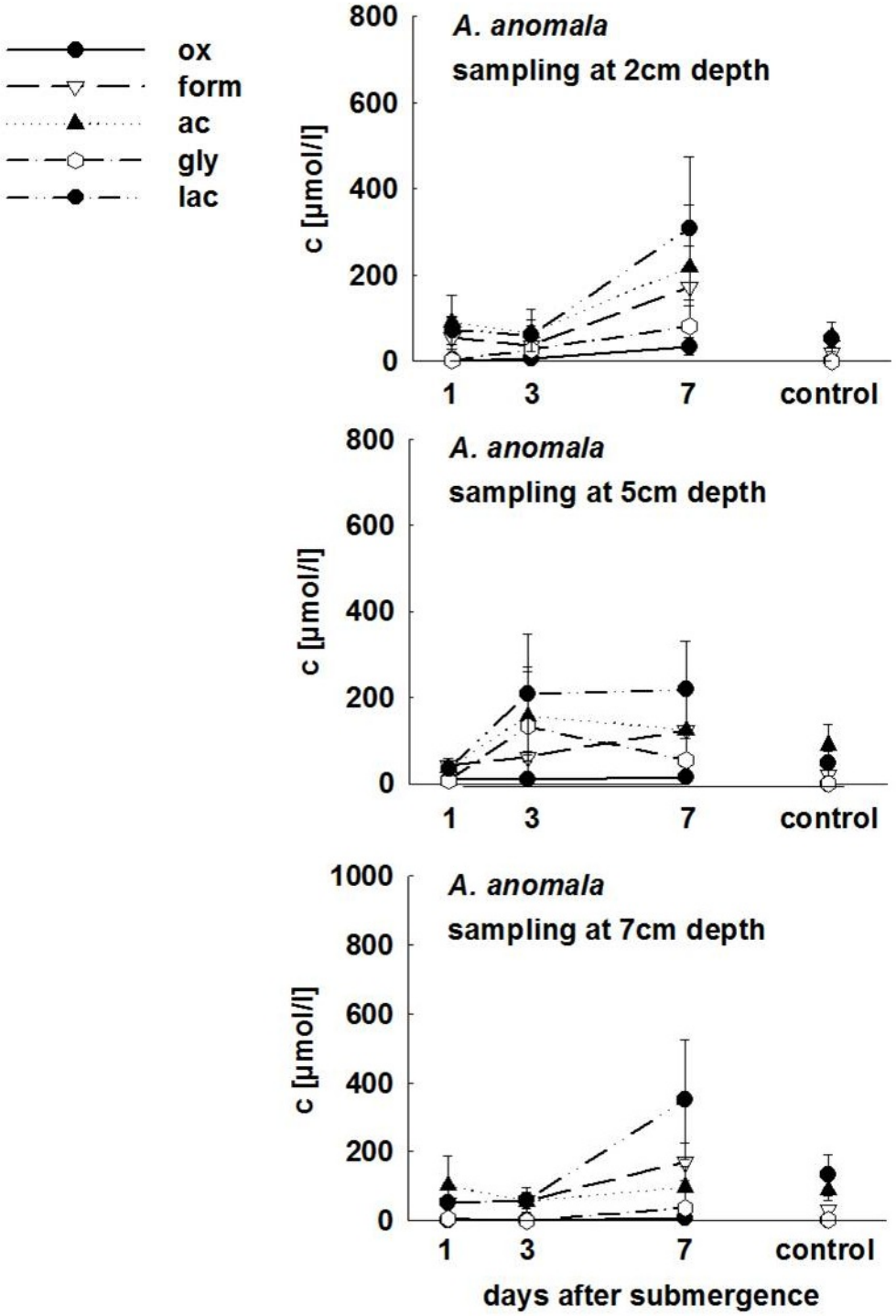
Fig. 4


ox
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ac
gly
lac
A. anomala
sampling at 2cm depth
A. anomala
sampling at 5cm depth
A. anomala
sampling at 7cm depth
c [µmol/l]
800
600
400
200
0
1000
1
3
7
control
days after submergence

Fig. 5

8.3 Manuscript 2

Dynamics of organic acid occurrence under flooding stress in the rhizosphere of three plant species from the water fluctuation zone of the Three Gorges Reservoir, P.R. China

Christina M. Schreiber[1,2*], Bo Zeng[1], Vicky M. Temperton[2], Uwe Rascher[2], Marian Kazda[3], Ulrich Schurr[2], Agnes Höltkemeier[4], Arnd J. Kuhn[2]

[1]Key Laboratory of Eco-Environments in Three Gorges Reservoir Region (Ministry of Education), School of Life Sciences, Southwest China University, Chongqing 400715, P.R. China

[2] Research Centre Juelich, IBG-2: Plant Sciences, D-52425 Juelich, Germany

[3]Institute of Systematic Botany and Ecology, University Ulm, D-89069 Ulm, Germany

[4] Research Centre Juelich, IBG-3: Agrosphere, D-52425 Juelich, Germany

*Corresponding author:
Christina M. Schreiber
Research Centre Juelich, IBG-2: Plant Sciences, D-52425 Juelich, Germany
c.schreiber@fz-juelich.de
Tel.: +49 (0) 2461 61 5493
Fax: +49 (0) 2461 61 2492

1. Keywords: root exudation, soil solution, anoxia, submergence, micro suction cups

2. Abstract

The effects of flooding on rhizospheric organic acid concentrations of three abundant flooding tolerant plant species (*Alternanthera philoxeroides* Mart., *Arundinella anomala* Steud., *Salix variegata* Franch.) from the water fluctuation zone of the Three Gorges Reservoir (TGR, Yangtze River) were investigated. Soil solution samples of 8 low molecular weight organic acids were obtained from rhizotrons using micro suction cups during 3 weeks of waterlogging, after 6 weeks flooding and after a 1 week recovery. To estimate the contribution of water temperature and microbial community, plants in sterile glass bead substrate and original Yangtze sediment were submerged in laboratory at +10°, +20° and +30°C.

Waterlogged plants did seldom express a significantly different pattern of rhizospheric organic acid (OA) composition compared to control plants. Flooding caused no burst of organic acid concentration in soil solution: All species express a silencing strategy. Average OA levels were higher in *A. anomala* rhizosphere than in the other two species, but increased again after resurfacing in all species. Temperature had a stronger influence in sediment than in sterile setup. In contrast to field measurements, succinate, malate and citrate were detected in the sterile setup. Microbial contribution appeared to have great influence on increasing OA occurrence.

3. List of abbreviations

a	*A. philoxeroides*
Ac	Acetate
ADH	Alcoholdehydrogenase
ANOVA	Analysis of Variance
ar	*A. anomala*
CE	capillary electrophoresis
Cit	Citrate
Form	Formate
GLM	General Linear Model
Gl	Glyoxylate
Lac	Lactate
LDH	Lactatedehydrogenase
Mal	Malate
MSC	micro suction cups
OA	organic acids
Ox	Oxalate
PAR	Photosynthetically Active Radiation
PEEK	Polyetheretherketone
s	*S. variegata*
SOD	Superoxidedismutase
Suc	Succinate
TGD	Three Gorges Dam
TGR	Three Gorges Reservoir
Tris	Tris(hydroxymethyl-)aminomethan
WFZ	Water Fluctuation Zone

4. Introduction

The construction of the Three Gorges Dam has caused large changes to the ecosystems along the Yangtze River banks in the People's Republic of China (Fearnside 1988; Park et al. 2003; Wu et al. 2004). The created reservoir is about 600km in length and 1500km^2 in area. For power generation and high water protection, the natural flood pulse is changed from summer to winter and raised to a maximum of 175m with a water fluctuation level of 30m. This results in drastic changes to the existing ecosystems, with higher banks now being flooded regularly and lower banks being flooded for a time period of up to 6 months. The temperature regimes of the river have changed, the timing of reproduction for plants now being summer instead of winter. But keeping vegetation cover on the banks is important for erosion protection (Allen 1979; Schiechtl and Stern 1996; Liu et al. 2004; Wang et al. 2005). In the higher levelled river banks, plants will be able to survive flooding periods and contribute to erosion protection (Wang et al. 2001; Gyssel et al. 2005). In the lower areas, plants, mostly annuals, will immigrate from the upper zones between flooding periods. Choosing flooding resistant plants for re-cultivation may lead to a downshift of the dividing line between these two areas of the fluctuation zone and provide more erosion protection (Gray 1998) and green-keeping.

Plants which are already known for their resistance to flooding (Luo et al. 2008) are now being considered for large scale planting, with emphasis on the different strategies to stay productive under new flooding conditions. This research aims not only to further understand possible survival strategies, but also to identify general functional attributes which are linked to flooding resistance and which can be used to screen for well-suited species for re-vegetating projects on flooded riverbanks.

Plants have developed different strategies to cope with submergence inside their own organism, like down-regulation of turnovers, and forming of aerenchyma, a tissue with large air spaces which allow better gas diffusion in plants (Justin and Armstrong 1987; Jackson and Armstrong 2008). But for plant survival during flooding, the plant roots form key plant tissues to investigate (Gyssel et al. 2005), not least because their growth and perseverance is the main factor for soil fixation and stabilization (Angers and Caron 1998). Water and nutrient uptake, oxygen release, exudation of pH-relevant substances (H^+, O_2, an-/organic ions, organic acids (OA)) and carbohydrates, amino acids, peptides, proteins and lipids influence pH and redox potential and provide energy supply for soil microorganisms (Jones 1998, 2004; Ryan and Delhaize 2001; Hinsinger et al. 2003; Farrar et al. 2003; Walker 2003; Blossfeld and Gansert

2007, Blossfeld et al. 2010). OA exudation is of special interest. It serves as nutritional input for the soil microbiota and availability (desorption, solubilisation, chelation) of metals (Harter and Naidu 1995; Hinsinger 2001) and may provide indication about plant root state, since strongest stimuli for increased OA exudation are nutrient deficiency, toxic cations and anoxia (Smucker and Erickson 1987; Neumann and Römheld 2000) and exudation is positively related to root growth and health (Prikryl and Vancura 1980). Monitoring OA exudation during stressful conditions and comparison to the normal state may provide information about plant performance, since it is strongly influenced by these factors. Additionally, OA exudation also means carbon loss, which can become crucial depending on plant environmental state, and it therefore represents a very interesting feature to observe under the given challenge of plants being flooded.

Under aerobic conditions, plant roots and mycorrhizal fungi are the main source for organic acids in soil solution (Jones 1998). Submergence leads to slow diffusion rates of CO_2 and O_2 (10^4 times lower than in air, Tiedje et al. 1984) and therefore even in waterlogged soil, anoxic conditions will build over time. Lactate/ethanol fermentations, methanogenesis, acetogenesis as anaerobic respiration pathways then dominate in soil microbiota (Yao et al. 1999) and also in plant root tissue, with ethanol, lactate and alanine as main end products, which can even reach a phytotoxic level (Drew 1997; Neumann and Römheld 2000; Dat et al. 2004). Anaerobic bacteria and archaeae now become the main source of OA (Dannenberg and Conrad 1999). Depending on the present electron acceptors, which are, in turn, dependent on redox potential and pH, several pathways of organic matter decomposition are possible with different in- and outgoing components. Depending on the prevalent conditions, for example, Fe(II) is oxidized to Fe (III) aerobically by bacteria like *Acidithiobacillus ferroxidans*, and is, in turn, reduced under anoxia by bacteria like *Geobacter* spp. and others who proceed OA to CO_2 and CH_4 (Weiss et al. 2003; Küsel et al. 2003). Anoxia and acidification are associated with the reduced forms of nutrients and therefore strongly lowered availability for microbial reduction or plant uptake (Neumann and Römheld 2000; Scheffer and Schachtschabel 2002). This situation becomes more complicated since the rhizosphere has a high spatial heterogeneity due to changes in moisture, particle size and adsorption potential. Composition of microbial communities changes along a root (Marschner et al. 2004) and root exudation does also depend on age of plant and root itself, soil moisture, temperature and active root growing zones (Strobel 2001; Bertin et al. 2003; Bais et al. 2006). If now different plants produce different conditions in their rhizosphere based on their exudation patterns, the

question arises as to how this may be beneficial for plant survival during flooding and/or for a faster return to productivity after flooding and therefore better growth, reproduction and competitiveness.

To measure the influences of plant and microbial OA exudation at the root-soil interface, soil solution has to be sampled from the rhizosphere and bulk soil. The minimally invasive micro-suction-cup technique (Göttlein et al. 1996; Vetterlein and Jahn 2004; Dessureault-Rompré et al. 2006; Shen and Hoffland 2007) allows sterile sampling also under field conditions which were used on-site in China. This method of soil solution sampling provides qualitative snapshots of the current rhizosphere situation at defined time points and with high spatial resolution, which is needed to provide better interpretation of results (Jones 1998). The use in rhizotrons in fixed patterns to the roots allows the tracking of exudates over relatively long time periods with little impact, which makes it possible to simulate flooding events with rhizotrons. In field experiments it is often not possible to quantitatively distinguish between plant exudation and microorganism consumption of root exudates since both are closely linked (Jones 1998) and spatial and temporal variations in plant activity have a direct influence on microbial activity. On the other hand, laboratory culture without microorganisms (MO) will not show real exudation patterns due to lack of MO feedback (Grayston et al. 1997; Jones 2003), especially when conducted in hydro-culture due to differences in root development (Schulz and Vetterlein 2007). But reactions in MO-free environment can be compared and quantified with complete root system- and productivity analysis in sterile substrates (glass beads or similar to allow close to normal root growth). Despite sampling time in the rhizotrone setups being always the same, due to weather influence or other, exudation peaks occur which are easily missed without continuous measuring (Dinkelaker 1993; Jones 1998, 2000; Strobel 2001). Also estimating exudation rates instead of total production is still difficult because it does not occur over the whole root surface and is therefore underestimated when related to the whole root system (Ryan and Delhaize 2001). Comparison between complete root system analysis and sampling with higher spatial resolution is therefore needed. The subsequent use of capillary electrophoresis (CE) allows direct analysis of the obtained samples and therefore provides an effective tool for soil solution analysis (Dabek-Zlotorzynska and Keppel-Jones 2000; Göttlein 2005).
The three chosen plant species (*Alternanthera philoxeroides* MART., *Arundinella anomala* STEUD., *Salix variegata* FRANCH.) are common in the Three Gorges Reservoir area. Their

ability to cope with flooding periods longer than 3 months (Luo et al. 2008) makes them suitable objects for the given task. *S. variegata* and *A. anomala* are native to the region, while *A. philoxeroides* is a neophyte originating from South America, but prospering well in the region. All provide different qualifications in habit and growth. *S. variegata* is a small tree species growing up to 2m with strong but slowly growing roots. *A. anomala* is a perennial grass with a very fast-growing, strong root system which is highly suited for keeping soil fixed (Tenten 2010, in preparation). *A. philoxeroides* is a very versatile plant, adjusted to higher temperatures but also able to survive lower temperature regimes, extremely fast growing with very stable root stocks but delicate adventive roots. The plant can re-grow vegetatively from pieces of one node and two leaves, therefore redistribute at high speed, and also build swim carpets for longer time periods. All three species do have low nutrient demands and populate the sandy riverbanks of Southwest China, after each flooding period re-colonizing the sediments the rivers leave behind. All of them are able to form aerenchyma in roots and shoots (Chen et al. 2007), *A. philoxeroides* with completely hollow stems providing most.

This study aims to provide an insight in OA dynamics, heterogeneity and temperature dependence in the rhizosphere of the three plant species influenced by flooding events. Occurrence patterns of OA in the rhizosphere are discussed, which varied during the flooding regime and also between distinct species and used soil substrates.

5. Material and Methods

5.1. Flooding experiment (field conditions)

Setup of rhizotrons

To allow controlled access to the rhizosphere, rhizotrons were built from plant pots to allow close to natural growth conditions, since rhizotron plantings in low-volume boxes may influence root growth and architecture. The pots had a total volume of 1.2dm³ (12 cm soil depth, ~7cm pot radius) and closed on one side with a 35° tilted plexiglass plate to allow roots to grow along visible to the observer and to be sampled via micro-suction cups (MSC). 15 holes, which would host the MSC (Fig. 1) during sampling, were drilled in square arrangements with a distance of 2.8 cm in-between and sealed between measurements with cling wrap, pressed to the plexiglass surface with light-proof black rubber foil.

Access and sampling method with micro-suction cups, experimental design for flooding periods (Rhizotron) controls, time schedule

MSC consist of a fibre which houses a PEEK-tube with hole (diameter <2µm to prevent MO entering), sealed with acrylate glue (MiniRhizon, Rhizosphere Research Products, Wageningen, Netherlands). Once connected to a vacuum chamber (with pump, -400mbar) and inserted into the rhizotron plexiglass plate's holes, they collect soil solution to Eppendorf tubes in the vacuum chamber. It has to be taken care of in soils which are not water-saturated that sampling volumes can differ due to soil moisture. Plants in experiment were kept well watered to minimize local concentration irritations due to water loss. Micro-suction cups were inserted for each measurement, fixated with tape, and the holes were sealed again after sampling with cling wrap. Water loss through the holes was negligible due to the extremely high water holding capacity in unmoved sedimental soil (quicksand like). Sampling time was 5-10 minutes to collect 70-100µl of soil solution (5 drops) from each of 15 sampling spots of one rhizotron, afterwards samples were directly frozen. Before each sampling, the MSC were dried by air-pressure to avoid dead volume.

Rhizotron sampling in natural substrate (Yangtze sediment)

Plants were collected from the riverside 3 weeks prior to the start of experiments; the roots were washed carefully and replanted into sedimental soil (collected at Yangtze riverside in Chongqing, N29°28’31.1”, E106°31’29.7”, 213m NN). For the pilot determination, a set of 9x2

plants in rhizotrons (Fig. 1), two of each species for each time period, was submerged and sampled after 1, 3 and 7 days complete flooding. Since sampling under water was impossible due to too strong disturbance of soil (oxygen entry) while moving pots, these sets were one-time-used. Sampling for the main flooding experiment started with beginning of waterlogging and continued after the 42 day flooding period (day 0,1,3 and 7). Plant sets contained 3 of each species for flooding, 2 as control sets. Low number of replicates is due to the extremely large amount of obtained samples. These were submerged for 42 days in 2m deep pools in the experimental garden of SWU, The pools were flooded by slow water inflow from the bottom. For sampling, plant sets were very slowly and carefully lifted to the surface with a special pot-fishing-rod to avoid soil disturbance. The 6 control plant sets were sampled parallel to treatments. They were placed beside the pools exposed to the natural climatic conditions. A plant-free pot (only Yangtze sediment) was sampled accompanying each treatment, yet OA concentrations were mostly below detection limit here (most likely due to the low nutrient and organic carbon content, compare table 1-3) and could thus not be compared. It was taken care of that the collected plants of each species were about the same age and size. To avoid high noise in results due to sampling at fixed spots without the possibility to choose the sampling position in relation to the roots freely, the roots were photographed during each sampling and the samples were sorted into categories aligned to distance to root. For the following treatments three classes were established, with d (distance to root) <2mm, 2mm<d<5mm and d>5mm. Oxygen in the substrate close to roots was monitored in a parallel experiment with the same plant species by N. Tenten (2010, in preparation) using permanently installed optodes (PRESENS, Regensburg, Germany). The microclimatic conditions (water- and air temperature and max. PAR/day, Fig. 2) were monitored to keep track of possible climatic influences on plant performance. Table 4 gives an overview of the treatments.

5.2. Temperature dependence of OA dynamics in a substrate independent setup and original Yangtze sediment setup (laboratory conditions)

Plant individuals for the substrate-independent setup were grown for 3 weeks in sand with nutrition irrigation (10% Hoagland solution). Then they were sterilized in 3% H_2O_2 and rinsed with deionized water and replanted into pots with 250g glass substrate to accustom for one week under irrigation with 10% Hoagland. The tops of the pots were sealed with parafilm and

black foil to prevent later algae infection from air. From all of the three species 12 pots with one individual each were put in each temperature treatment (1m submergence, temperature controlled at 10, 20 and 30°C, provided through water cooling circulation (cryostat, JULABO, Seelbach, Germany) and fish tank heating sticks (DEHNER, Rain, Germany). Temperature of non-submerged control sets was given by the air-conditioned room at 20°C). Provided Light (PAR) was ~550 µmol/m²s at water surface. To avoid later algae infection through the whole experimental time, water was filtered (fish-tank filter Aqua Top) and led through UV-light (5l/min). Deionized water was used and fertilization took place every week through injection of 10ml 10% Hoagland directly into the pots to avoid algae growth in surrounding water. Water was aerated with normal air through bubble stones outside the pots, pure CO_2 was added through a diffusor (CO_2-Diffusor, DEHNER, Rain, Germany) every second to third day to enable photosynthesis and control pH at 7.6 (common value of Yangtze River water in 2007, measured every season, total 4 times. Compare also Chen et al. 2002).

