Rapid ethylene release and stimulated ethylene production after de-submergence of plants: An analysis with a continuous flow system in line with a laser-driven intracavity photoacoustic ethylene detector

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Introduction

Ethylene plays a key role in several morphological responses to flooding of the soil or total submergence e.g. lysogenous aerenchyma formation (Drew et al., 1979; Jackson et al., 1985), stem swelling and lenticel hypertrophy (Jackson and Palmer, 1981), epinastic leaf curvature (Reid and Bradford, 1984), re-orientation of extension from prostate to erect (Hansen and Bendixen, 1974; Voesenek and Blom, 1989a) and elongation of the petiole, internode, coleoptile, rachis and lamina of various species (Ku et al., 1970; Musgrave et al., 1972; Musgrave and Walters, 1974; Métraux and Kende, 1983; Samarakoon and Horton, 1981; Laan and Blom, 1990; Voesenek and Blom, 1989a; 1989b; Voesenek et al., 1990). Evidence increasingly suggests that at least some of these hormonally regulated responses are adaptive and promote plant survival and reproduction (Ridge, 1987; Van der Sman et al., 1991; Voesenek, 1990). In Rumex, occurring in flooding gradients of river flood plains, ranging from seldomly flooded dykes to beaches subjected to frequent and prolonged inundation (Blom, 1990; Blom et al., 1990), shoot-atmosphere contact is crucial for survival. As in other species this contact is achieved through increased rates of extension by the submerged shoot, leading to earlier re-surfacing by the apex and young leaves. However, the efficiency by which the re-surfaced shoot aerates the root system and determines survival depends on the porosity of intervening root and shoot tissues (Voesenek et al., 1991). Ethylene plays a major role in the regulation of submergence induced petiole and/or internode elongation in Rumex, especially in those species found in wet field sites. The rise in the endogenous ethylene concentration in Rumex plants observed shortly after submergence (Voesenek and Blom, 1989a, b; Van der Sman et al., 1991) is probably resulting from a reduced outward diffusion, leading to entrapment (see Musgrave et al., 1972), and from an increase in biosynthesis (Van der

Sman *et al.*, 1991). According to Musgrave *et al.* (1972) de-submergence results in a rapid dissipation of accumulated ethylene into the atmosphere until a lower equilibrium concentration is reached. This paper deals with the kinetics of ethylene release and production in *R. palustris* after de-submergence of intact plants, measured in a continuous flow system in line with a highly sensitive laser-driven photoacoustic detector (Bicanic *et al.*, 1989; Harren *et al.*, 1990a; 1990b; Voesenek *et al.*, 1990). The importance of intact plants instead of excised plant parts in ethylene physiology was stressed recently by Morgan *et al.* (1990). The use of relatively high flow rates, in combination with a sensitive detection, enabled us to distinguish between ethylene release by plants due to previous entrapment and that evolved more gradually as a consequence of increasing production.

Methods

Photoacoustic detection of ethylene

Alexander Graham Bell (1881) discovered the photoacoustic principle, i.e. the transformation of light energy into acoustic energy. Interest in the photoacoustic effect was stimulated by the invention of powerful lasers. In the set-up used for the experiments described, a mechanically chopped infra-red laser beam was directed into a photoacoustic cell (PA cell) containing ethylene. Due to absorption of energy, ethylene molecules excited in this way are transformed into the rotational level of a higher vibrational state at atmospheric pressure. De-excitation in the infra-red region generally occurs via non-radiative decay. This relaxation process increases the kinetic energy and temperature of the gas molecules in the PA cell. An increase in temperature in a (semi)closed vessel with a constant volume (PA cell) increases the pressure. When the infra-red laser beam is chopped at an audio frequency, pressure fluctuations of the same frequency occur inside the PA cell. These fluctuations can be detected with sensitive microphones placed inside the PA cell. A longitudinal acoustic resonance in the PA cell was obtained by careful tuning of the chopper frequency. The effect may be likened to resonance in an open organ tube achieved when the length of half the acoustic wave equals the length of the pipe. Additional increases in signal were achieved by placing the PA cell inside the laser cavity. This set-up has given a sensitivity of 6 picoliters of ethylene per liter (Harren, 1988; Harren *et al.*, 1990a).

