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Micro-aerobics: when rice plants lose their resistance against oxygen*

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Abstract
Photoacoustic determination of ethane, ethanol and acetaldehyde releases from 14 d old rice seedlings leads to the conclusion that rice seedlings start suffering significant lipid peroxidation under micro-aerobic conditions. To produce micro-aerobic conditions in otherwise normal atmospheres, the oxygen concentration has been reduced to a value between 0.3 and 0.05% (v/v). The defense of the rice seedlings against oxygenic radicals becomes insufficient under these almost anaerobic conditions. The findings presented here are relevant for the clarification of what causes non-survival of rice seedlings under prolonged submergence.

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(Some figures in this article are in colour only in the electronic version.)

1. Introduction

1.1. General

It is advantageous if a scientist has at his disposal a method that permits direct observation of the dynamics of key processes. With its non-invasive character, and its extremely high sensitivity to detect the emission of specific well-defined trace gases *in real time*, the photoacoustic technique represents such a method which allows revealing plant responses under stress situations.

This technique was applied to rice seedlings under micro-aerobic gas-phase conditions. Here, the plants suffer from scarcity of oxygen in an almost anaerobic situation. Trace gas emission of ethanol and acetaldehyde, but especially the release of ethane traces, were measured to reveal the occurrence of radical oxygen species (ROS) and the onset of injury by lipid peroxidation. In the normal about 21% of oxygen in the atmosphere, healthy plants are sufficiently protected against the aggressive nature of oxygen. If, for example some ROS are formed during respiration in the dark, scavengers are available to remove these dangerous species sufficiently before serious injury occurs. In rice seedlings, until the level of oxygen decreases to an astonishingly low concentration of 0.3%, no anaerobic fermentation is observed within the first 8 h of treatment [1]. Fermentation is recognized by the release of ethanol and its precursor acetaldehyde. At oxygen levels of 0.15% and below, a significant rise in acetaldehyde emission is found above its fermentative value. The enhanced acetaldehyde release is accompanied—or even preceded—by emission of ethane, the signal molecule of lipid peroxidation [2]. This micro-aerobic behavior is the subject of the present paper.

1.2. The technical development behind the micro-aerobic measurements

In the Nijmegen laboratory, trace gas measurements in biological systems were started by investigating ethylene emission by plants in stress situations [3, 4]. A CO_2 waveguide laser was utilized in an intra-cavity photoacoustic setup. Among biologically interesting gases, for ethylene, the still unsurpassed photoacoustic detection limit of 0.006 ppbv (parts per billion by volume) was reached [5]. A well-defined gas mixture was flowed over biological samples, at the rate of typically 1 l h^{-1}, and on-line analyzed in a photoacoustic detection cell. In this cell, the flowing gas was exposed to a chopped infrared CO_2 laser beam tuned to a laser line

* Dedicated to Professor Anna Giardini, University of Rome, at her retirement.

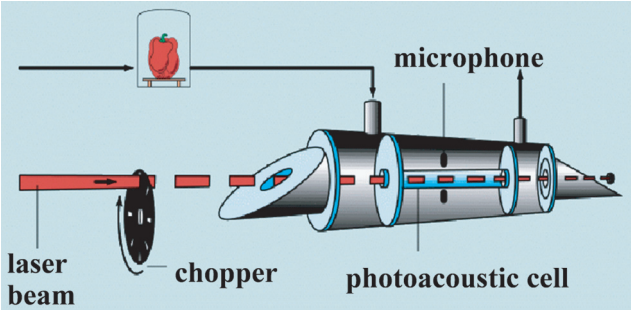

Figure 1. General outline of the experimental set-up. The infrared laser beam enters the photoacoustic cell from the left and is modulated by the chopper. The acoustic resonator becomes active if its gas content includes a trace gas component that absorbs the incoming laser light. Practically all absorbed light energy is transmitted to the acoustical oscillation. The chopping frequency is chosen to yield an acoustical oscillation in resonance with the resonator pipe. The oscillation is detected by sensitive microphones. In our actual experiment, the photoacoustic cell is placed inside the laser cavity to attain laser power in the watt-range. The red pepper fruit in the cuvette was the first plant system studied by us thoroughly, where we found the sharp postanoxic acetaldehyde peak that eventually led to the conclusions of this paper [6].

where ethylene showed a strong absorption. The resulting heating manifested itself by pressure changes at the chopping frequency which were measured by sensitive microphones (figure 1).

