Laser-driven Photoacoustic Spectroscopy: What We Can Do with it in Flooding Research


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Photoacoustic spectroscopy is a highly sensitive technique for measuring low molecular weight gases such as the plant hormone ethylene. Due to its high sensitivity (10 pl I -1 ethylene), photoacoustic spectroscopy can be combined with flow-through systems that avoid the need for enclosing excised plant parts in small volumes for head-space analysis. In this way, artifacts introduced by various accumulation techniques can be avoided and ethylene production monitored at short intervals in air or other gas mixtures as it flows out of a cuvette enclosing all or part of an intact plant. The principles of this technique are described. Three case studies demonstrate the application of photoacoustic spectroscopy in flooding research. These studies concentrate on accurate measurement of endogenous ethylene concentrations in submerged shoots and roots, root ethylene production under subambient oxygen pressures and the simultaneous measurement of ethylene production and leaf growth. In addition, the qualitative and quantitative methods previously used to measure the gaseous plant hormone ethylene are briefly reviewed. Finally, the future prospects of photoacoustic spectroscopy in flooding research are discussed. © 1997 Annals of Botany Company

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INTRODUCTION

In many flooding-resistant plants, the simple, unsaturated two carbon volatile ethylene is of importance for the initiation of anatomical and morphological adaptations to waterlogged or submerged conditions. It is well-suited for this task since it is a gas under ambient atmospheric conditions (Abeles, Morgan and Saltveit, 1992) and it is metabolized only very slowly in plant tissues (Hall, 1991). Both characteristics make this gas ideal to signal a change from well-drained to waterlogged or submerged conditions (Ridge, 1987). Provided that some oxygen is available, ethylene production continues in tissues surrounded by water. However, due to an approx. 10000 times slower diffusion rate of ethylene in water compared with in air, concentrations of this gas will increase dramatically in submerged plants by entrapment (Jackson, 1985). An enhanced level of ethylene is the first step in a still largely unknown cascade of reactions, ultimately leading to adaptive responses such as adventitious root formation (Visser, 1995), stimulated shoot extension (Voeseke et al., 1993) and development of lysigenous aerenchyma (Jackson et al., 1985). In order to study these processes it is very important to make accurate measurements of endogenous ethylene concentrations and of production levels of whole plants or parts of it. These measurements are needed to (a) relate changes in ethylene to the physiological response under study and (b) increase understanding of regulation of ethylene biosynthesis under flooded conditions.

The first qualitative analysis of ethylene was described by Gane in 1934 (Gane, 1934). He demonstrated chemically that apples produce this gas. Subsequently, various techniques such as bioassays, straight chemistry, gravimetric analyses, manometric techniques and physico-chemical colorimetric assays were used to analyse ethylene levels more quantitatively. Gradually, sensitivity increased from approx. 100 pl I -1 in 1939 to 0.01 pl I -1 in 1957 (for review see Abeles, 1973). A major milestone was the introduction of the gas chromatograph (Burg and Stolwijk, 1959; Huelin and Kennett, 1959). The sensitivity of a gas chromatograph depends strongly on the type of detector. A thermal conductivity detector can determine 10–100 pl I -1 ethylene (Burg and Stolwijk, 1959), whereas a flame ionization detector has a sensitivity of 1 nl I -1 (Bassi and Spencer, 1989). Even higher sensitivity can be obtained when a photoionization detector is used (0.1 nl I -1; Bassi and Spencer, 1989). Even at these sub-parts per million sensitivities, which approach those of plants themselves, quantification of ethylene production rates by plants is mostly performed by head-space analysis of accumulated ethylene evolved by excised pieces of tissue enclosed in small incubation vials. This procedure can disturb ethylene production by wounding, disruption of transport processes, gravitropic disorientation and changes in gas composition.
around the tissue (Brailsford et al., 1993). Additionally, such an accumulation method is not suited for long-term measurements since excision disrupts normal developmental and metabolic processes. Similarly, short-term measurements are disrupted by wound ethylene production. These problems can be avoided by the use of a flow-through system in combination with a large chamber in which intact plants are enclosed. One of the first flow-through systems designed to measure ethylene continuously is described by De Grief and De Proft (1978). They used a large vessel to enclose their plants which was flushed with purified air scrubbed of background hydrocarbons. The outflowing gas was passed through a column characterized by a high adsorption capacity for ethylene. Heating of the column released ethylene quickly, which subsequently was measured with a gas chromatograph equipped with a flame ionization detector. Ethylene concentrations as low as 0.01 nL L\(^{-1}\) could be detected reproducibly (De Grief and De Proft, 1978).

