

RICE AND *PHRAGMITES*: EFFECTS OF ORGANIC ACIDS ON GROWTH, ROOT PERMEABILITY, AND RADIAL OXYGEN LOSS TO THE RHIZOSPHERE¹

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Young *Phragmites* plants were grown in two cocktails of monocarboxylic acids (C₁–C₅) at pH 6, where the concentration of each acid was innocuous and the total undissociated (potentially toxic) concentrations were 0.35 mmol/L and 0.42 mmol/L. Rice plants were subjected to 1.5 mmol/L acetic acid at pH 4.5 (undissociated concentration = 1.05 mmol/L). In *Phragmites*, each cocktail curtailed root growth especially and induced premature shoot senescence. In both species, after 3–5 d of treatment, radial oxygen loss (ROL) from apical regions of adventitious roots, and from *Phragmites* laterals, was reduced to very low values and associated with cell wall lignification and suberization in the surface cell layers. At later stages of treatment, rice responded to acetic acid in similar ways to *Phragmites*, with the development of intercellular and callus type occlusions in the gas space system, vascular blockages, and the failure of laterals to emerge. The results are relevant to the supply of oxygen from *Phragmites* roots to sediments for the phyto-purification of waste waters, to the efflux of methane and carbon dioxide from wetlands, and to rice cultivation.

Key words: lignification; organic acids; *Phragmites*; phytotoxins; radial oxygen loss; rhizosphere; rice; root permeability.

In *Phragmites australis* (Cav.) Trin. ex Steud., individual lower monocarboxylic acids (and sulphide) have been shown, in laboratory experiments, to induce almost all the symptoms associated with reed decline in the field (Armstrong, Afreen-Zobayed, and Armstrong, 1996; Armstrong, Armstrong, and Van der Putten, 1996; Armstrong et al., 1996b; Armstrong and Armstrong, 1999). These include stunting and death of roots and shoots, failure of laterals to emerge, bud death, premature senescence of shoots, blockages within the gas space and vascular systems, lower levels of starch in rhizomes and cell wall lignification and suberization of surface cell layers of laterals and apical regions of adventitious roots. The lower the pH of the rooting medium, the greater is the concentration of the lipid-soluble, toxic, undissociated organic acid molecules (Hollis, 1967; Jackson and Taylor, 1970; Rao and Mikkelsen, 1977; Lynch, 1978, 1982) and the more severe are the symptoms (Armstrong and Armstrong, 1999). Commonly, mixtures of many organic acids can occur in the field, including die-back sites. They are produced from the decaying underground parts of the plant (Kovacs et al., 1989; Armstrong and Armstrong, 1999) and may be generated from other sources of decaying organic matter. For example, at a eutrophic site in the Czech Republic, bud death and reed decline have been associated with a strongly reducing organic layer in the surface of the lake sediment (Cízková, Strand, and Lukavská, 1996) and with mixtures of organic acids in interstitial waters (Cízková et al., 1999). So far, however, there has been no direct evidence that cocktails of dilute organic acids, at a comparatively high pH of 6 (as can occur in the field) and such that the concentration of each acid is innocuous, can be harmful to the reed. In this study, the concentration of each acid in a cocktail was 1 mmol/L at pH 6,

with the undissociated concentration being ~0.06 mmol/L. It has previously been shown that monocarboxylic acids individually at this concentration are not toxic to *Phragmites* (Armstrong and Armstrong, 1999).

Various diseases of rice have also been associated with toxicity due to sulphide and organic acids. For example, in Aki-ochi, or “autumn decline,” symptoms include early flowering, discoloration of roots, and progressive decline of the plant. The lower organic acids, including acetic, propionic, and butyric have been shown variously to reduce the uptake of P, K, Si, Mn, Mg, Ca, and NH₄-N (Mitsui et al., 1954; Takijima, Shiojima, and Arita, 1960; Tanaka and Navasero, 1967; Rao and Mikkelsen, 1977) and to reduce root respiration (Tanaka and Navasero, 1967). Rao and Mikkelsen found that individually these acids at 10 mmol/L and pH~4 caused the death of seedlings within 1–2 d; at 1 mmol/L (pH 4.6) the seedlings showed signs of desiccation. Increased organic acid toxicity at lower pH has also been shown to apply to rice seedlings (Tanaka and Navasero, 1967; Rao and Mikkelsen, 1977). So far, however, there appears to have been little documentation of any anatomical effects of organic acids on rice.

In wetland plants, the passage of atmospheric oxygen via the internal gas space system of the plant to the underground organs is vital to maintain aerobic metabolism, while the radial diffusion of oxygen out of the permeable parts of the root system into the rhizosphere protects those vulnerable parts by promoting oxidations of potential phytotoxins, e.g., FeII, MnII, sulphide, and organic acids commonly existing in flooded soils (Armstrong, 1970; Trolldenier, 1988; Conlin and Crowder, 1989; Armstrong, Armstrong, and Beckett, 1992; Begg et al., 1994; Saleque and Kirk, 1995; Wang and Peverley, 1999). Currently, there is still much interest in the efflux of oxygen from the roots of *Phragmites*, *Typha*, and other aquatic macrophytes, which aids water purification in both natural and artificial reed beds by inducing nitrification in the rhizosphere (Reddy, Patrick, and Lindau, 1989). Furthermore, radial oxygen loss (ROL) from root to rhizosphere inhibits methanogenesis (Oremland, 1988; Conrad, 1989), promotes CH₄ oxidations within the rhizospheres (Epp and Chanton, 1993; Gilbert

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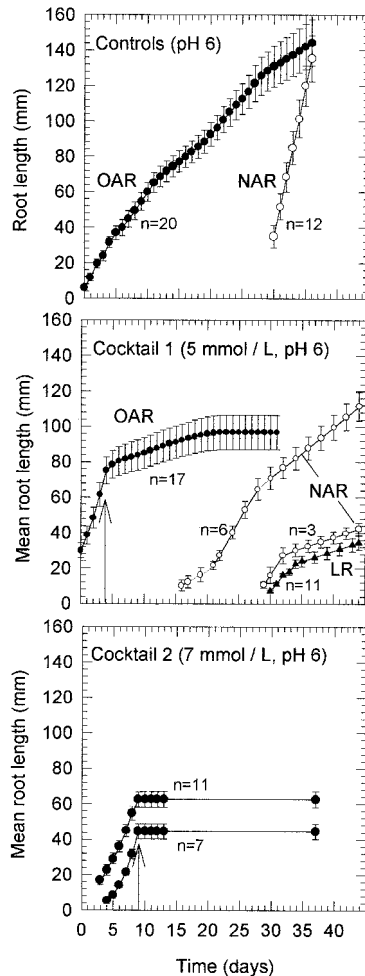