The ethylene success, the subsequent extension to other biologically interesting gases utilizing different lasers for photoacoustic detection, and the additional application of sensitive mass-spectrometric techniques with minimal fragmentation during ionization (PTR–MS, proton transfer reaction–mass spectrometry) led to the recognition of the Nijmegen laboratory as Life Science Trace Gas Facility for Biology, by the European Union under the 4th, 5th and 6th framework; practical detection limits for various gases can be found on www.ru.nl/tracegasfacility.

In addition to investigations of many aspects of ethylene in the plant life cycle, fermentation processes were studied via the emissions of ethanol and acetaldehyde in plants and fruits under low oxygen conditions [6, 7]. Oxidative stress and pathogen attack have been investigated via lipid peroxidation markers [8, 9]. The measurement of nitrogen fixation potential in heterocyst cyanobacteria via the acetylene assay clarified the effect of varied oxygen levels in this process [10, 11]. In the rice rhizosphere, methane oxidation and diffusive methane transport through flooded rice plants into the atmosphere were quantified [12]. The respiration of insects was monitored by measuring the dynamics of methane, water and carbon dioxide emissions [13, 14].

The hereafter discussed trace gas measurements in rice seedlings were performed with a laser-photoacoustic setup. The used laser was a CO-laser in its normal (ethanol and acetaldehyde) and its overtone (ethane) version [1, 2, 13], [15–18]. A biomedical application of such a laser system for detecting ethane in exhaled breath is found in [19]. The lowest detection limit reached for ethane achieved with this system is 0.1 ppb (see hereinafter).

The trace gas detection technique practiced in the Nijmegen laboratory has two drawbacks that form a barrier for general application. The needed costly equipment is not commercially available, and it is not trivial to operate. For the study reported here, the CO-lasers have been homemade by experienced glassblowers. They are cooled by some liters per hour of liquid nitrogen flushed through a jacket around a 1.16 m long discharge tube. In order to achieve the highest detection sensitivity, and to realize laser action at many hundreds of CO transitions, skillful tuning of the lasers is needed while the photoacoustic cell is mounted in an intra-cavity setup. The system has to work stably round the clock. All this requires a small team of dedicated and experienced operators. Even the operation of the much simpler CO_2 laser for measurements in biological laboratories has encountered serious difficulties without continuous technical assistance; a way out of this dilemma is offered by the restriction to a single type of trace gas like ethylene, thus giving up the intrinsic flexibility of the laser system. This restriction allows a significant lowering of costs and renders the system much easily operable (see, e.g., www.sensor-sense.nl).

In future, gas lasers, so far mainly used in photoacoustic studies, will probably be replaced by much smaller and eventually less costly, solid-state laser devices. Recent studies based upon solid-state laser technology look promising [20–25]. It is instructive to follow the improvement of the detection limit for ethane in the last few years. For ethane in air, the lowest detection limit obtained for the ‘CO overtone-laser-based spectrometer’ has been about 0.1 ppb volume, e.g. [26]. Kuhnemann *et al* [27] have already reported a detection limit of 0.5 ppb for an ‘optical parametric oscillator (OPO)-based detector’ in 1998. In 2002, with a similar OPO system van Herpen *et al* [28] achieved 10 ppt. Only 2 years later, von Basum *et al* [29] reached the state-of-the-art value of 3 ppt; if the integration time increased from 1 s to 3 min, a detection limit of 0.5 parts per 10^{12} was found. In spite of this impressive success story, the drawback still remains that complete systems of high sensitivity and facile operation are not yet available commercially. A review of recent developments is found in [30].

