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ORIGINAL ARTICLE

Patterns of peroxidative ethane emission from submerged rice seedlings indicate that damage from reactive oxygen species takes place during submergence and is not necessarily a post-anoxic phenomenon

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Abstract Using ethane as a marker for peroxidative damage to membranes by reactive oxygen species (ROS) we examined the injury of rice seedlings during submergence in the dark. It is often expressed that membrane injury from ROS is a post-submergence phenomenon occurring when oxygen is re-introduced after submergence-induced anoxia. We found that ethane production, from rice seedlings submerged for 24–72 h, was stimulated to 4–37 nl gFW^{-1}, indicating underwater membrane peroxidation. When examined a week later the seedlings were damaged or had died. On de-submergence in air, ethane production rates rose sharply, but fell back to less than 0.1 nl gFW^{-1} h^{-1} after 2 h. We compared submergence-susceptible and submergence-tolerant cultivars, submergence starting in the morning (more damage) and in the afternoon (less damage) and investigated different submergence durations. The seedlings showed extensive fatality whenever total ethane emission exceeded about 15 nl gFW^{-1}. Smaller amounts of ethane emission were linked to less extensive injury to leaves. Partial oxygen shortage (O_2 levels <1%) imposed for 2 h in gas phase mixtures also stimulated ethane production. In contrast, seedlings under anaerobic gas phase conditions produced no ethane until re-aerated: then a small peak was observed followed by a low, steady ethane production. We conclude that damage during submergence is not associated with extensive anoxia. Instead, injury is linked to membrane peroxidation in seedlings that are partially oxygen deficient while submerged. On return to air, further peroxidation is suppressed within about 2 h indicating effective control of ROS production not evident during submergence itself.

Keywords Ethane emission · Lipid peroxidation · Non-invasive technique · Oxygen shortage · Photoacoustic detection · Prolonged hypoxic and anoxic conditions · Real time measurements · Submerged rice · Survival tests and damage assessment

Abbreviations
RH Relative humidity
ROS Radical oxygen species

Introduction

Total submergence of young plants in the field is a major problem for rainfed lowland rice farming in many parts of Asia (Zeigler and Puckridge 1995). Although it is frequently assumed that injury to plants

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from submergence is caused by anaerobiosis (e.g., Ito et al. 1999; Agarwal and Grover 2006) there is increasing evidence that the anaerobic state (anoxia) is not a prerequisite for the damage. A key observation leading to this conclusion has been the absence of a strong post-submergence burst in acetaldehyde, after submergence in water initially in equilibrium with air, in the dark; this burst is to be expected after tissues are rendered anoxic for >0.5 h (Boamfa et al. 2003). This seeming absence of anoxia indicates that submergence can damage rice plants severely even when the plants retain some oxygen, i.e., under hypoxic conditions. In this context, it is pertinent to consider whether reactive oxygen species (ROS) could play a role in the injury caused under such relatively mild conditions of partial oxygen shortage. The conventional view would be that submergence imposes anoxia and that when de-submerged rice plants renew contact with oxygen in the air, an increase in ROS production coupled with inadequate scavenging, damage or kill cells by unregulated peroxidations (Ella et al. 2003; Jackson and Ram 2003). These views are supported by electron paramagnetic resonance studies and by measurements of products of lipid peroxidation, showing increased amounts of ROS in post-anoxic plants and evidence of peroxidative attack as a consequence (Crawford et al. 1994; Thongbai and Goodman 2000; Ella et al. 2003). However, if ROS play a part in damaging plants that have experienced non-anoxic submergence, this conventional picture would obviously require revision.

Ethane production has been recognized for many years as a sensitive indicator of membrane peroxidation (Riely et al. 1974); it was also observed during post-anoxia in plants (Elstner and Kunze 1976; Pfister-Sieber and Brandle 1994). Accordingly, our approach was to use ethane emission as a marker of ROS activity. Among polyunsaturated fatty acids, linolenic (C18:3) acid is vulnerable to ROS peroxidation and known to be the source of peroxidative ethane (see details in Halliwell and Gutteridge 2003). To minimize artifacts and secure detailed kinetics of ethane release we employed a CO-overtone laser (Bachem et al. 1993), combined with a photoacoustic detector of high sensitivity, capable of monitoring ethane emissions continuously and non-destructively (Martis et al. 1998). The high sensitivity of the system and its capability for in line flow analysis of ethane emission allow us to make frequent non-destructive analyses from quite a modest amount of plant material. In general, gas chromatographs equipped with, e.g., mass spectrometers or flame ionization detection lack sensitivity for ethane at these levels. As a result samples need to be accumulated/enriched, which can have a severe drawback on the time resolution and/or experimental conditions (densely packed plant material, accumulation/shortage of CO_2, etc.). The laser-based system enabled us to monitor ethane released both during and after submergence. We also examined plants made anoxic with flows of nitrogen gas, compared a submergence tolerant cultivar of rice (FR13A) with a less tolerant one (CT6241) and used plants with different starting-times for submergence since submergence beginning in the morning causes more damage than that starting in the afternoon as a consequence of depleted sugar levels (Ram et al. 2002). We show that, during submergence in water containing some oxygen, large ethane emission signals indicate major damage to cell membranes prior to any reconnection with the aerial atmosphere; this, in turn, leading to leaf damage and death of plants in the days following de-submergence.

