BEYOND HYPOXIA: RICE SEEDLINGS BETWEEN LIPID PEROXIDATION AND FERMENTATION.

Joerg Reuss and Frans Harren, Trace Gas Facility, Radboud University Nijmegen, Faculty of Science, Institute for Molecules and Materials, Department of Molecule and Laser Physics. Revised 21/11/'12

Abstract. A parallel analysis of ethane & acetaldehyde trace gas emission from rice seedlings under oxidative stress yields real time information on the onset of the fermentation process upon decreasing the oxygen concentration; at slightly higher oxygen concentrations, lipid peroxidation is observed due to failing defense against reactive oxygen radicals. This lipid peroxidation is shown to become a determining factor for the survival chance of the rice seedlings exposed to flash floods.

1. Introduction. This paper is based on measurements performed in the Physics Laboratory of the Radboud University Nijmegen, in the years between 1998 and 2005. The research was largely financed by the EU project RICE FOR LIFE (ERB3514-PL95-0708, directed by Dr. Michael Jackson, Bristol), and by the Dutch Academy of Science KNAW project 95BTM04-Indonesia. It deals with rainfed lowland rice, in Asia often hit by temporary flash flooding that can lead to total submersion of the seedlings and to subsequent complete loss of a crop locally, Ito et al. 1999. The Nijmegen task in this project was to possibly find out the reason why certain rice cultivars show a higher tolerance of long term flooding. The trace gases ETHANE and ACETALDEHYDE are emitted by rice seedlings under conditions simulating the stress encountered in flooded fields. For the rice cultivars CT6241 and FR13A it was found that their different tolerance against flooding is connected to the effectiveness to deal with reactive oxygen species. This connection has recently been found, too, to be important for waterlogging-tolerant & -susceptible pigeon pea genotypes, Sairam et al. 2009; Kumutha et al. 2009. In the present work, the properties of CT6241 and FR13A are further exploited to clarify how the lipid peroxidation process takes place in real time especially under micro-aerobic conditions, i.e. at oxygen concentrations as low as 0.05 % v/v.

The two processes, fermentation & lipid peroxidation (LIPOX), occur almost simultaneously at low oxygen concentrations and both lead to the release of acetaldehyde, whereas the emission of ethane originates unequivocally from LIPOX. Micro-aerobic conditions are realized in the gas phase and during submergence. Complete anaerobiosis in the gas phase is discussed, too, because during re-admission of air after anaerobiosis short micro-aerobic episodes are observed.

Abbreviations: FTG – fast trace gas detection; FW – fresh weight; fwhm – full width half maximum; Lipox – lipid peroxidation; nl/g h = nl h^{-1} g^{-1}FW – nanoliter per gram fresh weight per hour; ROS – reactive oxygen species; SD – standard deviation; SOD – superoxide dismutase; v/v – volume per volume.

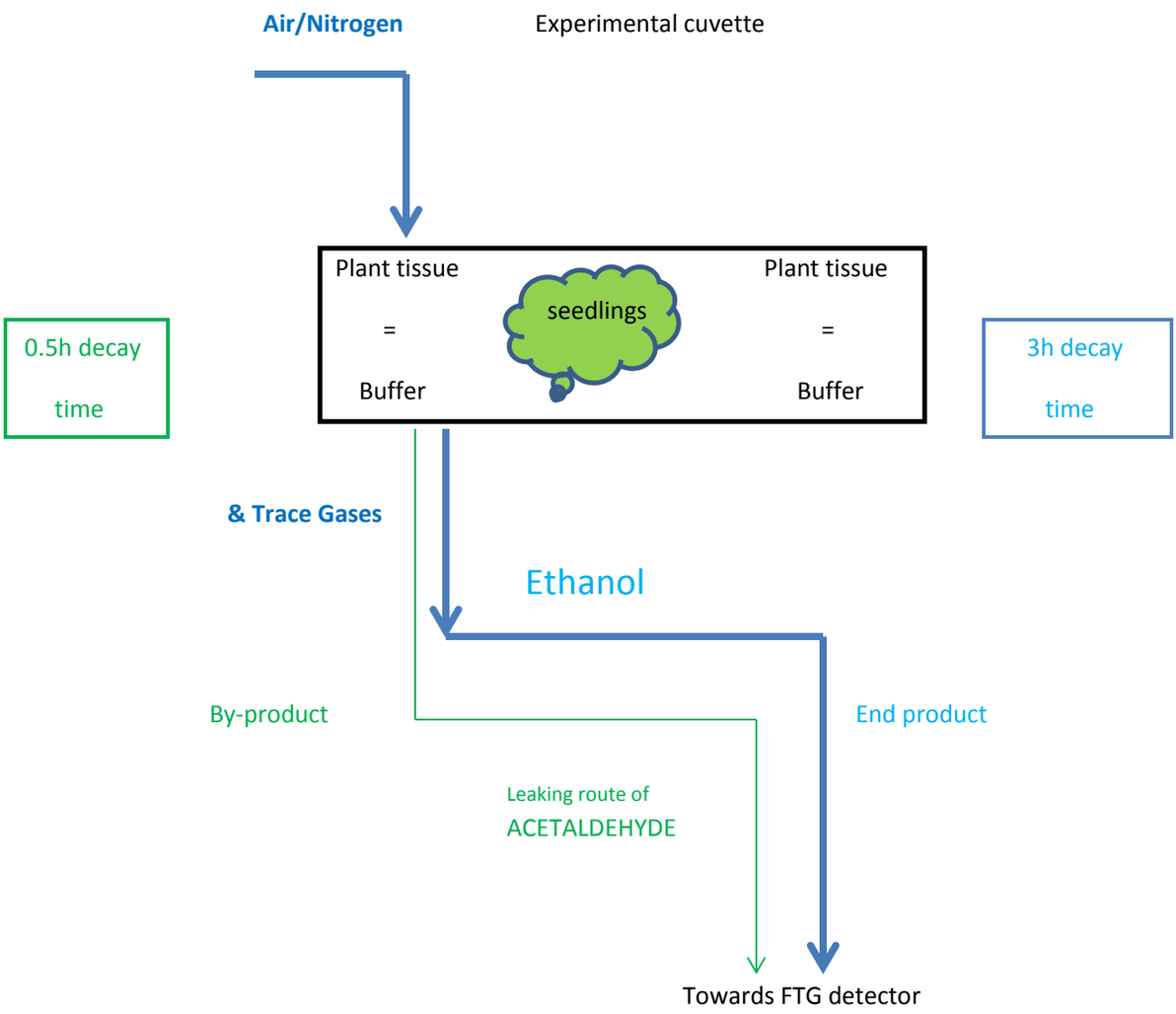

Fig. 1. Pathway of acetaldehyde and ethanol, produced during & after hypoxic stress and flowing towards the FTG detector. Trace gases are released from plant tissue into the gas-phase with indicated retention times. - In case of submergence experiments, the seedlings are completely under water, i.e. the buffer consists of tissue and submergence water.

1.1 FTG detection

Sensitive Fast- Trace- Gas detection is the important technical element at the basis of the investigations described in this work. It allows studying the dynamics of LIPOX in plant cells, in real time. The restriction to trace gas detection comes forth from the intention to follow fast processes by the emission of relatively small and rather inert molecules that can be unequivocally and non-invasively measured by photo-acoustic detection techniques. Fast means to allow detection at least within minutes and preferably within seconds. Measurements have used photo-acoustic detection equipment mounted intra-cavity, inside the cavity of a powerful CO laser, (Urban 1995, Zuckermann et al 1997, Martis et al. 1998, Bijnen et al. 1998,-Reuss & Harren 2008). Gas phase measurements were performed in a flow system, where nitrogen or air (with a reduced oxygen content) passes over the investigated plants in a

cuvette, thereby taking up emitted gas traces; these are subsequently analyzed in the FTG detector through which the gas flow is directed, Fig. 1.

1.2 Uniqueness of C_2H_6

In the context of this paper, ethane is unique in that it originates exclusively from LIPOX processes, at least under the natural conditions here discussed. It is the small and rather inert product of reactive oxygen species (ROS) attacking linolenic acid. The fact that ethane comes only from linolenic acid does not form a severe handicap; linolenic acid occurs in all plant leaves and in some oil seeds, e.g. soybean, rapeseed, and linseed oils; linoleic acid is sufficiently widespread to render ethane a rather general tracer molecule to detect lipid peroxidation in plants, Halliwell & Gutteridge 1999. FTG detection of ethane monitors LIPOX in a non-invasive manner so that living tissue can be observed over long periods. Other small carbon hydrates like ethylene (C_2H_4) are also products of LIPOX and easily detectable. Their origin from LIPOX must, however, be ascertained e.g. by parallel measurement of ethane.
In this work, ethane has been observed originating from rice seedlings under oxygen deprivation, Santosa et al. 2007. The detection limit has been about 100 ppt v/v. This detection sensitivity has been recently improved by two orders of magnitude, thus opening exciting research avenues, von Basum et al. 2004.

1.3 Between LIPOX and fermentation

The most important natural oxidant is atmospheric oxygen. Plants and animals are adapted to oxygen and depend on it. Its dangerous aspects are felt strongly, when the oxygen concentrations become anomalously high or low: too high leads to burning of carbon hydrates; too low leads to asphyxia. Plants can endure low concentrations of oxygen astonishingly well. They switch to alcoholic fermentation, if oxygen runs low, and stay alive at least during limited stress periods, using up their sugar reserves.