An alternative approach to laser spectroscopy for trace gas detection is based upon mass-spectrometric methods. Their weak point is the fragmentation of trace molecules upon ionization by electron bombardment, the technique usually applied. The fragmentation renders the disentangling of mass spectra from gas mixtures with many unknown molecular components very difficult indeed. In recent years, the introduction of PTR for ionization has significantly diminished the fragmentation difficulties. Low-cost PTR–MS instruments are commercially available nowadays (www.ptrms.com). The remaining fragmentation limits their practicality for trace gas detection to molecules with masses below about 100 amu. For the detection of, e.g., acetaldehyde traces, PTR–MS is much easier, less costly and more sensitive a method than the photoacoustic technique [7]. Its practice still requires judicious technical know-how.

1.3. Rice problems

Rice is a staple food for people in Asia. Supporting about 700 million people, 25% of the total area cultivated for rice

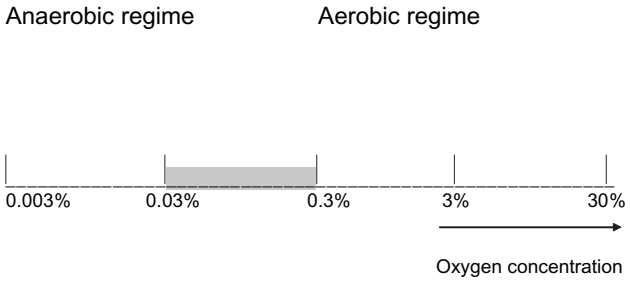

Figure 2. The micro-aerobic regime, for rice between 0.03 and 0.3% of oxygen. The regime (gray bar) is characterized by sufficient oxygen to form ROS abundantly, but insufficient oxygen for providing complete defense against ROS attack. It is the region where heavy lipid peroxidation occurs and ethane is observed, coupled with partial ROS scavenging and non-fermentative acetaldehyde emission. It is mainly this oxygen range where new phenomena were observed. The anaerobic regime extends below 0.003% of oxygen. The aerobic regime is centered on 20% of oxygen; for rice no fermentation is observed within the first 8 hours of treatment for oxygen levels higher than about 0.3%.

is potentially vulnerable to flooding. Young seedlings of this rainfed low-land rice often suffer irregular flooding, and losses are severe if submergence lasts for periods longer than about a week [31]. As part of the European Union INCO-DC program 'Rice for Life', it has been investigated by the Nijmegen group what causes fatal injury by submergence, in order to stimulate the breeding of more tolerant species. Submergence was mimicked in gas-phase experiments, imposing hypoxic or anaerobic conditions on rice seedlings. The advantage of gas-phase experiments derives from the fact that the conditions can be better controlled. The practical relevance of the conclusions has been checked by submergence tests [2, 32]. In the course of this investigation, it became clear that interesting and eventually injurious processes occur when rice seedlings are exposed in the dark to micro-aerobic (i.e. almost anaerobic) oxygen levels below 0.3%, a domain that perhaps was not considered before with deserved attention (figure 2). Phenomena signaled by ethane releases and by acetaldehyde and ethanol releases are discussed in the following.

2. Rice seedlings under oxygen stress

2.1. The anaerobic phase and postanoxic effects

In the anaerobic limit (zero-oxygen level), plants provide the necessary energy for cell maintenance by alcoholic fermentation. Fermentation is rather inefficient as compared to aerobic respiration; about 6% alone of the ATP molecules produced from sugars by aerobic dark respiration becomes available. Plants adapt to this shortfall by switching off energy-consuming processes that are not strictly necessary for short-time survival. With oxygen lacking, there is no danger of injurious ROS and therefore the replacement of ROS-scavenging enzymes is interrupted. This adaption is the principal hypothesis in our description of the processes that lead to the observed trace gas releases under postanoxic and micro-aerobic conditions [6, 31].