Materials and methods

Plant material, germination and plant culture

Seed of the submergence tolerant *Oryza sativa* L. cv. FR13A, and a susceptible line, CT6241, was supplied by Dr. S. Sarkarung (IRRI, International Rice Research Institute, http://www.irri.org/; Thailand Office Bangkok, Thailand). Seeds were surface sterilized with 1% sodium hypochlorite solution for 10 min, washed under running tap water for 5 min and placed in 110-mm-diameter glass Petri dishes lined with filter paper wetted with 15 ml of tap water. The Petri dishes were placed in the dark at 30°C and RH 65%. Sprouted seedlings with 1-cm-long coleoptiles were transferred to culture trays (30 × 20 × 15 cm) filled with black 'Lacqtene' low-density polyethylene grains and nutrient solution [Yoshida et al. 1976; major nutrients: 0.849 mM KH_2PO_4, 0.123 mM K_2HPO_4, 1.428 mM NH_4NO_3, 0.754 mM $CaCl_2{\cdot}2H_2O$, 0.513 mM K_2SO_4, 1.644 mM $MgSO_4{\cdot}7H_2O$, minor nutrients: 9.5 μM $MnCl_2{\cdot}4H_2O$, 18.89 μM H_3BO_3, 0.156 μM $CuSO_4{\cdot}5H_2O$, 0.152 μM $ZnSO_4{\cdot}7H_2O$, $7.484{\cdot}10^{-5}$ μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ and 35.75 μM Fe.EDTA), pH 5.0]. The strength of the nutrient solution was raised gradually from 25% of full strength for the first 2 days, followed by 50% for the next 2 days and finally to 100% after 8–10 days. The culture trays were aerated with air flowing through perforated silicone rubber tubing at the base of the tray. After 1 day, the pH of the solution in the culture trays was adjusted to 5.0. On the following day, the solution was renewed and the procedure repeated every 2 days. The plants were grown under a 12 h light/12 h dark regime at 28/22°C (light source Philips SON-T Agro400, PPFD:

700 μmol m^{-2} s^{-1},) and a relative humidity of 60–65%. The pH of the solution around the roots increased from 5.0 to 5.8 over 16 h and typical shoot/root ratios (fresh weight) at the start of experiments were 1.72 ± 0.06 (FR13A) and 1.37 ± 0.04 (CT6241). On average, a total weight of 0.30 and 0.25 g and a length of 22 ± 3 and 16 ± 3 cm per seedling corresponded to FR13A and CT6241, respectively, determined at the start of the measurements. For each set of measurements, ten healthy 14-days-old seedlings of about equal length were chosen.

On-line detection of ethane

Ethane emitted by rice seedlings was detected by using a CO-laser-based photoacoustic detector (Fig. 1). A similar set-up was used to detect extremely low concentrations of acetaldehyde and ethanol in relation to fermentation processes by cucumber seeds (Leprince et al. 2000), red bell peppers (Zuckermann et al. 1997) and rice seedlings (Boamfa et al. 2003, 2005); methane and water emissions from cockroaches and beetles were measured as performed by Bijnen et al. (1996). For ethane detection, the laser was re-designed and adjusted to a mid-infrared wavelength region between 2.6 and 4.1 μm (Bachem et al. 1993; Martis et al. 1998; Santosa 2002; Santosa et al. 2003). Briefly, the trace gases are detected via their absorption of rapidly chopped infrared light, which generates pressure variations, resulting in acoustic energy detected by a miniature microphone. The intensity of the generated sound is proportional to the concentration of absorbing trace gas molecules.

To distinguish a specific trace gas from other gases we used the infrared tunability of the CO-laser. The laser was line-tunable over a large frequency range, i.e., over 165 laser lines with strong output power between 2.6–4.1 μm of wavelength. In this frequency range, amongst others, ethane and ethylene have a strong and characteristic absorption pattern (Santosa 2002) and each could be present in the detection cell. We unraveled the mixed absorption strength pattern using a multi-component matrix calculation algorithm (Meyer and Sigrist 1990). To determine the concentration of N gases, the microphone signal on $N + 1$ laser lines was sampled (the additional laser line was used to eliminate instrument effects). The ethane concentration