In the gas phase, micro-aerobic conditions concern very low oxygen concentrations, at and slightly above 0.05% v/v. It is the range where anaerobic fermentation becomes eventually replaced by LIPOX as the main contributing process for acetaldehyde emission, Boamfa 2005 and Santosa 2007, see also Reuss & Harren 2008.

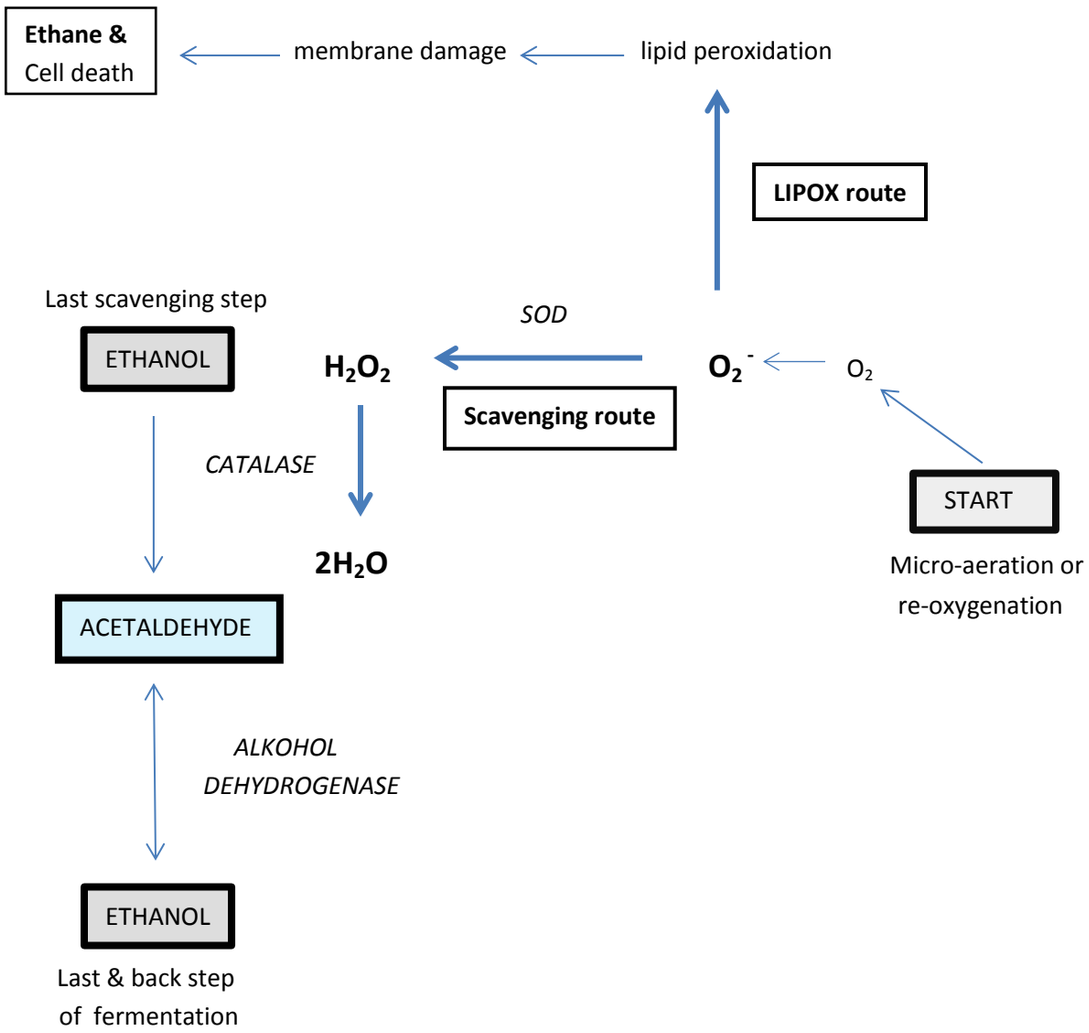

Fig. 2 P*roduction of "peroxidative" acetaldehyde along the scavenging route, and the route of the back-reaction of the last fermentation step that leads to "fermentative" acetaldehyde. This double production scheme was proposed by Monk et al. 1985. The LIPOX route leads to ethane production.*

LIPOX occurs as consequence of the presence of ROS, a dangerous and fortunately rare byproduct of oxygenic respiration. Living systems normally keep this byproduct under control, mainly by scavenging, i.e. transforming the radicals into less dangerous molecules. Enhanced concentrations of products of LIPOX are, however, observed during stress situations, when the scavenging defense becomes insufficient. Shortage of oxygen creates such a stress situation that is accompanied by LIPOX. – Improved scavenging attenuates LIPOX. In the presence of ethanol, efficient scavenging of ROS (i.e. the transformation of ROS into the less dangerous hydrogen peroxide, H_2O_2) yields a "peroxidative" acetaldehyde release, the final product of the scavenging route, Fig. 2. - Further lowering the concentration of oxygen leads eventually to fermentation, i.e. production of ethanol and its precursor, the "fermentative" acetaldehyde. - The stress by ROS, the failing and re-establishment of defense against ROS and the alternative provision with energy via fermentation form a combination of events that is addressed here by parallel FTG observation of ethane & acetaldehyde emitted by rice seedlings under shortage of oxygen. - We have only studied seedlings of two rice cultivars; other cultivars have been investigated by enzymatic techniques and yield results not contradicting our findings, Ushimura et al. 1995.

2 Rice seedlings exposed to various hypoxic conditions and beyond, in the dark.

2.1 Gas-phase (post-)anaerobic treatment. We first discuss gas-phase results obtained under extreme conditions: during anaerobiosis no ethane release whatsoever is observed; however, post-anaerobic emission occurs: a sharp ethane peak is measured after periods of anaerobiosis of 8h or more, Fig. 3.1, attesting an episode of LIPOX.

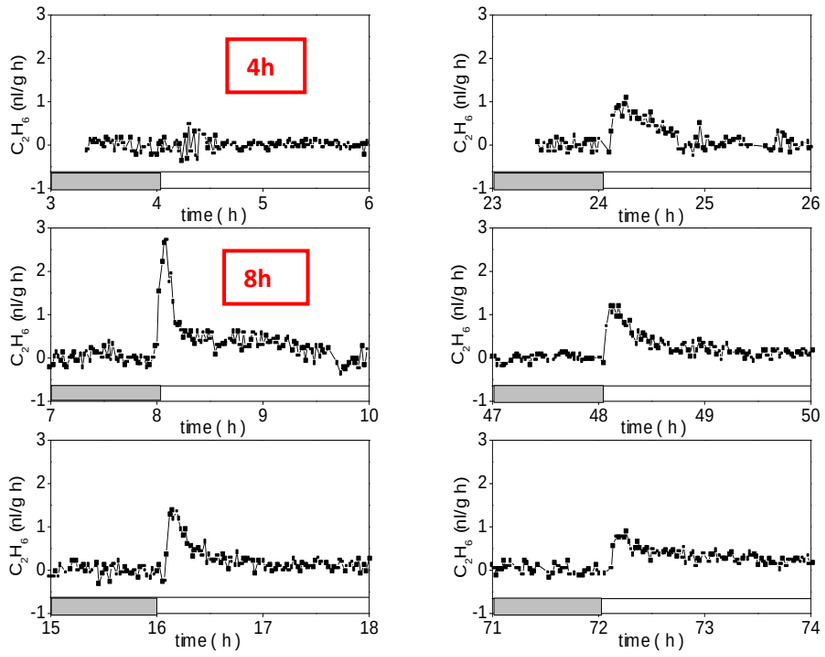

Figure 3.1 *Ethane emission rates (nl/g h) from the tolerant cultivar FR13A. Rice plants are subject to anoxia in the dark, starting in the morning. The grey bar at the bottom of each panel indicates that the plants are under anaerobic conditions (nitrogen flow) starting at t = 0; the light bar signals (return to) normal air conditions. From Santosa 2002*

The ethanol emission rate due to fermentation, measured during anaerobiosis, shows a continuous increase for about 10 h of anaerobiosis, or else until an air flow replaces the nitrogen flow through the cuvette containing the rice seedlings, Fig. 3.2. A measured rate of 1 µl h^{-1} g^{-1} FW v/v corresponds to the ethanol concentration of 10^{-4} % v/v in a gas flow of 1 l/h. - Note that the measured ethanol rate is not the production rate of the rice seedlings in the cuvette; part of the produced ethanol accumulates dissolved in the seedling tissue, from where it diffuses very slowly into the surrounding gas flow. The observed slow continuous increase is evidence for a build-up of alcohol concentration in the plant tissue that acts as a buffer, Fig.1. Only when the signal levels off after many hours, the measured rate corresponds to the production rate - After the return to aerobic conditions, fermentation stops and the ethanol accumulated in the tissue is observed to be slowly released into the gas-phase, with a retention time of about 3 h half-time period, Fig. 3.2.B. – During the fermentation process, acetaldehyde as precursor of ethanol starts to be emitted slightly earlier than ethanol; its emission rate is smaller than the value observed for ethanol. When the emission rates for both compounds show saturation after about 6 ÷ 8 hours of anaerobiosis, the emission rate is about 50 times stronger for ethanol than for acetaldehyde, Fig. 3.2, E÷G. Note that the measured emission rate of acetaldehyde reaches saturation about 6 times faster than ethanol; at the end of anaerobiosis, also a much shorter retention time in the plant tissue is observed than for ethanol, Fig.3.2, B ÷ D.