Plant individuals for the Yangtze sediment setup were collected from the riverbanks, washed and re-planted carefully into parafilm-sealed sediment-filled pots. They were not fertilized but otherwise treated like described above for the substrate-independent setup.

Three plants of each species and temperature set were harvested after 1, 3 and 5 weeks submergence and 1 week recovery outside the tanks. Harvest ensued through carefully transferring the content of the pot of 1 individual completely into filter units (NALGENE, Thermo Fisher Scientific, Denmark) nylon membrane, 2µm pore size) and rinsing with deionized water (volume documented to take dilution into account). The substrate was filtered under vacuum to gain the diluted soil solution. Solution was then concentrated by freeze drying and analyzed via capillary electrophoresis for organic acids. Substrate was weighed wet, vacuumed and oven-dried for 24 hours at 80°C to estimate remaining solution. Plant individuals were parted in roots and shoots (roots were analyzed with WinRhizo® root scanning.) and after drying (48hours/80°C) weighed to estimate achieved biomass and relate to measured acid concentrations. Please refer to Table 4 for an overview of all experimetal setups.

5.3. CE analysis of soil solution for organic acids

Capillary electrophoresis is an effective tool for the analysis of organic acids in small sample volumes. It was used for the determination of organic acids with a salicylate electrolyte

(Bazzanella et al. 1997). The system G1600A (Agilent, Böblingen, Germany) was used, including a diode array detector and using fused silica capillaries (Polymicro, Phoenix, USA, 75µm inner diameter, 64.5 cm total length, 56 cm effective length). The electrolyte contained 7.5mM salicylic acid, 15mM TRIS, 0.5mM dodecyltrimethylammonium hydroxide and 0.3mM $Ca(OH)_2$ and was freshly prepared every day. Separation ensued at 30kV and 25°C. Quantification was performed by using external aqueous standard solutions of the acids (5, 10, 20µmol) and internal standardization by phenylacetic acid in samples. Precision was best in a concentration range from 0-40µmol/l with linear calibration curves. Samples were diluted accordingly and measured 2 times after flushing the capillary with 0.1 NaOH for 5 min, water for 1 min and electrolyte for 5 min. During analysis, up to 8 low molecular organic acids were identified from the soil solution samples (oxalate, formate, succinate, malate, acetate, glyoxylate, lactate and citrate).

5.4. Substrate characteristics

For the substrate-independent (MO-free) experiment glass beads were used as substrate (diameter 425-850µm), watered with 10% Hoagland solution. The plants in their natural habitat are faced with a lot of sediment input from Yangtze River, which is the worlds most sediment carrying river (Higgit and Lu 2001; Yang et al. 2006). The original substrate is a very fine sediment (87% fine sand <20µm diameter, Tab. 1) with almost quicksand properties (releases water slowly, but fast when experiencing movement), the nutrient content is very low (compare cations, Tab. 2, and carbon content, Tab. 3. Pool water pH was 8.2 ±0.3, soil pH 7.9±0.4. All of the considered plant species are capable of growing productively and reproducing in this substrate.

5.5. Microclimate logging

Measurement was taken by a microclimate station located directly at the pools, where the submerging of the waterlogging-, short- and long-time flooding experiment took place. PAR (Sykes quantum sensor), air temperature, water temperature and rainfall were logged (Minicube Mini 32, Jiří Kučera - Environmental Measuring Systems, Brno, Czech Republic).

5.6. Data analysis

We tested mean values for organic acid concentrations using general linear model (GLM, Type 4 sum of squares) Repeated Measurements ANOVA for time series data with statistical significance defined at $P < 0.05$ unless otherwise stated. Here we tested for overall effects of different experimental treatments (Between Subjects Effects, i.e. irrespective of time: species (*A. anomala, A. philoxeroides, S. variegata*) and distance of sampling spot to roots (<2mm, 2-5mm and >5mm) in the flooding experiment, species and temperature (level: 10, 20 and 30°C) in the substrate independent and Yangtze sediment setup), as well as effects of time on the effects of different experiment factors on the organic acid concentration. Interaction of factors time*species and time*distance was tested for the flooding experiment, time*species and time*temperature for the substrate independent setup. Time intervals between measures were not identical. The Greenhouse-Geisser correction was used to adjust significance values if assumption of sphericity was not met. Games-Howell post hoc test was used since not all sets showed homogeneity of variance after Levene's test ($P < .05$).

Statistical analysis was done using PASW Statistics 18, graphical presentation was done using Sigmaplot 10.0 (PASW: SPSS Inc., Chicago, USA. Sigmaplot: Systat Software Inc., London, UK).

6. Results

6.1. Flooding experiment

Microclimatic influence

Since all measurements were conducted under field conditions (pools and control sets exposed to free air and weather), the microclimatic changes have to be taken into account when dealing with the results of the soil solution analysis. Fig. 2 shows the average day temperature of air and water and the daily maximum PAR during the experiment.

Noticeably between 21st and 28th of May, PAR and temperature declined strongly, which was likely to influence performance of the resurfaced plant sets after their temperature-buffered submergence period. Over the whole experiment period existed a tendency to warm up (averaging about +5°C). During the measurement periods there was no detectable rainfall (not shown) which could have lead to additional hypoxic effects in the dense soil of control plant sets.

Pilot determination of organic acid concentrations in the rhizosphere after 1, 3 and 7 days of complete submergence

To characterize spatial distribution of OA levels in the time after a flooding event, a first experiment included spatially explicit sampling of sets of plants that were submerged for 1, 3 and 7 days. Soil solution was extracted at different distance to the roots and analyzed separately. The general state of rhizospheric OA occurrence was documented in long-term periods (up to 6 weeks). OA in the soil compartment of the different plant sets with their high soil water content could be assumed to be well mixed: An example of concentration differences related to distance from root for *A. philoxeroides* is shown in Fig. 3. A similar situation was found in the other species (not shown), where no significant differences between the distance classes occurred (compare also time*distance effects, Table 5). Because OA-values did not significantly differ between distances classes, it was not further discriminated between different distances in the following.

All plant sets were sampled directly after resurfacing, following submergence for 1, 3 or 7 days. Figure 4 shows concentrations of 8 OA in the rhizosphere of the three species. The overall amount of detected acids was slightly increasing in the rhizosphere of

A. philoxeroides (Fig. 4a) over time, with the strongest increase in lactate (LAC) (from <50 to more than 400µmol/l) and acetate (AC) (from <50 to more than >180µmol/l). In the rhizosphere of *S. variegata* (Fig. 4b), the amount of glyoxylate (GL) (>700µmol/l), lactate (LAC) (>600µmol/l) and formate (FORM) (>250µmol/l) increased significantly after 7 days of submergence from values below 100µmol/l after 3 days. *A. anomala* sets (Fig. 4c) showed a similar trend, however total amounts were lower. Changes over time were significant for FORM, AC, GL and LAC (Table 5, flooding: pilot determination). There was no immediate reaction in the rhizosphere of all three species, but a slow response. There were only minor findings of oxalate (OX), succinate (SUC), malate (MAL) and citrate (CIT).

Flooding experiment: Organic acid dynamics in the rhizosphere during waterlogging, followed by submergence and recovery

In the following experiments we simulated natural flooding, beginning with a 23 day period of root submergence (waterlogging) with eventually increasing hypoxia. This was followed by a 42 day period of complete submergence (2m water table) and 7 day recovery outside of the water basin in aerating soil conditions.

Flooding Experiment: Waterlogging

In this first period, a 23-day waterlogging period was surveyed. Soil solution samples were taken before start of waterlogging, and then on day 6 and 23 during waterlogging. Control plants outside the pools under natural growing conditions were sampled simultaneously.

The soil solution from *A. philoxeroides* showed concentrations of 80-100 µmol/l (LAC, FORM and GL) in the beginning of treatment in both control and treatment plant (Fig. 5a,b). This changed after 7 days, when the values rose up to 10 times as high (please note the Y-axis scaling) in the waterlogged plants, compared to the control plants. At the end of the waterlogging period, the pattern of the waterlogged plants reached a state close to that of the control plants again. *S. variegata* showed a very high value of FORM before start of the treatment, while other OA were in the range of the control set. Also in the following, the waterlogged sets showed a similar pattern like the control sets (Fig. 5c,d) with FORM peaks up to 1800 µmol/l and other OA in the range of 100-200µmol/l. Overall the level of OA appearance apart from FORM was in the same range like the level found in sets of *A. philoxeroides*.

In the whole treatment, a clear effect over time on the measured results appeared for OX, FORM, SUC, MAL, AC and CIT. Also between the species significant differences appeared (time*species, Table 5, flooding: waterlogging). Distance did not account for a similar strong effect on the setup.

The sets of *A. anomala* (Fig. 5e,f) showed a slightly but not significantly increasing concentration of OA concentration for all acids, except FORM, which rose up to more than 1000µmol/l in both treatment and control sets. Also here, the waterlogged sets and the control sets showed similar exudation patterns (except GL), while the overall amounts of measured OA were in the same range than in the sets of the other two species.

Flooding experiment: Recovery after full submergence

After the waterlogging period, the plant sets were completely submerged for 42 days. The submerged sets were brought to the surface carefully afterwards to avoid soil movement and were then immediately sampled. Due to the water holding capacity of the soil, almost no water emerged through the sampling holes. At that point, *A. philoxeroides* and *S. variegata* had lost their original leaves. *A. philoxeroides* lost them after about 14 days flooding, *S. variegata* lost them continuously through the 42 days until just few yellow leaves were left. With resurfacing, *A. philoxeroides* already showed small newly formed leaves emerging from nodes close to the soil surface. Also the fragile stem tips sported buds or already small young leaves. The stems, which elongated up to 60cm within the first week of flooding through violent aerenchyma forming, were very shallow and fragile at resurfacing, but either they strengthened quickly within 1-2 weeks, or, if damaged, died and re-grew from the highest intact node. The majority of *A. anomala*'s older leaves died, but younger leaves inside the leave formation survived and grew up to 5cm within the first two weeks of resurfacing. *Salix variegata* was found producing new roots from stem meristems during flooding and also new leaves in only 2-6 days after resurfacing. All plants survived the flooding period.

The sets of *A. philoxeroides* (Fig. 6a) showed medium values directly after and 1 day after flooding, decreasing below 300µmol/l towards day 3, then rising again to about 800µmol/l (LAC and FORM being dominant). *A. philoxeroides* differed significantly from its control and both other species in this treatment (Table 5, flooding: recovery) which was not the case during short flooding and waterlogging.

In *S. variegata* (Fig. 6b), the control sets showed the highest amounts of OA (up to 2600µmol/l LAC, 500µmol/l AC and 200µmol/l FORM). Directly after resurfacing these are the only detectable ones, but in a lower concentration (800/250/350µmol/l). These amounts then declined until the 3rd day where all concentrations were below 200µmol/l. Only very small amounts of OX, SUC, MAL and CIT were detected. Overall the levels were slightly higher than in *A. anomala* (~100µmol/l, Fig. 6c).

While the control sets of *A. anomala* showed very high concentration values of present OA (up to 2500µmol/l, Fig. 6c), the resurfacing sets concentrations were below 500 µmol/l for the first 3 days, increasing towards day 7 (>1000µmol/l) and reaching a composition close to that of the control sets.

Changes over time in all three species were more significant here in FORM, AC, GL and LAC than in the other treatments. Overall, strongest effects take place in the recovery treatment, they were weaker during pilot determination or waterlogging, especially regarding differences between species.

6.2. Temperature dependence of OA dynamics in a substrate independent setup and original Yangtze sediment setup during 35 days flooding

This experiment was designed to show if temperature and flooding duration have an influence on OA production, independent of pre-existing microbial communities. Plants were harvested in each temperature-controlled basin and from the control set after 7, 21, and 35 days of submergence and after a 7 day recovery period. The total OA amounts in the harvested and rinsed substrates were determined. The OA content, relating to the dryweight of the respective root systems [µmol/root DW[g]] at the 4 different measuring times is shown for FORM and AC in Fig. 7.

Glass bead substrate

Control plants of *A. philoxeroides* showed lowest overall OA level (<30µmol/g DW) with highest contents after 21 days experimental time for FORM. AC appears strongest after 7 days (20µmol/g DW). Small amounts of OX and SUC were found[1].

[1]data shown in appendix (Fig. 7+8)

Compared to *A. philoxeroides*, *S. variegata* control sets did express two (AC) to four times (FORM, MAL[1], LAC[1]) higher values after 35 days. In *A. anomala*, MAL[1], AC and LAC[1] were predominant OA's (up to 40 µmol/g DW) in all measurements.

In the temperature controlled submergence treatments, the weakest reactions were shown by *A. philoxeroides* sets, where the 10°C stage (Fig. 7a) showed almost the same pattern as the control plants, esp. the AC peak at day 7 and the appearance of LAC[1]. The 30°C stage showed lowest values with OX[1], FORM, SUC[1] close to detection limit, and AC and LAC[1] around 10µmol/g DW. Significanct effects of temperature over time on OA concentration (Fig. 7, Table 6) were seldom found.

Plant survival was 100% in *A. philoxeroides* (all sets), with fastest recovery (new forming of lost leaves) in 10°C and 20°C setup. Plants in 30°C setup did not loose their leaves while submerged and showed strongest stem elongation (>50cm compared to >35cm (20°C) and >20cm (10°C) and most stable stems, stems in 10°C set were becoming slimy and very easily breakable), but lost their leaves after resurfacing and then started forming new leaves from low nodes and stem tips.

S. variegata in 10°C setup showed no detectable amounts of OA in the first two weeks except small values of MAL[2], AC and LAC[2]. Overall level was low (<10µmol/g DW, except LAC[2]) and in the 30°C setup even lower (<5µmol/g DW). While OA levels decreased in control sets, they increased after resurfacing in the other treatments, strongest in 10°C and 20°C setup (Fig. 7b). 4 of 15 *Salix* individuals in the 30°C setup died, here most leaves were lost or yellowed in all individuals. In 20°C setup also leaves were lost, but this effect was gradually receding down to 10°C setup. All individuals in 10 and 20°C treatment survived.

A. anomala showed in 10°C setup after 21 days highest production of MAL[2], AC, LAC[2] (150-200µmol/g DW. MAL[2], AC and LAC[2] were consistently appearing in all temperature sets, other OA's were found sporadically. Overall level of production was twice as high as in *S. variegata*. OA levels, esp. FORM and LAC[2], rose after resurfacing (Fig. 7c) without significant differences between temperature treatments while controls decreased. 2 plants of the 30°C treatment died, all other individuals survived and recovered.

Original Yangtze sediment

The overall OA levels were tenfold higher than in the glass bead substrate. OX, SUC and CIT were not present in detectable amounts. In all *A. philoxeroides* sets (Fig. 7d) a rise of FORM,

AC and LAC[2] occurs towards day 21 of submergence, the control set expressing highest values (~600 µmol/g DW) followed by 30°C>20°C>10°C, then a decrease towards week 5. MAL occurrence is very low. After resurfacing, values increase (FORM, AC, LAC[2]), strongest in the 30°C and 10°C sets (~1000µmol/g DW), and almost not at all in control sets.

S. variegata sets show less intensive changes over time (max. ~250µmol/g DW, Fig. 7e), but also here after resurfacing values increase (FORM, AC, LAC[1]), strongest in the 10°C sets.

In *A. anomala* sets the same effect is stronger than in the other two species for the 10°C stage and weakly present for the other temperature treatments (Fig. 7f)

All plants survived the treatments and recovered. All three species in the 10°C setup kept most of their leaves and started re-growing fast. *S. variegata* in 20° and 30° lost almost all leaves. Some *A. philoxeroides* individuals in 30°C sets showed stronger stem elongation than in glass bead substrate (>70 cm after 1 week, had to be kept from reaching the water surface).

[2]data shown in appendix (Fig. 7+8)

7. Discussion

In the three study species, organic acid (OA) levels in the rhizosphere only slowly increased within the first days of submergence. OA concentrations in rhizosphere solution did not exceed 500 µmol/l and no immediate response, i.e. burst of OA, was detected after flooding (Fig. 4 and 5). During waterlogging, exudates showed great variations, which could be related to changes in air temperature and irradiance. Highest OA values appeared after the 42 days flooding period. Root exudates in microorganism free substrate generally showed no significant differences between temperature levels, but between plants. Low concentrations of malate (MAL), succinate (SUC), oxalate (OX) and citrate (CIT) could be detected in the microorganism free substrate, while in the original sedimental soil the OA concentrations were tenfold higher and formate (FORM), acetate (AC) and lactate (LAC) showed highest concentrations. Resurfacing resulted in a strong rise in OA levels in both set-ups.

In the following we focus on the direct effects of complete submergence and temperature on the rhizosphere as well as plant performance under waterlogging and the return to productivity after flooding.

Effects of complete submergence and temperature

In the new water fluctuation zone of the Three Gorges Reservoir (TGR), the most important environmental factors influencing root exudation and micro organism (MO) consumption are anoxia and temperature. Anoxic conditions build up within 1-6 days of submergence in pots of *A. anomala* and *Hermarthria compressa* (Tenten 2010, in preparation), and we assume that similar conditions will apply for *S. variegata* and *A. philoxeroides*. Hypoxic and anoxic conditions influenced root exudation of the three species, but no exudation bursts were observed, as described for example for *Medicago sativa* and *Lotus corniculatus* (Barta 1986) or *Zea mays* (Xia and Saglio 1992). The lack of an immediate response of OA concentration to submergence pointed towards a silencing strategy of the studied species (Fig. 4 and 5). The pilot determination with 1, 3 and 7 days submerged plants did not show significant differences between *A. philoxeroides* and *A. anomala*. In *S. variegata* the values were lower (50%) during the first three days, followed by an increase (Fig. 4), so that all species showed values which exceeded control plant level after 7 days. Main components in OA composition were the fermentation products AC, FORM and LAC. In all three species, this was not a sudden

process but OA levels slowly increased; no sudden exudation outburst caused by changed environmental conditions did take place. These outbursts would also have shown in increased fermentative activity due to increased nutrient entry for fermenters (Voesenek et al. 2006), but more rapidly. Lack of O_2 and other electron acceptors like ferric iron in this nutrient-poor sediment may also limit sudden strong reactions (Dannenberg and Conrad 1999; Hori *et al*. 2007). A silencing strategy was already described for other salix species (e.g. *S. alba*), which are known to be able to decrease respiration rates (Chirkova 1978).

Our experiments showed no clear temperature effects on root exudation (Fig. 7), as known from tomato, where amino acid excretion increased at 30°C (Rovira 2005), but on general rhizospheric turnovers (compare microclimate, Fig. 2, to Fig. 5, increasing light and temperature), where microorganisms also contribute (Jones 1998). Our findings from the substrate-independent setup supported fast reactions after the end of a flooding experience, like *S. variegata* rhizosphere expressing OA strongest after resurfacing, including MAL and CIT, which indicated intact aerobic root turnovers. Lowest values of all OA were found in the 30°C set-up, where exudation also did not restart after resurfacing

Waterlogging does not hamper plant performance much

There was no immediate response to waterlogging, and also no significant differences in the general patterns during the waterlogging period between treatment and control. FORM, GL, LAC, AC and other OA generally increased in all species, control and treatments, towards day 7 of waterlogging and then decreased., which hinted to a major influence of microclimatic factors. Compared to Fig. 2, these patterns correlated with the PAR, which was high until March 29th and then declined to a lower level in the following 2 weeks. All three species are as riparian plants adapted to light (Loesch 2003), and thus low PAR values will result in lower photosynthetic activity and carbon fixation. If these general turnovers are lower, also root exudation will decrease in times of non-optimal light supply. Even a higher temperature level in week 3 did not lead to increased activity, so a stronger correlation to light is to be assumed.