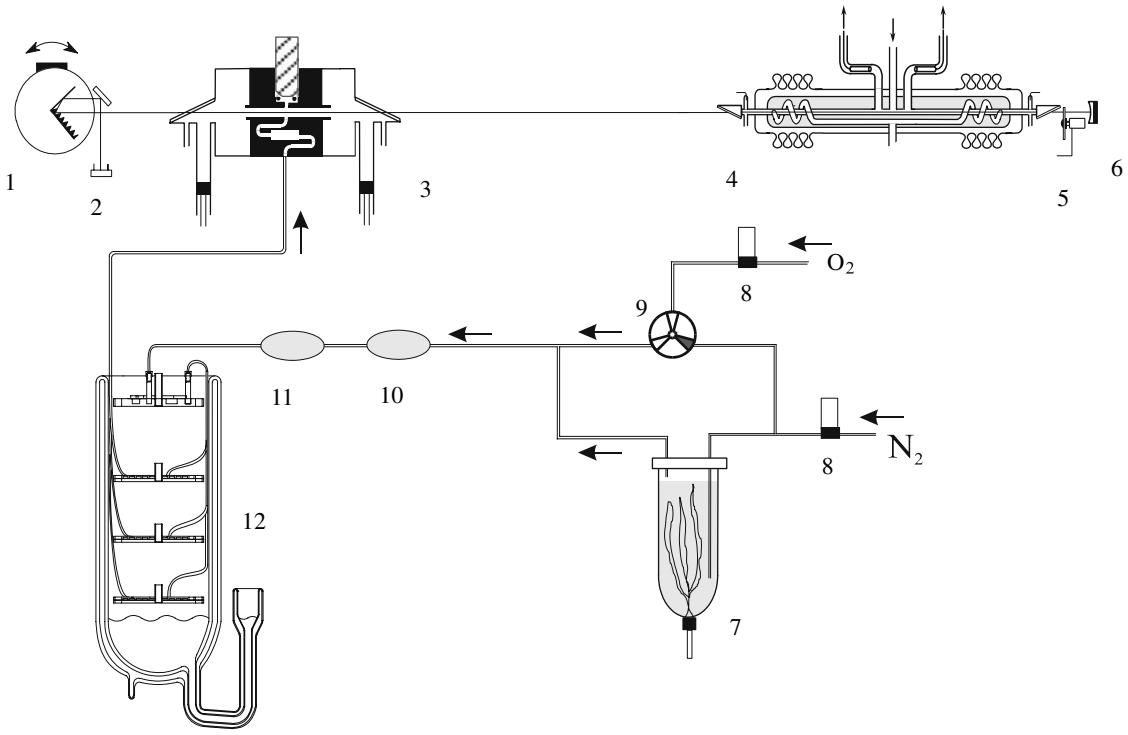

Fig. 1 Experimental set-up. The CO-laser extends from its grating (*1*) to an end-mirror (*6*) and contains a laser discharge tube (*4*) for light generation, a photoacoustic gas detection cell (*3*) and a chopper for light modulation (*5*). The laser power is monitored by (*2*). Ethane emitted by the rice seedlings in the sampling cuvette (*7*) was transported in a gas flow, regulated by mass flow controllers (*8*) to the detection cell (*3*). Before, KOH was used to remove CO_2 (*10*), $CaCl_2$ to remove water vapor (*11*) and a cold trap to remove other, volatile, spectroscopically interfering gases (*12*). Variation in the oxygen composition of the carrier gas changed the acoustic behavior of the detection cell. To avoid this artifact, a stream of pure oxygen flow and one of nitrogen were recombined in a variable ratio using a valve system (*9*) to ensure that O_2 concentrations in the acoustic detector remained unchanged even when the plants in were subjected to different concentrations of oxygen

was then calculated taking into account interfering signals of ethylene and pentane, i.e., $N = 3$. The absorption coefficients of these gases for the four relevant laser lines are given in Table 1.

The emitted trace gases were transported by a carrier gas flow (flow rate 1.5 l h^{-1}) from the sampling cell to the detection cell. Before entering the detection cell, the carrier gas was led through scrubbers to remove spectroscopically interfering gases produced by the plants. A KOH scrubber was used to remove CO_2, a $CaCl_2$ scrubber to remove water and a cooling trap (at 120 K) to remove ethanol and acetaldehyde. Since it is important for photoacoustic detection that a carrier gas of constant composition is used, oxygen was mixed to the nitrogen either before or after the sampling cuvette, for aerobic or anoxic conditions, respectively, in order to obtain a constant oxygen concentration in the detector and thus to maintain the same ethane sensitivity for changing conditions in the sampling cuvette (Fig. 1). We have achieved a minimum detectable ethane concentration in the air below $1{:}10^9$.

Submergence tests

The rice seedlings were inserted into a long narrow glass cuvette (35 cm length, 3 cm diameter, volume: 250 ml, Fig. 1), which had three ports: a gas inlet and an outlet port on top and a third port at the bottom to drain water. We used aerated tap water [equilibrium partial pressure 21 kPa (21%) oxygen], referred to as floodwater, to submerge the plants completely, leaving only a small gas volume at the top of the cuvette.

During the submergence period the plants were kept in the dark by wrapping the cuvette with aluminum foil. No gas exchange took place with the surroundings since all ports were closed. Submergence treatments were started either in the morning (10.00 a.m.) or in the afternoon (4.00 p.m.) with submergence periods up to 3 days. At the end of submergence the amount of ethane released from the plant was measured in two steps. First, the ethane produced during the submergence period and accumulated in the water was measured by bubbling nitrogen gas through the cuvette for more than 2 h, and measuring the discharged ethane. No oxygen was present in the gas flow, at this stage, to avoid any post-anoxic effect. Then, the water was drained. During drainage (30 min) the seedlings were kept under nitrogen gas. Subsequently, the effect of return to normal conditions was observed by adding 20% oxygen to nitrogen flowing through the cuvette. We monitored post-stress ethane release from the seedlings that still remained under darkness.

Gas phase anoxia and hypoxia tests

To compare with the submergence results, batches of ten rice seedlings were subjected to gas phase anaerobiosis by flowing 1.2 l h^{-1} of dry nitrogen gas through the cuvette. The cuvette contained 25 ml of water to cover the roots. The period of anaerobiosis varied from 4 h up to 3 days, starting either early in the morning (10.00 a.m.) or later in the afternoon (4.00 p.m.). The ethane release was monitored during and after the anaerobiosis.