Since acetaldehyde under anaerobic conditions is not an end product, its emission rate depends on its reaction velocity to produce the final product ethanol; for a higher reaction velocity less acetaldehyde leaks out of the fermentation circuit into the nitrogen flowing towards the FTG detector.

At re-aeration after a prolonged anaerobic period, the post-anaerobic release peak of ethane shown in Fig. 3.1 attests a short LIPOX episode. This episode should be accompanied by a scavenging action of ROS, at least after the seedlings have resumed the production of the needed enzymes of their defense system. This is what one observes; for instance after 8h of anaerobiosis and delayed by about 1 h with respect to the sharp ethane peak, a broad acetaldehyde emission peak (about 1.5h after re-admission of air, at t_2) tells us that the defense against ROS is working. It works so efficiently that LIPOX becomes terminated. To keep control the scavenging process continues for some hours, Fig.3.2 E. After anaerobic periods of (or longer than) 8h, the post-anaerobic acetaldehyde release rate does not return to the pre-treatment level; the broad emission peak is followed by plateau at a value that increases for longer duration of anaerobiosis, Fig.3.2 F÷H. Presumably, the consequence of some irreversible injury becomes

felt, experienced by the plant tissue during prolonged anaerobic treatment. Leaf injury has been investigated seven days after the end of the imposed anaerobiosis. After 8h without oxygen, foliar dehydration and necrosis was already found for more than 20% of the leaf surface for the FR13A cultivar and for more than 40% for the CT6241 cultivar, Santosa et al. 2007. The high post-anaerobic plateau values of Fig. 3.2 F&G - that are observed following the broad post-anoxic peak - may also at least partially originate from threats other than LIPOX.

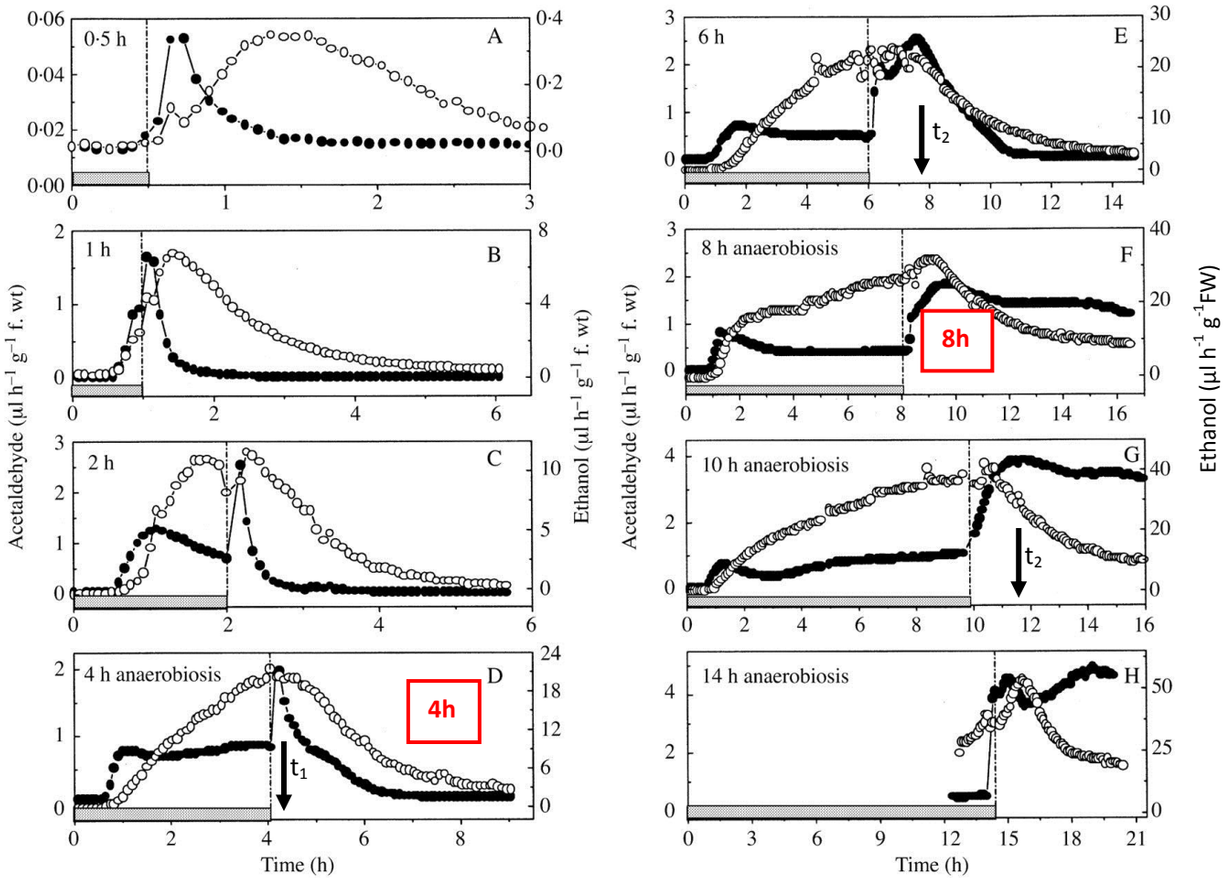

Fig. 3.2. Effect of anaerobic treatment, and subsequent return to normal air; ethanol (open circles) and acetaldehyde (closed circles) emissions from single batches of three 14-d-old FR13A rice seedlings, measured by on-line laser photo-acoustics. The plants were placed in the dark and given an anaerobic treatment for 0·5 h (A), 1 h (B), 2 h (C), 4 h (D), 6 h (E), 8 h (F), 10 h (G) or 14 h (H) by enclosing them in a cuvette supplied with nitrogen gas flowing at 2 l h^{-1} (grey horizontal bars). Thereafter, the plants were returned to a flow of air (2 l h^{-1}), while the ethanol and acetaldehyde output was continued to be measured. t_1 indicates the time when a sharp immediate post-anaerobic acetaldehyde peak occurs, and t_2 a time when the broad post-anaerobic acetaldehyde peak occurs. From Boamfa 2005

Already after less than 1 h of anaerobiosis a sharp high immediate post-anaerobic acetaldehyde peak rate (fwhm of 20 min) is measured at t_1, Fig. 3.2B÷D. According to Monk et al. 1985, there are **two** processes that can be held responsible for this peak rate, Fig. 2. The **first** is the back-step of the two-way process: acetaldehyde + NADH ↔ ethanol + NAD^+, enzyme alcohol dehydrogenase. Right at the end of anaerobiosis, NAD^+ is present to allow this reaction to run backwards. However, it takes energy to arrive at acetaldehyde via the enzyme alcohol dehydrogenase; energy might be lacking after a long period of anaerobiosis. If the citric cycle is re-established in the presence of oxygen, lack of energy might

no longer form a problem.
The **second** pathway is connected to ROS scavenging and yields peroxidatic acetaldehyde:
$NADH + O_2^{\bullet -} + H^+ \rightarrow NAD^\bullet + H_2O_2$, enzyme SOD, & ethanol $+H_2O_2 \rightarrow$ acetaldehyde $+ 2H_2O$, enzyme catalase.
This second pathway is responsible for the broad post-anaerobic acetaldehyde peak at t_2.

The results shown in Fig. 3.2 sustain the two-process-picture: two events are observed together after 6h of anaerobiosis, Fig.3.2 E, the first at time t_1 producing the high immediate peak and the second at t_2 producing the broad peak about 1.5 h after re-admission of air, resulting in a double peak. Controlling Fig.3.2D one discovers that the broad second peak is already announced by a broad shoulder; similarly, in Fig.3.2F a sharp initial rise forms a reminder of the first sharp and high peak. Still we have mentioned no clue yet that the two components of the double peak come from two <u>different</u> production pathways. Help comes from the additional observation of differences between the emission rates of the two investigated cultivars, see also section 2.4. Here suffices to state that for the two post-anoxic acetaldehyde events the first sharp peak at t_1 should have an origin different from the origin of the second broad peak at t_2, because at t_1 cultivar CT6241 emits more acetaldehyde than FR13A and at t_2 FR13A emits more acetaldehyde than CT6241. As the event at t_2 corresponds to scavenging as measure of defense against LIPOX, the event at t_1 should be connected to the back-step reaction of fermentation, Fig.2. With this conclusion, all (post-)anaerobic emission features of Figs. 2.1&2.2 find an explanation.

<u>2.2 Gas-phase micro-aerobic treatment</u>.

While it is quite simple to produce completely oxygen-free (anaerobic) conditions, it is very difficult and we did not succeed to work in a controlled way with an atmosphere containing less than 0.05 % v/v of oxygen. At zero oxygen no ethane is formed at all (since LIPOX needs at least some oxygen), whereas between 0.1 % and 0.8 % of oxygen, during the first 2 h of treatment already LIPOX occurs creating a continuous release of C_2H_6, Table 2.