Fig. 1. *Phragmites*: growth of adventitious roots from control and organic acid treatments. Cocktail 1: acetic, propanoic, n-butyric, iso-butyric and caproic acids: each 1 mmol/L; total concentration = 5 mmol/L; total undissociated (toxic) concentration = 0.35 mmol/L. Cocktail 2 contained the above acids together with formic and valeric acids, each also 1 mmol/L; total concentration = 7 mmol/L; total undissociated (toxic) concentration = 0.42 mmol/L. Each culture solution was at pH 6. OAR = original adventitious roots; NAR = new adventitious roots; TLR = thick lateral roots (atypical); arrows refer to start of acid treatments. Bars = ± 1 SE.

and Frenzel, 1995), and can thereby reduce potential efflux of CH_4 from the plants by 34% for *Phragmites* (Grunfeld and Brix, 1999) and by >80–90% for rice (Holzapfel-Pschorn, Conrad, and Seiler, 1986; Frenzel, Rothfuss, and Conrad, 1992). Natural and cultivated wetlands contribute 40–50% of total emissions of CH_4 to the atmosphere, accounting for 7–9% of global warming. In vegetated wetlands >90% of CH_4 emitted from rice paddies (Banker et al., 1995) and ~62% from *Phragmites*-dominated habitats (Grunfeld and Brix, 1999) can pass into the atmosphere via the root \rightarrow internal gas space \rightarrow shoot pathway; in certain species this is augmented during the growing season by convection (Chanton et al., 1993; Sorrell and Boon, 1994; Whiting and Chanton, 1996; Grunfeld and Brix, 1999). Thus, factors affecting root permeability, ROL, and the porosity of the gas-space system of the plant are clearly important. So far, there appears to have been no published work investigating the possible effects of phytotoxins on root permeability to oxygen and its radial loss to the rhizosphere.

This study seeks to test three hypotheses: (1) that cocktails of the lower monocarboxylic organic acids, where the concentration of each acid is innocuous, may nevertheless be harmful to *Phragmites*, (2) that rice responds to organic acid toxicity in ways similar to *Phragmites* by inducing cell wall thickening in the hypodermal layers of adventitious root apices and in the epidermis of laterals, and blockages in the internal gas space and vascular systems, and (3) that in rice and *Phragmites*, organic acids, by inducing cell wall thickening in normally permeable regions of the root system, thereby also induce impermeability to oxygen and reduce ROL to the rhizospheres.

MATERIALS AND METHODS

Plant material—*Phragmites*—Overwintered 1-yr-old plants (original shoot height = 450–600 mm and rhizome length \sim 100 mm), which had been raised from seed collected from plants along the Humber estuary, were used as the source of plant material.

Rice—Seeds of cv. Norin 36 were germinated in the summer on moist tissue in shallow trays covered in polythene in a propagating frame with natural light; $T = 20^\circ\text{C}$. Germination occurred in \sim 1 wk and the trays were then transferred to the open bench in the glass house for 3–4 wk until the shoots were \sim 6–8 cm high. The seedlings were then transplanted into buckets (volume = 15 L) containing moist John Innes compost No. 2 (John Innes Institute, Norwich, UK); the water table was gradually raised over 2 wk, until the soil was flooded to a depth of \sim 2 cm. Conditions were: $T = 16^\circ\text{C}$ (minimum) and 29°C (maximum), with natural light, and an 18-h day length.

Treatments—*Phragmites*—The old roots were trimmed back to 1–2 cm and

Fig. 2. *Phragmites*: fresh, hand-cut, transverse sections (TS) of adventitious and fine lateral roots and of portions of whole lateral roots after 3 d (A–D) and 10 d (E–L) of exposure to cocktail 2 (formic, acetic, propanoic, n-butyric, iso-butyric, caproic and valeric acids, each 1 mmol/L; total concentration = 7 mmol/L; total undissociated (toxic) concentration = 0.42 mmol/L). Control medium contained no organic acids. Each culture solution was at pH 6. All sections stained with phloroglucinol + concentrated HCl to show lignification, red. Control adventitious roots (not shown) were unlignified up to 20 mm from apex. (A) Transverse section of adventitious root 1 mm from apex with cell wall lignification of hypodermis. Most of the root cap material had sloughed off. Bar = 0.27 mm. (B) High power view of outer layers of A. Bar = 0.13 mm. (C) Transverse section of adventitious root 3 mm from apex with cell wall lignification of hypodermal layers and of some cells of cortex, some cortical intercellular blockages and premature lignification of endodermis, protoxylem vessels, and medulla. Bar = 0.13 mm. (D) High power of outer layers of (C). Bar = 0.07 mm. (E) Transverse section of adventitious root 5 mm from apex with cell wall lignification of hypodermis, parts of cortex and stele and some intercellular blockages of cortex; also, blockages of protoxylem and phloem. Bar = 0.13 mm. (F) High power of outer layers of (E). Bar = 0.07 mm. (G) High power of stele and inner cortex of (E). Bar = 0.07 mm. (H) Transverse section of lateral from cocktail 2 treatment root \sim 6 mm from apex with pronounced lignification of epidermal cell walls. Bar = 0.07 mm. (I) Transverse section of lateral from control treatment \sim 6 mm from apex; note absence of lignification of epidermal cell walls; cf. (H). Bar = 0.07 mm. (J) Part of an entire lateral root showing cell wall lignification of epidermis, which is more pronounced subapically. Bar = 0.67 mm. (K) Apical region of entire lateral root from control treatment; note absence of lignification of epidermal cell walls; cf. (J) and (K). Bar = 0.27 mm. (L) High power of apical region of (J). Bar = 0.07 mm.



old, dead stems shortened to ~100–130 mm and the plants were transferred to black polythene-covered glass tubes (height = 400 mm; diameter = 50 mm) containing 25% Hoagland's solution to resume root and shoot growth under growth room conditions; the shoots received continuous lighting from the side; $T = 18^{\circ}\text{C}$; photosynthetically active radiation (PAR) = 80–100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The plants were secured in the tubes with the old culms emergent and the rhizomes submerged under ~40 mm of culture solution; they were arranged randomly in line, parallel to a bank of lights, and their positions changed every alternate day to ensure that, as far as possible, they experienced identical external conditions.

After 12–14 d, when the roots were ~1–30 mm and shoots 100–150 mm long, plants of similar size were selected and divided into three groups of eight plants each; their rhizomes and roots were immersed, as described above, in the following culture solutions, each having as the base 25% Hoagland's solution in stagnant agar (0.05% w/v) degassed by autoclaving: (1) control, containing no acids; (2) cocktail 1, containing a mixture of five lower volatile monocarboxylic organic acids (acetic, propionic, n-butyric, iso-butyric, and caproic) each at 1 mmol/L concentration (total undissociated concentration = 0.35 mmol/L); (3) cocktail 2, containing seven acids (formic, acetic, propionic, n-butyric, iso-butyric, caproic, and valeric) each at 1 mmol/L concentration (total undissociated concentration = 0.42 mmol/L). The pH of each culture medium was 6, adjusted by the addition of NaOH solution and using a portable pH meter (Camlab Ltd., Cambridge, UK). Half of the plants from each treatment received only one dose of the acid medium, which was left unchanged between days 1 and 5 for adventitious root ROL and anatomical assays. For the remaining plants, the media were renewed every alternate day; these plants were used for monitoring growth and senescence.