To induce hypoxia, batches of rice seedlings were sealed in flasks containing mixtures of nitrogen and oxygen generating initial oxygen concentrations of 0.1, 0.25, 0.5 or 0.75% (v/v).

Survival and leaf damage

After each experiment the seedlings were returned to their growth cabinet. Survival and leaf damage were scored after 7 days of recovery. Survival of plants was indicated by the emergence of new leaves and continued growth. Plant survival was quantified as the percentage of plants surviving either submergence or gas phase anaerobic conditions. The extent of foliar damage caused by the treatments was scored as the percentage of leaves that emerged prior to treatment and subsequently became shriveled and brown.

Results

Ethane emission and injury during and after submergence

Ethane emitted by plants during submergence accumulated in the surrounding water. To measure the

Table 1 Selected laser lines and absorption coefficients of relevant gases used for measuring ethane

Laser lines $P(J'')_{v''}$	Wavelength (cm^{-1})	Ethane ($atm^{-1}\ cm^{-1}$)	Pentane ($atm^{-1}\ cm^{-1}$)	Ethylene ($atm^{-1}\ cm^{-1}$)
$P(10)_{22}$	3,097.7480	0.19 ± 0.1	0.109 ± 0.006	0.21 ± 0.01
$P(8)_{24}$	3,006.9494	9.0 ± 0.1	0.53 ± 0.04	0.18 ± 0.02
$P(13)_{24}$	2,988.4669	5.2 ± 0.5	3.2 ± 0.6	1.9 ± 0.2
$P(9)_{25}$	2,954.5848	9.4 ± 0.9	7.7 ± 0.7	0.15 ± 0.02

amount, the floodwater was sparged with nitrogen gas for 3–5 h to transfer the dissolved ethane to the laser detector. Figure 2a shows a 3.5 h time course of ethane being removed in this way from 230 ml of floodwater during the sparging and after the cultivar FR13A had been submerged for 24, 48 or 72 h in the dark, starting in the morning. The total amount of ethane produced during submergence can be calculated from the area under each emission plot.

In Fig. 2b, a more detailed view is shown for a particular case of 24 h submergence shown in Fig. 2a. The associated expansion of the ethane emission axis allows the minimum detectable amounts of ethane we can measure to be made visually clear. Ethane emission

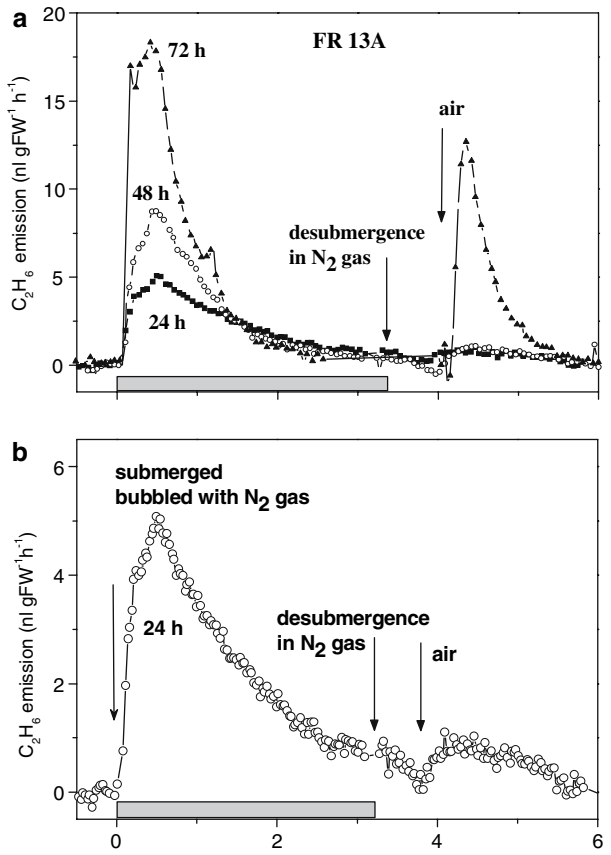

Fig. 2 **a**, **b** Ethane produced by seedlings of rice cultivar FR13A during and after 24, 48 or 72 h submergence in the dark in water initially in equilibrium with air. *Ethane data* for the first 3.5 h of the graph represents ethane produced during the preceding submergence period. It was quantified at the end of submergence by sparging the floodwater with a stream of nitrogen gas to sweep dissolved ethane into the laser detector. This ethane-stripping was complete after 3.5 h (*grey bar*). The seedlings were then desubmerged (*first arrow*) under nitrogen gas and returned to air within a few minutes (*second arrow*). The pattern of post-submergence ethane release over the subsequent 2.5 h (3.5–6 h) is shown in real time. **b** Enlarged version of the 24-h submergence curve shown in **a**. After 29.5 h, the post-submergence signal has dropped below 0.1 nl gFW^{-1} h^{-1}

continued to be monitored after submergence was halted by draining the sparged floodwater. Drainage took 30 min, during which time the water was temporarily replaced with nitrogen gas. Then, after a short delay of 30 min, a flow of 20% oxygen in nitrogen was introduced (Fig. 2, see arrow: rice seedlings in air). The response to this re-aeration (right side of Fig. 2a and b) was an almost instantaneous release of ethane that could be measured directly. Ethane emission increased at first, reached a peak and then declined to values below 0.1 nl gFW^{-1} h^{-1} (Fig. 2b). This transient post-submergence emission was completed after about 2 h.