The rhizosphere of *A. philoxeroides* and *A. anomala* showed a strong rise of FORM in the rhizosphere after 1 week of waterlogging. FORM is described as a dominant exudate under anoxia and important in the fungal nitrous emission pathway (Ryan et al. 2001; Ma et al. 2008), but also a terminal electron acceptor under methanogenesis, one of the most common

anerobic respiration pathways. FORM together with AC is the end product of fermentation of citrate, oxaloacetate and pyruvate, and is also produced anaerobically from H_2 and CO_2 by anaerobic MO (acetagens, sulphate reducers and methanogens, Horn et al. 2003). High values in our experiments imply hypoxic conditions and available precursors of fermentation during the first days of waterlogging. This is further supported by other fermentation products like LAC and AC which were also found in higher concentrations. Hypoxic conditions may have been overcome later by newly developed aerenchyma, and indeed fermentation products decreased after two weeks of waterlogging. Additionally, under more aerobic conditions, FORM can then be oxidized to CO_2 by bacteria by formate dehydrogenase (Alves et al. 2004). Thus, there is no indication that anoxic conditions during waterlogging severely affected metabolism of the three species.

Return to productivity

After the 42 day flooding period, high levels (up to 1000µmol/l) of fermentation-related OA's (mostly AC, GL, LAC) were found in the rhizosphere of all species. Other OA did not accumulate in the rhizosphere, which could be due to reduced excretion or increased microbial consumption (Fig. 6). *A. anomala* and *S. variegata* pass oxygen into their rhizosphere after resurfacing from submergence (Schreiber, unpublished data). With a new entry of oxygen into the soil and beginning aeration through soil surface and plant roots, aerobic degradation of these fermentation products may account for OA decrease over the next 3 days. The control plant sets, especially of *S. variegata* and *A. anomala,* showed significant higher values of OA in their soil solution (1000-3000µmol/l, Fig. 6), due to access to direct sunlight and resulting better growth and stronger turnovers. Seven days after re-surfacing, OA level of the treatments reached same values as control plant. This points toward normalizing rhizosphere processes during the recovery of the plants and OX, MAL and CIT were again present in detectable amounts; they are known root exudates which are utilized fast by soil microbiota (Jones 1998). This also indicated that the roots were again contributing to rhizospheric turnovers and microbial utilization after the flooding period. The findings in the substrate-independent and original sediment setup supported this with generally increasing exudation values 7 days after re-surfacing. All studied species seem to be able to return to normal productivity within a few days after a flooding period.

Field to lab: sediment and glass bead substrate

The average concentration of exudates in the substrate-independent MO-free laboratory set-up was far lower than OA in the natural soil solution. Without soil microbiota interaction, OA levels in the substrate may indeed be lower than otherwise (Grayston et al. 1997; Jones 2003). Nevertheless, OX, MAL, SUC and CIT were detectable in far higher amounts (App. Fig. 8+9) in relation to the other OA than in the field experiment (Fig. 4, 5, 7: Products of the microbial utilization of these exudates (FORM, AC, GL, LAC) were represented far stronger). They are obviously consumed too fast in field experiments to be detected with the current method. The effect of temperature (higher temperature caused higher fermentation rates) supports this as it is clearly found in the natural substrate, but not in the MO-free setup (Tab. 7).

Without MO contribution, *A. anomala* showed the strongest exudation in the control sets. FORM, AC and LAC were still dominant, which leads to the conclusion that indeed not all detected values in field measurements originated only from microbial fermentations, but also from roots. LAC dominated in the flooded sets, which is explained through fermentation processes inside root cells (Neumann and Römheld 2000) and the deposition of LAC and ethanol outside the root apex due to its toxicity.

OA levels in the natural sediment were mostly tenfold higher. This must not directly be linked to root exudation, but to MO activity and exudation together. In the beginning, fermentation products accumulated (compare also pilot determination, Fig. 4), but with continuing anoxia overall levels receded again. According to Chen and Qualls (2002), increased LDH (lactate dehydrogenase) and ADH (alcohol dehydrogenase) activity is known from roots of the wetland species *Lepidium latifolium* for the first 7 days of anoxia, while pyruvat decarboxylase and cytochrome c oxidase remain stable. Thus, higher fermentation inside root cells also leads to exudation of the products, which are then utilized by soil microbiota. Since the species in question can endure very long submergence periods, it can be assumed that after a certain time turnovers are shut down as far as possible during prolonged flooding. Less nutrition for fermenters in the nutrient-poor sediment explains receding OA levels which level out (for example anaerobic conversion of CO_2 to acetate, which is then further processed. Conrad and Klose 2006). Resurfacing, and strengthening metabolism, re-starts fermentation and respiration in the still anoxic root surrounding, thus the observed rise in fermentation products. A plant contribution to fermenter nutrition explains the higher level compared to the MO-free environment.

Survival: reaction and strategies

During longer flooding intervals, the slow rise of fermentation-related soil solution OA content in the rhizosphere of all three species pointed towards degradation processes and not active plant reaction. Since OA exudation and other exudations (carbohydrates, amino acids) are linked in naturally growing roots (Prikryl and Vancura 1980), the silencing strategy of the plant roots helps avoiding depletion of carbohydrates during longer submergence. According to Ye (2010), *S. variegata* can survive flooding even longer than *A. anomala* due to more carbohydrate reserves (starch, sugars) in stem and roots. The plants returned to productivity very fast, which implies existence of a mechanism to tackle oxidative stress. A high capacity of SOD (Superoxidedismutase) or other oxygen radical captors mediated resistance to anoxia may be crucial prerequisite for a fast recovery, as described in Chen and Qualls (2002) for *L. latifolium*. Higher activity of SOD was found in anoxic than in aerated plant parts, only slowly decreasing during longer anoxia. The ability to maintain root structure (*S. variegata, A. anomala*) or to discard and re-form roots fast (*A. philoxeroides*) also promote fast recovery. From an applied perspective, *A. anomala* and S. *variegata* seem to be able to stabilize soil as their root structures successfully survive submergence. The delicate roots of *A. philoxeroides* in contrast do not provide strong hold. Its strategy of rapid stem elongation (no real growth, a common response in plants adaptable to flooding according to Jackson and Colmer 2005; Voesenek 2006; Mommer et al. 2006), which normally either helps to reach the surface or enables breaking of stem parts by the water current, is beneficial for its survival, but not for soil stabilization.

Conclusions and Outlook

The survival strategy of the three species during flooding is down-regulating under complete submergence. Recovery with renewed growth was enhanced in cold water, which may allow a better performance during future flooding events in the Water Fluctuation Zone of TGR region, which will take place in winter with 10-15°C lower water temperatures than during summer flooding. Since the roots of *Salix variegata* and *Arundinella anomala* stay alive during longer flooding events, they can be taken into consideration for re-cultivation and stabilization of the river banks. *Alternanthera philoxeroides* survives, but does not supply stable root mass.

8. Acknowledgements & Grants

This study was supported by German Academic Exchange Service (DAAD, grant 332 4 00 580, D/07/40075), National Natural Science Foundation of China (grant 30770406) and Program for New Century Excellent Talents in University (grant NCET-06-0773). We thank Ai QiaoLi and Ren Qiu Fang from the School of Life Sciences, Southwest China University Chongqing/Beibei, for their help with the experimental setup in China.

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10. Figures

Fig. 1 Pot rhizotron for use in the field experiments. 15 holes in the 35° tilted plexiglass plate allow soil solution sampling via microsuction cups. The transparent side is sealed with black rubber foil between samplings to avoid water loss and light entry.

Fig. 2 Microclimatic aspects: max. PAR/day, average air temperature/day (24h) and average water temperature/day (24h), logged by Minicube Mini32 directly at sampling location, time schedule of experiment (shaded areas)

Fig. 3 Mean concentration of 8 organic acids (OA [µmol/l]) in three different distances from roots (<2m, 2mm<x<5mm and >5mm) in the soil solution of the rhizosphere of *A. philoxeroides* (control set of pilot determination experiment). Sampling was done by microsuction cups. Error bars indicate the standard deviation of the mean

Fig. 4 Flooding experiment, pilot determination: Concentration of main root exudates (8 OAs [µmol/l]) in the soil solution of the rhizosphere of *A. philoxeroides, S. variegata* and *A. anomala* during a flooding period of 1, 3 and 7 days in pools with 2m water depth. Sampling of soil solution was done by microsuction cups (MSC) in rhizotrons with original sediment. Control plants in rhizotrons with original sediment were kept close to the pools under natural conditions at the original location and were also sampled by MSC. Error bars indicate the standard deviation of the mean

Fig. 5 Flooding experiment, 23 days waterlogging period (above: treatment, below: non-submerged control sets). Concentration of main root exudates (8 OAs [µmol/l]) in the soil solution of the rhizosphere of *A. philoxeroides*, *S. variegata* and *A. anomala* during waterlogging. Sampling of soil solution was done by microsuction cups (MSC) in rhizotrons with original sediment. Control plants in rhizotrons with original sediment were kept close to the pools under natural conditions at the original location and were also sampled by MSC. Error bars indicate the standard deviation of the mean

Fig. 6 Flooding experiment, 7 days recovery after 42 days submergence: Concentration of main root exudates (8 OAs [µmol/l]) in the soil solution of the rhizosphere of *A. philoxeroides, S. variegata* and *A. anomala* during recovery. Sampling of soil solution was

done by microsuction cups (MSC) in rhizotrons with original sediment. Control plants in rhizotrons with original sediment were kept close to the pools under natural conditions at the original location during the flooding treatment. After treatment, treated plants and control plants were kept under identical natural conditions for 7 days recovery, sampled by MSC on day 0, 1, 3 and 7. Error bars indicate the standard deviation of the mean

Fig. 7 Monitoring of organic acids (FORM, AC) in the rhizospheric soil solution of the 3 species at 3 different temperatures. a-c: Setup in glass beads, roots and pots disinfected with H_2O_2, d-f: Setup in original Yangtze sediment, no disinfection. Plants were submerged for 35 days and sampled after 7, 21, 35 and 42 days by rinsing and filtering the substrate. Please note the different y-axis scaling. Error bars indicate the standard deviation of the mean. Asterisks mark significant values ($P < .05$). Additional data: Fig. 7 (LAC, MAL, glass substrate (a-c) + sediment (d-f)), Fig. 8 (OX, SUC, CIT, glass substrate), available in appendix

11. Appendix

Fig. 8 Monitoring of organic acids (MAL, LAC) in the rhizospheric soil solution of the 3 species at 3 different temperatures. a-c: Setup in glass beads, roots and pots disinfected with H_2O_2, d-f: Setup in original Yangtze sediment, no disinfection. Plants were submerged for 35 days and sampled after 7, 21, 35 and 42 days by rinsing and filtering the substrate. Please note the different y-axis scaling. Error bars indicate the standard deviation of the mean. Asterisks mark significant values ($P < .05$)

Fig. 9 Monitoring of organic acids (OX, SUC, CIT) in the rhizospheric soil solution of the 3 species at 3 different temperatures. a-c: Setup in glass beads, roots and pots disinfected with H_2O_2. Plants were submerged for 35 days and sampled after 7, 21, 35 and 42 days by rinsing and filtering the substrate. Please note the different y-axis scaling. Error bars indicate the standard deviation of the mean

12. Figures

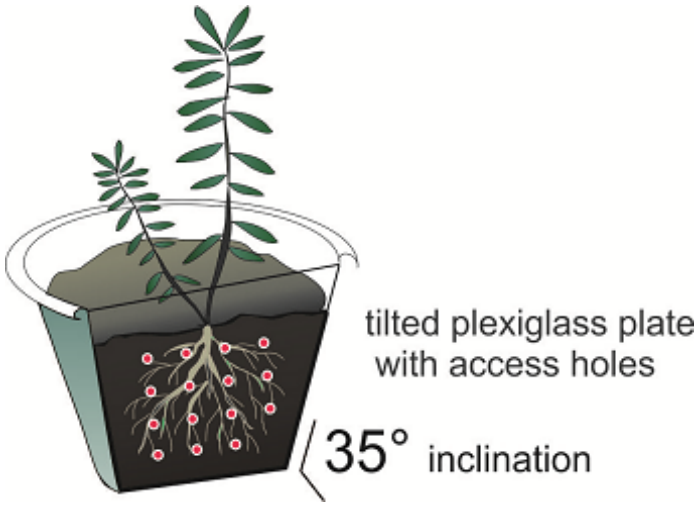


Fig. 1

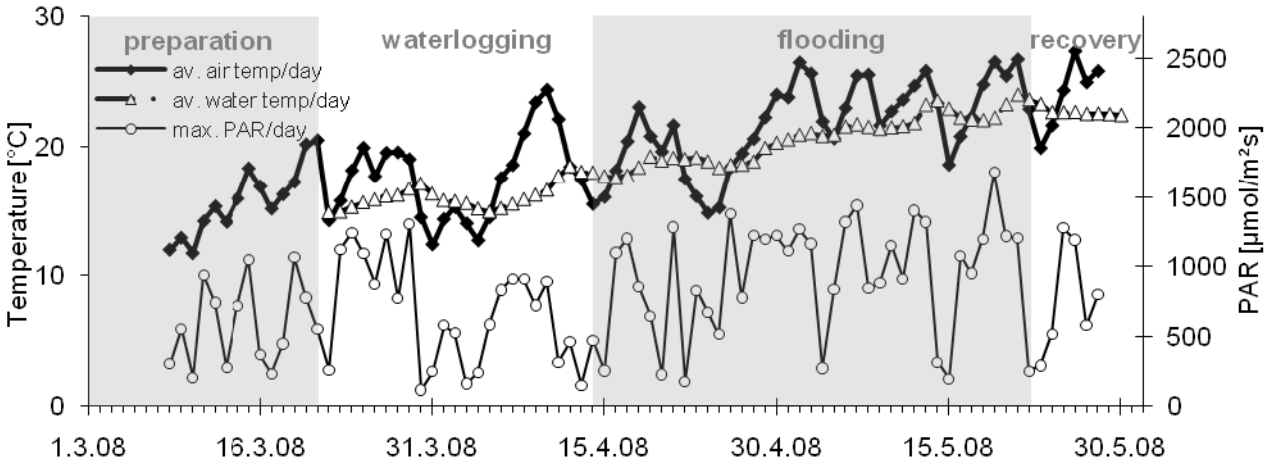


Fig. 2

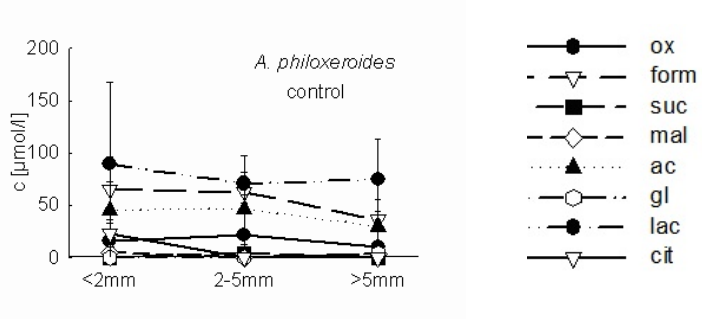

200
150
100
50
0
c [µmol/l]
A. philoxeroides
control
<2mm
2-5mm
>5mm
ox
form
suc
mal
ac
gl
lac
cit

Fig. 3

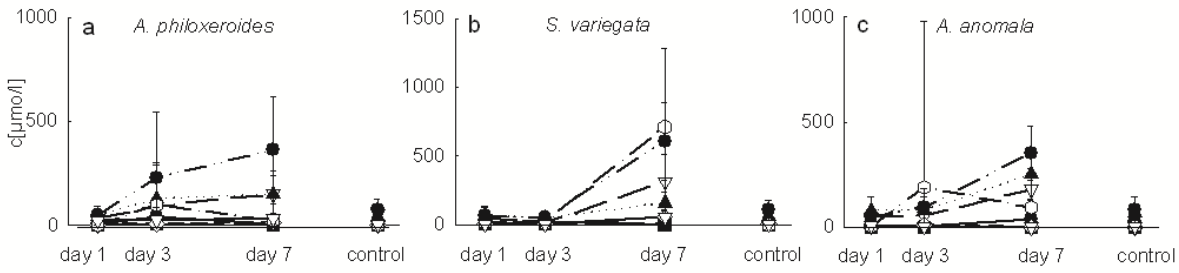
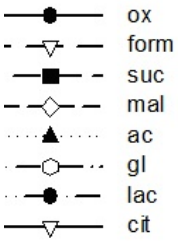

1000
500
0
c[µmol/l]
a
A. philoxeroides
day 1
day 3
day 7
control
1500
1000
500
0
b
S. variegata
day 1
day 3
day 7
control
1000
500
0
c
A. anomala
day 1
day 3
day 7
control

Fig. 4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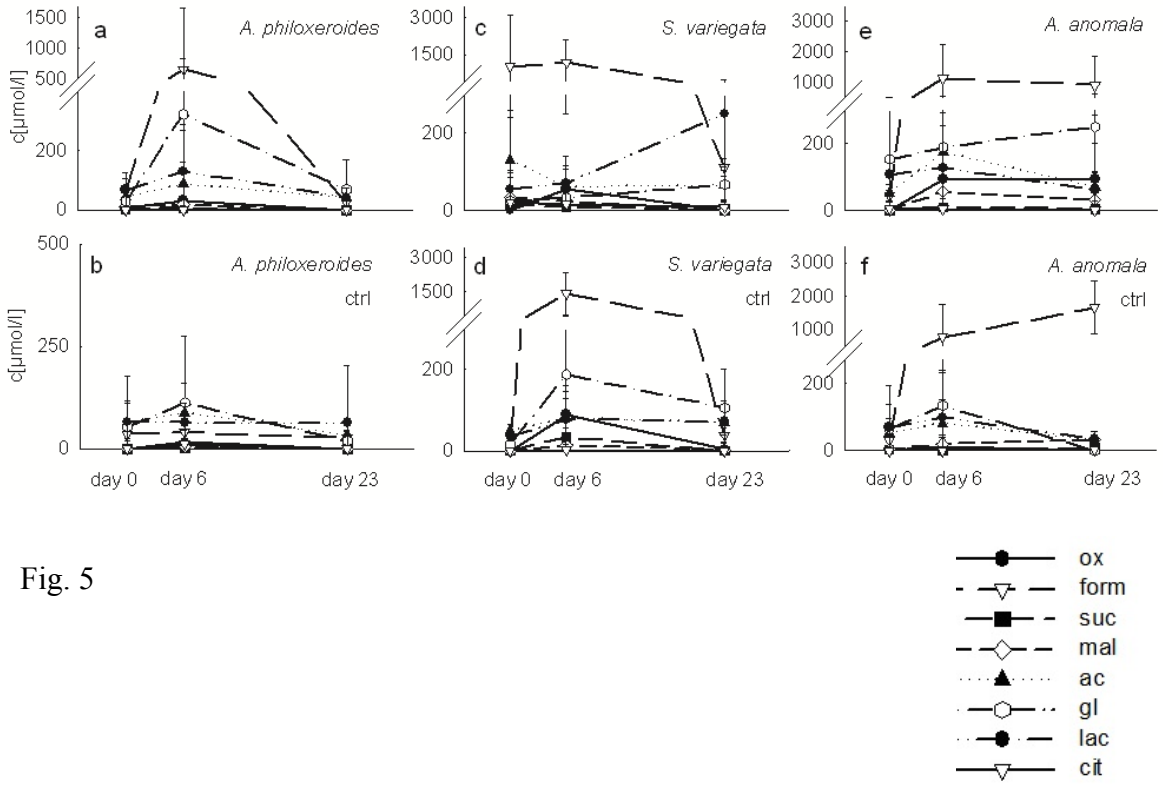
a
A. philoxeroides
b
A. philoxeroides
ctrl
c
S. variegata
d
S. variegata
ctrl
e
A. anomala
f
A. anomala
ctrl
c[µmol/l]
1500
1000
500
200
0
3000
2000
1000
250
day 0
day 6
day 23
ox
form
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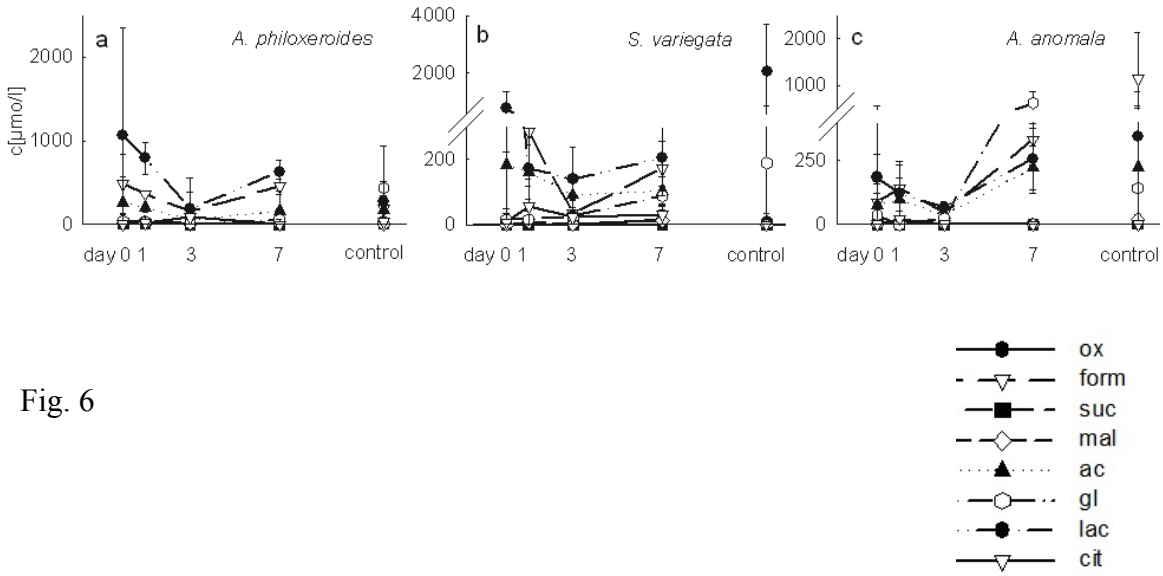
ac
gl
lac
cit