However, this elegant method has the disadvantage that short-term kinetic studies on ethylene release are difficult to perform since a considerable time is needed for sufficient ethylene to be absorbed onto the trapping column. To achieve short-term kinetics, it is necessary to measure ethylene every few minutes directly in the out-flowing air of a flow-through system.

This article presents case studies concerning the application of a laser-driven photoacoustic ethylene detector in flooding research. The method overcomes all of the above-mentioned problems and measures ethylene to levels as low as 10 nL L\(^{-1}\) directly and almost continuously in the outlet-flowing air of a sample chamber connected to a flow-through system.

**METHOD**

The transformation of light energy into acoustic energy, the so-called photoacoustic effect, was reported in 1880 by Alexander Graham Bell (Bell, 1880). He showed that thin disks of very different materials such as selenium, carbon, and hard rubber emitted sound when exposed to rapidly interrupted beams of sunlight. Thereafter, interest in the photoacoustic principle declined to imperceptible levels for almost a century. A renaissance of interest in this phenomenon in physics was initiated by Kreuzer (1971) who used powerful laser light in photoacoustic spectroscopy to measure very low levels of pollutants in gases. More recently, photoacoustic set-ups have been used for plant (eco)physiological research that contain three essential components: a tunable laser, a photoacoustic cell and a flow-through system with associated computer (Fig. 1).

**Tunable laser and photoacoustic cell**

So far, the highest sensitivity with respect to ethylene detection has been achieved with a CO\(_2\)-laser emitting wavelengths between 9 and 11 μm. Ethylene molecules show a highly specific absorption pattern in this infrared wavelength region (Harren et al., 1990; Voesenek et al., 1992). When absorbing monochromatic light at 10.53 μm (the so-called 10P14 CO\(_2\) laser line), ethylene molecules are excited to a higher energy level. Excited ethylene molecules fall back to their original ground state via non-radiative decay. De- excitation or relaxation increases the kinetic energy and temperature of all the gas molecules around the excited ethylene molecules. According to the laws of Boyle and Gay-Lussac, an increase in temperature of a gas will lead to a rise in pressure in a semi-closed volume. When we now block the laser beam the pressure in the photoacoustic cell will decrease to its original value. Rapid and repeated interruption of the laser beam is performed by a mechanical chopper. Periodic pressure changes (at audio frequency) will then occur and can be detected by sensitive microphones. The magnitude of the microphone signal is proportional to the number of absorbing ethylene molecules. The sensitivity of the photoacoustic system can be improved further by...
contriving a longitudinal acoustic resonance in the photoacoustic cell obtained by careful tuning of the chopper frequency. The effect is comparable to resonance in an open organ pipe achieved when the length of half the acoustic wave equals the length of the pipe. An additional increase in sensitivity may be obtained by increasing the power of the exciting laser beam to approx. 100 W. This has been achieved by placing the photoacoustic cell inside the laser cavity. These adjustments have resulted in an ethylene sensitivity of less than 10 pl l⁻¹ (Harren et al., 1990).

Flow-through system

Our experiments with plants or parts of plants were performed in a flow-through system with a constant flow rate between 0.5 and 5 l h⁻¹. The flow-rate was controlled automatically during every ethylene measurement with the aid of a flow controller. Before passing over plant material within a glass cuvette, air or another gas mixture was cleaned of trace hydrocarbons. The catalyst contains platinumized Al₂O₃ pellets heated to 400 °C. Under these conditions, nearly all hydrocarbons were oxidized to carbon dioxide and water. The gas-flow was then split into eight channels each connected to a flow controller and onto the sampling cuvette (volume 200–600 cm³). After passing through the cuvettes gas flowed through an electronically controlled series of eight valves and into the photoacoustic cell. When not in line with the photoacoustic cell, each valve vented the flow into the atmosphere. Before entering the photoacoustic cell, carbon dioxide and water were removed from the gas-flow with KOH and CaCl₂ scrubbers.