As the period of submergence was lengthened from 24 to 48 h or 72 h the amount of ethane accumulated in the floodwater increased with time for both cultivars. This daily increase in total ethane emission was greater in submergence-intolerant CT6241 than in submergence-tolerant FR13A. The amount of ethane produced and thus released into the water by both cultivars was always larger when submergence started in the morning as compared to the afternoon start (Table 2).

Table 2 also shows the amount of ethane released and integrated over the first 2 h after de-submergence. Although the maximum rates of release were high during this time, the peak was short-lived (Fig. 2). The total ethane produced in 2 h after de-submergence was thus much smaller than during the preceding 24, 48 or 72 h submergence treatment (Table 2). Nevertheless, the effects of the different treatments were similar in arithmetic sign to those underwater. Thus, CT6241 always produced more ethane than FR13A, the amount being larger when submergence started in the morning compared to the afternoon and when submergence duration was lengthened from 24 to 48 h or 72 h (Table 2).

The number of plants that survived submergence and the extent of damage to full-grown leaves were related to the total amount of ethane produced (Table 2). In general, survival became less probable with the duration of submergence and thus with larger amounts of ethane produced. Similarly, for the same period of submergence, fewer plants survived when submergence started in the morning compared to the afternoon in association with a larger total ethane emission. Finally, for many treatments, more plants of cultivar CT6241 died than those of FR13A and this is linked to the larger amount of ethane it produced as compared to FR13A. Independent of starting time, duration of submergence, and cultivar, total fatality and 100% leaf damage were observed whenever total ethane release (i.e., the sum of submergence and post-submergence production) surpassed approximately 15 nl gFW^{-1}. We termed this amount as the "lethal limit".

Table 2 Ethane-emission accumulation (nl gFW^{-1} ± SD) during submergence of FR13A and CT6241 in the dark, the ethane production during 2 h following de-submergence, and the sum of both values

Submergence period	Cultivar	Ethane emission during submergence (nl gFW^{-1} ± SD)		Ethane emission after de-submergence (nl gFW^{-1} ± SD)		Total ethane production (nl gFW^{-1} + SD)		Survival (% ± SD)		Leaf damage (% ± SD)	
		Morning	Afternoon	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon
24 h	FR13A	7.3 ± 0.8*,**	3.9 ± 0.3*	1.2 ± 0.1*,**	0.8 ± 0.4	8.5 ± 0.8*,**	4.7 ± 0.5*	60 ± 30*,**	100 ± 0*	80 ± 10*,**	30 ± 5*
	CT6241	12.7 ± 0.1**	9.4 ± 1.1	1.7 ± 0.1**	0.9 ± 0.4	14.4 ± 0.2**	10.3 ± 1.2	20 ± 20**	90 ± 10	95 ± 5**	40 ± 5
48 h	FR13A	8.6 ± 0.9*,**	4.4 ± 0.5*	2.3 ± 1.1*	1.8 ± 0.7*	10.9 ± 1.4*,**	6.2 ± 0.9*	0 ± 0**	90 ± 10*	100 ± 0**	55 ± 10*
	CT6241	21.0 ± 1.8**	12.8 ± 1.5	4.0 ± 0.9**	6 ± 4	25 ± 2**	19 ± 4	0 ± 0	0 ± 0	100 ± 0	100 ± 0
72 h	FR13A	14 ± 3*,**	9 ± 2*	9 ± 3*,**	3.2 ± 1.5*	23 ± 4*,**	12 ± 3*	0 ± 0**	30 ± 20*	100 ± 0**	80 ± 10*
	CT6241	37 ± 15	33 ± 7	10.6 ± 1.4**	8 ± 3	47 ± 15	41 ± 7	0 ± 0	0 ± 0	100 ± 0	100 ± 0

Survival scores and extent of leaf damage are shown and were scored (means of three replicates ± SE) after 7 days. Leaf damage is scored as the damage percentage of the old leaves. No survival is observed for a total ethane production larger than 15 nl gFW^{-1}, irrespective of cultivar starting time of submergence. We have undertaken two sample *t* test to show statistically meaningful differences ($P < 0.05$) between appropriate pairs of means

*Significant difference between cv FR13A and cv CT6241 at each time of analysis (e.g., 24 h)

**Significant difference between morning and afternoon submergence for the same cultivar

Gas phase anoxia and hypoxia

In contrast to the substantial amounts of ethane produced by submerged plants, no ethane was directly observed from plants of either cultivar during exposure to an anaerobic environment created by a flow of nitrogen gas in the dark. However, as soon as 20% oxygen was restored, ethane evolved strongly. Examples of post-anoxic ethane production are shown in Fig. 3, for CT6241 treated for 4–72 h without oxygen. Post-anoxic ethane production after 8 h or longer periods of anoxia shows a rapid peak. This was followed by a long tail lasting several hours. The tail signifies an almost constant elevated output of ethane and precludes the determination of the total amount of ethane emitted during the post-anoxic interval. Ethane emission data integrated only over the first 1.5 h of post-anoxia are presented in Table 3, with related survival and leaf damage percentages. These demonstrate that CT6241 evolved more post-anoxic ethane than FR13A and that FR13A was less damaged by anoxia than CT6241 although the difference is less consistent than that seen in the aftermath of submergence.