The CO_2 waveguide laser emits wavelengths between 9 and 11 μm in the infra-red and produces radiation at 90 discrete laser lines. Various gases such as ethylene, ozone, ammonia and hydrogen sulphide are characterized by fingerprint-like absorption spectra at the CO_2 laser wavelengths. The strongest ethylene absorption was observed at a wavelength of 10.53 μm (10P14 CO_2 laser line), a much weaker absorption was observed at 10.51 μm (10P12 line). During one measurement cycle absorptions and the accompanying microphone

signals were determined on both laser lines and recalculated to correct for background signal. A stepmotor-driven grating regulated transfer between both laser lines. The laser power on both lines was maximized during every measurement cycle with the aid of a computer-controlled piezo.

The PA cell was connected to a continuous gas-flow system in which air was first led into a catalyst to remove all hydrocarbons at a temperature of 350 °C. Thereafter, the flow was split between eight flow controllers, which regulated the flow ($0.2 - 4.5$ L $\cdot$ h^{-1}) before it entered one of the eight glass cuvettes in which plants could be placed. Eight computer controlled valves connected each cuvette in turn with the PA cell. When not in line with the PA cell, the air in a cuvette was vented to the atmosphere. Before entering the PA cell the air was led through a scrubber filled with KOH pellets to remove CO_2.

Experimental set-up

Rumex palustris, predominantly occurring on flood plain sites with frequent and prolonged floods, was used in the experiments here and plants were grown out of seed collected in the field. Growth conditions are described in Voesenek and Blom (1989a). At the end of the growth period the plants had five true leaves, whereas a shoot fresh weight of 1.65 ± 0.27 g and an internal gas volume of the shoot of 0.349 ± 0.067 mL was reached after the experiments.

The kinetics of ethylene release from *R. palustris* after de-submergence were followed using the continuous flow system described earlier. Plants were transferred to the flow-through cuvette (600 mL) and exposed to a continuous photosynthetic photon flux density of 50 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} at 25 ± 1 °C. After 24 h acclimatization the plants were completely submerged in tap water for 24 h. Towards the end of this submergence period, while the plants were still below the water level, the ethylene production rate (nl $\cdot$ h^{-1} $\cdot$ plant^{-1}) was determined. The ratio of the mean ethylene production (n=36) divided by the individual production level was used to correct the ethylene release after de-submergence. This correction was based on the assumption that high ethylene production rates, with all other influencing variables constant, will lead to high internal ethylene concentrations during submergence and thus automatically to high release peaks after de-submergence (Jackson, 1985). In summary, the ratio corrects for inter plant variation in ethylene production under water.

The position of the plants in the cuvettes could be varied vertically over a height of 14 cm by sliding a rod connected, through a water-tight seal, to the underside of a plant pot in which a *Rumex* plant was growing. After the 24 h of submergence, plants were moved upwards in the cuvette until the whole shoot protruded above the water surface. The plants were pulled downwards and submerged again after 2–3, 5, 11, 15, 22, 45, 60, 90, 180, 360, 720, 1200, 1440, 1620, 1980, 2400, 3180, 3600, 6900, 9060 or 18000 s. One individual was used for every de-submergence period (2–4 replicates). An injection of a known concentration of gas (*e.g.* ethylene) into a continuous flow system results in a characteristic time course of response as it passes through the detector, *i.e.* a

steep, almost instantaneous rise, followed by an exponential decay curve, in which the slope of this decay is determined by the flow rate in the system. A sudden release of ethylene by a de-submerged plant should give the same pattern of response. The area under this peak ($nL \cdot h \cdot l^{-1} \cdot plant^{-1}$) multiplied by the flow rate ($2 \ L.h^{-1}$) corresponds with the ethylene release ($nl \cdot plant^{-1}$) of the plant immediately after de-submergence. The calculated ethylene release was multiplied with the already mentioned production correction ratio.

When the amount of ethylene released was greater than the presumptive exponential decay curve, the extra gas was deduced to be the consequence of extra synthesis during the de-submergence period (Van der Sman et al., 1991). This newly synthesized ethylene was deducted from the total. The remaining represented ethylene released from entrapment. The ethylene release after de-submergence was plotted in a graph against the exposure times to the atmosphere. A non-linear regression procedure (procedure nlin; SAS Institute Inc. Cary, 1985) was used to fit a function through both entrapment and production data.