Table 2 .The total amount of ethane accumulated during 2h in the gas space of the cuvette, containing initially the indicated oxygen concentration in nitrogen. The cultivar CT6241 produces more than FR13A From Santosa et al. 2007 & Santosa 2002

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

At these extremely low oxygen concentrations, the acetaldehyde emission of the seedlings changes dramatically. i) For seedlings e.g. of the CT6241 rice cultivar at 8 h after the beginning of the micro-aerobic treatment one observes an acetaldehyde emission rate of 0.3 µl h^{-1} g^{-1} FW at 0.25 % v/v of oxygen, a value slightly higher than the aerobic value 0.10 µl h^{-1} g^{-1} FW; ii) at an oxygen concentration down to 0.05 %, this rate assumes the 10 times bigger value of 3.4 µl h^{-1} g^{-1} FW; iii) the anaerobic rate then falls back to 0.52 µl h^{-1} g^{-1} FW, see third column of Table 3. Nothing of this excursion to an intermediate high is seen in the emission rate of ethanol: the rate of ethanol emission is observed to rise steadily from practically the zero-fermentation value at 0.3 % v/v to about halfway at 0.1 % v/v and

further to the nearly the full-fermentation value at 0.05 % v/v of oxygen, see fifth column of Table 3. In Fig.4 is shown how these micro-aerobic rates for acetaldehyde and ethanol develop in the first 8h.
The diverging trends at micro-aeration of the emission rates of acetaldehyde and ethanol connect to the dual origin of acetaldehyde, Fig. 1. The intermediate high of acetaldehyde is attributed to the shortcoming of residual aerobic respiration to provide sufficient energy for the living tissue: an austerity program is put in force cutting down the production of SOD. As consequence, dangerous O_2^- appears together with its protonated form $HO_2^\bullet$, attacking membrane lipids and propagating LIPOX, Halliwell & Gutridge. Residual SOD & CATALASE still are active. The result is a) the production of ethane signaling LIPOX and b) the appearance of an extra amount of non-fermentative acetaldehyde.

	Acetaldehyde ($\mu L\,h^{-1}\,g^{-1}$ f. wt)		Ethanol ($\mu L\,h^{-1}\,g^{-1}$ f. wt)		CO_2 ($\mu L\,h^{-1}\,g^{-1}$ f. wt)	
O_2 (%)	FR13A	CT6241	FR13A	CT6241	FR13A	CT6241
0·00	$0·90 \pm 0·13^{a}$	$0·52 \pm 0·04^{A}$	55 ± 6^{j}	55 ± 5^{j}	115 ± 16^{t}	93 ± 20^{t}
0·05	$5·5 \pm 1·0^{b}$	$3·40 \pm 0·15^{B}$	47 ± 9^{k}	46 ± 2^{k}	150 ± 40^{u}	193 ± 2^{u}
0·10	$4·4 \pm 0·4^{c}$	$3·1 \pm 0·5^{c}$	31 ± 2^{l}	31 ± 4^{l}	100 ± 7^{v}	154 ± 13^{V}
0·15	$2·60 \pm 0·16^{d}$	$1·60 \pm 0·10^{D}$	$9·8 \pm 1·9^{m}$	$14·0 \pm 0·7^{M}$	124 ± 8^{x}	163 ± 11^{X}
0·20	$0·77 \pm 0·12^{e}$	$0·64 \pm 0·10^{e}$	$3·2 \pm 0·3^{N}$	$6·0 \pm 1·3^{n}$	140 ± 6^{y}	149 ± 8^{y}
0·25	$0·80 \pm 0·14^{f}$	$0·33 \pm 0·09^{F}$	$5·8 \pm 0·1^{o}$	$4·5 \circ 0·8^{o}$	115 ± 9^{z}	180 ± 40^{z}
0·30	$0·35 \pm 0·05^{g}$	$0·14 \pm 0·05^{G}$	$1·43 \pm 0·06^{p}$	$2·30 \pm 0·08^{P}$	138 ± 11^{w}	156 ± 9^{w}
0·50	$0·10 \pm 0·02^{h}$	$0·10 \pm 0·02^{h}$	$0·86 \pm 0·15^{r}$	$0·9 \pm 0·1^{r}$	117 ± 8^{q}	102 ± 10^{q}
20·9	$0·14 \pm 0·04^{i}$	$0·10 \pm 0·02^{i}$	$0·75 \pm 0·04^{s}$	$0·75 \pm 0·04^{s}$	140 ± 20^{a}	201 ± 6^{A}

All values are means with standard errors of 4–5 individual experiments. For each volatile at each O_2 concentration, means for the two cultivars showing different superscript letters are significantly different

Table 3. Effect of 8h anaerobic, micro-aerobic and aerobic treatment in the dark on the rates of acetaldehyde, ethanol and CO_2 emission rates of three 14-d-old FR13A and CT6241 rice plants, measured at the end of the treatment. From Boamfa et al 2005 & Boamfa 2005

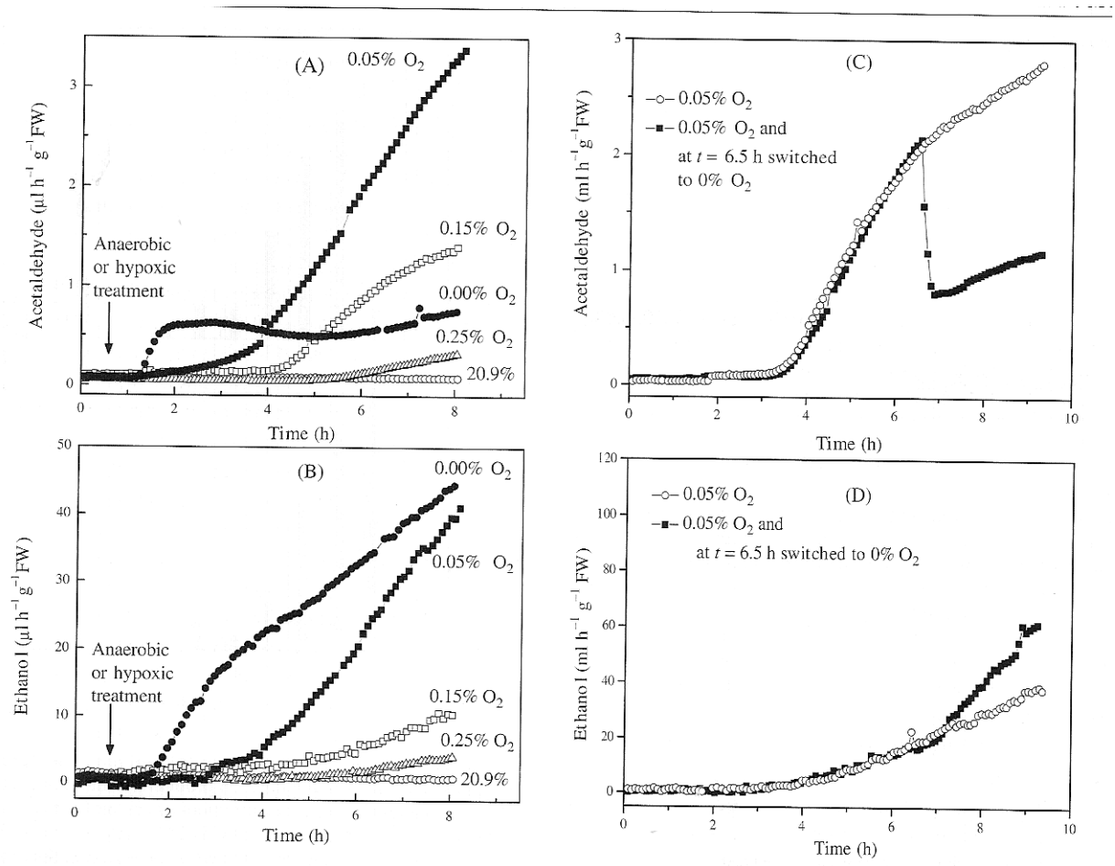

Fig. 4 *Acetaldehyde and ethanol emission rates, under aerobic, anaerobic and micro- aerobic conditions, applied to the rice cultivar CT6241. Left: the rates during an 8h treatment for acetaldehyde, A, and ethanol, B. Right: A micro-aerobic treatment starts at t = 0h. The plunge in the acetaldehyde emission rate, C, and the change of gradient in the ethanol emission rate, D, are caused by a change to anaerobic conditions. From Boamfa et al. 2005 & Boamfa 2005*

In Fig.4C is shown what happens, if complete anaerobiosis is imposed following a micro-aerobic treatment of 6 h: the production of non-fermentative acetaldehyde stops within 15 min since without oxygen no ROS and no H_2O_2 is formed and thus the scavenging route for the production of acetaldehyde becomes non-active. What appears as a sudden transition in Fig.4C is the switching from the 0.05% curve to the 0.00% curve in Fig. 4A. At this switching moment, the non-fermentative acetaldehyde amounts to 2/3 of the total emission rate. - Similarly, for the ethanol rate the change of gradient at 6.5 h in Fig. 4D represents the switching from the 0.05% curve to the 0.00% curve in Fig. 4B. Due to its much longer retention time in the buffer tissue the transition appears to be more gradual for ethanol. - Fig.4 demonstrates the new quality of observing in real time processes that take place in plants under changing environmental conditions.