When anoxia started in the morning, the strongest post-anoxic ethane peak was observed for 8 h of anoxia for both cultivars; however, if anoxia started in the afternoon (less subsequent damage to the seedling), the maximum post-anoxic effect was seen only after 48 h of anoxia. These differences are reflected in survival and leaf damage scores; high mortality occurred after shorter anoxic treatments when these started in the morning compared to the afternoon. As expected, CT6241 emitted more post-anoxic ethane than FR13A and suffered more damage from shorter anoxic exposures.

To test the notion that partial oxygen shortage could be responsible for stimulating ethane production during submergence, seedlings were sealed for 2 h in the dark in flasks containing initially 0, 0.1, 0.25, 0.5 or 0.75% (v/v) of oxygen in nitrogen gas. The presence of such small amounts of oxygen was found to support ethane production while anaerobic treatments and air (Table 4) failed to induce an ethane signal significantly above background. Furthermore, submergence-intolerant cultivar CT6241 formed about twice as much ethane as the more tolerant cultivar FR13A, reflecting the findings for submerged plants.

Discussion

We used ethane emission as a readily measurable marker to assess the role of lipid peroxidation in damage caused to rice seedlings by complete submergence.

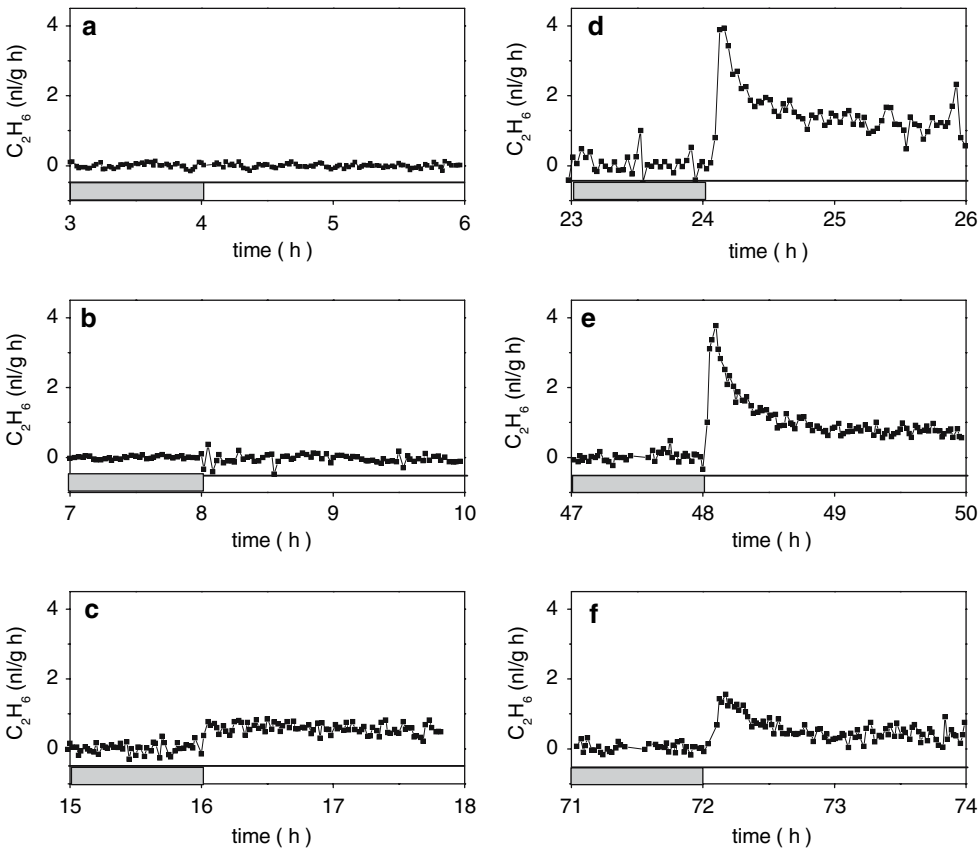

Fig. 3 a–f Rates of ethane production by seedlings of a submergence-intolerant cultivar of rice (CT 6241) subjected to a flow of anoxia-inducing nitrogen gas for 4 h (**a**), 8 h (**b**), 16 h (**c**), 24 h (**d**), 48 h (**e**) or 72 h (**f**) starting in the afternoon. The *grey bar* in the base of each graph shows when the plants were anoxic. The *light bar* shows when the plants were in air

Table 3 Ethane emission (nl $gFW^{-1} \pm SD$) by seedlings of two rice cultivars (FR13A and CT6241), exposed to a 100% nitrogen gas phase in the dark, after returning seedlings to air following increasing periods of oxygen-free conditions (anoxia)