During anaerobiosis in the dark, no ethane has been observed (table 1); this marker molecule of lipid peroxidation

Table 1. Effect of different initial concentrations of oxygen on the amount of ethane accumulated during the first 2 hours, from 2-week-old seedlings of rice cultivars FR13A and CT6241, under gas-phase conditions. The low levels of oxygen stimulate ethane output above that of anaerobic or well-aerated plants. The ethane values are means of biological duplicates with standard errors (SD), or single estimates. An asterisk (∗) indicates a significant difference ($p < 0.05$) between cv FR13A and cv CT6241. The results (nl $= 10^{-9}$ l) are normalized for 1 g fresh weight of seedlings, nl g^{-1} FW (from [2]).

Initial O_2 concentration	Ethane produced during 2 h (nl g^{-1} FW ± SD)				
	0%	0.1%	0.25%	0.5%	0.75%
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

is not formed due to the absence of ROS that cannot be generated anoxically [2]. The situation changes very much under micro-aerobic, i.e. near-anaerobic conditions, where lipid membranes are being attacked by ROS and ethane is emitted, as will be discussed hereinafter.

On the other hand, dark anaerobic conditions lead to fermentation and besides ethanol, its precursor acetaldehyde is observed [1]; the production rate of acetaldehyde levels-off after an anaerobic period of about 1 h (figures 3(A) and (B)).

If after an anaerobic treatment in the dark, lasting for 8 h or longer, air is admitted again; a sharp postanoxic production spike of ethane often with a long tail is observed (figure 4). When this ethane spike becomes observable, the seedlings are already moribund [2, 33]. The long tail is considered as evidence of continued lipid peroxidation, often referred to as originating from a chain reaction [34].

The high mortality observed accompanying the ethane spike contrasts with the postanoxic acetaldehyde spike occurring already after 1 h of anaerobic treatment, with greater than 80% survival being seen when periods of anaerobiosis last for 8 h or less. The postanoxic acetaldehyde spike on top of the slowly varying fermentative acetaldehyde production is considered to arise from a beneficial scavenging process, whereby superoxide O_2^- is converted to less dangerous hydrogen peroxide [1, 6, 31]. The production of this non-fermentative acetaldehyde is thought to be the result of a two-step reaction. The first step is promoted by the enzyme superoxide dismutase (SOD) and yields H_2O_2 from O_2^- and O_2; H_2O_2 oxidizes ethanol in the second step to form acetaldehyde and H_2O under the catalyzing action of catalase (CAT). Hereafter, we shall refer to this reaction as the SOD–CAT process.

2.2. The micro-aerobic treatment of rice seedlings

Micro-aerobic gas phase conditions in the dark yielded ethane signals too weak to be detected in real time. Santosa *et al* [2] resorted to cumulative ethane measurements where batches of rice seedlings are sealed in flasks containing mixtures of nitrogen and oxygen. Only the initial oxygen concentration is then well defined. Values equal to 0.1, 0.25, 0.5 and 0.75% (v/v) were chosen. After 2 h of accumulation, a clear integrated ethane peak has been found when the resulting gas mixture in the flask is washed through the detector by nitrogen flow (table 1). The micro-aerobic ethane signals show that