Fig. 5


a
A. philoxeroides
b
S. variegata
c
A. anomala
c[µmol/l]
4000
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cit

Fig. 6

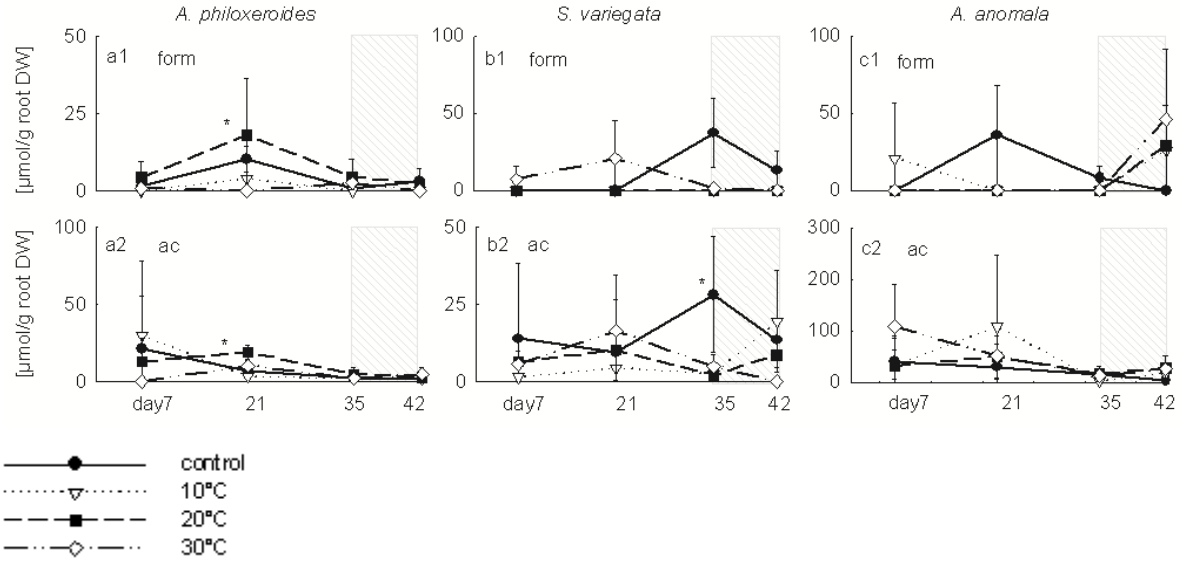


Fig. 7 A-C

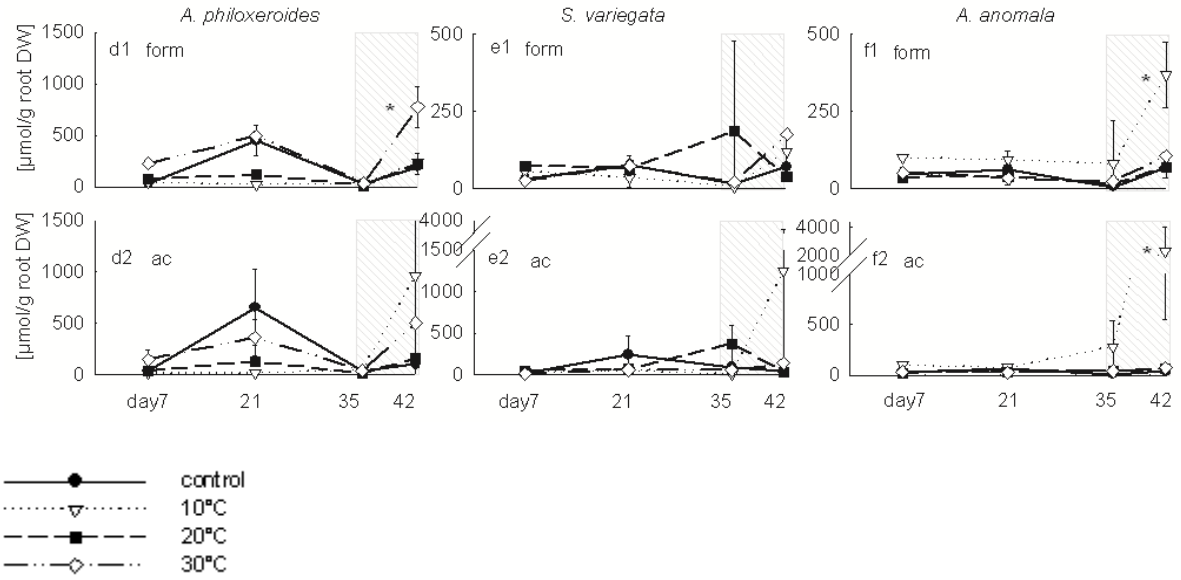
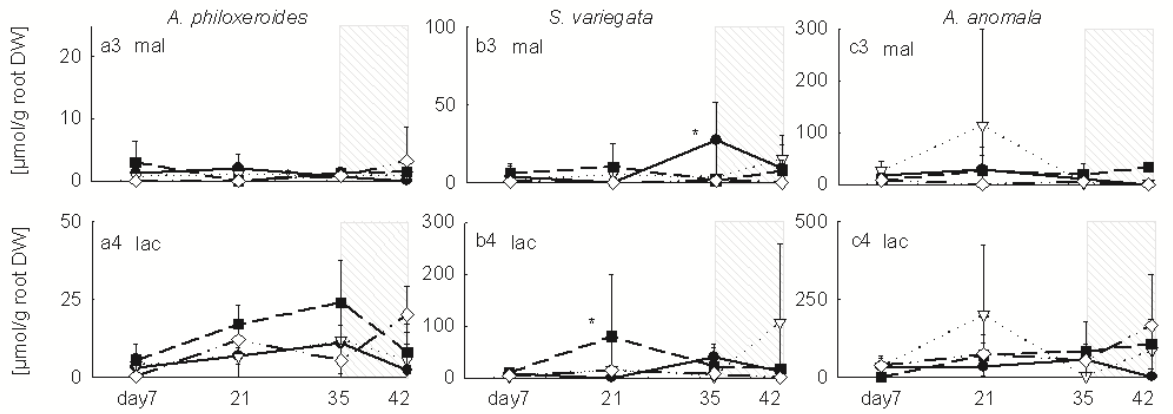
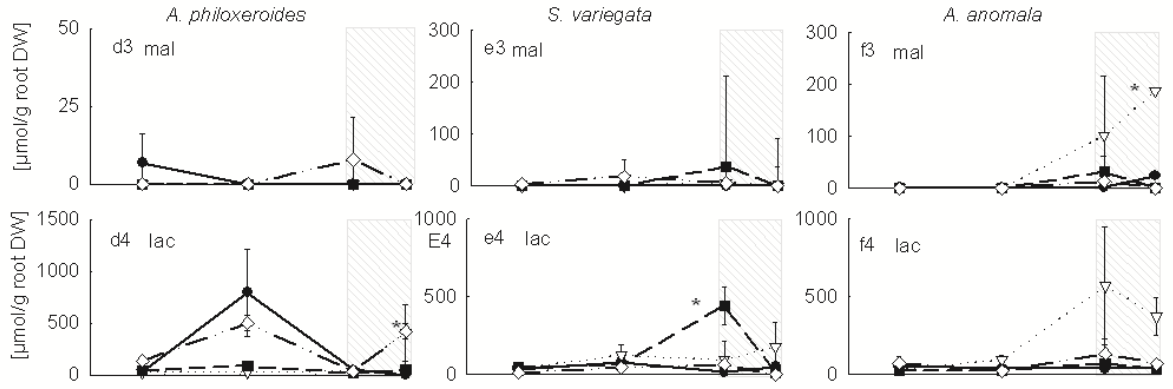


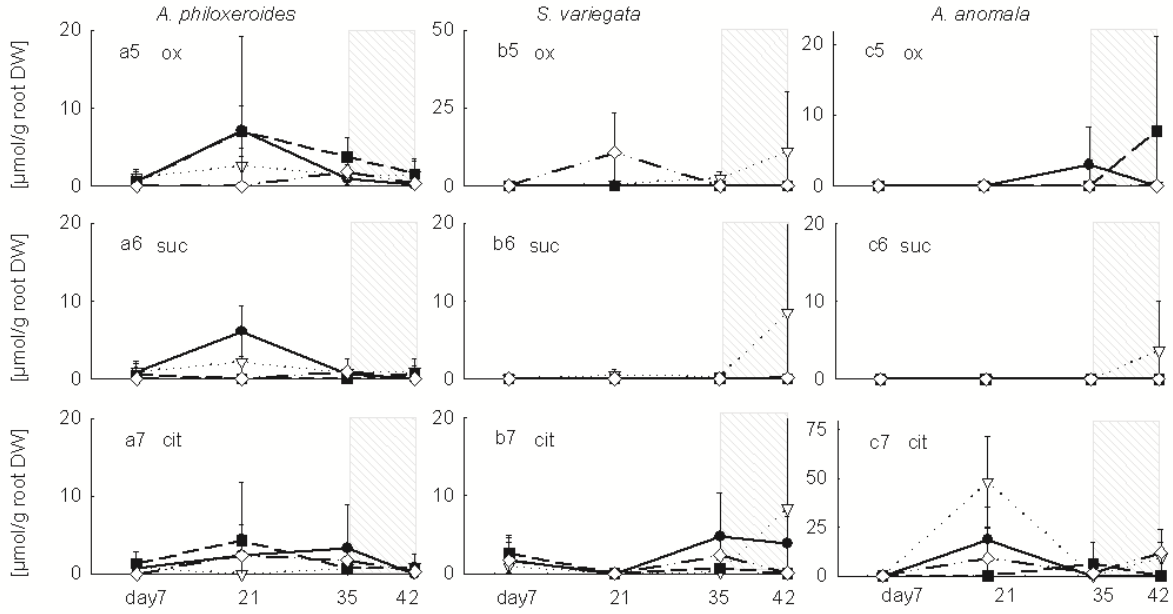
Fig. 7 D-F



App. Fig. 8 A-C



App. Fig. 8 D-F



App. Fig. 9 A-C

8.4 Manuscript 3

Running head: Rhizosphere dynamics in high resolution

Rhizosphere dynamics of 2 short-time flooded plants species from the water fluctuation zone of Three Gorges Reservoir, observed by means of high-resolution minimal-invasive rhizobox techniques

Christina M. Schreiber[1,2*], Bo Zeng[1], Stephan Blossfeld[2], Uwe Rascher[2], Marian Kazda[3], Ulrich Schurr[2], Agnes Höltkemeier[4], Arnd J. Kuhn[2]

[1]Key Laboratory of Eco-Environments in Three Gorges Reservoir Region (Ministry of Education), School of Life Sciences, Southwest China University, Chongqing 400715, P.R. China

[2]Institute of Bio- and Geosciences (IBG-2): Plant Sciences, Research Center Jülich GmbH, Leo-Brandt-Str., D-52425 Jülich, Germany

[3]Institute of Systematic Botany and Ecology, University Ulm, D-89069 Ulm, Germany

[4]Institute of Bio- and Geosciences (IBG-3): Agrosphere, Research Center Jülich GmbH, Leo-Brandt-Str., D-52425 Jülich, Germany

Bo Zeng	bzeng@swu.edu.cn
Stephan Blossfeld	s.blossfeld@fz-juelich.de
Uwe Rascher	u.rascher@fz-juelich.de
Marian Kazda	marian.kazda@uni-ulm.de
Ulrich Schurr	u.schurr@fz-juelich.de
Agnes Höltkemeier	a.hoeltkemeier@fz-juelich.de
Arnd Kuhn	a.kuhn@fz-juelich.de

*Corresponding author:

Christina M. Schreiber

Institute of Bio- and Geosciences (IBG-2): Plant Sciences, Research Center Jülich GmbH, Leo-Brandt-Str., D-52425 Jülich, Germany

c.schreiber@fz-juelich.de

Tel.: +49 (0) 2461 61 5493

Fax: +49 (0) 2461 61 2492

Abstract

The rhizosphere of two flooding resistant plant species (*Arundinella anomala* Steud., *Alternanthera philoxeroides* Mart.), which originate from Three Gorges Reservoir area (P.R. China), has been examined for reactions to waterlogging and submergence. Spatio-temporal rhizospheric dynamics were monitored in the original sedimental substrate by means of a dual-access floodable rhizobox, which allows monitoring of oxygen- and pH-dynamics (planar optodes) non-invasively and in high temporal and spatial resolution as well as simultaneous low-invasive soil solution sampling.

Roots could be observed easily in situ during growth and exposure to flooding. The floodable rhizobox is therefore considered a valuable tool for root reaction monitoring also under flooding conditions. Analysis of samples for low molecular weight organic acids (LMWOA) was done by capillary electrophoresis.

During waterlogging, both species exuded oxygen into their rhizosphere and showed diurnal rhythms of rhizospheric acidification. PH of the rhizosphere of growing root tips decreased up to 0.8 units that corresponded to higher LMWOA values.

The plants maintained diurnal rhythms during flooding and the rhythms rapidly gained maximum amplitude again after resurfacing. We thus conclude that the root system was fully functioning during flooding and that flooding poses no threat to the physiology of the root system of the study species.

1. Keywords: planar optodes, pH- and oxygen monitoring, anoxia, *Arundinella anomala* Steud., *Alternanthera philoxeroides* Mart.

2. Abbreviations

ac	Acetate
CE	capillary electrophoresis
cit	Citrate
form	Formate
gl	Glyoxylate
lac	Lactate
LMWOA	Low molecular weight organic acids
mal	Malate
OA	organic acids
ox	Oxalate
suc	Succinate
TGR	Three Gorges Reservoir

3. Introduction

The construction of the Three Gorges Dam at the Yangtze River (P.R. China) created a water fluctuation zone 45 to 75m above the original water level. Only species well-adapted to long-term flooding will be able to grow on the riverbanks and replace the original vegetation. Two flooding-resistant riparian species (*Alternanthera philoxeroides* Mart. and *Arundinella anomala* Steud.), originating from the concerned area, are now examined for a better understanding of survival mechanisms during flooding events, their abilities to stabilize the surrounding soil, prevent soil erosion and keep river banks vegetated to mitigate soil runoff (*Allen* 1979*; Schiechtl* and *Stern* 1996; *Liu* et al. 2004; *Wang* et al. 2005).

A healthy, well-developed root system with strong resistance to anoxia is crucial to survive flooding. Plants influence their rhizosphere: Water and nutrient uptake, oxygen release and exudation of pH-relevant substances (H^+, O_2, an-/organic ions, low molecular weight organic acids (LMWOA) and carbohydrates, amino acids, peptides, proteins, lipids) influence pH and redox potential and provide energy supply for soil microorganisms (*Jones* 1998, 2004; *Ryan* and *Delhaize* 2001; *Hinsinger* et al. 2003*; Farrar* et al. 2003; *Walker* 2003; *Blossfeld* and *Gansert* 2007). Abiotic factors already account for a heterogeneous pH distribution in soil (*Hinsinger* et al. 2003; *Kirk* 2004; *Scheffer* and *Schachtschabel* 2002), and roots contribute further to heterogeneity, for example by H+ or OH- excretion during either ammonium or nitrate uptake (*Marschner* 1995, *Marschner* and *Römheld* 1983), but also when stressed. An example are P and Fe deficiencies (*Kirk* and *Kronzucker* 2005; *Marschner* 1995; *Walter* et al. 2000; *Kania* et al. 2003).

Oxygen loss by roots also contributes to soil pH changes. Oxygen exudation under waterlogged conditions is known from aerenchymatic plants (*Mainiero* and *Kazda* 2005), as is different organic acid (OA) exudation under stress conditions. Dependent on growth status, age, nutrition, but also on abiotic stress factors like temperature, drought or anoxia (*Jones* 1998; *Strobel* 2001; *Bertin* et al. 2003; *Rovira* 2005, *Bais* et al. 2006), plant roots show increased OA exudation. Minimizing carbon loss is an important feature when enduring stress like submergence, since already up to 40% of the photosynthetically produced low molecular weight carbon molecules are excreted into the rhizosphere under normal growth conditions (*Bais* et al. 2006), Since OA exudation and healthy plant growth are positively linked (*Pikryl* and *Vancura* 1980), monitoring OA occurrence in the rhizosphere may lead to conclusions

about root health status. Being able to monitor these changes in high resolution will help to decode rhizospheric turnovers.

To gain better insights into the reactions of these root systems, a new non-invasive approach to investigate rhizospheric reactions has been tested for completely submerged plants. The plants were grown in floodable rhizoboxes which allowed temperature-controlled submergence and microorganism-free soil-solution sampling. At the same time, planar pH- and O_2-sensitive foils (*Blossfeld* and *Gansert* 2007; *Blossfeld* et al. 2010; *Huber* et al. 2001; *Klimant* et al. *2001*; *Jensen* et al. 2005; *Frederiksen* and *Glud* 2008) were used to monitor changes in the direct root surrounding. This technique provided a high time- and spatial resolution of ongoing changes in the rhizosphere of stress-exposed plants.

The plant species discussed here do not react in drastically increased OA exudation under flooding stress like e.g. *Medicago sativa* and *Lotus corniculatus* (*Barta* 1986) or *Zea mays* (*Xia* and *Saglio* 1992), but show a silencing strategy (Schreiber et al. in preparation). They are known for being able to resist up to 6 months of complete submergence (*Luo* et al. 2008) and are common in the natural water fluctuation zone of Yangtze River. *Arundinella anomala* Steud. is a perennial grass species with a very fast-growing, strong root system, which is highly suited for keeping soil fixed (*Tenten* 2010, in preparation). *A. philoxeroides* is native to South America and was imported as a forage plant (*Yan* et al. 2001), an extremely fast growing weed with very stable root stocks but delicate adventive roots. This neophyte can re-grow vegetatively from pieces of one node and two leaves and build swimming carpets, and therefore was quickly distributed the area.

With this study we also aimed to understand how these perseverant species avoid fast anoxic death, and to explore root strategies for a faster return to productivity after flooding and therefore better growth, reproduction and competitiveness.

4. Material and methods

Rhizobox

Plants and substrate were collected at Yangtze riverside (Chongqing, N29°28’31.1”, E106°31’29.7”, 213m NN). The substrate is a very fine sediment (87% fine sand <20µm diameter) with low nutrient content. Both species are able to grow productively in this substrate.