For measuring ROL from fine laterals, an additional group of nine plants was grown in 25% Hoagland's solution until adventitious roots were 180–290 mm long and the longest laterals 15–18 mm. (It was not possible to use plants from the main treatments as, here, the laterals from the cocktails were much shorter than those of the controls.) The cocktails were added during the period of ROL measurement (see relevant section).

Rice—When the shoots were ~30 cm high, individual tillers were removed, the roots cut back to 1–2 cm, washed, and each was transferred into a glass tube with the shoot base and root system submerged, as described for *Phragmites*, and kept in the growth room ($T = 22^{\circ}\text{C}$; PAR = 80–100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$); here the nutrient medium was 25% Yoshida solution (Yoshida et al., 1976), renewed on alternate days.

When the longest new roots were ~100 mm long, the plants were divided into two groups of six, for separate treatments, the base of each rooting medium being 25% Yoshida solution (Yoshida et al., 1976) in 0.05% w/v agar, degassed by autoclaving: (1) controls, at pH 4.5, without the addition of acetic acid; (2) 1.5 mmol/L acetic acid at pH 4.5 undissociated concentration = 1.05 mmol/L. For half the plants from each treatment the media were left unchanged between days 1 and 5 for root ROL and initial anatomical assays. The media of the remaining plants were changed every alternate day until day 14 for final anatomical examinations.

Growth—For *Phragmites*, shoot growth was recorded on at least nine shoots per treatment on each of the 4 d preceding treatments and during the first 4 d of the treatment period; adventitious root growth increments were measured until day 37 by marking the positions of the tips of selected roots (at least 17 per treatment) on the glass container and measuring the distances between marks using digital callipers (RS Components Ltd., Corby, Northamptonshire, UK). The growth of new adventitious roots and of the thick laterals from the cocktail 1 treatment was also monitored as were the onset of shoot senescence and the percentage of shoot senescence, viz. (number of senescing leaves/total number of leaves) \times 100, the latter being measured on day 22.

Anatomy—On alternate treatment days 1–6, for both species, transverse sections were made of selected adventitious roots and laterals from plants used in the ROL measurements. Further studies were made on *Phragmites*

after 10 d, and on rice plants after 14 d of continuous exposure to acetic acid and on the controls, including transverse sections of rhizome, root-rhizome junctions, and adventitious roots. *Phragmites* sections and entire laterals from both species were stained with phloroglucinol and concentrated hydrochloric acid to detect lignification (confirmed with aniline hydrochloride) and were occasionally stained with Sudan 111 to detect suberization. Sections of rice adventitious roots and rhizomes were left unstained as there was comparatively little lignification. Specimens were photographed using an Olympus BX40 photomicroscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Radial oxygen loss from roots—*Phragmites*—Radial oxygen loss along adventitious roots was measured using sleeving cylindrical Pt cathodes in conjunction with Ag-AgCl anodes, after the method of Armstrong and Wright (1975). Radial oxygen loss profiles were taken from ~3 mm subapically to the points at which laterals emerged and were measured on at least two roots from each plant per treatment, between days 3 and 5 after the start of the treatment period. This time was chosen because apical root wall lignification and suberization in the cocktail treatments were detectable at this stage (see RESULTS: ANATOMY). For ROL measurements, the control or treatment media were replaced with freshly deoxygenated 0.05% w/v aqueous agar containing 1/4-strength Hoagland's solution.

The ROL from lateral roots was detected by means of bare Pt wire cathodes (length = 50 mm; diameter = 0.37 mm), used in conjunction with Ag-AgCl anodes. The cathode was loosely coiled around the adventitious root in the region where the laterals were 10–15 mm long. The ROL was measured with the roots in freshly deoxygenated control-type medium for at least 8 h; this medium was then drained from the base of the tube so as not to disturb the electrode and then either cocktail 1 or 2 was added and ROL measurements were resumed. Electrodes were also inserted in the media in positions remote from the roots to measure background O_2 diffusion rates.

It was necessary to confirm, if possible, that reduced ROL from adventitious roots and laterals was a function of root wall impermeability and not related to an absence of oxygen within the adventitious roots. After ROL measurements were completed on *Phragmites* from the toxin treatments, apical 30-mm regions of adventitious roots were removed, with the plants still intact, and the sleeving Pt electrode moved around the cut end, to detect O_2 diffusing from the cut end. Also, in both species, attempts were made to blow air, via a stem base, through the rhizome and out of the cut ends of roots submerged under water.

Rice—The ROL from the apical and subapical regions of adventitious roots was measured using the same method as that described above for *Phragmites*. Measurements were taken 3–4 d after the roots had received a single dose of 1.5 mmol/L acetic acid at pH 4.5; for the control roots the medium was also at pH 4.5.

RESULTS

Growth—*Phragmites*—Cocktail 1 had the effect of stunting the growth of the original adventitious roots and fine laterals (Fig. 1, Table 1). With the cocktail 2 treatment, the adventitious roots stopped growing almost immediately and there was no production of new adventitious roots. After 20 d of treatment, those laterals that had emerged were extremely stunted, being only 0.5 mm long compared to 14 mm for the controls (i.e., from the region on the root where the laterals were longest). With cocktail 1 the laterals were also stunted, ~5 mm long. However, cocktail 1 was not sufficiently toxic to prevent the growth of new adventitious roots, which appeared in two flushes; also thick laterals of indefinite length and resembling adventitious roots were produced on adventitious roots that had stopped growing. We have previously noticed this effect in response to butyric acid (Armstrong and Armstrong, 1999); here this may well have been in response to n- and iso butyric acids in cocktail 1.

TABLE 1. *Phragmites*: effects of cocktails of organic acids on growth, radial oxygen loss from roots and shoot senescence.