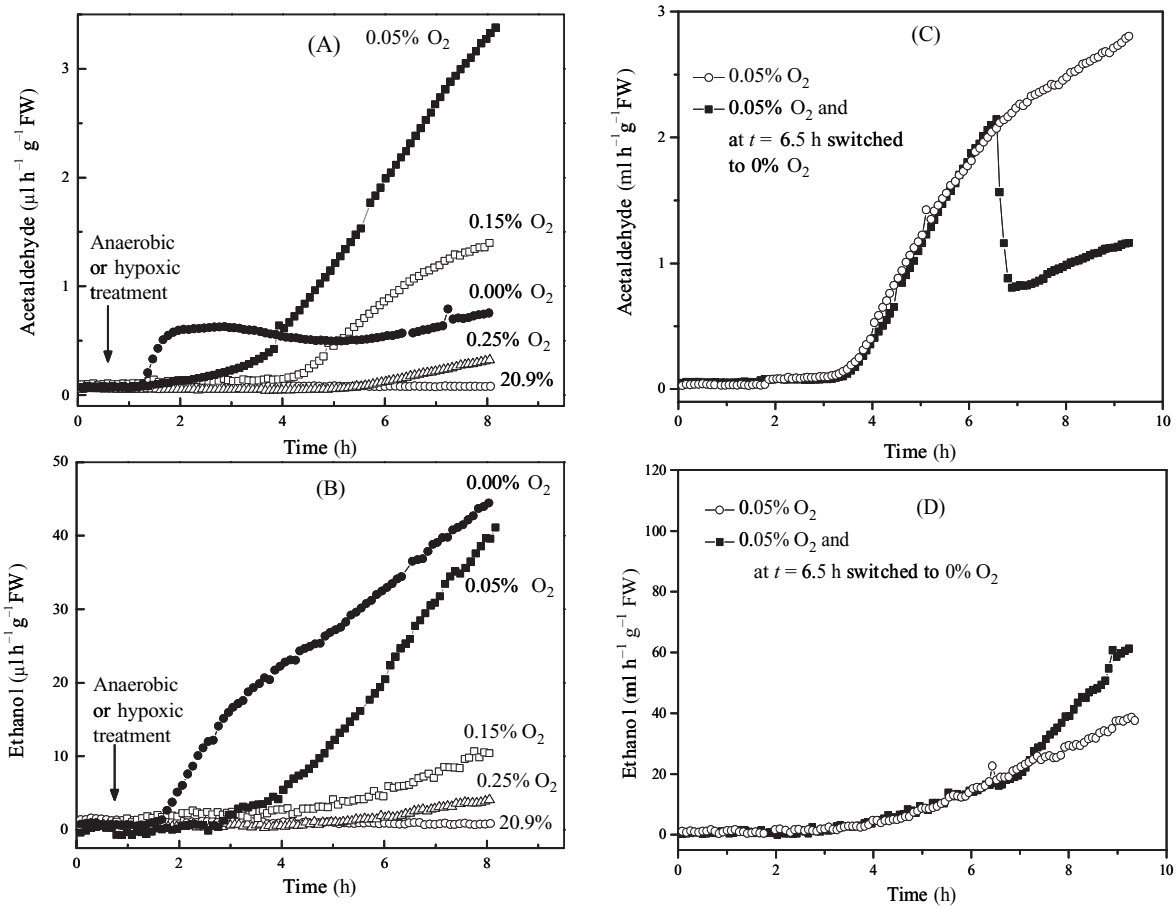

Figure 3. Acetaldehyde and ethanol emission rates, under aerobic, anaerobic and micro-aerobic conditions, applied to the rice cultivar CT6241. Left: the effect of 8 h treatment, compared to a continued aerobic situation, for acetaldehyde, A and ethanol, B. Note the huge rise in acetaldehyde release for 0.05% of oxygen. Right: the plunge in acetaldehyde production rate at the transition from micro-aerobic to anaerobic conditions, C, has no counterpart for ethanol, D. In C and D, the micro-aerobic treatment starts at $t = 0$ h. From [1]; μl h^{-1}g^{-1}FW, microliter per hour per gram fresh weight.

under these extremely low oxygen levels lipid peroxidation occurs, leading eventually to the death of the plants.