Post-micro-aerobic conditions (i.e. return to normal air) after 8h of treatment at 0.05% of oxygen lead to a pronounced peak of the acetaldehyde emission rate, reaching its maximum after about 1.5h, Fig. 5. Already during the 8h of micro-aerobic conditions, the acetaldehyde emission rates from FR13A batches of three 14-day-old rice seedlings were extremely high. This trend extends thus into the post-treatment period; after 8h of treatment the post-treatment emission for 0.05% O_2 assumes a peak value at t= 10h four times higher than the value of the anaerobic post-treatment peak, Fig. 5, though the micro-aerobic ethanol emission at t = 8.5h is lower than the anaerobic emission, Fig.4B, and no pronounced post-anaerobic ethanol emission is observed, Fig. 2.1F. The post-micro-aerobic process responsible for the big acetaldehyde release appears to be disconnected from fermentation.

What makes the 0.05% oxygen concentration so special to let the seedlings emit the observed maximum of non-fermentative acetaldehyde? We start this discussion by following what happens if we lower the oxygen concentration from normoxic values to zero. Under normoxic conditions, there is no

fermentation and no ethanol is present in the tissue of the seedlings. The plants are in the dark and respire aerobically. Possible low production of ROS is taken efficiently care of by scavenging. But then on lowering the oxygen concentrations to values as low as 0.15% the seedlings suffer increasingly from lack of ATP energy carriers; the production of important enzymes decreases; yet ROS have to become cleared off; some fermentation starts to occur. The ethanol production still remains far below anaerobic values, Fig.4B. The micro-aerobic emission rate of acetaldehyde attains values clearly above anaerobic emission rates, after 5h of treatment, Fig.4A. Some ROS escape the scavengers and produce ethane, Table 2. At still lower oxygen concentrations like 0.05% the situation becomes dramatic. The ethanol emission approaches anaerobic rates, Fig.4B. The by now mainly non-fermentative acetaldehyde emission rate becomes four times the anaerobic rate, still rising after 8h of treatment, Fig. 5. This is the highest micro-aerobic emission rate we have measured – but it is also the lowest oxygen concentration we could produce in a stable manner. Note that between 0.15% and 0.05% the oxygen concentration drops by a factor 3, whereas the acetaldehyde rate rises by a factor 4, for FR13A, Fig. 14. Thus, a factor 3 drop in available oxygen yields about 4 times more of the scavenging product acetaldehyde, 8h after the start of the micro-aerobic treatment; all this happens at emission levels far above anaerobic values, Fig. 5. A greater fraction of the available oxygen at the lower concentration goes into the ROS&H_2O_2 route and produces much more acetaldehyde, probably because the respiration route becomes increasingly barred by shortage of needed enzymes.

In this picture fits the observation of the high post-micro-aerobic acetaldehyde peak, since a higher fraction of the oxygen in the inrushing air is initially ending up on the ROS&H_2O_2 route. Remarkably this high peak is followed by a drop down to the aerobic pre-treatment acetaldehyde level, i.e. 8h of micro-aerobic treatment at 0.05% of oxygen yield seedlings without irreversible damage, contrasting the post-anaerobic results, Fig.5.

A last comment on these astonishing observations concerns what is expected to happen at still lower oxygen concentrations. If no oxygen is left available, no ROS&H_2O_2 are produced to form peroxidatic acetaldehyde, i.e. neither high emission rates at the end of the treatment nor high post-treatment peaks are observed. One cannot determine what the real critical oxygen concentration is for the highest amount of oxygen transformed into ROS, because measurements below 0.05% were not successful. We expect, however, that below 0.05% of oxygen the threat of cell death will still grow, because the two injurious trends still may gain: the fraction of oxygen producing ROS may still augment & the defense against ROS may further deteriorate. The most reliable micro-aerobic results are collected in Table 3, showing the measured averaged rates at the end of an 8h treatment, with error bars; note that between 0.15% and 0.05% of oxygen, the emission rate of ethanol (acetaldehyde) increases by about a factor 5 (2), for the cultivar FR13A. The transition from oxygen-powered to fermentation-based energy provision is underway and causes a free coming higher fraction of oxygen to produce the “final” rise of acetaldehyde emission while the total of available oxygen is dwindling: the scavenging process still works! This “final” rise would NOT indicate the final point of highest danger; this point would be the failing of the scavenging process, signifying the end of defense and free play for ROS causing irreversible damage.

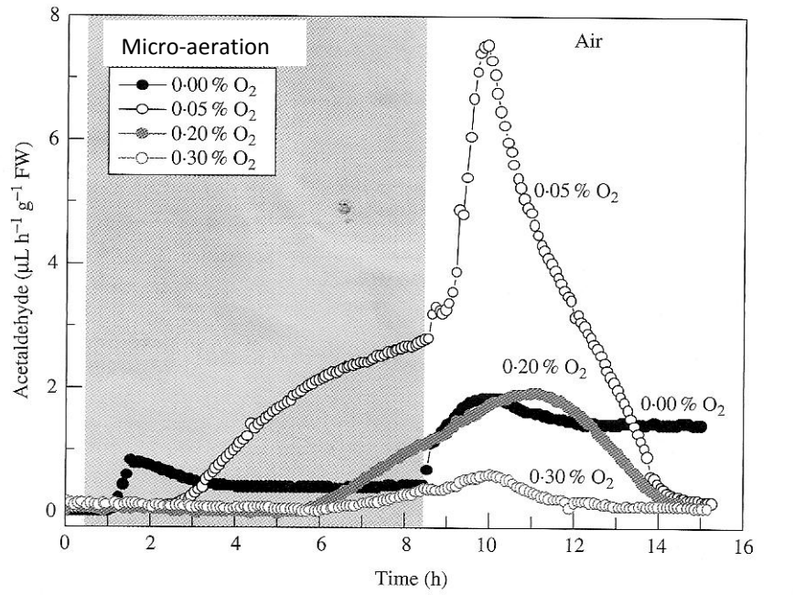

Fig. 5 *The post-treatment acetaldehyde emission of FR13A seedlings, returned to air after 8h of micro-aerobic exposure at various low oxygen concentrations. Boamfa et al. 2005& Boamfa 2005*

2.3 Submersion treatment

Next we discuss the most important results of this paper for practical purposes; one wants to understand what determines the survival chance of rice seedlings under flooding conditions and why the cultivar FR13A fares better than CT6241 under submersion. All what has been learned for hypoxic gas phase conditions has prepared us to understand the crucial role played by LIPOX. To measure the effect of submersion, mainly initially aerated tap water was used that completely covered the rice seedlings sealed in a long and narrow cuvette during the time of trace gas accumulation.

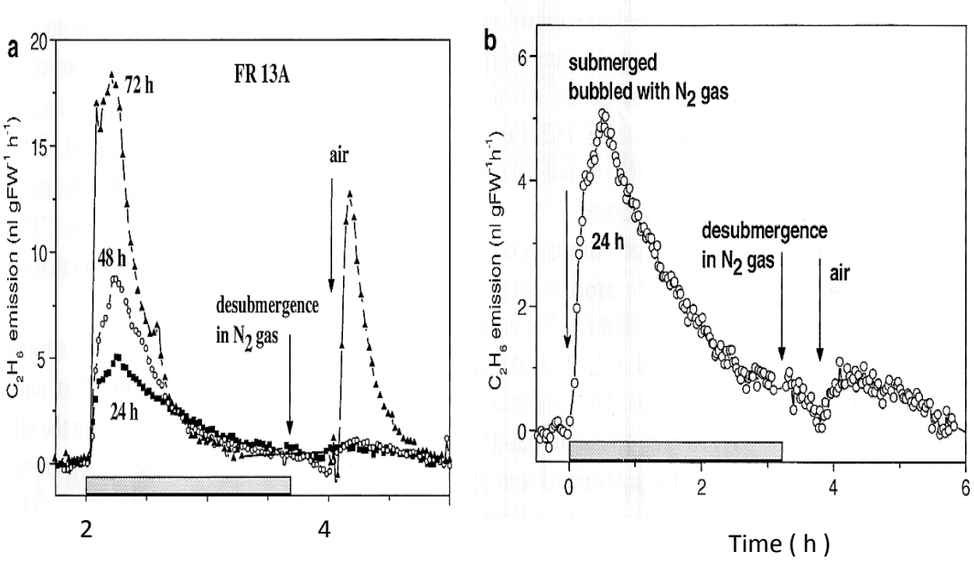

Fig. 6a. Ethane produced by seedlings of cultivar FR13A during and after 24, 48 & 72h submergence in the dark in initially aerated water. Ethane-stripping (by bubbling N_2 through the submergence water) was completed after 3.5h (grey bar). When the in N_2 gas de-submerged seedlings were exposed to an air flow (second arrow), the post-submergence emission rates of ethane indicate a temporary LIPOX episode, starting at 4.2h. 6b. Enlarged view of the 24h submergence curve shown in a. After 29.5h, the post-submergence signal has dropped below 0.1 nl gFW^{-1} h^{-1} From Santosa et al. 2007 & Santosa 2002

To measure release of ethane from submerged plants one is fortunately assisted by the fact that the unstirred submersion water acts as a buffer collecting ethane during submersion; at the end of the chosen collection period, the water becomes stirred by bubbling nitrogen gas through it. This gas loaded with ethane is flown through the FTG detector to determine the total accumulated ethane content. The results show a continuous ethane production under submersion in the dark, Figs. 6&7 and Table 4.