	Control without organic acids	Cocktail 1 + 5 organic acids (each 1 mmol/L) Total undiss.conc. = 0.35 mmol/L	Cocktail 2 + 7 organic acids (each 1 mmol/L) Total undiss.conc. = 0.42 mmol/L
Growth per adventitious root (mm) over 4 d pre- ceding onset of treat- ments	(a) 21.8 ± 2.7 (17) (b) 16.6 ± 2.8 (17)	‡48.7 ± 4.5 (17)	‡34.3 ± 2.8 (17)
Growth per adventitious root (mm) over 4 d after onset of treatments	(a) 20.0 ± 2.5 (17) (b) 16.3 ± 3.6 (17)	‡7.7 ± 1.6 (17)	‡0 (17)
+ Fine lateral root length per root (mm) 20 d after start of treatments	13.8 ± 0.4 (30)	5.42 ± 0.71 (30)	0.51 ± 0.03 (30)
Typical radial oxygen loss 20 mm from adventitious root apex (ng O ₂ ·cm ⁻² ·min ⁻¹), 3–5 d af- ter start of treatments	80	2.5	0
Typical radial oxygen loss from amid fine lateral roots after 2.7 d of treat- ment (ng O ₂ /min)	20–25	0–0.5	0
Growth per shoot (cm) over 4 d preceding onset of treatments	(a) 5.2 ± 0.8 (13) (b) 4.1 ± 0.7 (13)	7.7 ± 1.0 (13)	5.1 ± 2.3 (9)
Growth per shoot (cm) over 4 d after onset of treat- ments	(a) 10.9 ± 3.9 (13) (b) 2.5 ± 0.7 (13)	‡3.9 ± 0.8 (13)	‡0.3 ± 0.1 (9)
Approx. onset of shoot se- nescence: day after start of treatments	30–31	21–23	6
Percentage of shoot senes- cence, 22 d after start of treatments [(no. senescing leaves/total no. leaves) × 100]	<2	25	49

Note: Cocktail 1 contained acetic, propionic, n-butyric, iso-butyric and caproic acids; cocktail 2 contained the same acids as 1, but with the addition of formic and valeric acids. The base for each cocktail was 25% Hoagland's solution in 0.05% w/v agar; undiss. conc. = concentration of undissociated (toxic) acid molecules. Values are given as means ± 1 SE and significant differences between an organic acid and its respective controls (*t* tests or Mann-Whitney rank sum test) are indicated as follows: † *P* = 0.007; ‡ *P* ≤ 0.001 where (a) = control relative to cocktail 1; (b) = control relative to cocktail 2; + from mid-lateral zone. Numbers in parentheses are the sample sizes. Curtailment of shoot growth was more pronounced than expected with cocktail 2, but here the shoots were relatively taller.

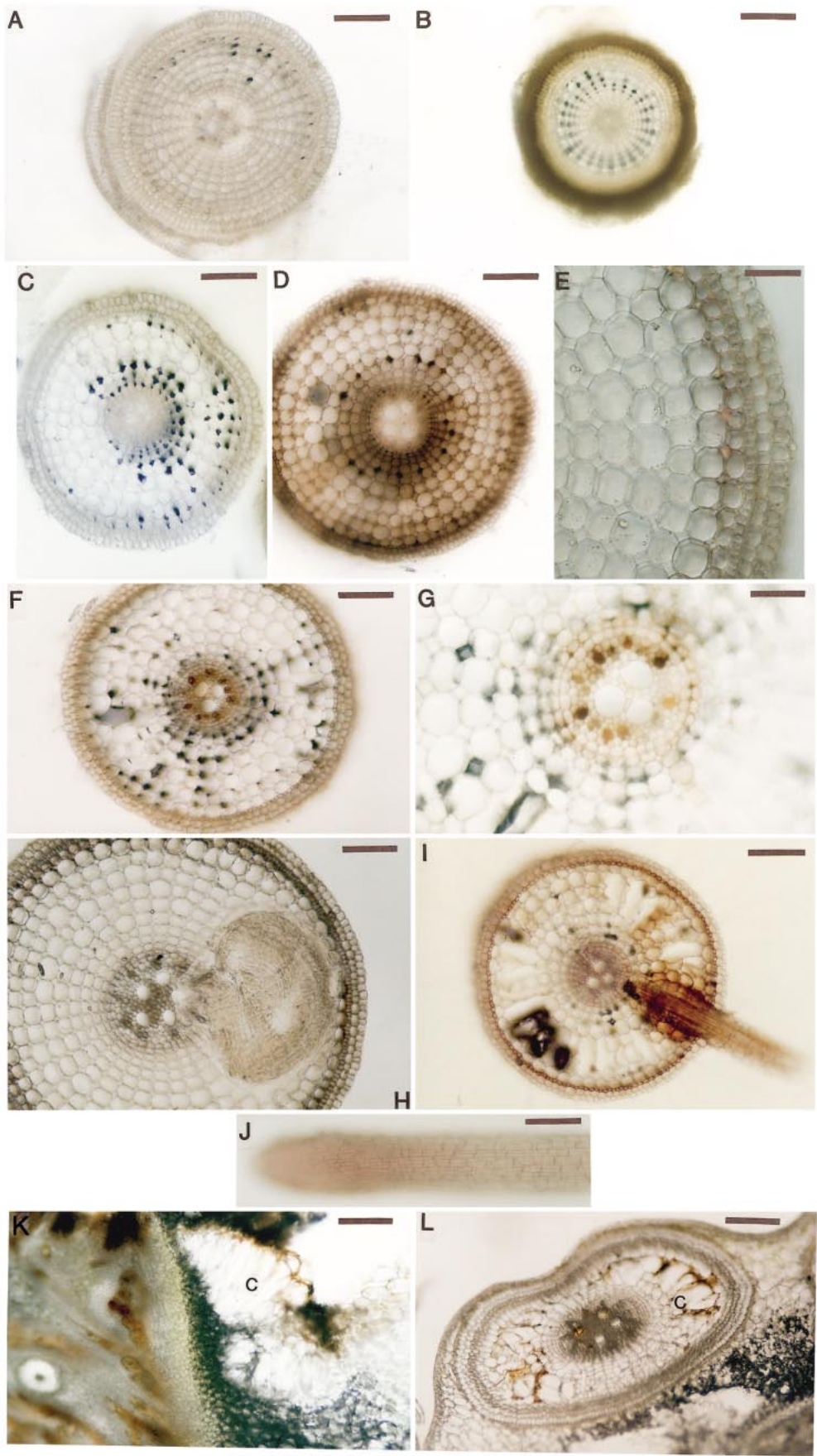
Shoot growth was also retarded by the organic acid treatments, but the effects were less marked than for the roots (Table 1). Shoot growth almost ceased within 7 d when the plants were subjected to cocktail 2, while with cocktail 1 growth continued until the end of the experimental period (day 31), but was far slower than the controls. With both cocktails there was premature senescence of the shoots; for cocktail 2 the onset was far earlier and the degree of senescence far greater than for cocktail 1 (Table 1). With cocktail 2 a dark purple mottling of the leaves started to develop within the first 6 d of treatment. There was virtually no senescence of the controls at the end of the experiment.

Rice—Growth of roots and shoots was not measured in this study; as mentioned in the introduction, it has been documented that organic acids, including acetic, can inhibit the growth of rice. In this study, also, it was noted that in plants

exposed to acetic acid for 2 wk, adventitious and lateral root growth was much inhibited; there was also death of adventitious root apices and death of laterals that had emerged prior to treatment and premature shoot senescence.

Anatomy—Cell wall lignification, as detected using phloroglucinol and hydrochloric acid was invariably confirmed with aniline hydrochloride and, in all the cases tested, was associated with some degree of suberization.