Period of anaerobiosis	Cultivar	Ethane emission (nl $gFW^{-1} \pm SD$)		Survival (% ± SD)		Leaf damage (% ± SD)	
		Morning	Afternoon	Morning	Afternoon	Morning	Afternoon
4 h	FR13A	0.07 ± 0.04*,**	0.14 ± 0.08*	93 ± 5*,**	100 ± 0	20 ± 5	20 ± 10
	CT6241	0.3 ± 0.1**	0.02 ± 0.02	87 ± 10**	100 ± 0	25 ± 10	20 ± 10
8 h	FR13A	0.67 ± 0.17*,**	0.07 ± 0.03*	80 ± 20*,**	100 ± 0*	50 ± 10*,**	20 ± 10*
	CT6241	1.76 ± 0.07**	0.0 ± 0.06	9 ± 5**	90 ± 5	95 ± 5**	40 ± 10
16 h	FR13A	0.51 ± 0.01*,**	0.14 ± 0.03*	0 ± 0*,**	100 ± 0*	100 ± 0*,**	40 ± 10*
	CT6241	0.88 ± 0.03**	1.04 ± 0.17	18 ± 5	16 ± 0	80 ± 10	80 ± 10
24 h	FR13A	0.33 ± 0.07*	0.28 ± 0.11*	40 ± 20*,**	60 ± 10	90 ± 10*,**	80 ± 10
	CT6241	1.3 ± 0.5**	2.1 ± 1.0	0 ± 0**	60 ± 10	100 ± 0**	80 ± 10
48 h	FR13A	0.51 ± 0.07*,**	0.8 ± 0.1*	0 ± 0	0 ± 0	100 ± 0	100 ± 0
	CT6241	1.23 ± 0.13**	2.0 ± 0.2	0 ± 0	0 ± 0	100 ± 0	100 ± 0
72 h	FR13A	0.58 ± 0.08*,**	0.66 ± 0.09*	7 ± 0	20 ± 0	95 ± 5*,**	80 ± 10*
	CT6241	0.65 ± 0.05**	0.91 ± 0.17	0 ± 0	0 ± 0	100 ± 0	100 ± 0

The post-anoxic emission rates are integrated over the first 1.5 h after the start of re-aeration. Percentage survival and extent of leaf damage after 7 days recovery from anoxia also shown

*Significant difference ($P < 0.05$) between cv FR13A and cv CT6241 either for morning or for afternoon submergence treatments at each time of analysis (e.g., 24 h)

**Significant difference between morning and afternoon submergence for the same cultivar

A cultivar that is unusually tolerant of submergence (FR13A) was compared with a susceptible one (CT6241); it was found that, overall, greater tolerance of submergence (i.e., longevity) goes with less ethane production, i.e., with less lipid peroxidation.

Submergence

Plants submerged in the dark in unstirred water that contains normal levels of dissolved oxygen are likely to experience partial shortage of oxygen. This arises

Table 4 Effect of different concentrations of low levels of oxygen on the amount of ethane produced over 2 h by 2-week-old seedlings of rice cultivars FR13A and CT6241, under gas-phase conditions

Initial O_2 concentration	0%	0.1%	0.25%	0.5%	0.75%
	Ethane produced over 2 h (nl g^{-1}FW + SD)				
CT6241	0.3 ± 0.1*	1.2	1.0 ± 0.15	2.1*	1.9
FR13A	0.2 ± 0.1	0.55	0.65 ± 0.1	1.2	0.6

The low levels of oxygen stimulate ethane output above that of anaerobic and well-aerated plants. The ethane values are means of biological duplicates ± SE, or single estimates

*Significant difference ($P < 0.05$) between cv FR13A and cv CT6241

because slow gas diffusion rates in water decrease the inward flux of oxygen to rates that fail to match the plant's own normal oxygen consumption in the dark. Under conditions closely similar to ours, Boamfa et al. (2003) reported that dissolved oxygen concentrations decreased to 50% of the starting value during the first 2 h of submergence followed by a slower decline to 25% of normal oxygen after 24 h. Thus, after 1 day underwater external oxygen is still available to the plants.

Many biochemical changes take place in cells that are partially oxygen deficient (hypoxic). Examples include aerenchyma formation (Jackson et al. 1985), stimulation of ethylene production together with slower elongation in roots and an enhanced tolerance to later and more severely stressing anoxia (Saglio et al. 1988). Such changes are preceded by an, as yet, unexplained sensing of oxygen deprivation and by subsequent signal transduction pathways leading to altered patterns of gene expression and product processing or stabilization (Bailey-Serres and Chang 2005). These events are usually seen as part of a reorganization of cellular biochemistry that increases tolerance to possible later development of anoxia should oxygen deprivation subsequently intensify. Work with a human hepatoma Hep3B cell line (Chandel et al. 1998) indicates that hypoxia, induced by 1.5% external O_2, promotes the formation of signaling molecules effecting changes in gene expression of hypoxic cells. These signals include ROS generated by mitochondria as O_2^- . In plants, too, hypoxia (albeit very severe, 0.003%, oxygen and very dim illumination of 0.22 μmol m^{-2} s^{-1}) has been shown to promote an increase in ROS that in turn appears to activate alcohol dehydrogenase (ADH), a key enzyme in anaerobic energy metabolism (Fukao and Bailey-Serres 2004). Paradoxically, however, ROS like $OH^{\cdot}$ and $HO_2^{\cdot}$ can also highly damage plant membranes as a consequence of their ability to initiate a peroxidative-chain reaction affecting polyunsaturated fatty acids such as linolenic acid (Blokhina et al. 2003). Thus, control of ROS levels in hypoxic cells is critical to the survival of stressed cells if their adaptively useful role as signaling molecules is not to be overtaken by their potential to harm the cells by means of uncontrolled peroxidations.