As next step in the measurement, the tap water is removed and the seedlings are de-submerged under a nitrogen atmosphere; 1 h hereafter, the nitrogen is replaced by an air flow to measure the post-submergence post-anaerobic release rate of ethane, as shown in Fig.6. The post-submergence amount of ethane is found significantly smaller than the amount accumulated during submergence, Table 4.

Thus, submerged plants are subjected to damaging LIPOX all the time, and not only and not even mainly after de-submergence, a view previously hold, Ella et al. 2003. The better a cultivar is protected by an efficient defense system against ROS, the higher are its chances to survive a period of submergence by flooding.

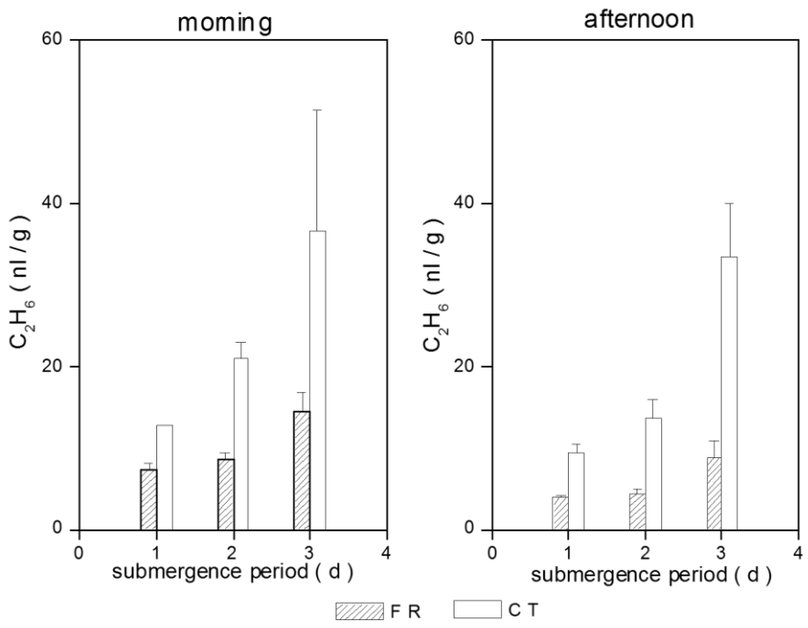

Fig.7 *Total ethane production, accumulated during the submergence period. Total ethane production per unit fresh weight from two different cultivars, submergence tolerant (FR13A) and intolerant (CT6241). The plants were either submerged in the morning, or in the afternoon after a day of photosynthetic energy supply. From Santosa 2002*

Treatment duration	Ethane during treatment nl gFW^{-1} ± SD	Ethane after treatment nl gFW^{-1} ± SD	Survival % ± SD	Leaf damage % ± SD
24h FR	7.3 ± 0.8	1.2 ± 0.1	60 ± 30	80 ± 10
24h CT	12.7 ± 0.1	1.7 ± 0.1	20 ± 20	95 ± 5
48h FR	8.6 ± 0.9	2.3 ± 1.1	0	100
48h CT	21.0 ± 1.8	4.0 ± 0.9	0	100
72h FR	14.0 ± 3.0	9 ± 3	0	100
72h CT	37 ± 1.5	10.6 ± 1.4	0	100

Table 4 Integral ethane emission during and after submergence in the dark starting in the morning, and damage score. Except for 0% survival and 100% leaf damage, for all measurements the differences between the cultivars FR13A & CT6241 were statistically significant. Survival was scored 7 days after return to air. Leaf damage was scored for old leaves. From Santosa et al. 2007 & Santosa 2005, simplified

For (tap) water in equilibrium with normal air, at 30^0C, the oxygen density is 0.24 mol m^{-3}. This is about the density at gas phase conditions at 0.5% v/v of oxygen in nitrogen, Setter et al 1996. Thus, at the start

of submergence in tap water the seedlings already experience micro-aerobic oxygen densities. During submergence in the dark in a sealed cuvette, the initial oxygen density decreases due to respiration of the seedlings, Fig.8. In addition, not only the density of oxygen dissolved in the stagnant submersion water counts, but also the greatly reduced speed of diffusion in solution determines the availability of oxygen during submergence. Rice seedlings submerged in stagnant tap water experience indeed LIPOX immediately and continuously, confronting cell death, Fig. 7 and Table 4. Tap water forms a similar but harsher environment than 0.05% oxygen gas phase conditions. One deals here, too, with the critical condition where LIPOX takes place, and fermentation is about to start. Deduced from post-submergence acetaldehyde and ethanol emission, fermentation has been observed only if before submersion the oxygen content of the submersion water had been reduced almost to zero by bubbling nitrogen through the water for 6 h. Fermentation has been suppressed again, when after the bubbling of nitrogen illumination was applied to the submerged rice seedlings and oxygen was produced by photosynthesis, Fig.9. It seems justified to conclude that anaerobic fermentation does not form an important factor limiting the survival of rice seedlings under flooding.

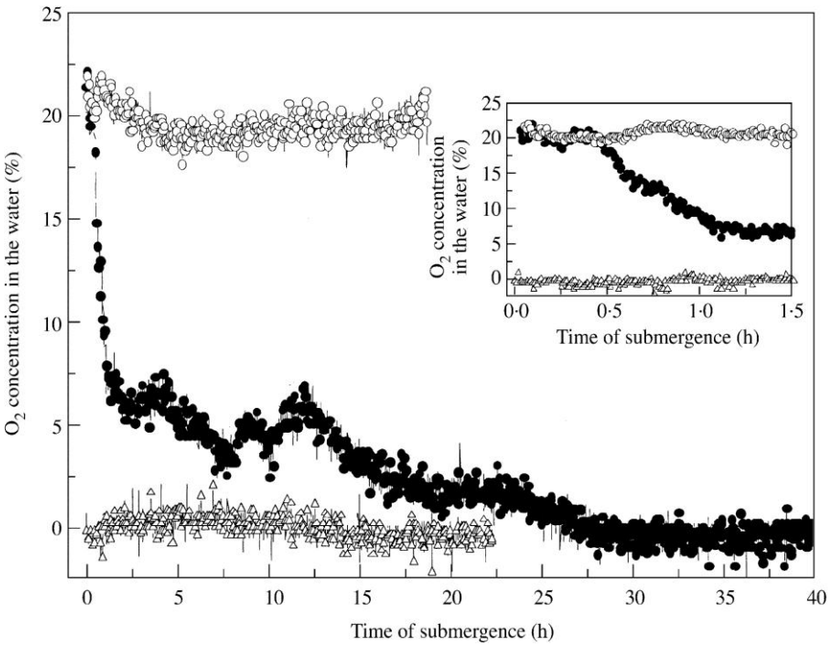

Fig.8. Changes in oxygen concentration in water surrounding a batch of three 14-d-old FR13A rice seedlings submerged for 16 h under different conditions. Filled circles, initially aerated water in the dark, open triangles, initially oxygen-free water in the light. The concentration of oxygen in water without plants and initially in equilibrium with air are shown with open circles. The inset gives enhanced detail of changes in oxygen concentration in the first hour. From Boamfa et al. 2003 & Boamfa 2005

2.4 FR13A vs. CT6241, two rice cultivars.

The acetaldehyde emission is compared for the two cultivars, to extract 2 empirical rules r_1 & r_2 from the experiments.

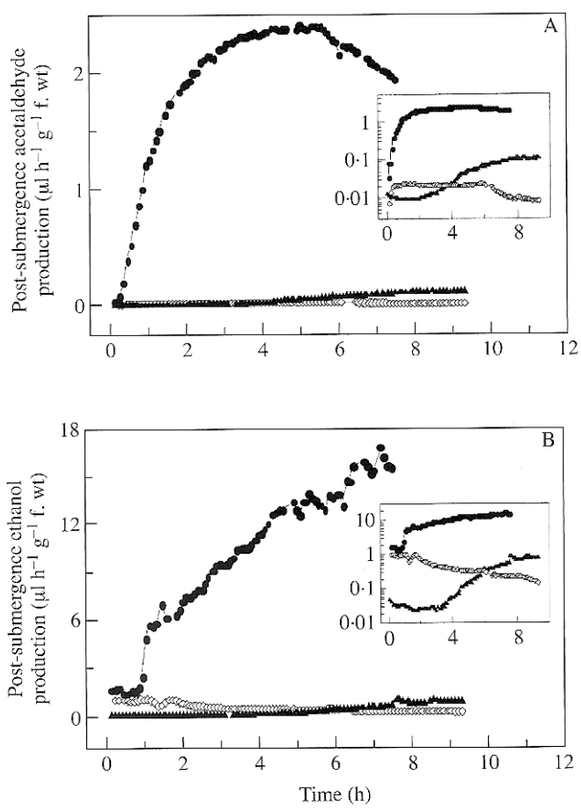

Fig.9. Production of acetaldehyde and ethanol for a batch of three 14-d-old F13A seedlings after 16 h of submergence ending at t = 0. The plants were de-submerged in air. Filled circle, (i): submergence water initially de-oxygenated, in the dark. Filled triangles,(ii): submergence water initially aerated, in the dark. Open diamonds, (iii): as filled circles, but under light conditions, 500 µmol m^{-2} s^{-1}. A strong ethanol signal from fermentation is only observed in case (i); the inserts show the same results on a log-scale. From Boamfa et al. 2003 & Boamfa 2005

r_1. Without LIPOX, under fermentation conditions, CT6241 shows the higher acetaldehyde emission rates (CT>FR); it is the "leakier cultivar", i.e. a higher percentage of acetaldehyde leaks away before the last step reaction of alcoholic fermentation, Fig.2. One deals here with a two-way reaction. A priori it is unclear whether the back-step reaction, too, shows CT >FR. However, our discussion in section 2.1 suggests that also for the back step reaction CT>FR is valid.

r_2. FR13A as the better scavenger wins the price for the highest acetaldehyde production under LIPOX conditions (FR>CT), if no fermentation takes place, or if the main contribution to the acetaldehyde release is of this non-fermentative "peroxidatic" origin. This is a one-way reaction.