The rhizobox consists of an aluminium body with access from two sides. The front side allows soil solution sampling through inserted tubes, which contain guiding sleeves (raster plates) for steel capillaries (0.6mm diameter). Two of these raster plates form a unit, with a filter on the inner side directly contacting the rhizosphere (pore size <0.2µm to prevent microorganism (MO) contamination of samples) and divided by a septum to provide watertightness. The holes in the inner plate narrow to 0.4mm diameter to prevent capillaries from penetrating the filter. This setup allows installation of capillaries at defined points and MO-free soil solution sampling.

The rhizobox was closed on the backside with 3mm plexiglass to observe root growth and allow reading of the pH- and O_2-optodes (10x30mm each, one pH- and O_2-optode per plant). Optodes were fixed to the inner side of the glass with silicone glue in direct contact to the rhizosphere. Inbetween measurements, tinfoil protected the optodes from light entry and possible photobleaching. A plexiglass tank was mounted on top of the box allow flooding of the setup (25-30cm water depth). Cooling elements were fixed with thermal paste onto the front side to control temperature. The rhizoboxes were filled with sediment, the plexiglass taken off and the plants carefully transferred onto the substrate surface, always kept moist before refitting the plexiglass. It was taken care that no air caverns formed in the substrate when closing the rhizobox. The boxes were tilted at a 35° angle allow the root growth along the glass and the optodes. One individual of each species was investigated. Since the plants were kept in their original substrate, no fertilization was applied. After one week adaptation, the plants were waterlogged for 2 days (day 1+2), then flooded completely for 2 days (day 3+4), followed by another 2 days of waterlogging (day 5+6). The rhizoboxes were kept at 20°C air temperature. Light supply consisted of a PAR of 500µmol/m²s from 7am to 9pm each day. All experiments were conducted at SW University Chongqing/Beibei, P.R. China.

Planar optodes

The pH- and O_2-measurements were made using planar optodes (PRESENS, Regensburg, Germany), a non-invasive optical technique (*Blossfeld* and *Gansert* 2007). This measurement is based on different fluorescence decay times of pH- or O_2-sensitive indicator dyes compared to reference dyes (compare *Gansert* and *Blossfeld* 2008; *Gansert* et al. 2006; *Huber* et al. 2001; *Klimant* et al. 2001). An optical glass fiber, connected to a light source and measuring device (pH-1 mini, PRESENS, Regensburg, Germany) conveys the excitation light pulse

(470nm). The glass fiber is moved by step motors (compare *Blossfeld* and *Gansert* 2007) over a 10x30mm grid in 2mm steps (5 seconds per step, 1 measurement per second, resulting in 75 measuring points on an area of 10x30mm). A measuring cycle (25 min per rhizobox) was done every 2 hours. The average of each 5 measurements per measuring point was processed with SigmaPlot (Systat Software, Inc., San Jose, CA, USA) to create contour plots of pH- and O_2 distribution along the optode foils. The setup is shown in Fig. 1.

((Fig. 1))

Soil solution sampling

Ten steel capillaries were inserted permanently (otherwise water loss due to high water column) opposite the planar optodes (Fig. 2) to allow corresponding measurements, aligned in two rows with 5 capillaries every 5mm. Capillaries were sterilized before use (96% ethanol), and sampling was done two times each day (10:00, 18:00 o'clock) by applying negative pressure (~300mbar) with a vacuum chamber containing Eppendorf tubes. Sampling with each capillary took about 5 seconds, the sampling volume was 40-50µl. Before each sampling, the first drop (10µl) was discarded. The samples were frozen immediately and stored at -20°C.

Soil solution analysis

Capillary electrophoresis was used to determine organic acids in the soil solution samples with a salicylate electrolyte (*Bazzanella* et al. 1997). The system G1600A (AGILENT, Böblingen, Germany) was used, including a diode array detector and using fused silica capillaries (POLYMICRO, Phoenix, USA, 75µm inner diameter, 64.5 cm total length, 56 cm effective length). The electrolyte contained 7.5mM salicylic acid, 15mM TRIS, 0.5mM dodecyltrimethylammonium hydroxide and 0.3mM $Ca(OH)_2$, which was freshly prepared every day. Separation ensued at 30kV and 25°C. Quantification was performed by using external aqueous standard solutions of the acids (5, 10, 20µmol) and internal standardization by phenylacetic acid in samples. Precision was best in a concentration range from 0-40µmol/l with linear calibration curves. Samples were diluted accordingly and measured 2 times after flushing the capillary with 0.1 NaOH for 5 min, water for 1 min and electrolyte for 5 min. During analysis, up to 8 low molecular organic acids were identified from the soil solution

samples (oxalate (ox), formate (form), succinate (suc), malate (mal), acetate (ac), glyoxylate (gl), lactate (lac) and citrate (cit)).

Results

A. anomala showed a diurnal rhythm of rhizospheric acidification. Rhizospheric pH was lower during daytime than at night. It varied in a range from 6.0 to 6.5 from root to bulk soil in the afternoon (5 p.m., Fig. 2A), and in the dark period this spread receded to a range of 6.4 to 6.8 (compare 11 p.m., Fig. 2A). Oxygen was released from the root into its surrounding, this loss was clearly visible during daytime (mostly between 11 a.m. and 5 p.m.) while it receded completely at night (Fig. 2B).

((Fig. 2))

These rhythms lessened with the onset of complete submergence to a pH-spread of 6.4 to 6.5 at night and 6.2 to 6.5 in the afternoon (Fig. 3A). An oxygen loss was barely visible (Fig. 3B).

((Fig. 3))

During the recovery (day 5+6), these rhythms re-intensified almost to the level from before the flooding event (Fig. 4A+B), and the oxygen release increased strongly towards day 6 (Fig. 4B, lowest diagram, day 6). Interestingly, the root on the left side (compare Fig. 2, photograph) exuded oxygen during waterlogging (+5% in a 4mm zone around the root compared to bulk soil), the root on the right side did not. After re-surfacing, the oxygen release intensified to up to +10% in an 8mm zone around the root compared to bulk soil.

((Fig. 4))

OA occurrence was higher for ac in direct root vicinity, where it slightly intensified during flooding, but not for other OA (Fig.5). Form, gl, ac and lac were main contributors to organic acid composition in soil solution. Ox, suc, mal and cit were only sporadically found and are not displayed. No "burst" of OA occurrence could be observed directly after flooding.

((Fig. 5))

Diurnal rhythms of acidification were also found in *A. philoxeroides,* with lowest pH values (6.1) around the root in the measuring window at 1 p.m. and highest (6.8) in the early morning in bulk soil during waterlogging (day 1+2). No immediate reaction to the onset of flooding was observed, but continued flooding also caused a less intense spread of pH (6.3-

6.5, not shown). During recovery (waterlogging after flooding), a root grew into the measuring window with a visible acidification at its tip of 6.0 (Fig. 6).

((Fig. 6))

At the same time, a corresponding peak in OA concentration in the lower area of the measuring zone was observed (ac 90µmol/l, gl 50µmol/l, Fig. 7). During waterlogging and recovery, oxygen content around the root was 5-10% higher than in bulk soil.

((Fig. 7))

6. Discussion

The planar optode technique has already proven to be suitable under water-saturated and even moderate soil moisture conditions (*Blossfeld* and *Gansert* 2007; *Blossfeld* et al. 2009). Our results show that also under flooded conditions a high resolution of pH and oxygen measuring can be achieved. The floodable rhizobox with its two-way access has proven to be a suitable tool for rhizosphere monitoring even under flooded conditions. Using planar optodes as a non-invasive technique provides a new window to observe the spatio-temporal dynamics of root reaction.

Our study confirms earlier findings (*Jones* 1998, *Marschner* 1995): the root tip and root elongation zone were strongest zones of rhizospheric acidification and oxygen release. The pH of a growing root tip can be 1-2 units lower than the bulk soil (*Hinsinger* et al. 2003; *Kopittke* and *Menzies* 2004). Locally restricted uptake of positively charged ions (K, NH_4^+) is known to cause this effect along roots (*Bravin* et al. 2009; *Marschner* 1995; *Miller* and *Cramer* 2004). Other factors like the acid-growth mechanism (*Peters* 2004; *Versel* and *Pilet* 1986), which finds strongest acidification at strongest growing root zones, or the here observed oxygen loss might add to the acidifying effect. In the two studied species an oxygen contribution is likely, because the findings of organic acids in the direct root vicinity did not reach exceedingly high levels during flooding compared to the initial situation.

The investigated species did not express sudden reactions to submergence, as known from *Zea mays* or *Medicago sativa* (*Barta* 1986, *Xia* and *Saglio* 1992), which increase OA exudation when flooded. Also no direct acidifying reaction was observed, as it was in *S. variegata* (*Schreiber* 2010) with a drop of more than 2 pH units at an active root tip with

beginning submergence. Nevertheless, the general reaction during root growth is an acidification in the rhizosphere compared to bulk soil, no alkalization as known from ryegrass or alpine pennycress (*Fan* and *Neumann* 2004; *Peters* 2004). The existence of diurnal rhythms of rhizospheric acidification was clearly documented for both *A. anomala* and *A. philoxeroides* in this experiment. Diurnal rhythms, as described here, were already reported from *Medicago sativa* (*Jarvis* and *Hatch* 1985), *Vigna unguiculata* (*Rao* et al. 2002) and *Juncus effusus* (*Blossfeld* 2007). In contrast to our experiment, *Medicago* and *Vigna* also show overnight alkalization. A photosynthetically derived increase of oxygen loss from roots could be a source for protons through oxidizing Fe_2^+ (*Kirk* 2004), since oxygen concentration showed a similar diurnal rhythm as acidification, this is possible here. Also photosynthesis itself is a driving force for rhizospheric acidification (NH_4^+ to glutamine, *Foyer* and *Noctor* 2002), especially since in hypoxic soils, NH_4^+ is the main nitrogen source and NH_4^+ uptake and corresponding H^+ release may increase (*Brune* et al. 2000; *Marschner* 1995).

Photosynthesis indeed appeared to be not completely shut down during the 2 days submergence at least in *A. anomala,* because oxygen loss was still existent during submergence. Light was available for the plants during submergence, and thus limited (lack of CO_2 due to slow diffusion in water, *Tiedje* et al. 1984) photosynthesis may have been present. Well shaped aerenchyma in roots and shoots provide oxygen diffusion to roots (*Tenten*, in preparation). The oxygen loss after *resurfacing in A. anomala increased strongly which hints towards resumed* photosynthesis when exposed to air.

The reaction of soil microorganisms has not been specially regarded in this short experiment. However, eventual exudation bursts would then have shown in a rise of fermentation products which did not happen. Soil microbial communities must have been present though, since almost no ox, suc, mal or cit was found in soil solution samples. They are exudates known to be utilized fast by soil microbiota (*Jones* 1998), and indeed the discussed plants exude them as found in a sterile experiment in glass bead substrate (*Schreiber*, in preparation).

To estimate root health during submergence, in this 6-day-experiment our method proved to be a convenient tool to observe diurnal rhythms and oxygen losses. The plants return very fast to the same diurnal rhythms as before the flooding event, which leads to the conclusion that the event did not hamper their performance much. The habitus of the plants showed no visible damages in any form, and freshly growing roots in the recovery period supported the view non-permanent damage. Live roots protect soil and make these species an interesting subject to investigate further for erosion protection in Three Gorges Reservoir area. These plants have

already been proven not to react with dramatically increased carbon excretion, and at least for the LMWOA no such effect could be found here also. Avoidance of carbon depletion makes these species so perseverant (*Ye* 2010).

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9. Figure legends

Fig. 1 Floodable Rhizobox (temperature controlled) with two-way access. Front side: filtered soil solution sampling in high spatial resolution via steel capillaries. Backside at the corresponding area: Automated pH- and oxygen measurement with planar optode foils. A tank can be mounted to simulate flooding events.

Fig. 2 Spatio-temporal variations of pH (A) and O_2 [%air saturation] (B) in the rhizosphere of *A. anomala* during initial waterlogging (day 1). White arrows mark growing roots, also seen as photograph. Circles mark corresponding soil solution sampling spots.

Fig. 3 Spatio-temporal variations of pH (A) and O_2 [%air saturation] (B) in the rhizosphere of *A. anomala* during flooding (day 4). White arrows mark growing roots.

Fig. 4 Spatio-temporal variations of pH (A) and O_2 [%air saturation] (B) in the rhizosphere of *A. anomala* after resurfacing (day 5+6). White arrows mark growing roots.

Fig. 5 Concentration [µmol/l] of 4 organic acids in the soil solution of *A. anomala* on day 1, 3 and 6 (waterlogging, flooding, after resurfacing) of the experiment period over 2 rows of measuring spots (5mm vertical distance, compare Fig. 5) corresponding to Fig. 2-4.

Fig. 6 Spatio-temporal variations of pH (A) in the rhizosphere of *A. philoxeroides* and growing root tip after resurfacing (day 5+6). White arrows mark growing roots. Circles mark corresponding soil solution sampling spots.

Fig. 7 Concentration [µmol/l] of 4 organic acids in the soil solution of *A. anomala* on day 5 and 6 of experiment (waterlogging following 2 days flooding) over 2 rows of measuring spots (5mm vertical distance, compare Fig. 5) corresponding to Fig. 6.

Figures

Fig. 1

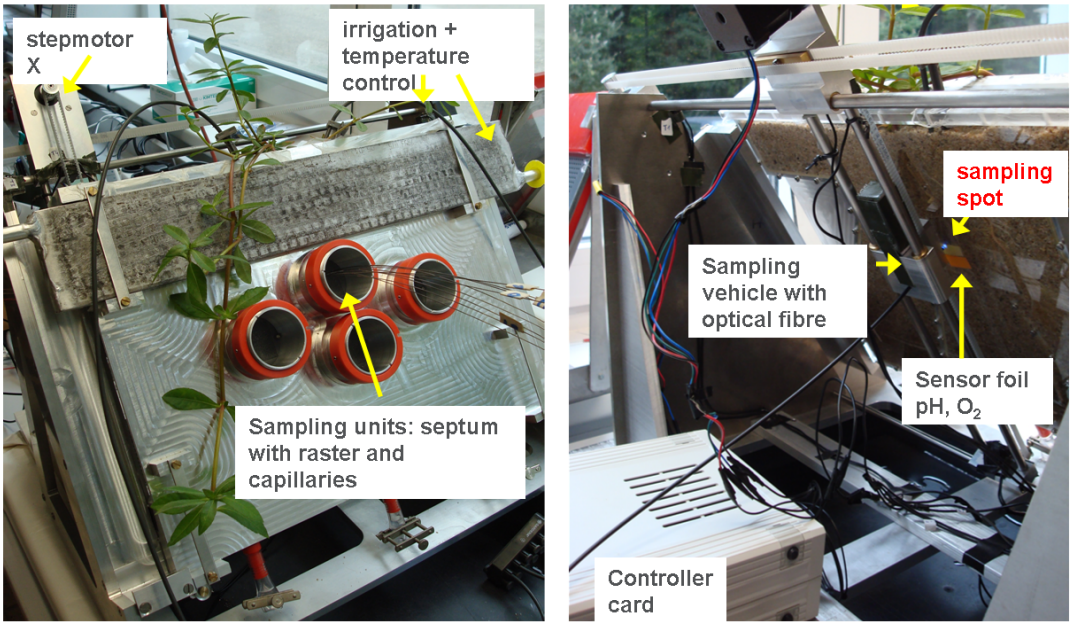


Fig. 2

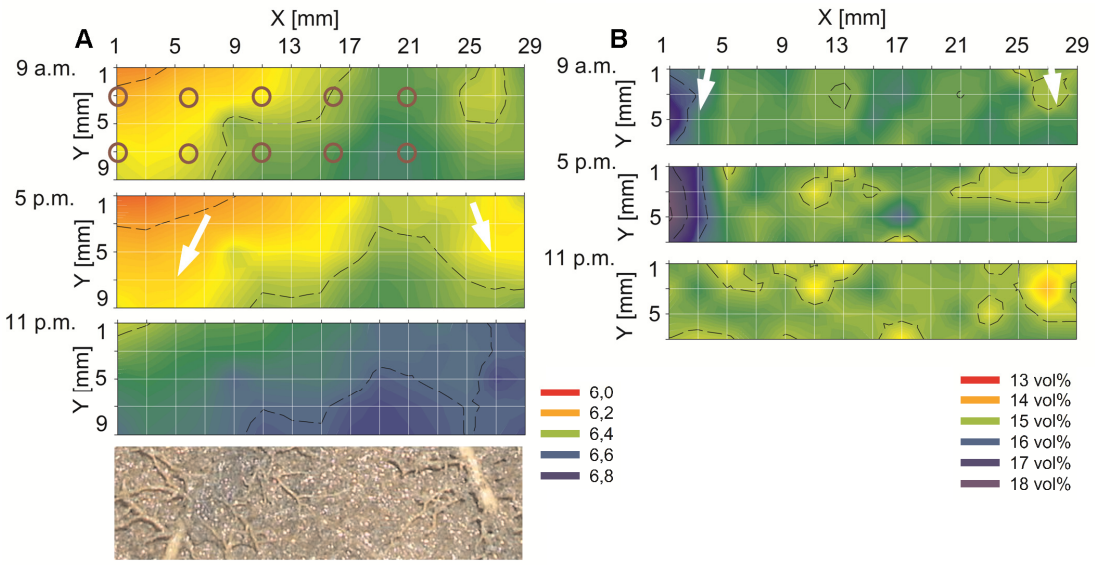


Fig. 3

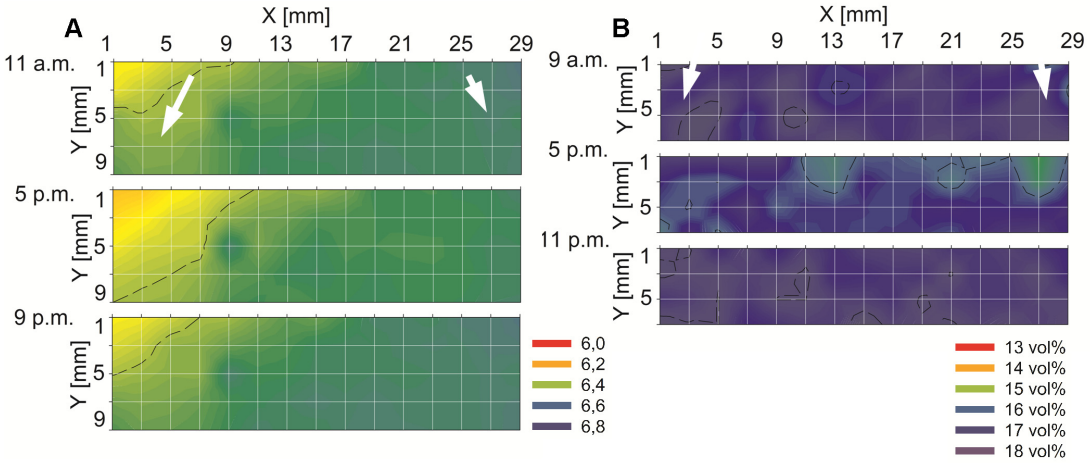

A
X [mm]
1 5 9 13 17 21 25 29
11 a.m.
5 p.m.
9 p.m.
Y [mm]
1 5 9
6,0
6,2
6,4
6,6
6,8
B
X [mm]
1 5 9 13 17 21 25 29
9 a.m.
5 p.m.
11 p.m.
Y [mm]
1 5
13 vol%
14 vol%
15 vol%
16 vol%
17 vol%
18 vol%