Submergence under our laboratory conditions creates micro-aerobic conditions, too; rice seedlings, submerged in the dark in unstirred tap water in a closed flask, produce ethane that has been accumulated for periods up to some days. The observed amount of ethane largely exceeds post-treatment emissions observed after the seedlings are returned to air, and increases with duration of submergence; the survival percentage of seedlings is zero after 2 or more days of this submergence treatment [2]. In figure 5, the results shown are for a 24 h submergence period. These laboratory measurements are supposed to mimic what happens in flooded rice fields.

For gas-phase measurements in the dark under micro-aerobic conditions, the acetaldehyde and ethanol results are shown in figure 3. The signals for the final product of alcoholic fermentation, ethanol, and for acetaldehyde appear to be mutually decoupled for increasing, but still nearly zero oxygen levels. Starting from its high anaerobic level, the ethanol production rates drop with enhancing oxygen levels, in agreement with the expected; the more the oxygen surrounding the seedlings, the less the fermentation. The acetaldehyde production rates, however, are largest for about 0.05% of oxygen with a value far above the anaerobic level, and then decrease to zero at 0.3%. This acetaldehyde overshoot is taken to originate from the non-fermentative process that also is responsible for the postanoxic acetaldehyde peak, the SOD–CAT process discussed hereinbefore [1]. Again, the acetaldehyde overshoot indicates the beneficial process of superoxide removal.

Boamfa *et al* [1] have shown that under micro-aerobic gas-phase conditions fermentation starts with a time delay of a couple of hours. Consequently, neither fermentative acetaldehyde nor ethanol is formed during the first 3–4 h, figures 3(A) and (B). As we have seen, a significant C_2H_6 release is already observed during the first 2 h of micro-aerobic treatment (table 1). Thus, lipid peroxidation starts while fermentation is still absent. Probably partial scavenging occurs in this period. But non-fermentative acetaldehyde, our marker molecule for scavenging processes, is not produced either, because during the early stages of the micro-aerobic treatment there is no ethanol substrate that is needed for the second step of the SOD–CAT process.

2.3. Two checks

Check 1. Under micro-aerobic conditions, the ethane emission from a relatively submergence-tolerant rice cultivar (FR13A) is consistently lower than that from

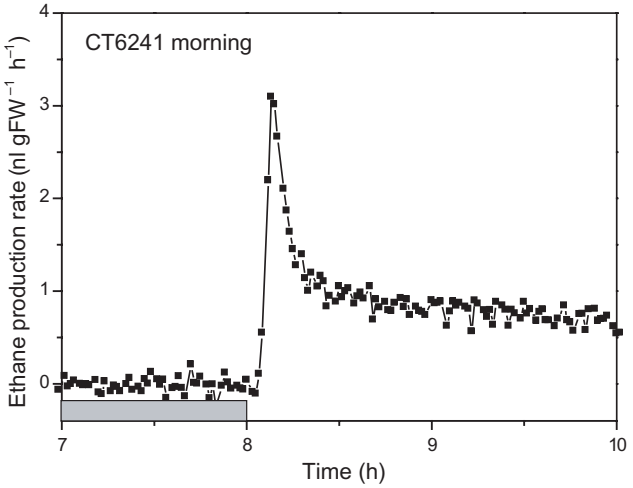

Figure 4. The postanoxic ethane release, after 8 h of anaerobiosis. CT6241 seedlings were exposed to an atmosphere without oxygen after a dark night in which part of their carbon hydrate reserves exhausted due to respiration. From [33]; nl gFW^{-1}h^{-1}, nanoliter per hour per gram fresh weight.