Phragmites—In the controls no lignification or suberization was detected throughout the experimental period in the apical 20 mm of adventitious roots or in the epidermis of laterals. However, after only 24–30 h in cocktail 2 and 2–3 d in cocktail 1, there were some signs of lignification and suberization of the walls of the surface cell layers of the apical 10 mm of adventitious roots, including those of the root cap and of the



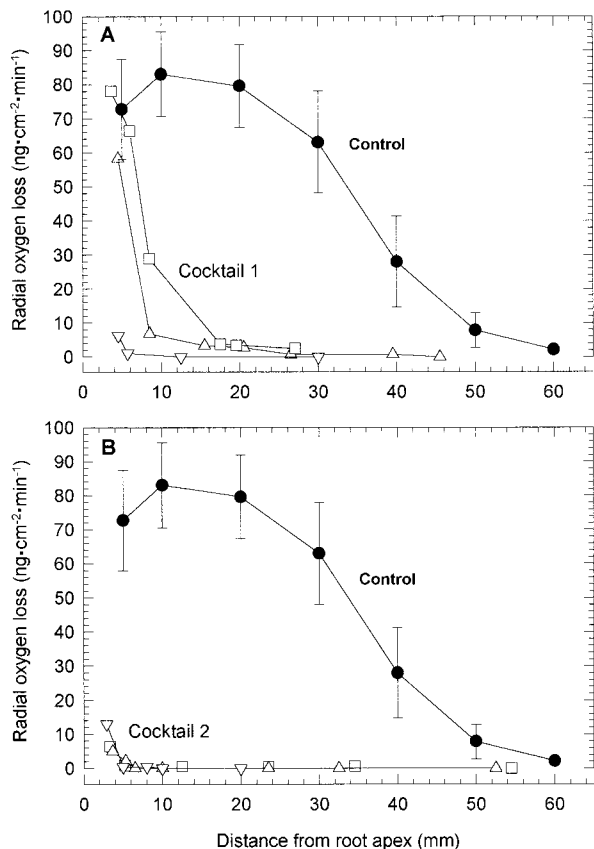


Fig. 4. *Phragmites*: effects of cocktails of organic acids on profiles of radial oxygen loss from apical regions of adventitious roots after 3–5 d of treatment. Length of roots = 90–170 mm. Control roots (●) received no organic acids, $N = 7$, bars = ± 1 SE. Cocktail 1 treatment (Fig. 4a: □, ▽, and △) and cocktail 2 treatment (Fig. 4b: □, ▽, and △). Cocktail 1 contained acetic, propanoic, n-butyric, iso-butyric and caproic acids: each 1 mmol/L; total concentration = 5 mmol/L; total undissociated (toxic) concentration = 0.35 mmol/L. Cocktail 2 contained the above acids together with formic and valeric acids, all also 1 mmol/L; total concentration = 7 mmol/L; total undissociated (toxic) concentration = 0.42 mmol/L. Each cocktail was at pH 6.

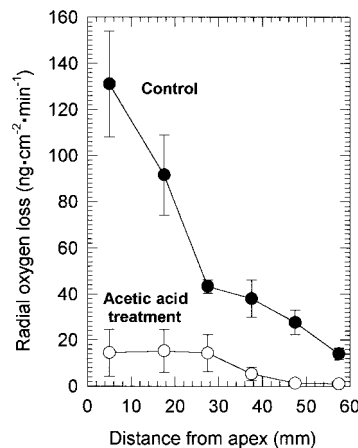


Fig. 5. Rice: effects of acetic acid on profiles of radial oxygen loss to the rhizosphere from apical regions of adventitious roots 5 d after roots received a single dose of 1.5 mmol/L acetic acid: undissociated (toxic) concentration = 1.05 mmol/L. Length of roots = 50–120 mm. Control roots (●) received no acetic acid ($N = 4$); ○ = acetic acid treatment ($N = 3$); bars = ± 1 SE.

epidermis of fine laterals. The effects became more pronounced as the experiment proceeded, especially with the cocktail 2 treatment (Fig. 2A–D, J–L). Although in the first 3–4 d the cortex and stele were apparently relatively unaffected, thereafter, particularly with cocktail 2, some of the cortical intercellular gas spaces and protoxylem and phloem became occluded, cortical cells became lignified, and there was premature lignification of the hypodermal layers and stele (Fig. 2E–G).

Rice—The adventitious root apices showed signs of being affected by the acetic acid within 24 h of the onset of treatment, with the yellowing of the outer cell layers of apical regions, including the root cap. After 6 d, even with the single-dose acid treatment, the brown discoloration was quite intense (Fig. 3B), possibly due to polysaccharide gums containing phenolic compounds. There were also premature cell wall thickening of the hypodermis, and blockages within a small proportion of the cortical intercellular spaces. In contrast to *Phragmites*, there was no obvious premature lignification within root apices at this stage.

After 2 wk of continuous exposure to acetic acid, obstructions in the cortical intercellular gas spaces of root apices

Fig. 3. Rice: fresh, hand-cut sections showing anatomical responses of roots and rhizomes to 1.5 mmol/L acetic acid at pH 4.5 (undissociated toxic concentration = 1.05 mmol/L). Sections were left unstained except for (J), which was stained in phloroglucinol and concentrated HCl to indicate lignification of cell walls (red). (A and C) Root not exposed to acid (control). (B, D, E) Six days after exposure to a single dose of the acid. (F–L) After 14 d of continuous exposure to acid, replenished on alternate days. (A) Transverse section of control adventitious root 2–3 mm from apex. Note absence of browning of outer cell layers; cf. (B). Bar = 0.13 mm. (B) Transverse section of adventitious root 2 mm from apex. Note browning of cells of root cap and some yellowing of epidermal cell layers; cf. (A). Bar = 0.27 mm. (C) Transverse section of control adventitious root 10 mm from apex. Note absence of browning of cell walls and absence of blockages in the cortical intercellular spaces. Bar = 0.13 mm. (D) Transverse section of adventitious root 10 mm from apex. Note browning of cell walls, especially of inner hypodermis (exodermis) and endodermis; also, brown blockages in some of the cortical intercellular spaces, cf. (C). Bar = 0.13 mm. (E) High power of outer cell layers of (D). Note blocked intercellular spaces. Bar = 0.07 mm. (F) Transverse section of adventitious root 15 mm from apex. Note browning of outer cell layers (especially exodermis) and browning and blocking of vascular elements. Bar = 0.13 mm. (G) High power of stele from (F) showing blockages within vascular elements and some intercellular spaces of the inner cortex adjoining the endodermis. Bar = 0.07 mm. (H) Transverse section of adventitious root 12 mm from apex with developing lateral root swollen and trapped within the cortex. Note disruption of cells of lateral root and thickened exodermis opposite the lateral; the exodermis is normally thin walled opposite a developing lateral. Bar = 0.13 mm. (I) Transverse section of adventitious root 25 mm from apex. Note swelling of lateral within the cortex and browning of lateral and adjoining cortex. Bar = 0.29 mm. (J) Whole lateral root tip from acid treatment. Note lignification of epidermal cell walls (red) especially at the apex; cf. Fig. 2J and L. (Laterals from the control treatment were unligified.) Bar = 0.27 mm. (K) Longitudinal section of rhizome–leaf base junction. Note development of callus, c, within aerenchyma. Bar = 0.27 mm. (L) Transverse section of root base within rhizome showing callus, c, occluding the root cortex, which is normally aerenchymatous. Bar = 0.27 mm.