Computer control

Various parts of the laser-driven photoacoustic set-up were placed under computer control, enabling fully automated collection of ethylene production rates of up to eight plants, each enclosed within a cuvette. A measuring cycle contains several computer-controlled steps. A photoacoustic run starts with the tuning of the laser to the ethylene-absorbing wavelength of 10.53 μm (10P14 laser line) by means of a stepmotor-driven grating. The laser power on the 10P14 line is then maximized by adjustment of the laser cavity with a piezo-electric element. Subsequently, 100 samples of the absorption at the 10P14 ethylene line are collected within 1 s. Samples encompassing the 95% confidence interval are used to calculate the mean ethylene absorption at the 10P14 line. Hereafter, the position of the grating changes to tune the laser to a non-ethylene absorbing wavelength (10.51 μm; 10P12 laser line). The laser power is again optimized, but now on the 10P12 line. Subsequently, 100 samples of the absorption strength are collected to quantify background noise. The mean absorption is calculated using the 95% confidence interval. Estimates of ethylene absorption and the background absorption, are then used to calculate the net ethylene signal expressed as a concentration (nl l⁻¹). This sequence takes approx. 1 min. The next step can be another photoacoustic run on the same cuvette or alternatively, a computer-controlled switch of a valve will connect another cuvette with the photoacoustic cell allowing a second plant to be examined. Thus, in an experiment with our automated laser-driven photoacoustic apparatus the order in which cuvettes are sampled can be controlled as can the number of times each cuvette is sampled. In addition, the computer software allows the inclusion of a delay before switching between cuvettes, or sampling them.

CASE STUDY 1: DETERMINATION OF ENDOGENOUS ETHYLENE CONCENTRATIONS IN SHOOTS AND ROOTS

Accurate measurements of endogenous levels of ethylene in plant tissues during submergence are extremely difficult and can easily be distorted by (a) uncontrolled loss of ethylene through exposing plant tissue to air or by reducing the boundary layer around a submerged plant as a result of water movement and by (b) handling-induced increases of ethylene production. Photoacoustic spectroscopy in line with a flow-through system avoids these difficulties. The principle of our measurement method is that on de-submergence or lowering of the water level, intercellular ethylene entrapped in the previous submergence period will rapidly diffuse to the surrounding air down a very steep concentration gradient. This assumption has been experimentally confirmed with data showing that de-submergence of the wetland plant Rumex palustris induces release of 90% of its entrapped ethylene within 1 min (Voeselek et al., 1993). De-submergence is performed within a cuvette without handling disturbance and without entry of any ethylene-containing outside air. The ethylene released from the plant flows to the photoacoustic cell for detection. Due to the characteristics of flow-through systems, a typical ethylene release peak comprising an initial steep rise followed by an exponential decay is observed. The slope of the exponential decay is determined by the flow-rate and the volume of the flow-through system. The area under the peak corresponds with the total amount of ethylene released by the plant.

Using these procedures with shoots of R. palustris that were spatially separated from the roots in a second compartment, revealed two peaks in ethylene release rather than the expected single ethylene peak (Fig. 2A). However, when a shoot of this plant was de-submerged in an environment of pure nitrogen the second peak was eliminated (Fig. 2B). This indicates that an oxygen-requiring step is involved in the production of this second burst in ethylene release. The enzyme 1-aminocyclopropane-1-carboxylic acid (ACC)-oxidase requires oxygen for the last step in the biosynthesis of ethylene that converts ACC into ethylene. We have concluded that the first peak in ethylene release is escape of entrapped ethylene, whereas the second peak represents de novo ethylene biosynthesis induced by de-submergence. In principle, there are two methods available to measure the amount of ethylene entrapped in submerged tissue. The first approach is to determine the height of the ethylene-release peak and compare it with a...
concentration just before de-submergence was calculated to be ethylene release data (□) were adjusted by arithmetic deduction to derived experimentally from ethylene injections (V) was fitted onto the spatially separated from the roots in two compartment cuvettes using calibration curve based on injections of known amounts of synthesis after de-submergence (O)- The area under the first ethylene release peak corresponded to 3-57 nl ethylene. The shoot had an reveal the size and shape of the ethylene peak generated by new the flow-through system. The area under the signal peak this is fitting a decay curve from the top of the release peak (see Figs. 2A). The decay constant can be determined ex­ ploitably by injecting a discrete volume of ethylene into the flow-through system. The area under the first ethylene release peak corresponded to 3-57 nl ethylene. The shoot had an internal gas volume of 0.45 x 10⁻³ l. Therefore, the endogenous ethylene concentration just before de-submergence was calculated to be 7933 nl l⁻¹. De-submergence in nitrogen to avoid the complicating impact of the second peak, which is new synthesis, revealed a very similar endogenous concentration (5540 nl l⁻¹).