Fig. 4

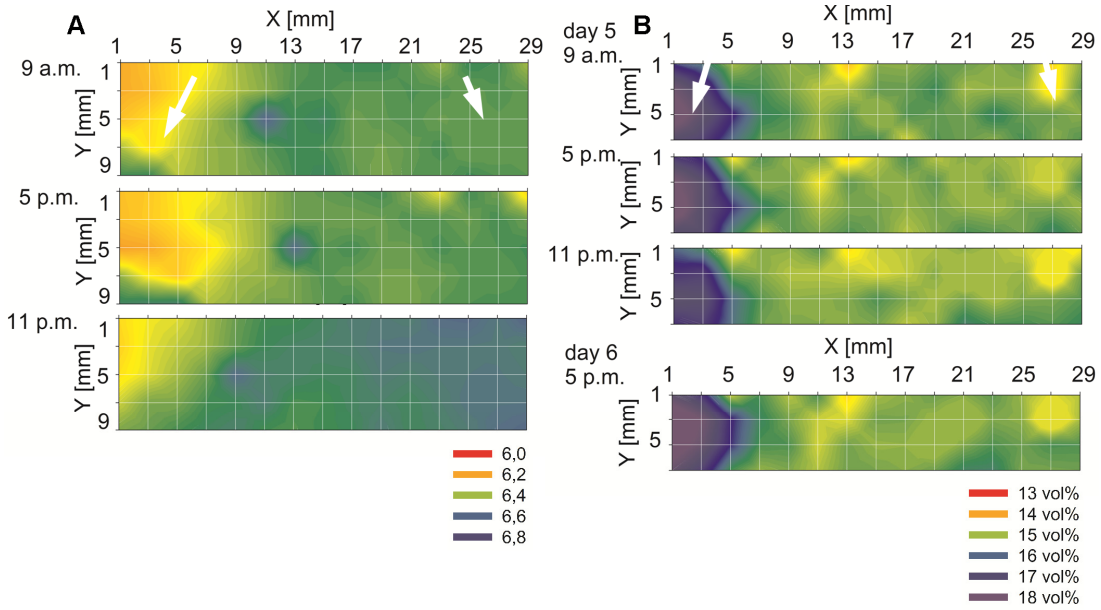

A
X [mm]
1 5 9 13 17 21 25 29
9 a.m.
5 p.m.
11 p.m.
Y [mm]
1 5 9
6,0
6,2
6,4
6,6
6,8
day 5
B
X [mm]
1 5 9 13 17 21 25 29
9 a.m.
5 p.m.
11 p.m.
Y [mm]
1 5
day 6
5 p.m.
13 vol%
14 vol%
15 vol%
16 vol%
17 vol%
18 vol%

Fig. 5

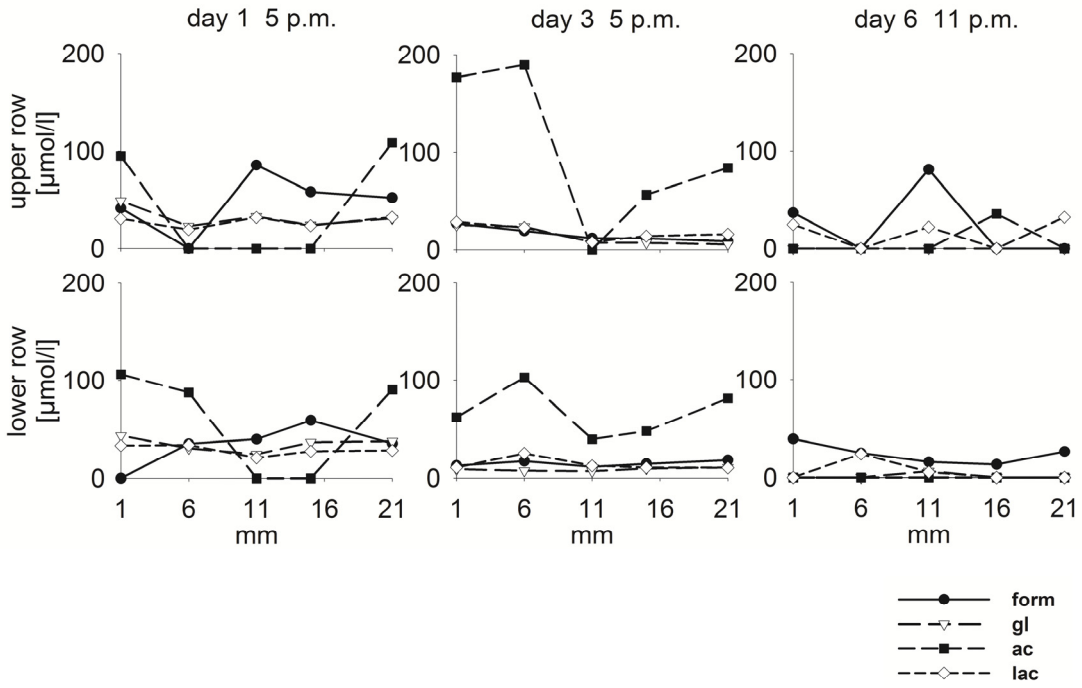

day 1 5 p.m.
day 3 5 p.m.
day 6 11 p.m.
upper row [µmol/l]
lower row [µmol/l]
200
100
0
1
6
11
16
21
mm
form
gl
ac
lac

Fig. 6

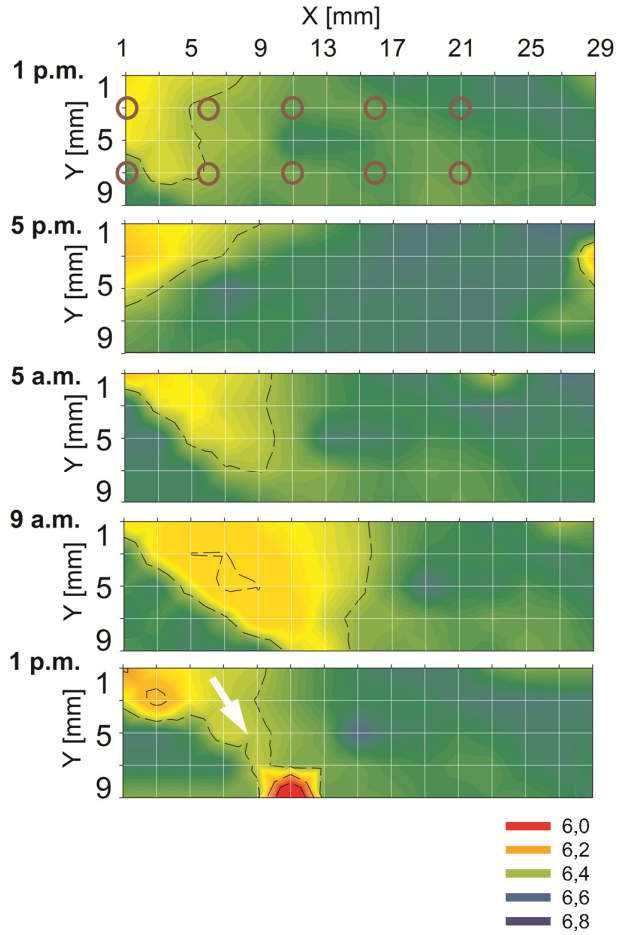
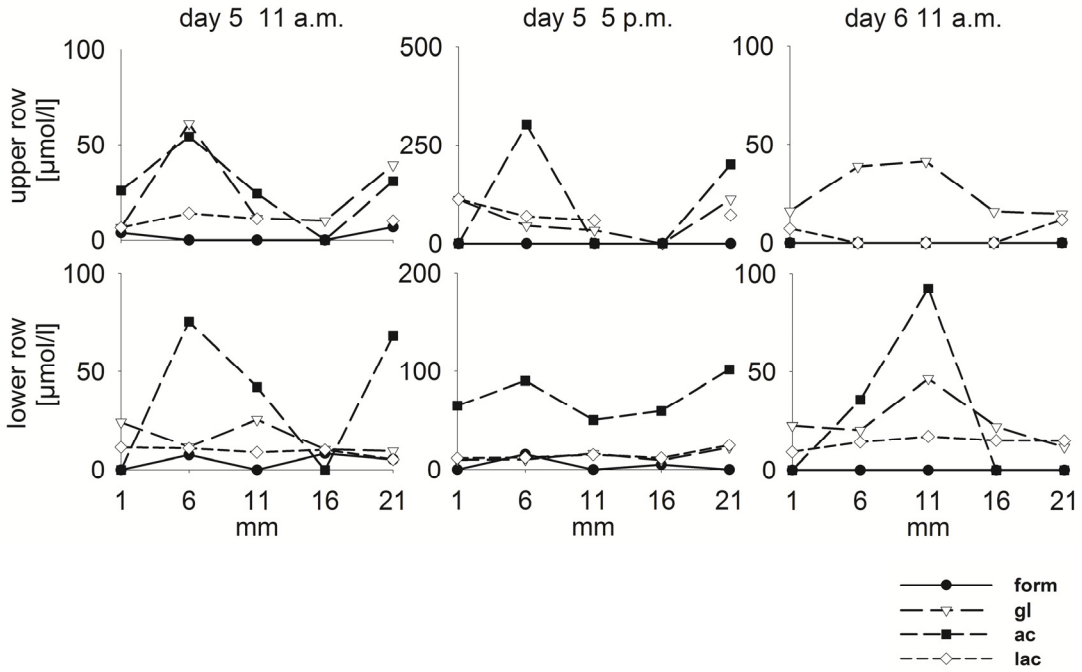

X [mm]
1
5
9
13
17
21
25
29
1 p.m.
5 p.m.
5 a.m.
9 a.m.
1 p.m.
Y [mm]
6,0
6,2
6,4
6,6
6,8

Fig. 7


day 5 11 a.m.
day 5 5 p.m.
day 6 11 a.m.
upper row [µmol/l]
lower row [µmol/l]
100
50
0
500
250
200
1
6
11
16
21
mm
form
gl
ac
lac

8.5 Manuscript 4

Surviving Submergence: Rhizosphere dynamics of flood-stressed plant species, observed by non- and low invasive methods

Running title: low-invasive rhizosphere investigation

C. Schreiber[1,2], Bo Zeng[2], U. Rascher[1], Marian Kazda[3], U. Schurr[1] and A. J. Kuhn[1]

[1] *Institute of Bio- and Geosciences (IBG-2): Plant Sciences, Research Center Jülich GmbH, D-52425 Jülich, Germany*

[2]*Key Laboratory of Eco-Environments in Three Gorges Reservoir Region (Ministry of Education), School of Life Sciences, Southwest China University, Chongqing 400715, P.R. China*

[3]*Institute of Systematic Botany and Ecology, University Ulm, D-89069 Ulm, Germany*

[4]*Institute of Bio- and Geosciences (IBG-3): Agrosphere, Research Center Jülich GmbH, D-52425 Jülich, Germany*

Corresponding author:
Christina Schreiber
Institute of Bio- and Geosciences (IBG-2): Plant Sciences
Research Centre Jülich
52425 Jülich
Germany
Fax: +49 2461 612492
Phone: +49 2461 615493
email: c.schreiber@fz-juelich.de

Abstract

The rhizosphere of three flooding-tolerant plant species (*Arundinella anomala* Steud., *Alternanthera philoxeroides* Mart., *Salix variegata* Franch.) from Three Gorges Reservoir (TGR) area, P.R. China, has been monitored for dynamics of pH, O_2 (planar optodes) and occurrence of low molecular weight organic acids in soil solution (LMWOA) and compared

to three less- and non-tolerant species (*Phalaris arundinacea* L., *Zea mays* L., *Hordeum vulgare* L.).

Plants were grown in sterile glass bead- and original sediment substrate in a dual-access floodable rhizobox at 10°C and 20°C for cycles of 2 weeks waterlogging, 2 weeks complete submergence and another 2 weeks waterlogging.

All observed species showed diurnal rhythms of rhizospheric acidification, which diminished during submergence, and recovered after resurfacing only in the tolerant plants, non-tolerant species did not survive. PH was higher (up to 8.1) in natural sediment than in sterile glass bead substrate. Oxygen content was almost depleted for *Z. mays* and *H. vulgare* already with beginning submergence, while all other species kept oxygen in their rhizosphere.

LMWOA occurrence did not differ between temperature treatments in glass substrate, but concentration of fermentation products was 3-5 times higher in 20°C sediment setup. Non-tolerant species showed a short increase of exudation with beginning anoxia.

1. Key word index

Arundinella anomala Mart., *Salix variegata* Franch., *Alternanthera anomala* Steud., *Zea mays* L., *Hordeum vulgare* L., *Phalaris arundinacea* L., planar optodes, root exudation, anoxia

2. Abbreviations

ac	Acetate
CE	capillary electrophoresis
form	Formate
lac	Lactate
LMWOA	Low molecular weight organic acids
mal	Malate
OA	organic acids
TGR	Three Gorges Reservoir
MO	Microorganisms

3. Introduction

On 26th October 2010, Three Gorges Reservoir reached its maximum water level of 175m for the first time. The construction of the Three Gorges Dam and its management, mainly focused on energy production and flood protection, caused drastic changes in the reservoir area on the banks of Yangtze River (Fearnside 1988; Park *et al*. 2003; Wu *et al*. 2004). The reservoir is about 600km in length and 1050km^2 in area with a flood pulse shifted from summer to winter and a 30m water fluctuation zone 100m above the original water level. The ecosystems in this zone, not yet adapted to flooding, are drastically changing and leave the riverbanks vulnerable to erosion. Flooding resistant riparian plant species from the lower river banks can be used to re-vegetate the water fluctuation zone and mitigate soil runoff (Allen 1979; Schiechtl & Stern 1996; Liu *et al*. 2004; Wang *et al*. 2005). Several local species are already known for their perseverance during submergence, but the mechanisms of their survival and the quality of their root systems during flooding events still have to be investigated to better understand and judge their applicability for this task.

Many plant species are adapted to short time flooding and react with fleeing strategies like rapid shoot elongation (Ye 2010), or building of aerenchyma (Drew, He & Morgan 2000, Jackson et al. 1999) which enables oxygen diffusion into the rhizosphere (Mainiero & Kazda 2005) to survive hypoxic episodes during flooding. Long-term flooding causes severe anoxia in the rhizosphere of plants. Several species are known to react to a stress event like flooding with increased root exudation (Jones 1998, Barta 1986), which, over time, leads to rapid carbon depletion in roots and eventually results in death of the plant. Connected to that is decreased hydraulic conductivity, root pressure and solute permeability (Birner & Steudle 1992), mostly caused by changes in transport properties of cell membranes. This effect has been found in several studies on maize or wheat (Everard & Drew 1989, Zhang & Tyerman 1991). Yet studies on flooding resistant species are few (Else et al. 1995 (*Lycopersicum*), Henzler et al. 1999 (*Lotus*)), especially if the whole plant is submerged, and more information is needed for a better understanding of how plant root systems stay alive during prolonged anoxia.

Plants influence their rhizosphere not only during stress events, they exist in a delicate feedback system: Competition with neighbour plants, interaction with microorganism community and abiotic soil environment stimulate root reactions. Growth, water and nutrient uptake, exudation of pH-relevant substances like protons, oxygen, CO_2, ions or organic acids,

carbohydrates etc. change their direct environment (Jones 1998, Strobel 2001, Bertin et al. 2003, Bais et al. 2006). Thus, plants are able to influence redox potential and pH of the surrounding soil and also provide nutrition for soil microbiota (Jones 1998, 2004, Ryan & Delhaize 2001, Hinsinger et al. 2003, Farrar et al. 2003, Walker 2003, Blossfeld & Gansert 2007). How and to which extent a plant reacts under stress conditions like flooding may be crucial for survival, since for example up to 40% of the photosynthetically fixed carbon is already lost into the rhizosphere under normal conditions (Bais et al. 2006).

Soil microbiota make use of this carbon source. Especially under anoxia, exudation and respiration do not originate from roots alone. Fermentations (lactate/ethanol), methanogenesis, acetogenesis etc. then dominate as energy sources (Yao et al. 1999). But distinguishing contributions of roots and MO is difficult, since neither works the same if separated (Grayston et al. 1997, Jones 2003). One approach to separate effects of root from MO metabolism is the comparison of sterile and non-sterile setups, taking temperature effects into account, since soil respiration is strongly declining with temperature (Boone et al. 1998).

To examine these processes under the special challenge of flooding, we used a dual-access floodable rhizobox at two different temperatures (10 and 20°C) for precise monitoring of rhizosphere dynamics. It allows automated non-invasive 24/7 pH- and O_2 monitoring from on side of the rhizobox as well as microorganism-free soil solution sampling from the opposite side. Planar optodes, pH- and O_2-sensitive foils that were in direct contact to the rhizosphere, allow high resolved monitoring of oxygen- and pH-dynamics (Blossfeld & Gansert 2007, Blossfeld et al. 2010, Huber et al. 2001, Klimant et al. 2001). The possibility of simultaneous low-volume soil-solution sampling enables analysis of various components in the rhizosphere. LMWOA exudation and growth are positively linked in a healthy plant (Pikryl & Vancura 1980), and increased LMWOA exudation is also a known plant reaction to environmental stress (nutrition, temperature, drought, anoxia etc. Jones 1998, Strobel 2001, Bertin et al. 2003, Rovira 2005, Bais et al. 2006). Therefore, analysis of LMWOA in low-volume soil solution samples via capillary electrophoresis (Bazzanella et al. 1997, Dabek-Zlotorzynska & Keppel-Jones 2000, Göttlein 2005) might help detect carbon fate and root health status.

The investigated species are already known for their perseverance during flooding (Ye et al. 2010). *Arundinella anomala* Steud. is a perennial grass species. Its fast-growing root system is highly suited to stabilize soil. *Alternanthera philoxeroides* Mart. is a neophyte originating from South America which was imported as forage plant and is now distributed widely. It grows very fast from root stocks or even single nodes with one or two leaves, and can even

build swimming carpets. *Salix variegata* Franch. is a small tree species, which, like the other two, is able to survive flooding periods from 3 to 6 months. All three species can survive productively on the nutrient-poor sediment of Yangtze River, one of the most sediment carrying rivers worldwide. *Zea mays* L. and *Hordeum vulgare* L., two non-tolerant cereals known well to literature (Birner & Steudle 1992, Fagerstedt & Crawford 1987, Menegus et al. 1989, Xia & Saglio 1992) are compared to these riparian species as a reference, as is a medium-tolerant species, *Phalaris arundinacea* L.. *P. arundinacea* is flooding tolerant through possession of anoxia-resistant rhizomes (Brändle 1991).

This study aims to improve understanding about rhizospheric processes, not only under sterile laboratory conditions but also in the original substrate. Understanding of survival mechanisms may lead to a more successful riverbank ecosystem management, especially in the case of TGR.

4. Material and Methods

The experiment cycle over 6 weeks (2 weeks waterlogging, 2 weeks flooding, 2 weeks recovery (waterlogged)) was conducted two times each for *A. anomala, A. philoxeroides* and *S. variegata* at 10° and 20°C, and once for *Z. mays*, *H. vulgare* and *P. arundinacea,* which were grown in washed sand and watered with 10% Hoagland solution, then transferred into the rhizobox at an age of 6 weeks after germination. *A. anomala, A. philoxeroides* and *S. variegata* were grown from cuttings (also 6 weeks) for the sterile planting, also grown in washed sand and watered with 10% Hoagland solution until transfer to the rhizobox. For the sediment planting they were collected directly at Yangtze river (Chongqing, N29°28’31.1”, E106°31’29.7”, 213m NN), as was the natural substrate, a very fine sediment (87% fine sand <20μm diameter). No extra nutrition was added here.

4.1 Dual access rhizobox

Plant roots were washed carefully with demineralised water and planted into the rhizobox (Fig. 1). One rhizobox (aluminium), contained three compartments (30cm height) to measure three plant individuals simultaneously. The roots were positioned so that two roots could be expected to grow along the planar optodes within the next week. The rhizobox was closed on

the backside with 3mm plexiglass to observe roots visually and allow reading of the pH- and O_2-optodes, and tilted at a 35° angle to promote root growth along the glass and the optodes. For the sterile setup, annealed glass beads (<0.2-1mm diameter) were used. The roots for this setup were sterilized carefully with H_2O_2 [3%]. After planting, one week in moderate soil moisture ensured adaptation to the rhizobox surrounding, the last 3 days gradually lowering the temperature of the cold treatments to 10°C. A 30cm tank was mounted on the rhizobox for later simulation of flooding. To control temperature, two cooling elements, connected to a cooling generator containing glykol, were fitted on the front of the box. An air pump provided air circulation within the tank in the waterlogged phase. Light was supplied with PAR=500µmol/m²s from 7am to 9pm each day. The substrate was kept waterlogged to avoid inhomogeneities in soil moisture and ensure comparable conditions. The setups in sediment substrate did not receive additional fertilization. The glass substrate was saturated with 10% Hoagland solution before start of experiment.