were sometimes more frequent, as were the browning and occlusion of vascular elements (Fig. 3F, G). Also, callus was detected within the aerenchyma of the root–rhizome junctions and the root bases located in the rhizome (Fig. 3K, L). As with *Phragmites*, the epidermis of laterals that had already emerged became lignified (Fig. 3J) and suberised, and there was also evidence of lateral root death (Fig. 3I). The failure of developing laterals to emerge (Fig. 3H) and their malformation and apparent death in the cortex seemed at least partly due to the premature cell wall thickening of the hypodermal layers opposite the lateral. Normally these regions remain as unthickened “windows” (Armstrong, 1992; Votrubova and Pecháková, 1997). These same effects have been described for *Phragmites* in relation to responses to phytotoxins (Armstrong, Armstrong, and Van der Putten, 1996; Armstrong, Afreen-Zobayed, and Armstrong, 1996; Armstrong and Armstrong, 1999). Neither the adventitious nor lateral roots of the controls showed any of these effects, even after 4 wk.

Radial oxygen loss from roots—Adventitious root apices—

The profiles of ROL from the apical regions varied for roots of differing lengths and according to their positions on the rhizome. Therefore, although at least six roots per treatment were tested, it was only possible to group together roots from the various treatments where these parameters were similar. However, within a treatment, the ROL profile patterns were the same. Measurements further up the roots were prevented by the emergence of laterals. For both *Phragmites* and rice, when a pressurized air flow was applied to shoot bases after ROL measurements had been completed, air freely bubbled from submerged roots whose apices had been excised, thus indicating a free diffusive pathway from shoot to root at this stage.

Phragmites—After 3–5 d of treatment, ROL values for control adventitious roots for the apical 2–30 mm were high, ~63–84 ng O₂·cm⁻²·min⁻¹; but declined to zero at ~60 mm behind the apex (Fig. 4, Table 1). For the cocktail 1 treatment the apical ROL values were variable, but the highest were 30–78 ng O₂·cm⁻²·min⁻¹ for the apical 4 mm; thereafter, they fell sharply to zero at ~30 mm behind the apex. For some roots from this treatment, apical values did not exceed 6 ng O₂·cm⁻²·min⁻¹. However, roots from the cocktail 2 treatment were even more affected; apical ROL values were consistently very low: 0–12 ng O₂·cm⁻²·min⁻¹, and reached zero, at 6 mm from the apex. In all treatments oxygen freely diffused from cut apical regions, indicating that there was adequate internal oxygen transport down the roots.

Rice—Five days after a single dose of the acetic acid, values of ROL were consistently lower for the apical 60 mm, compared to those of the controls (Fig. 5). For example, for the apical 5 mm, the mean ROL value for the acetic acid treated roots was 4.5 compared to 131 ng O₂·cm⁻²·min⁻¹ for the controls. At 40–50 mm from the apex, ROL values for the acid-treated roots were zero, compared to 20–34 ng O₂·cm⁻²·min⁻¹ for the controls.

Fine laterals: Phragmites—The values of ROL from the laterals were arbitrary since it was not possible to standardize the degree of proximity of the laterals with the coiled electrodes. Nevertheless, the results (Fig. 6, Table 1) clearly showed appreciable ROL from the control laterals and those

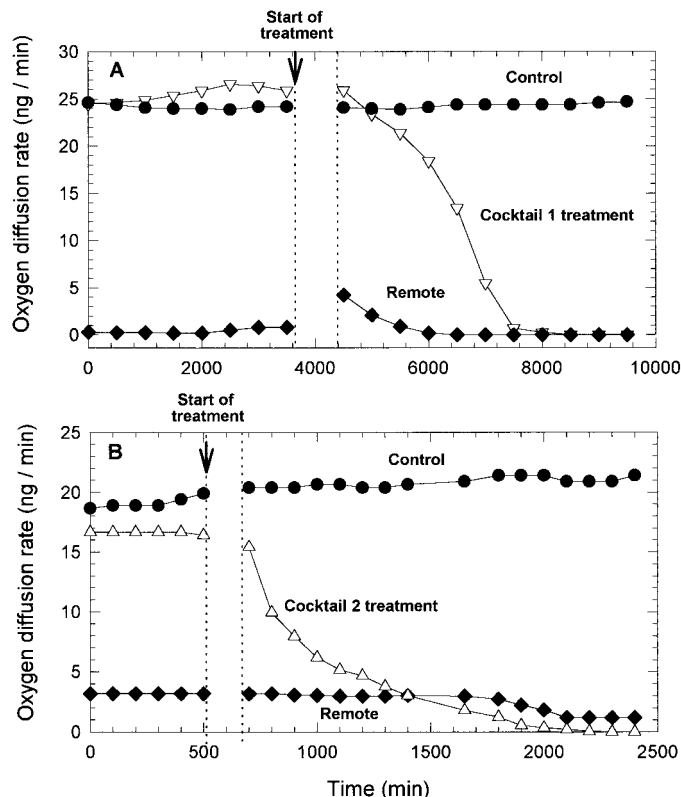


Fig. 6. *Phragmites*: typical effects of cocktails of organic acids on radial oxygen loss from fine lateral roots to the rhizosphere. Length of lateral roots (midlateral region) = 15–18 mm. Control roots (●) received no organic acids; ▽ = cocktail 1 treatment (Fig. 6a); △ = cocktail 2 treatment (Fig. 6b). Cocktail 1 contained acetic, propanoic, n-butyric, iso-butyric, and caproic acids: each 1 mmol/L; total concentration = 5 mmol/L; total undissociated (toxic) concentration = 0.35 mmol/L. Cocktail 2 contained the above acids together with formic and valeric acids, all also 1 mmol/L; total concentration = 7 mmol/L; total undissociated (toxic) concentration = 0.42 mmol/L. Each cocktail was at pH 6.

prior to treatment and that the addition of the organic acid cocktails caused the reduction of ROL virtually to zero within 1–3 d.