The results of the experiments shown in Fig. 2 and other similar experiments demonstrate that after 24 h of submergence, *R. palustris* accumulates ethylene up to a concentration of approx. 6000 nl l⁻¹. This is a 100-fold increase in concentration compared to non-flooded control conditions (Voesenek *et al.*, 1993). In other species, such as *Callitriche platycarpa*, *Nymphoides peltata* and *Oryza sativa*, internal concentrations reported for submerged plants, measured with traditional and thus problematic techniques, reach values of only 1000–2000 nl l⁻¹ (Musgrave, Jackson and Ling, 1972; Malone and Ridge, 1983; Métraux and Kende, 1983). However, the much higher concentration of 6000 nl l⁻¹ was described for *Ranunculus sceleratus* when submerged for 24 h (Samarakoon and Horton, 1984). Jackson and co-workers (1987) also reported relatively high internal ethylene concentrations (2200–11800 nl l⁻¹) in submerged shoots of rice plants. The significance of these enhanced endogenous levels lies in the growth stimulating action of ethylene for leaves, petioles, internodes, coleoptiles and/or mesocotyls of many amphibious and aquatic plant species growing in habitats with fluctuating water levels (reviewed in Voesenek and Van der Veen, 1994).

Species of the genus *Rumex* are spatially distributed in Dutch river floodplains according to their flooding resistance (Blom *et al.*, 1990). An important plant trait in relation to this differential resistance is a contrasting ability to stimulate shoot extension upon submergence. The wetland *Rumex* species *R. maritimus*, *R. palustris* and *R. crispus* possess this trait, whereas species from rarely flooded sites (e.g. *R. acetosa* and *R. acetosella*) do not (Voesenek and Blom, 1989; Banga, Blom and Voesenek, 1995). However, both elongating and the non-elongating species accumulate ethylene upon submergence as measured by photoacoustic spectroscopy (Voesenek *et al.*, 1993). Thus, enhanced shoot extension in *Rumex* species requires not only enhanced endogenous levels of ethylene, but also a specific tissue response.

Photoacoustic spectroscopy in line with a flow-through system was used to estimate endogenous ethylene concentrations in the primary root system of the wetland plant *R. palustris* submerged for 24 h in a solution of liquid agar to mimic waterlogged conditions. *Rumex palustris* was characterized by concentrations ranging from 1600 to 2300 nl l⁻¹. These measurements aimed to relate high ethylene levels in roots of flooded plants with the formation of adventitious roots. The initiation and outgrowth of this aerenchymatous type of roots is one of the major adaptations of wetland *Rumex* species to survive overwet soil conditions (Blom *et al.*, 1994; Visser *et al.*, 1995). Experiments with specific inhibitors of the ethylene biosynthetic pathway applied to *R. palustris* on stagnant agar, had demonstrated a reduction in the number of emerging adventitious roots (data not shown). Based on this evidence, we hypothesize that in *R
enhanced endogenous ethylene levels promote the induction of adventitious roots.