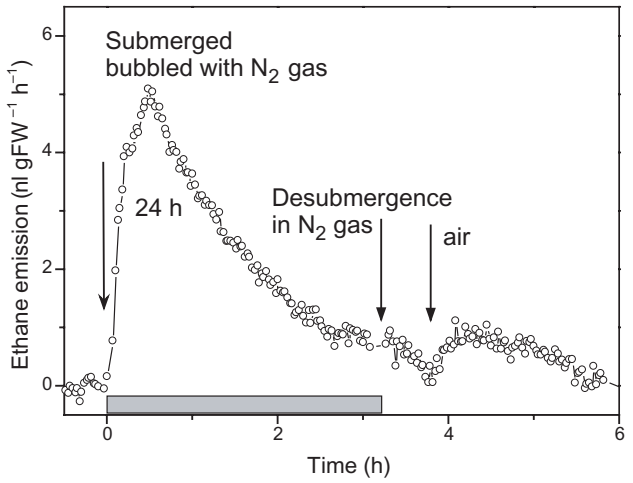

Figure 5. The ethane production by rice seedlings under submergence in the dark, accumulated during 24 h. For $t < 0$, the reference signal is shown measured before the submergence water is stirred by bubbling N_2 through it. The N_2 flow is analyzed in the photoacoustic measuring cell and a strong signal is observed. The integral under its area (above the gray bar) amounts to 7.3 nl gFW^{-1} and represents the amount of ethane accumulated. After de-submergence in N_2 the rice seedlings are exposed to air and the post-treatment ethane emission is measured in real time, $t > 3.8$ h. Together with the ethane accumulated during submergence, a total of 8.3 nl gFW^{-1} is found. For this figure, the rice cultivar FR13A was chosen; submergence started in the morning (from [2]).

the submergence-intolerant cultivar (CT6241) (table 1). The lower ethane-emitter lives longer being the cultivar with less lipid peroxidation. The release of ethanol and acetaldehyde under micro-aerobic conditions is compared for the two different rice strains and depicted in figure 6. Their ethanol signals do not show significant differences; however, concerning the overshoot signal of non-fermentative acetaldehyde in the micro-aerobic regime, FR13A emits significantly more than CT6241, i.e. submergence tolerance comes along with an enhanced beneficial scavenging activity.

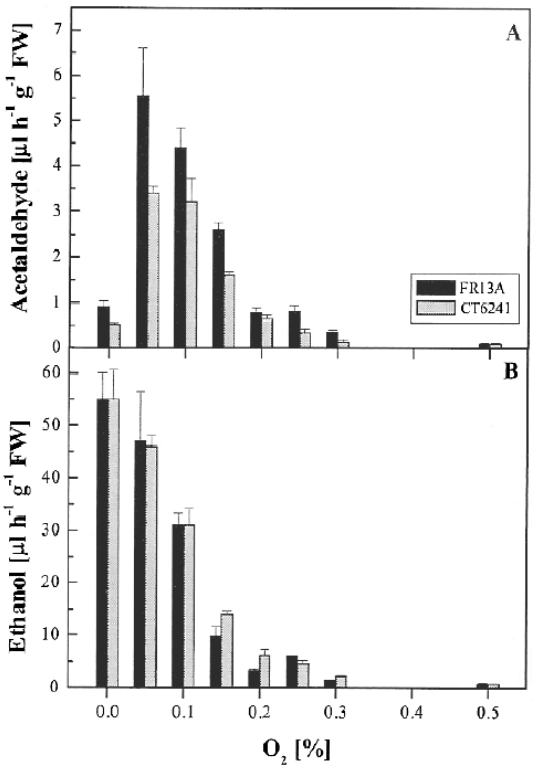

Figure 6. Effect of 8 hours of anaerobic and micro-aerobic gas-phase treatment in the dark on production rates for acetaldehyde (A) and ethanol (B). The measurements were performed on batches of three 14-days-old seedlings of CT6241 (gray bars) and FR13A (black bars). The production rates were measured at the end of the treatment. Standard errors of 4–5 individual measurements are shown (from [35]).

Check 2. In figure 3(C), the effect on the micro-aerobic acetaldehyde release is shown, if one switches from micro-aerobic to anaerobic conditions. Whereas the ethanol release keeps rising (figure 3(D)), the acetaldehyde emission plunges to less than half its value, demonstrating that at least one-half of its contribution comes from non-fermentative origin [1]. (After the switch, ethanol actually is emitted at a somewhat higher rate. ROS present under micro-aerobic conditions attack glycolytic enzymes [34]. The concentration of ROS drops at anaerobiosis and thus ethanol may be produced at a higher rate.)