	Anaerobic treatment	post-anaerobic observation
Short	no ethane emission acetaldehyde, CT>FR	no ethane emission acetaldehyde, CT>FR
Long	no ethane emission acetaldehyde, CT>FR	ethane, CT>FR acetaldehyde, FR>CT
	Micro-aerobic treatment	**re-aeration observation**
Short	ethane CT>FR acetaldehyde, not attempted	ethane, no measurements acetaldehyde, no measurements
Long	ethane, no measurements acetaldehyde FR>CT	ethane, no measurements acetaldehyde FR>CT
	Submergence	**re-aeration observation**
Long	ethane, CT>FR	ethane, CT>FR

Table 5. Ethane and acetaldehyde emission, compared for the two rice cultivars, during and after treatment. CT > FR (FR > CT) means that the cultivar CT6241 emits more (less) than FR13A.

These two rules help us to determine what causes certain trace gas emissions observed under the various conditions treated in this paper. In the next three sections we present the evidence for the rules under the three experimental conditions of this investigation. Table 5 helps to keep track during the discussion.

2.4.1 The two cultivars under (post-)anaerobic treatment.

During anaerobiosis no ethane is emitted, thus no LIPOX takes place and r_1 applies. Both cultivars show very much the same rate of ethanol emission, after more than 2h of continued oxygen deprivation, see Table 6; the acetaldehyde emission has been measured for both cultivars under the same conditions and shows higher values for the CT6241 cultivar whenever the differences are statistically relevant, see footnote of Table 6 and Fig. 11.

Table 6 . *Effect of up to 14 h anaerobic treatment in the dark or in the light on rates of acetaldehyde, ethanol and CO_2 production by batches of three 14-d-old FR13A and CT6241 rice seedlings, measured at the end of the anaerobic period*

	Production rates at the end of anaerobiosis (µL h^{-1} g^{-1}FW), Boamfa 2005					
	Acetaldehyde		Ethanol		CO_2	
Duration of anaerobiosis	FR13A	CT6241	FR13A	CT6241	FR13A	CT6241
Dark						
0 h	0·040 ± 0·013^{a}	0·05 ± 0·01^{a}	0·27 ± 0·06^{i}	0·35 ± 0·05^{i}	300 ± 30^{q}	285 ± 10^{q}
1 h	1·10 ± 0·05^{b}	1·45 ± 0·07^{B}	2·40 ± 0·04^{j}	2·8 ± 0·3^{j}		
2 h	1·00 ± 0·08^{c}	2·00 ± 0·03^{C}	20·9 ± 0·2^{k}	11·3 ± 1·0^{K}		
4 h	0·95 ± 0·06^{d}	0·90 ± 0·11^{d}	17·0 ± 1·3^{l}	15·5 ± 1·8^{l}	120 ± 15^{r}	95 ± 7^{r}
6 h	0·55 ± 0·02^{e}	0·52 ± 0·04^{e}	26 ± 2^{m}	22 ± 3^{m}		
8 h	0·90 ± 0·13^{f}	0·70 ± 0·13^{f}	55 ± 5^{n}	55 ± 6^{n}		
10 h	1·07 ± 0·07^{g}	1·7 ± 0·3^{G}	43 ± 4^{o}	47 ± 1^{o}		
14 h	0·90 ± 0·13^{h}	1·4 ± 0·3^{h}	40 ± 9^{p}	39 ± 6^{p}	100 ± 15^{s}	90 ± 5^{s}
Light (500 µmol m^{-2} s^{-1})						
2 h	0·075 ± 0·005^{x}	0·19 ± 0·02^{X}	2·20 ± 0·13^{t}	2·5 ± 0·2^{t}	Below detection	Below detection
12 h	0·050 ± 0·013^{y}	0·10 ± 0·02^{y}	8·0 ± 0·9^{u}	11 ± 1^{U}	Below detection	Below detection

All values are means with standard errors of 4–5 individual experiments. For each volatile at each time point, means for the two cultivars showing similar superscript letters are not significantly different at $P<0·05$ (Student's *t*-test).

The data for FR13A were already presented in Table 2 of an earlier paper (Boamfa *et al.*, 2003).

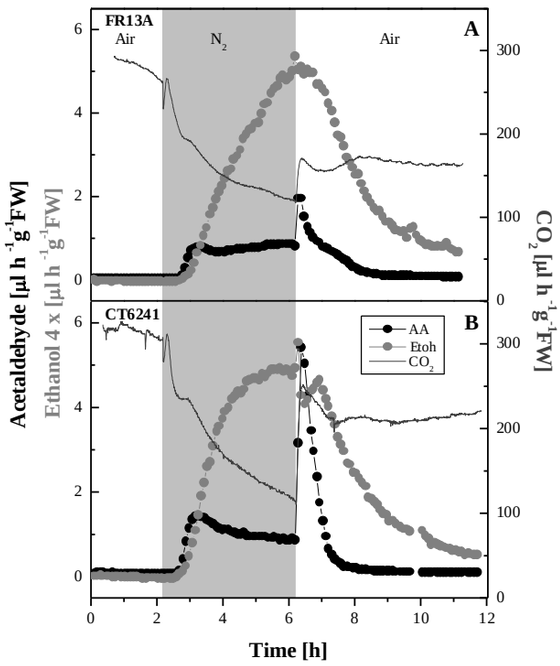

Fig.11. Effect of 4h anaerobic treatment and subsequent return to normal air flow on emission of ethanol (grey circles), acetaldehyde (black circles) and CO_2 (dark grey line) emissions from single batches of three 14-d-old FR13A (A) and CT6241 (B) rice seedlings. At t=0 h the plants were placed in air in the dark. At t= 2.1h, anaerobiosis was applied for 4 h followed by a 4 h re-aeration period. One representative measurement selected from 4 to5 independent experiments is shown. From Boamfa 2005

For short anaerobic periods, the post-anaerobic measurements of Fig.11 and Table 7 clearly show that the CT6241 cultivar emits more acetaldehyde. This is observed under circumstances without ethane emission, i.e.no LIPOX; we observe CT>FR; r_1 applies as discussed in section 2.1.

For long anaerobic periods (> 8 h), post-anaerobic ethane proves the occurrence of LIPOX, Fig.3.1 and Fig. 12. Here we observe FR>CT, Fig. 13 and Table 7; r_2 applies.