**CASE STUDY 2: ROOT ETHYLENE PRODUCTION UNDER SUBAMBIENT OXYGEN PRESSURES**

Submergence induces a dramatic change in the internal gas composition of flooded plants. One of these changes is a decline in the concentration of oxygen (Stünzi and Kende, 1989). Such a change can have a significant influence on the production rate of ethylene and thus on ethylene-mediated responses. Low levels of oxygen that cause tissue hypoxia can have opposing effects on the production rate of ethylene. The rate limiting step in the conversion of L-methionine into ethylene is ACC-synthase (S-adenosyl-L-methionine methylthioadenosine-lyase). The expression of certain genes of the multigene family coding for this enzyme can be stimulated in rice and tomato by reduced levels of oxygen (Zarembinski and Theologis, 1993; Olson, Oetiker and Yang, 1995). These enhanced levels of transcripts are also likely to be translated and the Theologis, 1993; Olson, Oetiker and Yang, 1995). These ACC-oxidase, the enzyme catalysing the last step in ethylene biosynthesis, requires molecular oxygen as a co-substrate to convert 1-aminocyclopropane-1-carboxylic acid into ethylene (Kende, 1993). Preliminary evidence indicates that both the mRNA levels coding for ACC-oxidase and the *in vitro* ACC-oxidase activity are enhanced in submersed shoots of *R. palustris* (data not shown) and also in shoots of tomato plants grown in flooded soil (English et al., 1995). Thus, the impact of reduced oxygen levels on the final ethylene production rate is still a research area with apparent contradictions. Increased production rates of ethylene in response to subambient partial pressures of oxygen have been described for nodal roots of *Zea mays*, roots of *Hordeum vulgare* and stems of deepwater rice (Jackson, 1982; Métraux and Kende, 1983; Jackson et al., 1984). However, hypocotyls of mungbean, coleoptiles of rice and fruits of banana demonstrated a reduction in ethylene evolution upon exposure to low levels of oxygen (Imaseki, Watanabe and Odawara, 1977; Raskin and Kende, 1983; Banks, 1985). Since all these results are based on head-space analyses of ethylene produced by excised tissue enclosed in small incubation vials we cannot rule out that artifacts introduced by this method may have influenced the final outcome of the experiments performed.

We used the photoacoustic flow-through set-up to avoid problems connected with head-space analysis in a study of ethylene production of primary roots of *Zea mays* under hypoxia-inducing environmental conditions. The method allowed the oxygen content of the flowing gas mixture to be changed without handling of the plant and/or opening of the cuvette. To adjust the set-up to this specific experiment two essential modifications were carried out. (a) After a switch from 21% oxygen to 3% oxygen the frequency of the chopper that interrupts the laser beam was adjusted to maximize the microphone signal. (b) In response to subambient oxygen concentrations most plant tissues shift in metabolism from oxidative phosphorylation to predominantly ethanolic fermentation. Ethanol, the end-product of this pathway, is volatile and can enter the photoacoustic cell. The broad absorption spectrum of ethanol in the infrared wavelength region masks the ethylene signal. To prevent this disturbance, gas leaving the cuvettes was passed through a cold trap before entering the photoacoustic cell. By selecting a temperature of approximately −100 °C, ethanol was frozen out, leaving ethylene, with its lower partial pressure to pass into the detector without freezing.