2.4. The point of no return

So far, we have discussed evidence that the capability of rice seedlings to survive a certain period of anaerobiosis appears to be related to their more or less developed defense mechanisms against the occurrence of ROS. In this section, we shall introduce a quantitative argument that will corroborate the plausibility of this point.

Consider the critical duration of submergence, D_c, for the two investigated cultivars. When submergence lasts longer than D_c, the point of no return is reached and the seedlings have been observed to perish. Non-survival is observed about a week after the seedlings are returned to air. Under the conditions of our submergence experiments, D_c typically amounts to 48 h. D_c has been found to depend strongly on the type of cultivar, CT6241 and FR13A, with $D_c(\mathrm{FR}) \approx 2D_c(\mathrm{CT})$ [2].

In addition, experiments have been performed with different starting conditions. Either submergence begun after a night in the dark, when the seedlings had consumed part of their reserves of carbon hydrates by respiration, or the starting point was taken in the afternoon after a day in the light, when the seedlings had built up their reserves of carbon hydrates. We found D_c(afternoon) $\approx 2\ D_c$(morning) [2].

It is possible to bring the results of the last two paragraphs together to a single point. For both the rice cultivars and for both the morning and the afternoon submergence treatments, the point of no return is reached when a total of about 16 nl gFW^{-1} of ethane is released by the seedlings; nl gFW^{-1} nanoliter per gram fresh weight. This finding strongly suggests that the lethal mechanism behind the non-survival of submergence of rice seedlings is the lipid-peroxidation process induced by micro-aerobic conditions.

As a last point, we mention that under gas-phase conditions the lethal limit after an anaerobic treatment, i.e. the 'total' minimum postanoxic ethane release concomitant with non-survival, is about ten times smaller than the quoted 16 nl gFW^{-1} [2]. Note that 'total' refers to an integral over the first 1.5 h after reaeration only, while the continued ethane production from the so-called chain reaction is clearly observed. Under gas-phase conditions mitigating osmotic effects are absent, which under submergence dilute the concentration of potentially dangerous agents and abate the chain reaction; compare figures 4 and 5.

3. Conclusions and outlook

An important conclusion from the investigation of rice seedlings is that under micro-aerobic gas phase conditions lipid peroxidation takes place unexpectedly. The capacity of certain rice cultivars to cope better with these dangerous and even lethal conditions coincides with their higher capacity to withstand longer periods of submergence. This significant difference among rice cultivars has been found to depend on the precise balance between ROS production and ROS inactivation by scavenging processes.

Considering what might happen to plants other than rice, one has to keep in mind that rice seedlings are exceptionally well adapted to submergence, i.e. to micro-aerobic conditions. The critical duration of submergence, introduced hereinbefore, D_c, is so much longer for rice than for most other seedlings that farmers inundate rice fields to get rid of weeds—which are generally believed to quickly suffocate under flooding. In the light of our results, it seems advisable to control this picture. A significant part of the advantage of submerged rice seedling may come from their more effective handling of ROS and not from their higher capability of anaerobic respiration. It seems possible that weeds with very short D_c values still have a critical ethane emission not too far from the rice value of 16 nl gFW^{-1}. In our opinion, this would demonstrate the point that their survival also is significantly influenced by their protective handling capability of ROS. In some plant species, however, efficient scavenging of ROS may still work sufficiently well when the oxygen level is down to values where ROS is no longer produced in injurious amounts. In that case, the plant survival under submergence could be a pure question of capability of anaerobic respiration. It seems worthwhile investigating rather in general, in our view, unpredictable behavior of various plants other than rice under their austerity program at extremely low oxygen levels.

Acknowledgments

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