4.1.1 pH- and O_2 monitoring

The planar optodes (PRESENS, Regensburg, Germany, 8x22mm each, except Fig. 5 (8x29mm)) were fixed to the inner side of the plexiglass with silicone glue in direct contact to the rhizosphere. Every compartment contained one pH and one O_2 optode. Between measurements, the boxes were covered with tinfoil on the plexiglass side to provide the optodes from photobleaching.

The optode approach is a non-invasive optical technique (Blossfeld & Gansert 2007). The measurement principle is based on the fluorescence decay time of pH- or O_2-sensitive indicator dyes (Gansert & Blossfeld 2008; Gansert et al. 2006; Huber et al. 2001; Klimant et al. 2001). The foils carry an analyte-insensitive luminophore as reference and an analyte-sensitive fluorophore with similar excitation spectra, but different decay times. The phase angle of excitating light changes depending on the ratio of intensity of reference luminophore and indicator fluorophore and directly reflects analyte concentration. A glass fiber, connected to a light source and measuring device (pH-1 mini, PRESENS, Regensburg, Germany) conveys the excitation light pulse (470nm). The glass fiber is moved by step motors (compare Blossfeld & Gansert 2007) over the 8x22mm grid in 2mm steps (5 seconds per step, 1 measurement per second, resulting in 44 measuring points on an area of 10x30mm). A

measuring cycle (25 min per rhizobox) was done every 2 hours. The average of each 5 measurements per measuring point was processed with SigmaPlot (Systat Software, Inc., San Jose, CA, USA) to create contour plots of pH-distribution along the optode foils. The setup is shown in Fig. 1.

4.1.2 Soil solution sampling

The front side of the rhizobox allows soil solution sampling through inserted tubes, which contain guiding sleeves (raster plates) for steel capillaries (0.6mm diameter). A sampling unit is formed by two of these raster plates with a filter on the inner side directly contacting the rhizosphere (pore size <0.2µm to prevent microorganism (MO) contamination of samples) and septum in-between to provide watertightness. The holes in the inner plate narrow to 0.4mm diameter to prevent capillaries from penetrating the filter.
The steel capillaries were sterilized with ethanol [96%] and rinsed thoroughly with demineralised water before installing them permanently into the sampling units. Due to the water column inside the rhizoboxes they could not be removed without water loss. For soil solution sampling they were connected to a low-pressure chamber, which enabled sampling of soil solution (30-50µl) directly into Eppendorf tubes. The first drop (empty volume) was discarded. Samples were directly frozen (-20°C). MO-free soil solution sampling was possible using this setup.

4.2 Capillary Electrophoresis

Capillary Electrophoresis (CE) was used to determinate low molecular weight organic acids with a salicylate electrolyte (Bazzanella et al. 1997). The system G1600A (Agilent, Böblingen, Germany) was used, including a diode array detector and using fused silica capillaries (Polymicro, Phoenix, USA, 75µm inner diameter, 64.5 cm total length, 56 cm effective length). The electrolyte contained 7.5mM salicylic acid, 15mM TRIS, 0.5mM dodecyltrimethylammonium hydroxide and 0.3mM $Ca(OH)_2$ and was freshly prepared every day. Separation ensued at 30kV and 25°C. Quantification was performed by using external aqueous standard solutions of the acids (5, 10, 20µmol) and internal standardization by phenylacetic acid in samples. Precision was best in a concentration range from 0-40µmol/l

with linear calibration curves. Samples were diluted accordingly and measured 2 times after flushing the capillary with 0.1 NaOH for 5 min, water for 1 min and electrolyte for 5 min. During analysis, up to 8 low molecular organic acids were identified from the soil solution samples (oxalate, formate, succinate, malate, acetate, glyoxylate, lactate and citrate).

5. Results

In glass beads and at 20°C, *A. anomala, A. philoxeroides* and *S. variegata* showed diurnal rhythms of acidification in the root vicinity throughout the whole experiment (Fig. 2). These rhythms were most intense during the initial waterlogging phase with an amplitude of pH of 0.3-0.4 (Tab. 1), with lowest pH values being around 6.6 and highest around 7. These rhythms grew weaker during flooding, and the amplitude receded to 0.2 units. They re-intensified again after resurfacing toward the initial values. *A. anomala* was an exception here since the roots grew strongly, and the active root zone eventually left the measuring window.

In direct comparison of a 10°C and 20°C treatment in glass bead substrate (Fig. 3) could the same rhythms be found, except for *S. variegata* in the 20°C treatment, where no active root grew along the optode. The amplitude of acidification recovered after submergence back to the initial state before submergence. The pH-spread was slightly greater (0.4-0.5, Tab. 1) than in the initial experiment (Fig. 2). The active root zone of *A. anomala* produced 0.6-1.0 units spread around its active root tips. *A. philoxeroides* showed only a narrow zone of influence in the 10°C treatment, while acidification in the 20°C treatment was not that much stronger, but more widespread through two roots, one newly growing from the left side during recovery. No great differences occurred between the two temperature treatments concerning pH.
The oxygen content (Tab. 2) was with 60-65vol% at the 20°C-treatment highest for *S. variegata* throughout the experiment, the other two species being 5 to 10% lower with a general decrease of 20vol% during flooding. Only a slight increase of 5vol% occurred after resurfacing and re-exposure of plants to air. The reactions in the 10°C treatment were similar to that of the 20°C treatment except for *S. variegata*, which is almost 20vol% lower than

during 20°C. Organic acid occurrence (Fig. 4, data for 10°C not shown) was also very similar between the two glass bead temperature treatments with acetate showing the strongest appearance (up to 200µmol/l, *A. philoxerides* cycle, Fig. 4). Malate was also present in respectable amounts of 100µmol/l in *A. anomala* and *A. philoxeroides*.

The 20°C cycle in sedimental soil (Fig. 5) showed a generally higher pH almost 1 unit above the glass treatments at 20°C (7.2-8). There were no great differences between species, but contours seemed less pronounced as in the glass bead treatments. The amplitude of pH dynamic was slightly less than in the glass- and 10° treatment. PH in 10°C sedimental substrate was much lower than in 20° substrate for all three species, but lowest for *A. anomala* (6.2-7.1, spread 0.7, otherwise spread was the same as in 20°C treatment, see Tab. 1). The 10°C sediment substrate treatment was also slightly lower in pH (always below 7.2, see Tab.1) than the 10°C glass bead substrate treatment (Upper limit 7.3-7.8, Tab.1).

A visible difference appeared in oxygen content (Tab. 2): Both treatments started with quite similar oxygen contents (55-60vol%). A slight decrease during flooding (except *A. anomala* at 10°) was followed by a stronger rise (10-20vol%) in the 10°C treatment, while values in the 20°C treatment did not increase again.

Formate is dominating the LMWOA detection in sediment setup (Fig. 6). In the 10°C treatment concentrations did seldom exceed 100µmol/l, but in the 20°setup form and also lac contributed to OA values of 1000µmol/l and more (Fig. 7). An accordance could be seen between elevated values and the root path of *A. anomala*. The influence of several roots was too strong in the other two species to determine a relation of exudation to one single root.

The flooding-resistant species were compared to less resistant species. *Z. mays* and *H. vulgare* showed an increase of exudation already in the first days of waterlogging (~200µmol/l, mostly acetate, data not shown) with beginning hypoxia in soil. *P. arundinacea* as a more adapted plant does not show such an increase, and no further OA exudation peak occurs. All three species show strong diurnal acidification activity with an amplitude of 0.5 to 0.8 (Tab. 1) in both 10°C and 20°C setup (Fig. 8), the 10°C treatment showing slightly lower overall pH values then the 20°C treatment during waterlogging and beginning submergence. These rhythms ceased with prolonged submergence in *Z. mays* and *H. vulgare* and did not recover with resurfacing, neither in 10 nor 20°C treatment. The plants did not survive. *P. arundinacea*, however, keeps its diurnal activity up (if weaker) and recovers without further problems.

This process of diminishing and re-intensifying or ceasing is mirrored by the oxygen depletion which took place during this treatment (Tab. 2). *Z. mays* and *H. vulgare* could not supply oxygen into their rhizosphere (5-10vol%), which became already visible during the initial waterlogging phase. The oxygen content dropped in the following submergence to 0-5vol% during flooding and did not rise again after submergence ended. In the rhizosphere of *P. arundinacea* at least 20-35vol% were maintained during the whole treatment, and the plant survived.

6. Discussion

The dual-access rhizobox has proven to be a practical tool for non-invasive and relatively easy controllable rhizosphere research even under flooded conditions, and it provides an excellent approach for intensive, non-invasive research on root-soil interactions since indeed a high resolution of pH, oxygen and OA sampling is achieved.

Diurnal rhythms of acidification and role of OA

All of our observed species, the flooding tolerant- as well as the non-tolerant ones, showed a pattern of diurnal acidification. This occurred in all substrates and as long as plants were alive, which proves a definite contribution of the plant to these rhythms.

Such diurnal patterns were already described for *Vigna unguicula, Medicago sativa* and *Juncus effusus* (Jarvis & Hatch 1985, Rao et al. 2002, Blossfeld 2007). Yet our findings do only show acidifying effects, no nightly alkalinization as reported from *Medicago* and *Vigna*. The root tips were zones of strongest acidification, as already confirmed in earlier findings (Jones 1998, Marschner 1995). The pH surrounding a growing root may be 1-2 units lower than that of the bulk soil (Hinsinger et al. 2003).

Rhizospheric acidification may be caused by several combined effects. Nutrient uptake means exchange of protons for cations, which react acidifying. Plants can even induce active proton extrusion under Fe-deficiency by increasing H^+-ATPase activity, which also leads to acidification of the rhizosphere (Zocchi & Cocucci 1990). While a plant is photosynthetically active, the gradient of O_2 partial pressure lets oxygen diffuse to the normally more hypoxic roots. Radial oxygen loss (ROL) can then act as an additional proton source, for example by oxidizing Fe_2^+ and freeing protons (Kirk 2004). Oxygen exudation is also protection from reduced substances in the root vicinity. For *A. anomala* we have already found a strong effect of increased ROL into an anoxic rhizosphere after two days flooding, accompanied by strong acidification (Schreiber et al. in preparation). The conversion of NH_4^+ to Glutamine can also contribute to rhizospheric acidification, since uptake of NH_4^+ against protons is a prevailing way of nitrogen uptake in wetland plants (Wagner 1991, Foyer & Noctor 2002).

Some plant species (*Lupinus albus*, Gerke et al. 1994, Hagström et al. 2001, Watt & Evans 1999) are also known to exude pH-relevant amounts of low molecular weight organic acids. Yet OA occurrence in this study was too low for them to have an effect on rhizospheric pH.

This can be seen in Fig. 5 and 7, where strongest exudation occurs in the 20° sediment setup, yet without pH reaction. The exuded amounts in the other treatments were so low that they can be considered insignificant for influence on pH in these species. Yet they can give a hint towards microorganism activity in the root vicinity. As mentioned, OA occurrence was highest in non-sterile sediment at 20°C temperature, which indicates that not only plants contribute to OA amounts in soil solution here, but also MO's. This is supported by the fact that we did find 3-5 times lower amounts of OA in the 20°C glass bead treatment. The fact that there were almost no temperature effects on acidification and OA exudation between the 10°C and 20°C MO-free treatment also supports this. MO's react strongly to temperature differences (Lösch 2004), but OA exudation of *A. anomala, A. philoxeroides* and *S. variegata* in sterile surrounding showed no temperature dependent relations (Schreiber et al. in preparation). Still all treatments showed regular rhizospheric acidification, and the tolerant species returned after two weeks submergence to the same status they had before that event. The diurnal rhythms of the non-tolerant *Z. mays* and *H. vulgare* ceased as the plants died. Therefore a regular rhythm of rhizospheric acidification can be seen as an indication for an intact root metabolism.

pH and oxygen

Oxygen dynamics follow diurnal rhizosphere acidification (and photosynthetic) rhythms, this is known from the three tolerant species (Schreiber et al., in preparation). Stronger ROL was observed during daytime, almost completely receding during the night. Therefore oxygen also plays an important role here in acidification along with high partial pressures of CO_2 in sediment during anoxia, which are normally buffered by the higher $CaCO_3$-content of the sediment. The normal pH of the aerated sediment is around 7.7, pH in glass bead substrate mostly between 6.5 and 7.5. The 10° sediment treatment had a far lower pH (1 unit) than the 20° treatment, despite the fact that the lower temperature should impede root and MO respiration and lower CO_2 partial pressure, also by increasing CO_2 solubility. But there was also a stronger oxygen loss (also increasing solubility in soil solution) than in the 20° setup, which could account for the difference.

All three tolerant species and *P. arundinacea* were able to release oxygen into the rhizosphere. Contrary to this*, Z. mays* and *H. vulgare* had the lowest oxygen contents in their rhizosphere during waterlogging (5-10vol%), and with beginning submergence oxygen content dropped close to zero and did not recover. With almost no aerenchyma and slow gas diffusion in water (10^4 times lower than in air, Armstrong 1979) photosynthesis and root

pressure of *Z. mays* and *H. vulgare* failed. The hydraulic conductivity of root systems of *Z. mays* and *Helius annuus* has been described to decrease under anoxia (Everard & Drew 1987,1989, Birner & Steudle 1992), while roots of *H. vulgare* loose potassium and chloride (Hiatt & Lowe 1967). Normally, water can diffuse into plant cells through the lipid bilayer or be conducted through aquaporins (Henzler et al. 1999). Under anoxia, membrane potentials change, decreasing root pressure, hydraulic conductivity and permeability to solutes, and make roots quite impervious. Without root pressure and solute transport, carbon assimilation fails and the plant dies.

Survival strategies and comparison

A. anomala, A. philoxeroides, *S. variegata* and even *P. arundinacea* have proven to be much better suited to survive flooding events than *Z. mays* and *H. vulgare*, which was to expect. With a stable root system and the ability to mobilize anaerobic metabolic pathways fast to prevent energy deficits and therefore membrane potential problems (Perata & Alpi 1993, Mommer et al. 2004) they are able to keep up root turnovers even in sudden anoxic conditions. Cutical thickness and general resistance was found lower in *A. philoxeroides* leaves which were build underwater, a process described by Frost-Christensen et al. (2003) to allow better gas exchange and therefore photosynthesis under water. Well developed aerenchyma in all three tolerant species (Colmer 2003) makes them well suited to survive flooding events in TGR area.

Z. mays and *H. vulgare* are definitely not suited for the challenging situation at TGR, but they were valuable for this work to actually assess the capabilities of the resistant species. The oxygen depletion in the rhizobox by the non-tolerant species might serve as an example, pronouncing the contrasts. Being able to compare completely different reactions with the same method improves interpretation and validation of the results.

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Tables

Table 1: Range of pH-values in the rhizosphere of *A. anomala, A. philoxeroides, S. variegata, Z. mays, H. vulgare* and *P. arundinacea*, measured by means of planar optodes directly at the root-soil interface in a 10°C and 20°C treatment, in glass bead substrate and sediment substrate, during 2 weeks waterlogging followed by 2 weeks flooding and another 2 weeks waterlogging, corresponding to Figures 2, 3, 5 + 8. Maximum was reached at about 5p.m., minimum between 4 and 8a.m.

wl = waterlogged, fl = flooded, rec = recovery (waterlogged)

(pH) indicates the plant has died, the pH is the average pH measured with the optode foil.

pH spread overview	sediment substrate					
	10°C			20°C		
	wl	fl	rec	wl	fl	rec
A. anomala	6.2-6.9	6.2-7.1	6.2-6.9	7.4-8	7.4-8	7.4-8
A. philoxeroides	6.9-7.2	6.8-7.2	6.8-7.2	7.2-7.6	7.8-8	7.6-8
S. variegata	6.9-7.1	6.8-7.1	6.7-7.1	7.7-8.1	7.4-8	7.4-8
	glass bead substrate					
	10°C			20°C		
	wl	fl	rec	wl	fl	rec
A. anomala	6.9-7.8	6.9-7.8	6.8-7.8	6.5-7.2	6.7-7.4	6.7-7.2
A. philoxeroides	6.8-7.1	7-7.4	6.9-7.4	6.5-7.1	6.5-6.9	7-7.7
S. variegata	6.6-7.3	6.7-7.3	6.6-7.1	6.9-7.3	7.2-7.8	6.5-6.9
Z. mays	6.6-7.2	6.8-7.6	# (7.6)	6.5-7.5	6.5-8.2	# (6.6)
H. vulgare	5.2-6.6	6.5-7.2	# (6.4)	6-6.7	5.8-6.5	# (6.4)
P. arundinacea	5.6-6.8	6.5-7	6.5-6.7	6.6-7.6	7.2-8.4	7.0-7.8

Table 2: Oxygen concentration [vol% air saturation] in the rhizosphere of *A. anomala, A. philoxeroides, S. variegata, Z. mays, H. vulgare* and *P. arundinacea*, measured by means of planar optodes directly at the root-soil interface in a 10°C and 20°C treatment, in glass bead substrate and sediment substrate, during 2 weeks waterlogging followed by 2 weeks flooding and another 2 weeks waterlogging, corresponding to Figures 2, 3, 5 + 8.
wl = waterlogged, fl = flooded, rec = recovery (waterlogged)

Soil oxygen content [%air saturation]	sediment substrate					
	10°C			20°C		
	wl	fl	rec	wl	fl	rec
A. anomala	70-75	70-80	75-90	50-55	45-50	45-55
A. philoxeroides	55-60	50-55	55-60	60-50	55-60	50-60
S. variegata	55-60	50-55	60-70	50-70	55-60	40-50
	glass bead substrate					
	10°C			20°C		
	wl	fl	rec	wl	fl	rec
A. anomala	50-65	45-55	45-60	50-60	30-40	30-45
A. philoxeroides	45-50	40-45	45-50	38-50	30-35	30-40
S. variegata	40-45	30-40	35-45	60-65	50-60	55-65
Z. mays	15-20	5-10	30-35	5-10	0-5	0-5
H. vulgare	25-30	15-20	30-35	5-10	0-5	0-5
P. arundinacea	35-40	20-25	70-75	20-30	20-30	30-35

Figures

Figure legends

Fig. 1 Dual access floodable rhizobox: Access from backside via planar optodes (yellow: pH-sensitive, orange: O_2-sensitive), access from the corresponding front side via steel capillary soil solution sampling. Temperature control and mountable water tank. Automated reading of optode foils was done with an optical fiber moved by a step motor system (not shown).

Fig. 2 pH in the rhizosphere of *A. anomala*, *A. philoxeroides* and *S. variegata*, measured using planar optodes directly at the soil-root interface in glass bead substrate at 20°C during 2 weeks. Three subsequent diagrams form a unit consisting of measurements at 5p.m., 0a.m. and 8 a.m., one unit per plant is shown for each treatment phase (waterlogging, flooding, recovery). Circles indicate corresponding soil solution sampling spots. White arrows mark position of roots.

Fig. 3 pH in the rhizosphere of *A. anomala*, *A. philoxeroides* and *S. variegata*, measured using planar optodes directly at the soil-root interface in glass bead substrate at 10°C and 20°C during 2 weeks. Three subsequent diagrams form a unit consisting of measurements at 5p.m., 0a.m. and 8 a.m., one unit per plant is shown for each treatment phase (waterlogging, flooding, recovery). White arrows mark position of roots.

Fig. 4 LMWOA occurrence in the rhizosphere of *A. anomala*, *A. philoxeroides* and *S. variegata*, measured by low invasive soil solution sampling in glass bead substrate at 20°C during 2 weeks waterlogging, submergence and recovery.

Fig. 5 pH in the rhizosphere of *A. anomala*, *A. philoxeroides* and *S. variegata*, measured using planar optodes directly at the soil-root interface in sediment substrate at 10°C and 20°C during 2 weeks. Three subsequent diagrams form a unit consisting of measurements at 5p.m., 0a.m. and 8 a.m., one unit per plant is shown for each treatment phase (waterlogging, flooding, recovery). White arrows mark position of roots.

Fig. 6 Exemplary measurements of LMWOA occurrence in the rhizosphere of *A. anomala*, *A. philoxeroides* and *S. variegata* done by means of low invasive soil solution sampling at 4p.m. in sediment substrate at 10°C during 2 weeks waterlogging, submergence and recovery.