Germinated caryopses of *Zea mays* with a primary root of approximately 10 mm long were sealed into a two-chamber cuvette. The primary root, still attached to the caryopsis, was enclosed in the lower chamber and exposed to a gas flow of 0.5 l h⁻¹ (see photograph in Brailsford et al., 1993). During the first and last 6 h of the experiment the primary root was exposed to air, whereas an intervening period of 16 h exposed the roots to either 3% oxygen or to pure nitrogen. The results demonstrated that, due to the high sensitivity of photoacoustic spectroscopy, it is possible to measure ethylene evolution continuously by a single root axis with a fresh weight of approx. 15 mg for many hours (Fig. 3A, B). All experiments showed a gradually declining production rate in air probably related to a handling-induced enhancement of the ethylene production at the start of the experiment. A reduced concentration of oxygen (3%) clearly stimulated the ethylene production in the primary root of *Zea mays* (Fig. 3A). A switch to complete anoxic conditions reduced the ethylene production to an undetectably slow rate (Fig. 3B). It can be concluded that molecular oxygen is required for ethylene production. On the level of a single cell there seems to be a paradox between the lack of ethylene production under anoxic conditions and the stimulation during exposure to subambient levels of oxygen. However, the explanation for the increased ethylene production under low levels of oxygen must be sought on the whole root level (Jackson, 1994). High levels of ACC may accumulate in the anoxic root tip or stele due to the absence of oxygen and/or to an anoxia-induced increase in ACC-synthase activity. This ACC can diffuse to better aerated cortical tissues where it can be converted into ethylene (Brailsford et al., 1993). The following observations are important in this context: (a) increased internal ACC levels may enhance the affinity of ACC-oxidase for oxygen (Yip, Jiao and Yang, 1988). (b) Partial oxygen shortage may enhance levels of mRNA coding for ACC-oxidase and increase *in vitro* ACC-oxidase activities (data not shown). Such an increase in the amount of ACC-oxidase can be an advantage under reduced oxygen levels for the following reason. A reduction in the concentration of molecular oxygen will reduce the number of conversions of ACC to ethylene per unit time. An increase in the number of ACC-oxidase molecules under these low oxygen conditions will, however, enhance the conversion rate since the number of oxygen molecules, even at 3%, exceeds, by far, the number of ACC-oxidase molecules. In conclusion, both the high ACC levels and the increased number of ACC-oxidase molecules may result in a situation where ethylene pro-
production rates, during periods of subambient concentrations of oxygen, are not changed or are even slightly stimulated. In order to understand differences between various plant species in the effect of oxygen shortage on ethylene production rates it will be necessary to determine their contents of ACC-synthase, ACC-oxidase and ACC.

High endogenous concentrations of ethylene in roots of maize, caused by physical entrapment or by a low oxygen-induced production increase, promote the premature collapse of cells in the root cortex ultimately leading to longitudinally interconnected gas-filled channels of lysigenous aerenchyma (for reviews see Armstrong, Brändle and Jackson, 1994 and Voesenek and Van der Veen, 1994). These diffusion and ventilation highways enable plants to maintain an endogenous gas composition that sustains metabolism in tissues with little or no direct access to oxygen in the immediate surroundings.

Case Study 3: Monitoring Ethylene Evolution and Leaf Growth Simultaneously

Because of the very high sensitivity of photoacoustic spectroscopy, ethylene levels can be measured directly in the outlet of a cuvette. This has allowed us to measure ethylene evolution simultaneously with leaf growth. A special cuvette was designed to combine photoacoustic measurements with growth registration by means of linear-variable displacement transducers (Fig. 4). The cuvette contains three compartments: one chamber for the roots, another for the shoot and a third chamber with a mechanism that allows the movement of a stainless steel wire (diameter 0.1 mm) connecting the transducer with a growing leaf but preventing the release of ethylene produced by the shoot. Two pulleys guide the wire through a small curved channel that during an experiment is filled with a saturated solution of ammonium sulphate [(NH₄)₂SO₄] thus functioning as a ‘water seal’ to prevent loss of ethylene. The resistance of the

Fig. 3. Ethylene production (nl g f.wt⁻¹ h⁻¹) of primary root of Zea mays (A) exposed to air for 6 h, followed by 16 h of 3% oxygen before returning to air [(■) 3% O₂, (□) control]. B. Exposed to air for 6 h followed by 16 h in nitrogen gas before returning to air.