In our work, significant ethane production during submergence is taken as evidence of ROS-mediated lipid peroxidation in hypoxic tissue under water. This is not an outcome of anoxic metabolism since we show that no ethane can be produced if oxygen is completely absent (Fig. 3). Table 2 and Fig. 2 show that there is an increase of accumulated ethane with duration of submergence, indicating that ethane is produced over the full period of submergence for up to at least 3 days. Submergence-tolerant FR13A appears better able to minimize underwater peroxidation than CT6241. This is seen in terms of less ethane being formed during the first 24 h of submergence and when inundation is prolonged from 24 to 48 and to 72 h. By varying the severity and length of the stress treatments and by comparing two cultivars of contrasting tolerance we have been able to compute from Table 2 and other data, how much ethane must be produced (nl gFW^{-1}) before the seedlings are killed (100% leaf browning and no new leaf production). This value is simply the total amount of ethane evolved during submergence and during the first 2 h after submergence. The limit is about 15 nl gFW^{-1} with the major part of the emission occurring during submergence itself. Regardless of the cultivar or treatment, seedlings die within a week once this total is exceeded. This value is thought to indicate the smallest amount of peroxidative damage that proves fatal.

One possible explanation for the difference in ethane production between these cultivars is that production of ROS-eliminating enzymes and anti-oxidants is smaller in CT6241. This is supported by the observation of a boost to the emission of acetaldehyde when rice seedlings were made hypoxic with 0.05–0.3% external oxygen, i.e., under micro-aerobic gas phase conditions (Boamfa et al. 2005). The emission was stronger from FR13A than from CT6241 and this was attributed to a more vigorous dismutation of O_2^- to H_2O_2 which in turn reacted with readily available ethanol to form acetaldehyde under the action of catalase. This pathway is thought to divert more O_2^- away from lipid peroxidation thus decreasing membrane injury and suppressing ethane production compared to submergence susceptible CT6241. Boamfa et al. (2005) also showed that acetaldehyde production was disproportionately high relative to that of ethanol in the micro-aerobic range.

We interpret this extra acetaldehyde as evidence of the action of protective SOD and catalase enzymes that collectively divert superoxide into acetaldehyde via H_2O_2 and ethanol. The observation of a strong and continuously rising acetaldehyde emission during micro-aerobic conditions reveals that the balance between ROS and its enzymatic scavenging system becomes perturbed at these small O_2 concentrations that typify submerged plants. Why this difference between cultivars occurs is unclear but it may relate to faster depletion of respirable reserves that characterizes submergence susceptible lines such as CT6241 (Ram et al. 2002). This may be an outcome of a faster rate of underwater leaf extension consuming more substrate (Jackson et al. 1987) thus leading to a greater energy crisis in the face of inhibited photosynthesis imposed by submergence and darkness. This may also explain the enhanced ethane production and damage if submergence starts in the morning. At that time respirable reserves are at a minimum (Ram et al. 2002).

Oxygen shortage under gas-phase conditions

The limited availability of oxygen during submergence can be mimicked to a certain extent by imposing micro-aerobic or anaerobic gas-phase conditions on the seedlings. Figure 3 shows that under complete lack of oxygen in the dark no ethane production is observed, i.e., no lipid peroxidation seems to occurs, presumably because of a total lack of oxygen acting as a precursor for ROS. This is in contrast to the situation in submerged plants and gives further support to the view that the injury to and the behavior of the submerged rice seedlings is not necessarily a consequence of anoxia. During the anoxic exposure it is reasonable to conclude that this severe stress compromises the ability of cells to produce enzymes and antioxidants (Blokhina et al. 2001, 2003; Jackson and Ram 2003). If true, a large burst in lipid peroxidation can be expected when anoxic rice seedlings are returned to air because the inevitable rise in ROS will go largely unchecked. In confirmation of this expectation, a large peak in ethane emission was indeed seen after re-aeration following more than 4 h without O_2. A similar effect, presumably with a similar explanation, is also seen when submerged plants are returned to air, although with some quantitative differences. One such difference is the inability of post-anoxic plants to restrict peroxidative damage to the first 2 h. In post-anoxic plants, peroxidative ethane production persists for considerably longer periods and this may indicate a chain reaction of lipid peroxidation that is a characteristic of the chemistry involved once the threshold for this chain reaction is exceeded (Halliwell and Gutteridge 2003).

Conclusions

We have shown that rice seedlings can be damaged fatally by submergence under conditions that do not necessarily induce anoxia. Absence of anoxia during this treatment is indicated by marked emissions of ethane. If the plants had been anoxic, such ethane production would be impossible. Ethane emission underwater is indicative of peroxidation of unsaturated fatty acids of lipid membranes by ROS. When total ethane emission exceeds approximately 15 nl gFW^{-1}, this is considered to be the outcome of a fatal amount of membrane attack. The generation of ROS and their incomplete scavenging appear to be responses to partial oxygen shortage since ethane production is shown to be promoted by micro-aerobic gas phase conditions (0.1–0.75% oxygen), but absent under anoxic and well-aerated conditions. Clearly, ROS involvement in submergence injury is not confined to post-anoxic situations as has been previously assumed. A submergence-tolerant cultivar (FR13A) formed less ethane than a susceptible cultivar (CT6241) and thus suffered less membrane injury. Previous findings (Boamfa et al. 2005) suggest this difference arises at least partially because a larger proportion of superoxide radicals is diverted into H_2O_2 as evidenced by the production of more acetaldehyde, generated in reactions involving superoxide dismutase, ethanol and catalase.

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