Fig. 7 Exemplary measurements of LMWOA occurrence in the rhizosphere of *A. anomala*, *A. philoxeroides* and *S. variegata*, done by means of low invasive soil solution sampling at 4p.m. in sediment substrate at 20°C during 2 weeks waterlogging, submergence and recovery.

Fig. 8 pH in the rhizosphere of *Z. mays*, *H. vulgare* and *P. arundinacea*, measured using planar optodes directly at the soil-root interface in sediment substrate at 10°C and 20°C during 2 weeks. Three subsequent diagrams form a unit consisting of measurements at 5p.m., 0a.m. and 8 a.m., one unit per plant is shown for each treatment phase (waterlogging, flooding, recovery). White arrows mark position of roots.

Figure 1

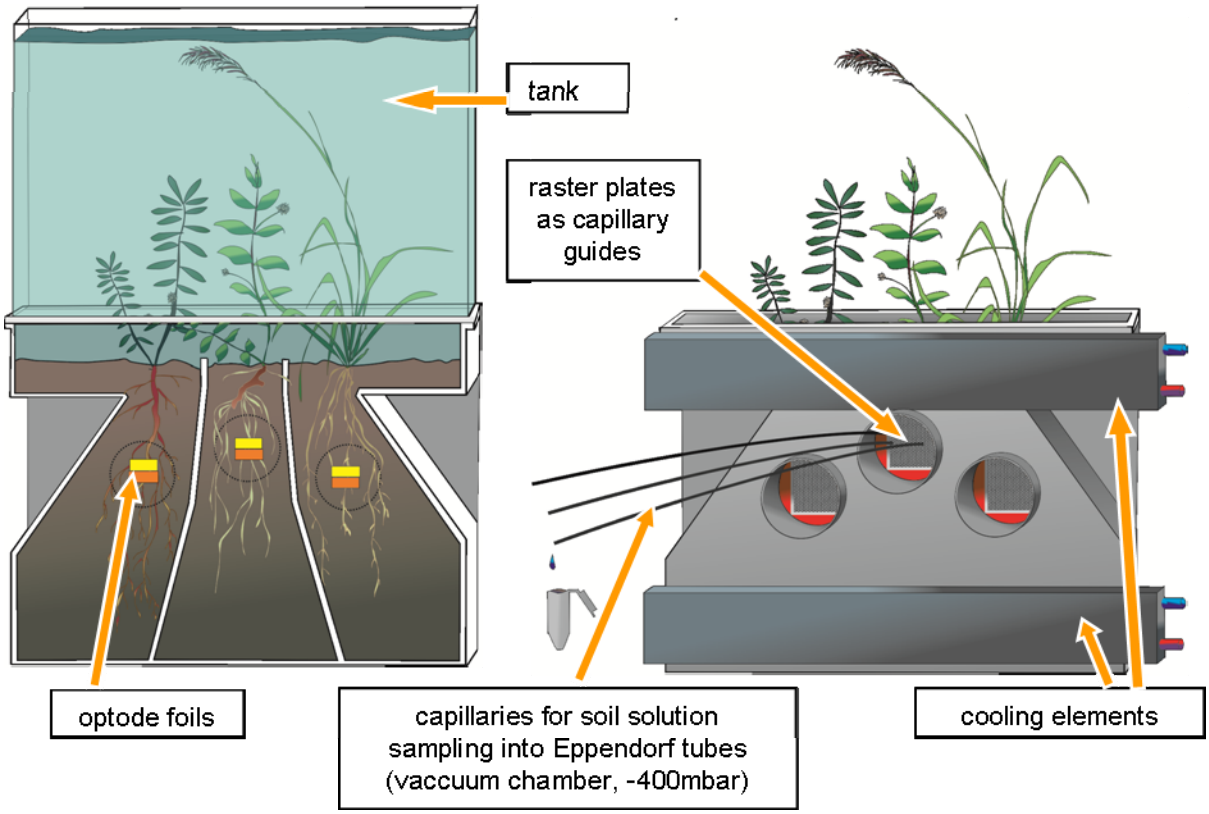


Figure 2

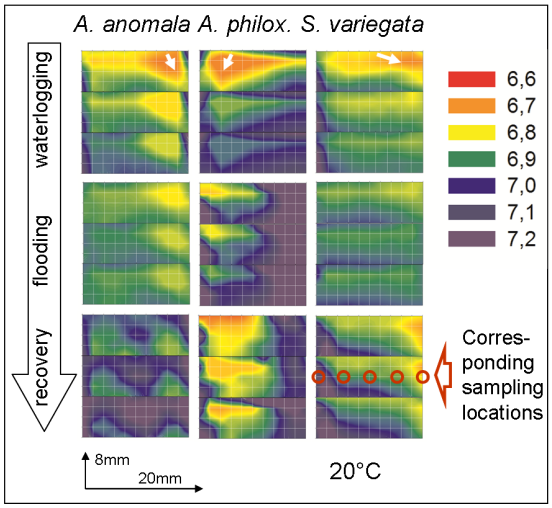


Figure 3

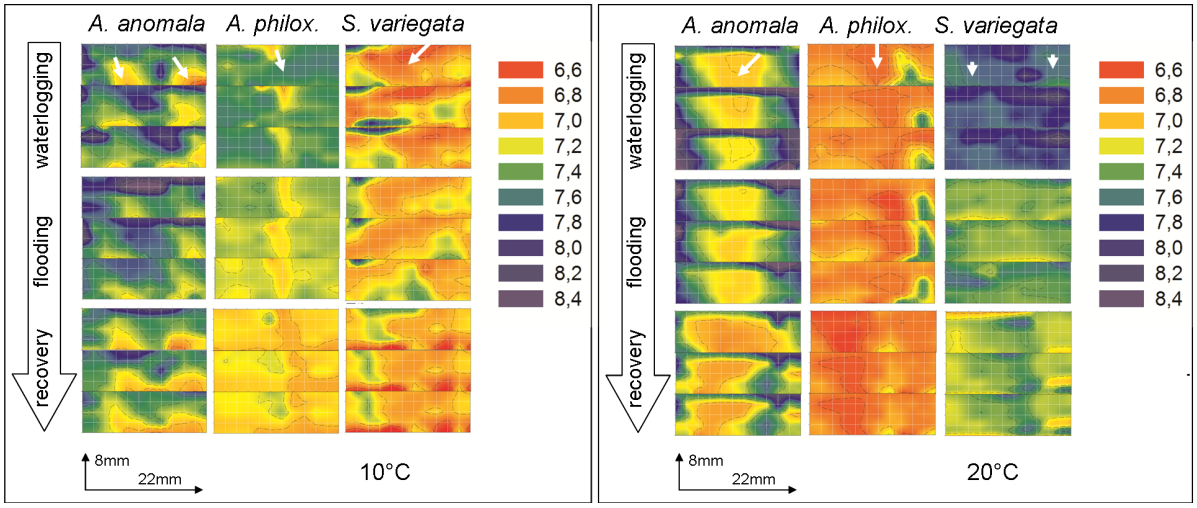


Figure 4

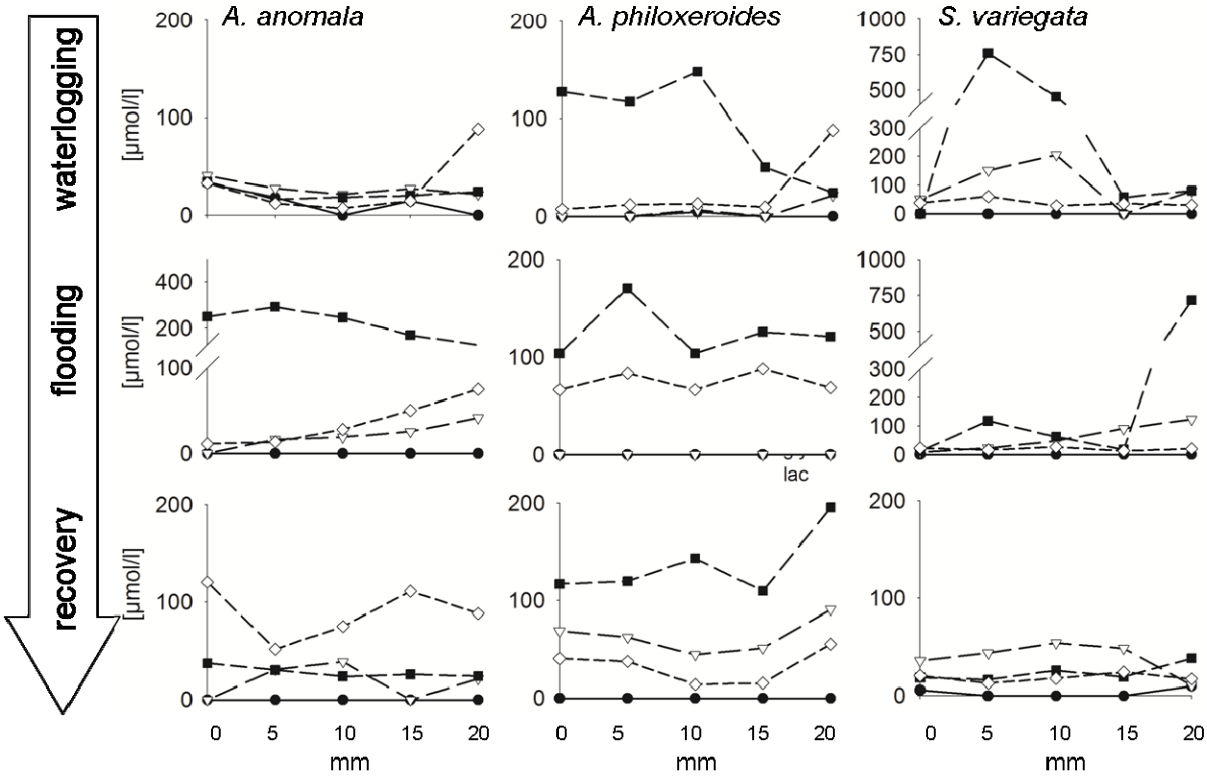


Figure 5

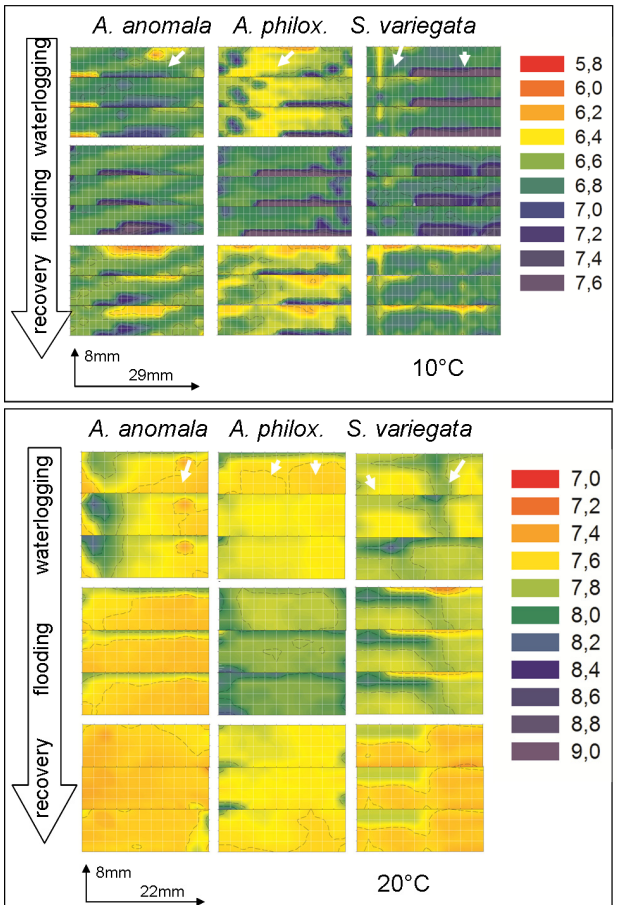


Figure 6

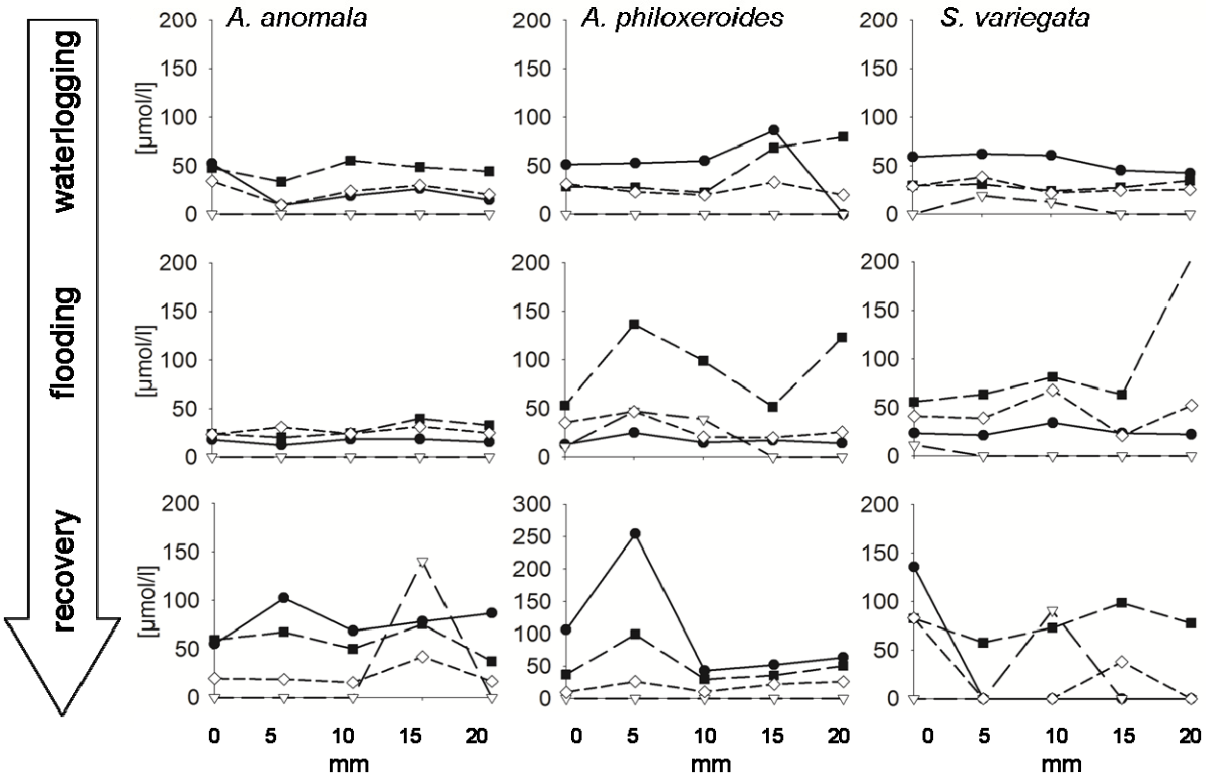


Figure 7

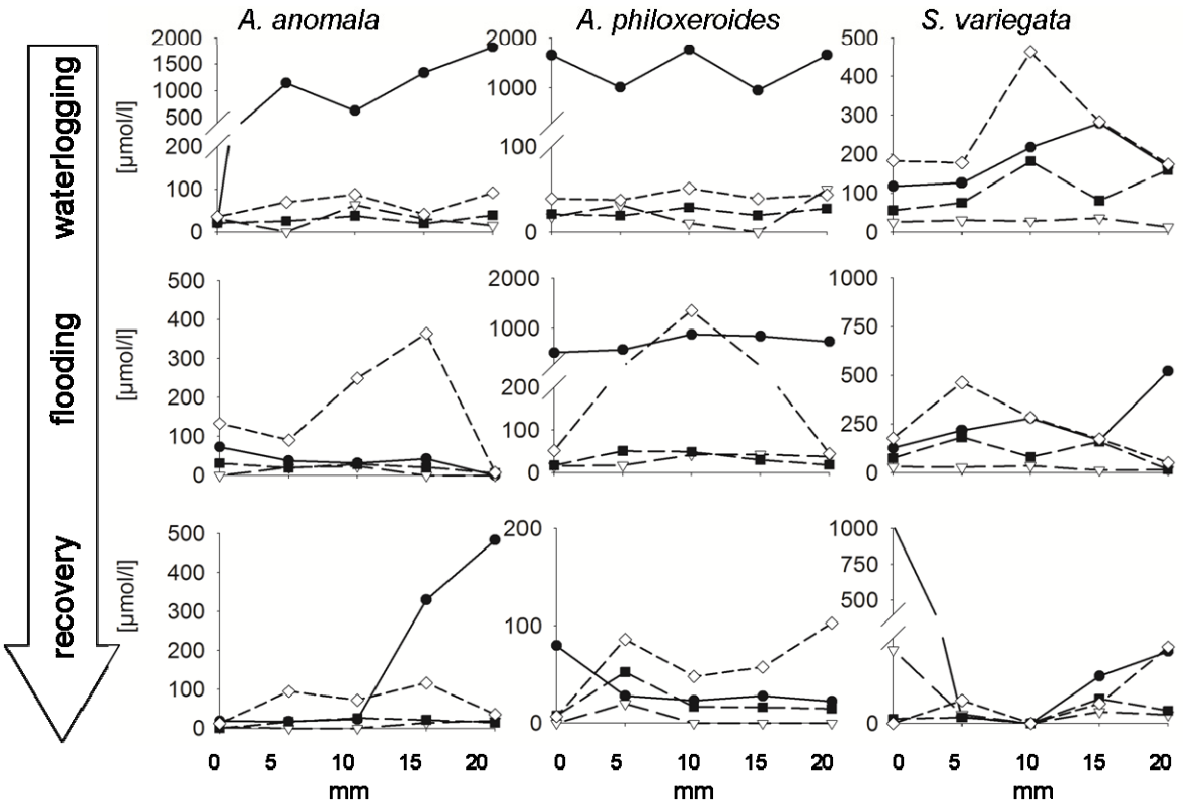
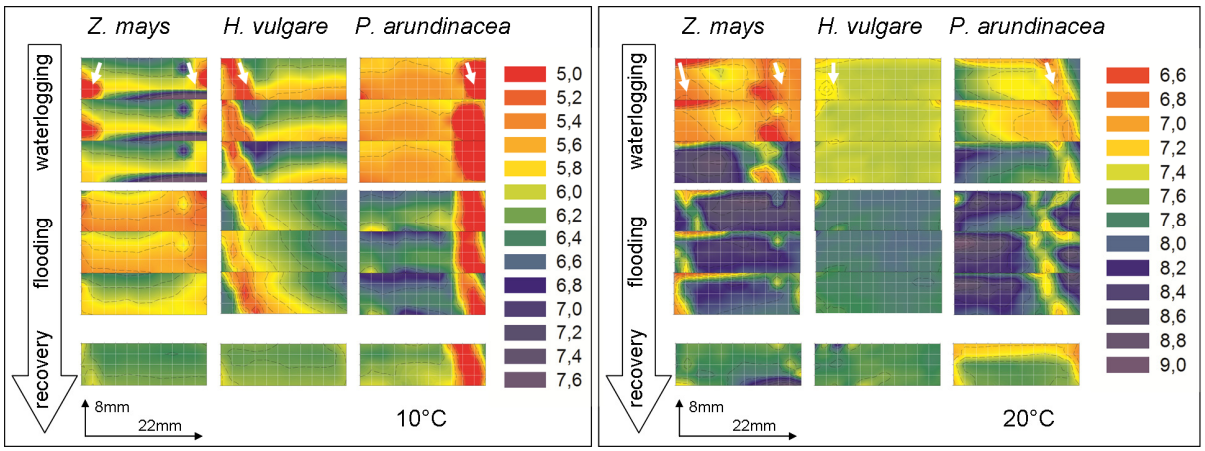


Figure 8



9. List of Abbreviations

MO	Microorganisms
TGR	Three Gorges Reservoir
TGD	Three Gorges Dam
LMWOA	Low Molecular Weight Organic Acids
OA	Organic Acids
PAR	Photosynthecially Active Radiation
MSC	Microsuction Cups
SWU	Southwest China University
PES	Polyethersulfone
PEEK	Polyetheretherketone
CE	Capillary Electrophoresis
HPLC	High Pressure Liquid Chromatography

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Hierrmit erkläre ich,

dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hiermit erkläre ich,

dass ich weder die vorliegende noch eine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden habe.