Fig. 4. Schematic presentation of experimental set-up for measuring ethylene production of an intact shoot simultaneously with elongation growth of the youngest leaf of Rumex palustris. Ethylene release was monitored by photoacoustic spectroscopy. Before compressed air entered the shoot compartment of the cuvette (SC; volume 500 ml) hydrocarbons were removed with a platinum catalyst (A) before entering a flow-controller (B) and the cuvette containing the plant. The out-flowing air was scrubbed of carbon dioxide and water before ethanol was frozen out with a cold trap (C) and passed into the photoacoustic cell (D). When necessary, shoots were submerged to level F. The roots are in a separate compartment of the cuvette (RC). A well-aerated nutrient solution (K) was pumped (J) through the root compartment. To prevent local depletion of oxygen in the rhizosphere the nutrient solution in the cuvette was circulated with a magnetic stirrer and flea (I). Oxygen-deficient solution conditions were realized by closing gate clips G and H. Growth of the youngest leaf was measured with linear displacement transducers. The wire connecting the transducer with the leaf tip was guided through a channel filled with a saturated solution of ammonium sulphate [(NH₄)₂SO₄] functioning as a ‘water seal’ (E) to prevent loss of ethylene.
two pulleys is very low, guaranteeing a smooth and continuous registration of leaf growth. This set-up is used to examine the following problem: low levels of oxygen (1–5%) stimulate the growth of the youngest petiole of *R. palustris*. This growth stimulation is not directly controlled by oxygen, but indirectly via ethylene action (Blom *et al*., 1994). However, it is not yet known whether this hypoxia-induced petiole growth is regulated via a stimulation of ethylene production in the shoot tissue of *R. palustris* or if decreased oxygen levels interfere with the perception-transduction chain for ethylene thus increasing the responsiveness of the petiole tissue towards the hormone. By simultaneously monitoring growth of the youngest leaf and ethylene evolution of the same shoot under ambient and subambient oxygen concentrations we hoped to answer this question. During the first exposure period to air, both leaf growth and ethylene evolution showed a circadian rhythm with the highest growth rates and the lowest ethylene evolution during the dark period (Fig. 5). Low oxygen levels completely reversed growth and ethylene evolution in relation to the dark/light regime since slow growth-rates and high ethylene production were now observed during darkness rather than in the light. The subambient oxygen level induced in shoots of *R. palustris* an increase in ethylene production, especially at night. However, the growth-rate of the youngest petiole was stimulated especially during daytime. This showed a lack of correlation between ethylene production and petiole growth under low partial pressures of oxygen. These results strongly suggest that reduced oxygen concentrations can stimulate petiole growth via an increased sensitivity of the petiole tissue to ethylene. To establish this effect more precisely, ethylene concentration-response curves are required under ambient and subambient concentrations of oxygen.

FUTURE PROSPECTS
The three case studies described above show that photoacoustic spectroscopy is a powerful technique for measuring small amounts and slow production rates of the hormone ethylene. Due to the very high sensitivity of the method, which is at least 1000 times more sensitive than the plant itself, it is possible to use a flow-through system in which concentrations of ethylene are necessarily extremely small. Experimental artifacts related to the technique of accumulation and head-space analysis and the use of excised tissue can thus be avoided. The three examples exploited the high time-resolution of the technique thereby demonstrating the suitability of photoacoustic spectroscopy for measuring fast changes in ethylene release.

Flooding of plants is often accompanied by rapid shifts in ethylene concentrations and production levels. This signals to the plant that its outside environment has changed dramatically and induces the development of adaptive responses to counteract the constraints of flooding. Therefore, we anticipate that photoacoustic spectroscopy will continue to be an important tool in the study of ethylene-induced responses to soil flooding and submergence.

A so-called photothermal deflection technique for using lasers to measure ethylene has recently been devised. With this it is possible to measure ethylene concentrations directly at the site of emission of a plant or plant organ and without the need to enclose that tissue in any way. Two laser beams, one from a CO₂ laser and one from a HeNe laser, are crossed just above (approx. 1.5 mm) the site of interest (e.g. the apex of a ripening tomato fruit). The infrared laser beam from the CO₂ laser excites ethylene molecules and consequently increases the temperature at the site of study by a few thousandths of a degree. This temperature increase changes the refraction index of the air at the site of detection and deflects the second laser beam in proportion to the concentration of ethylene (De Vries *et al*., 1995). This technique should enable ethylene production at local sites to be quantified on partially submerged plants without the need for tissue excision.

Photoacoustic spectroscopy is not restricted only to ethylene. Trace amounts of other volatiles of importance in flooding research such as acetaldehyde, methane, ethane and ethanol can also be detected. However, a different type of laser (CO-laser) is required to generate the appropriate wavelengths useful for the detection of these gases (Bijnen, 1995). With this technique greater insight will be gained into the energy metabolism of plants exposed to low levels of oxygen. For example, application of photoacoustic spectroscopy with a CO-laser can improve our knowledge of the production of highly toxic acetaldehyde upon re-aeration of plant tissue after flooding. This same technique can be applied to monitor ethane, a gas produced during membrane destruction due to the action of free oxygen radicals. They are produced when plant tissues shift from an anaerobic environment back to a fully aerated atmosphere.

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