DISCUSSION

For *Phragmites*, the results indicated that if sufficient numbers of acids are included, cocktails of dilute monocarboxylic acids can be harmful, even at pH 6, when the undissociated molarity of each acid has previously been shown to be innocuous (Armstrong and Armstrong, 1999). The stunting of adventitious and lateral roots, slowing down of shoot growth and premature cell wall thickening in surface layers of roots were apparent within the first 4 d of treatment with both cocktails, but more obvious with the more toxic cocktail 2, which contained two more acids than cocktail 1. Also, the onset of premature shoot senescence occurred 2 wk earlier with cocktail 2.

The toxicity of an organic acid cocktail will be directly related to the number and concentrations of acids present in the rooting medium and inversely related to pH. In the present study, it can be predicted that at a slightly lower pH of 5.9, rather than 6, cocktail 1 would have been as toxic as cocktail 2. The pH of the rhizosphere must also influence the toxicity of organic acids. It has already been demonstrated that the pH

of the rhizospheres of adventitious roots and laterals can be lower than that of the bulk soil, sometimes by more than two pH units, e.g., in rice (Begg et al., 1994; Saleque and Kirk, 1995), in *Phragmites* (Conlin and Crowder, 1989), and in *Cyperus involucratus*, *Eleocharis sphacelata*, and *Juncus ingens* (Sorrell and Orr, 1993). Similarly we have found (Armstrong and Armstrong, 1999) that the rhizospheres around the apices of young adventitious roots of *Phragmites* can be at pH 6.6–6.7, while the rooting medium is at pH 7.7.

It is interesting that rice responded anatomically to acetic acid as does *Phragmites* to the lower organic acids in general, and to sulphide (Armstrong, Afreen-Zobayed, and Armstrong, 1996; Armstrong, Armstrong, and Van der Putten, 1996; Armstrong and Armstrong, 1999), and in the present study to cocktails of dilute organic acids. These responses included premature cell wall thickening and some lignification of the normally permeable regions of the root system, occlusion within the vascular systems and the intercellular gas spaces of adventitious roots, and proliferations of callus that partly blocked the gas spaces within root–rhizome junctions and rhizomes. In rice, however, unlike *Phragmites*, there was comparatively little premature lignification, except in the epidermis of the laterals. The intercellular cortical spaces and vascular elements became occluded with yellowish-brown substances that darkened with time. Soukop et al. (Charles University, Prague, unpublished data) have found that this type of occlusion in *Phragmites* contains polysaccharide gums. Acetic acid has been shown to cause leakiness of cell membranes (Van Overbeek and Blondeau, 1954; Jackson and Taylor, 1970) and loss of ions from roots (Lee, 1976); we suggest, therefore, that such occlusions in both rice and *Phragmites* are due in part to phytotoxin damage to cell membranes. We interpret all of these anatomical symptoms as defense responses (Friend, 1981; Asada and Matsumoto, 1987) to prevent further ingress and spread of the toxins within the plants and possibly fungal infection. In *Phragmites* a predisposition to fungal infection has been associated with phytotoxin damage (Armstrong, Afreen-Zobayed, and Armstrong, 1996; Armstrong and Armstrong, 1999). Insofar as we are aware, this is the first documentation of anatomical effects of acetic acid in rice. Since Akiuchi disease of rice has also been linked to sulphide toxicity (e.g., Park and Tanaka, 1968), it would be interesting to know whether these same anatomical symptoms can be induced in rice by sulphide as they are in *Phragmites* (Armstrong, Afreen-Zobayed, and Armstrong, 1996).

For both *Phragmites* and rice, reductions of ROL from adventitious root apices and fine laterals were apparent at early stages of treatment. Since the internal aeration pathways allowed pressurized gas flow from the shoots to the apices of adventitious roots, we associate the reduced ROL with lowered permeabilities to oxygen induced by atypical cell wall lignification and suberization in the epidermis of the laterals and of the hypodermal layers of adventitious root apices; in some cases these were sufficient to reduce ROL to zero. At later stages, blockages that develop extensively within the gas spaces of adventitious roots, root–rhizome junctions, and rhizomes will impede longitudinal and lateral transport of oxygen to the laterals and to and within the adventitious root apex and contribute to reduced ROL to the rhizospheres and reduced respiratory oxygen supply. Reduced permeability and ROL from lateral root zones are likely to be particularly serious, since it is here that the oxidized rhizospheres are most extensive

(Armstrong and Armstrong, 1988) and the surface areas for absorption of water and nutrients are very large. One could therefore predict that reduced permeability to oxygen will be accompanied by reduced uptake of water and nutrients. This is supported by A. Soukopp (Charles University, Prague, unpublished data), who found that root wall lignification reduced permeability to water and iodate ions in *Phragmites*.

In rice, reductions in root permeability and ROL and impedances in the gas transport pathways produced by the acetic acid treatment could help to explain some of the symptoms of organic-acid-induced Akiuchi, including reduced root respiration and nutrient uptake, and the decreased ability of the roots to oxidize iron, reported by Takijima (1965). The vascular blockages reported in the present study could also help to explain the reduced uptake of nutrients in Akiuchi mentioned earlier.

It should be noted, however, that in wetland plants, including *Phragmites* and rice, the lignification of hypodermal layers in maturing subapical parts of adventitious roots, accompanied by reduced ROL to the rhizosphere, is a well-known, natural phenomenon, coinciding with the development of aerenchyma and laterals (Luxmoore, Stolzy, and Letey, 1970; W. Armstrong, 1971; J. Armstrong, 1992; Colmer et al., 1998; Armstrong et al., 2000). The latter are freely permeable to ROL and divert oxygen from the gas spaces within the main root. Thus, the normal development of impermeable layers in the basal and subapical regions of the root wall must be useful and help to conserve oxygen in the main root for supplying the laterals and the apical parts of the adventitious root. However, epidermal lignification of laterals and of the apical hypodermal layers of adventitious roots are atypical, since these are normally absorptive regions for salt and water uptake. Also, being permeable, they are vulnerable to attack from soil-borne phytotoxins, and so it is important that they are protected by oxidizing rhizospheres, as mentioned in the introduction.

Critically high accumulations of phytotoxins, arising from perhaps excessive fertilization or other means of eutrophication, can probably set in motion a complex series of reactions, and we present a scheme (Fig. 7) showing some of the possible interactions. We suggest that (a) the toxins will penetrate the rhizospheres, where the O₂ will be rapidly consumed during biological and chemical oxidation of reduced toxins such as Fe²⁺, Mn²⁺, and S²⁻ and in the microbial oxidation of organic acids; (b) if the metabolic breakdown of the phytotoxins in the rhizosphere is incomplete, the toxins will penetrate the vulnerable laterals and adventitious root apices, which will react by becoming less permeable, thus resulting in a diminution of ROL; (c) additionally, there may be a temporary proliferation of aerobic organisms in the rhizosphere stimulated by the presence of the phytotoxin substrates; (d) effects (a)–(c) will cause the shrinkage of the rhizospheres, thus bringing the source concentrations of phytotoxins closer to the roots, which may become overwhelmed. Reduced ROL from the roots will also result from blockages in the gas spaces of roots and rhizomes for diffusive transport, and in some species, e.g., *Phragmites*, of rhizomes for convective transport of oxygen. The shrunken rhizospheres will be ineffective in protecting the roots, resulting in an overall stunting of the root systems, a factor that will also contribute to a general reduction in rhizosphere oxidation. In extreme cases of dieback, root, rhizome, and bud death occur and finally localized death and decay of the plants, leading to further release of phytotoxins.