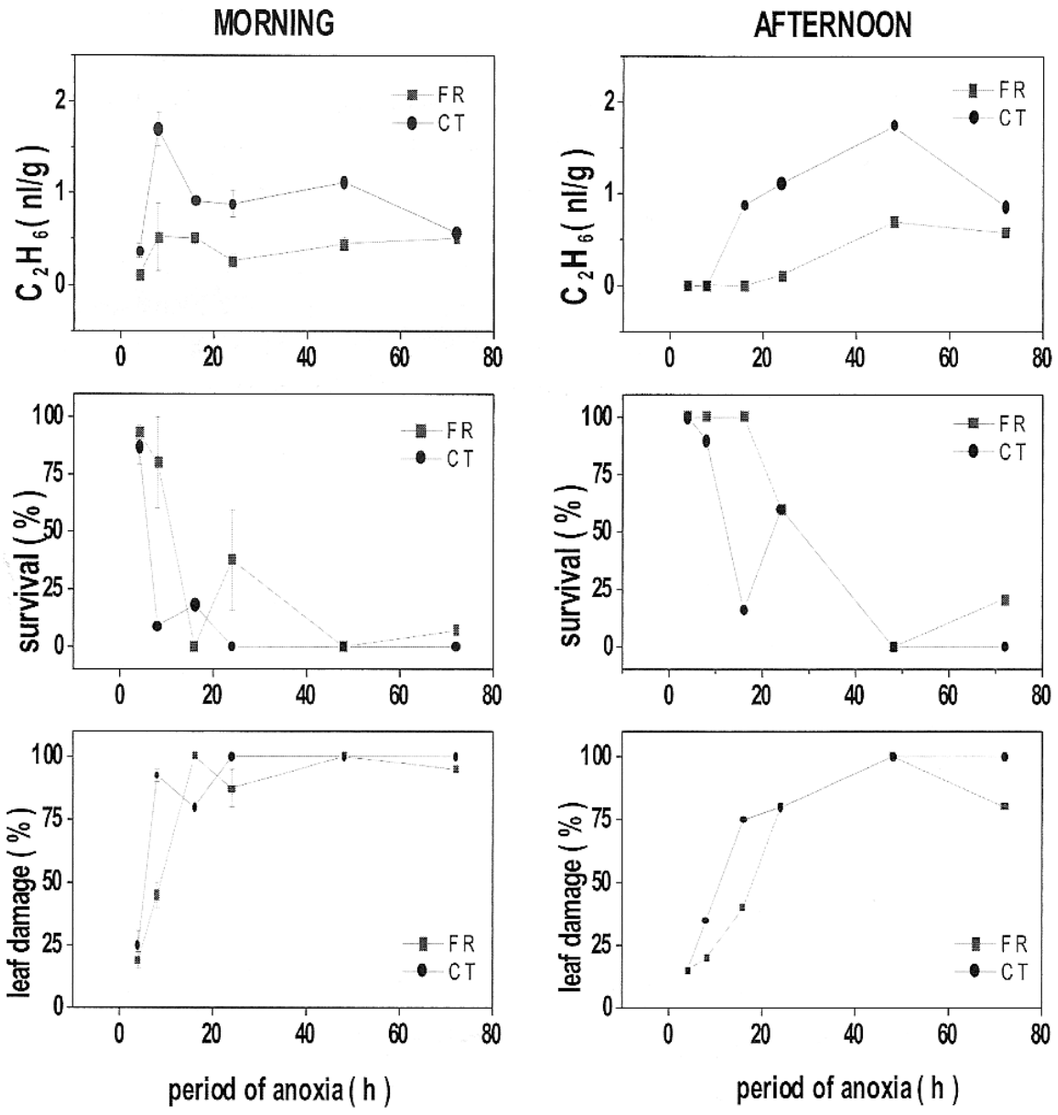

Fig. 12. Integrated post-anaerobic ethane production, survival and leaf damage for rice seedlings FR13A and CT6241, treated in the dark. Left panels and right panels show results for anaerobic treatment starting in the morning and in the afternoon, respectively. From Santosa 2002

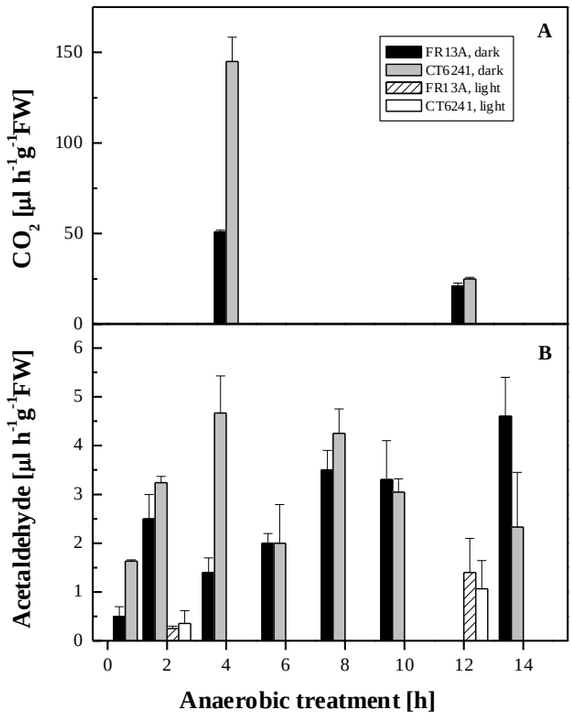

Fig.13 *During recovery in air after up to 14h of anaerobic treatment in the dark (or in light, 500 µmol m^{-2} s^{-1}) ,the increase rates of acetaldehyde production(B) are measured from batches of three 14-d-old FR13A (black bars) and CT6241 (grey bars) rice plants. The post-anaerobic increase was calculated as the difference between the post-anaerobic peak values and the production rates at the end of anaerobic treatment. All values are means with standard errors of 4 to 5 individual experiments. Note the change from grey bars being larger to black bars being larger, for long treatments. From Boamfa 2005*

Duration	FR13A	CT6241	see Caption	Illumination
1h	0.5 ± 0.05	1.6 ± 0.03	CT > FR	dark
2h	2.5 ± 0.5	3.2 ± 0.13	CT > FR	dark
4h	1.4 ± 0.3	4.7 ± 0.7	CT > FR	dark
6h	2.0 ± 0.2	2.0 ± 0.8	Double peak	dark
8h	3.5 ± 0.4	4.3 ± 0.5	≈	dark
10h	3.3 ± 0.8	3.0 ± 0.27	FR > CT	dark
14h	4.6 ± 0.8	2.3 ± 0.6	FR > CT	dark
2h	0.25 ± 0.05	0.4 ± 0.2	≈	light
12h	1.4 ± 0.7	1.0 ± 0.6	≈	light

Table 7 Post-anaerobic increase of acetaldehyde calculated as the difference between peak value and production rate at the end of the anaerobic treatment, µl h^{-1} g^{-1} FW. The duration of anaerobiosis is indicated in the first column. ≈ indicates that the rates for the two cultivars are not statistically different. A double peak has been observed for 6h, Fig. 2.1. FR > CT indicates that a higher post-anaerobic increase is observed for the FR13A cultivar, i.e. the acetaldehyde production mainly comes from ROS scavenging. The entry CT > FR means that the back step reaction of fermentation (Fig. 1) dominates. From Boamfa et al. 2005 & Boamfa 2005, modified.

2.4.2 The two cultivars under (post-)micro-aerobic treatment.

Under micro-anaerobic short time gas phase treatment there is a continuous ethane emission with significantly higher rates observed for CT6241 than for FR3A, Table 2. LIPOX is present at these small but non-zero oxygen concentrations. FR13A shows its superior scavenging properties: at the end of an 8 h micro-aerobic treatment and in the post-treatment phase, for all oxygen concentrations between 0.05% and 0.3% the acetaldehyde emission rate of FR13A is always higher, a clear case for r_2, FR>CT, Table 3 and Fig. 14. This occurs while the ethanol emission rates for both cultivars are practically identical, Table 3.

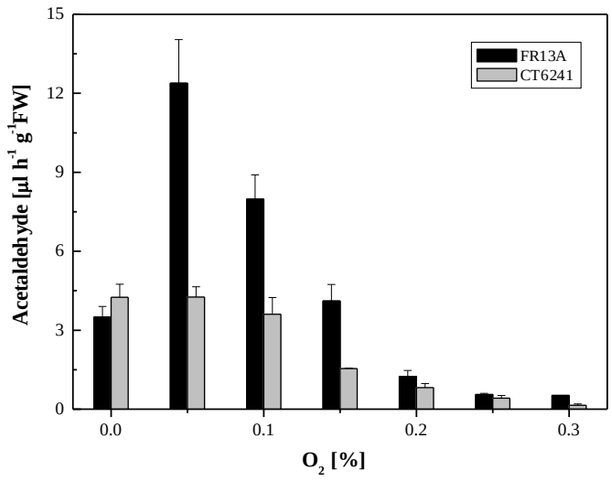

Fig.14 *During recovery in air from 8h of anaerobic (0% O_2) and of micro-aerobic treatment (0.05-0.3% O_2) in the dark, the change is observed from the CT6241 cultivar (grey bars) emitting more acetaldehyde to the clear prevalent emission by the FR13A cultivar (black bar).The rates of acetaldehyde production stem from batches of three 14-d-old rice plants. The post-treatment contribution was calculated as the difference between the post-treatment peak values and the production rates at the end of the treatment. All values are means with standard errors of 4 to 5 individual experiments. From Boamfa et al. 2005 & Boamfa 2005*

2.4.3 Two cultivars under (post-)submergence treatment.

For 16h submergence in initially aerated water, in the dark, no (or little) post-submergence acetaldehyde & ethanol has been observed, Fig.9; apparently hardly any fermentation takes place. During submergence under these conditions, a much higher ethane production for CT6241 is observed than for FRA13, Table 4; the ethane is accumulated during 1 or 2 or 3 days of treatment and shows a continuous increase. Thus, heavy LIPOX takes place in the absence of fermentation. This leads to zero survival of the seedlings after 48h of submergence, with slight survival advantages for FR13A after 1 day of submergence, Table 4. The post-submergence ethane measurements, too, show clearly higher emission rates for CT6241, Table 4. – Measurements to compare the acetaldehyde emission of the two cultivars under submergence were not attempted.

3. Conclusions

Micro-aerobic gas phase conditions seem to simulate rather well the oxygen stress that rice seedlings experience under submersion.

The submersion-susceptible rice cultivar CT6241 is observed to emit more ethane than the submersion-tolerant cultivar FR13A, under submersion conditions, Fig. 7 & Table 4, and under micro-aerobic conditions, Table 2. Ethane emission means that injurious LIPOX takes place, caused by ROS.

These observations suggest that LIPOX is the process that determines the survival chance of flooded rice seedlings, an idea sustained by the results showing that FR13A emits more peroxidative acetaldehyde than CT6241 under micro-aerobic conditions, Table 3 & Fig. 14. Peroxidative acetaldehyde emission indicates scavenging of ROS. FR13A possesses thus the more efficient defense against ROS, which if not scavenged causes LIPOX.

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