Some possible effects of phytotoxins within wetland plant roots, rhizomes, rhizospheres and soil

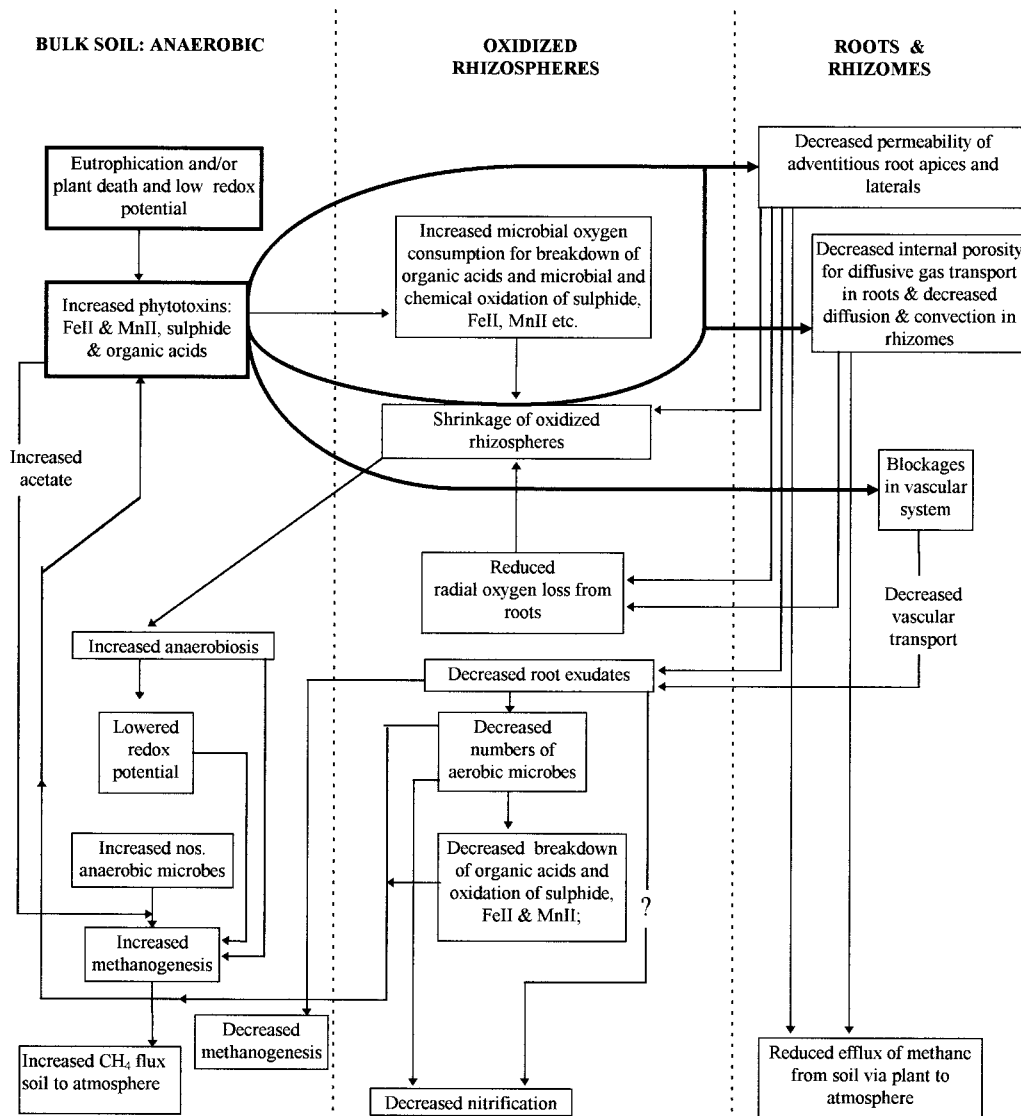


Fig. 7. Some possible effects of phytotoxins within wetland plant roots, rhizomes, rhizospheres, and soil.

Decreased rhizosphere oxygenation will be accompanied by reductions in aerobic microbes, including nitrifying bacteria (Hofmann, 1990) and methanotrophs (Gilbert and Frenzel, 1995), and increases in anaerobic microbes, including methanogenic bacteria. Rates of nitrification will decrease, whereas methanogenesis will increase, especially if acetate is among the phytotoxins present (Rothfuss and Conrad, 1993). Additionally reduced root permeability and biomass, shoot senescence, and blockages in the xylem will tend to decrease water uptake by the plants, and this will promote higher water tables and thereby increase rates of denitrification and methanogenesis. In relation to the latter, Sorrell et al. (1997) found that rates of methanogenesis were far higher at dieback sites than at healthy reed sites. Moreover, dieback sites tended to remain waterlogged and methanogenic during dry periods, whereas healthy sites became dry and inactive. They conclude that "reed wetlands may not be great generators of CH₄ while the

plants are healthy and actively growing, but become more intense CH₄ producers when the plants senesce and die" (p. 1984). If decreased root permeability to oxygen is accompanied by diminished permeability to CH₄, as seems likely, then, despite increased methanogenesis, less CH₄ may pass along the root → rhizome → shoot pathway into the atmosphere, due to decreases in root permeability and biomass and reduced diffusive gas transport and, in some cases, e.g., *Phragmites*, convective flow through the plant. Here, phytotoxin damage has been correlated with reduced convective flow at reed dieback sites, due to blockages in the gas space system and premature shoot senescence (Armstrong et al., 1996a). The possible effects of phytotoxins on CO₂ production and emission from wetlands have not been discussed here, but they may be similar to those effects on CH₄.

Although high phytotoxin levels will probably tend to reduce potential methanogenesis because of diminished root ex-

udation (Whiting and Chanton, 1993; Minoda and Kimura, 1994) correlated with blockages in the phloem and decreased root permeability and biomass, it seems likely that the overall effect of phytotoxins will be to increase rates of methanogenesis. However, since aquatic macrophytes are important emitters of CH₄, the question of the effects of phytotoxins on the overall long-term emissions of CH₄ from wetlands would appear to merit further investigation.

It would obviously be of great interest to know, from further practical investigations and modelling, the extent to which the reactions indicated in Fig. 7 and other related effects might take place. They are clearly relevant to the health and survival of reed and of other emergent aquatic macrophytes such as rice in localities that are prone to accumulations of phytotoxins, to the role of reed in the phytoremediation of waste waters by artificial wetlands, and to the emissions of CH₄ and other greenhouse gases via such plants from wetlands.

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