Results

De-submergence of 5 h (18,000 s) resulted in a characteristic release pattern of ethylene. Figure 1 presents this burst of ethylene from the plant expressed as concentration measured in the PA-cell. Immediately after re-surfacing, a large amount of ethylene was rapidly released from the plant to the atmosphere. A second, more gradual peak, reached a maximum 3 h after de-submergence. The absolute amount of ethylene released ($nl \cdot plant^{-1}$) can be calculated from such a graph when the concentration ($nl \cdot L^{-1}$) is multiplied by time (h) and flow rate ($L \cdot h^{-1}$). The release rate ($nl \cdot h^{-1} \cdot plant^{-1}$) at a certain moment can be calculated by multiplying ethylene concentration ($nl \cdot L^{-1}$) and flow rate ($L \cdot h^{-1}$).

The pattern of ethylene release of previously submerged R. palustris plants after short-time exposures (2 s–60 min) to the atmosphere is presented in Fig. 2 (open squares). Correction for newly synthesized ethylene was not necessary for the first 360 s, thereafter peak areas of ethylene release were adjusted as described in the methods section. Based on the assumption that diffusion of entrapped ethylene from a plant to the atmosphere is a first-order process, an exponential regression was fitted into the data (Fig. 2). This regression yielded a maximum ethylene release or asymptote of $1.42\pm0.09 \ nL \cdot plant^{-1}$. When this maximum level of released ethylene was divided by the internal gas volume of the shoot, a mean endogenous ethylene concentration in the plant shortly before de-submergence of $4.07\pm0.26 \ nl \cdot mL^{-1}$ could be calculated. Half of this ethylene in the plant was released within 18 s (t_{50}), whereas 90% "escaped" in a time span of almost 56 s.

From 720 s onwards the amount of newly synthesized ethylene increased dramatically. It should be borne in mind that this is ethylene production in
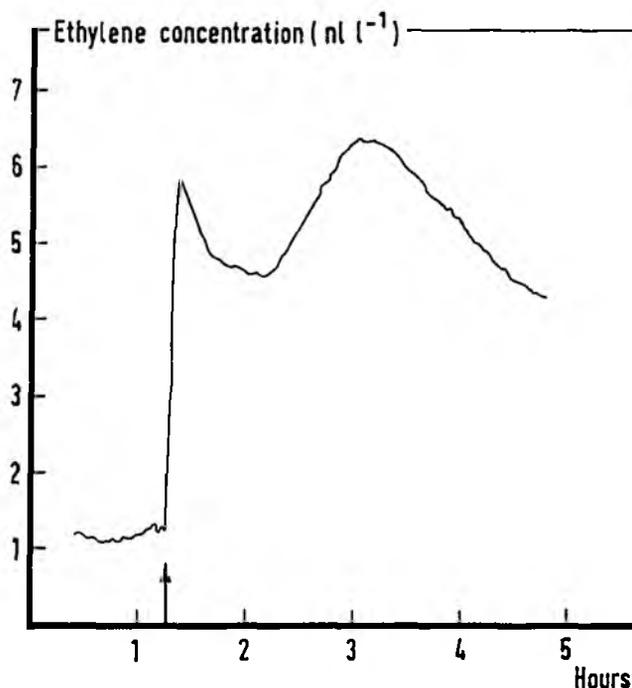
Figure 1. The pattern of ethylene release of one *Rumex palustris* plant after de-submergence (arrow) expressed as ethylene concentration ($nL \cdot L^{-1}$) measured in the photoacoustic cell connected with a continuous flow system.

addition to a basic level of production. When the maximum of release of the first rapid ethylene peak (1.42 nL $\cdot$ plant^{-1}) is subtracted from the total ethylene release, the remaining ethylene of the exposure times from 720–9060 s is restricted to newly synthesized ethylene. These data are plotted in Fig. 2 with closed squares. An exponential regression fitted into these data, shows clearly that interference of ethylene production with the first rapid release peak (first 60 s) can be ignored.

Discussion

The photoacoustic ethylene detector is unique that it can operate without a collection trap. Ethylene can be measured directly in the effluent of the cuvettes, despite high flow rates (up to 4.5 L $\cdot$ h^{-1}) in the system. Knowing the potential interference by oxygen, carbon dioxide and ethylene itself on the ethylene production by plants (Yang and Hoffman, 1984; Woltering *et al.*, 1988), the advantage of continuous flow systems instead of head space analyses in sealed containers is obvious. Direct detection of ethylene in the outlet flow of a cuvette is a prerequisite to study rapid changes in ethylene production such as the release of ethylene after de-submergence. The highly sensitive laser-driven photoacoustic detector in an intracavity set-up has created the possibility to measure these rapid changes in ethylene release. Such measurements would have been impossible with collection traps and traditional gas chromatographic
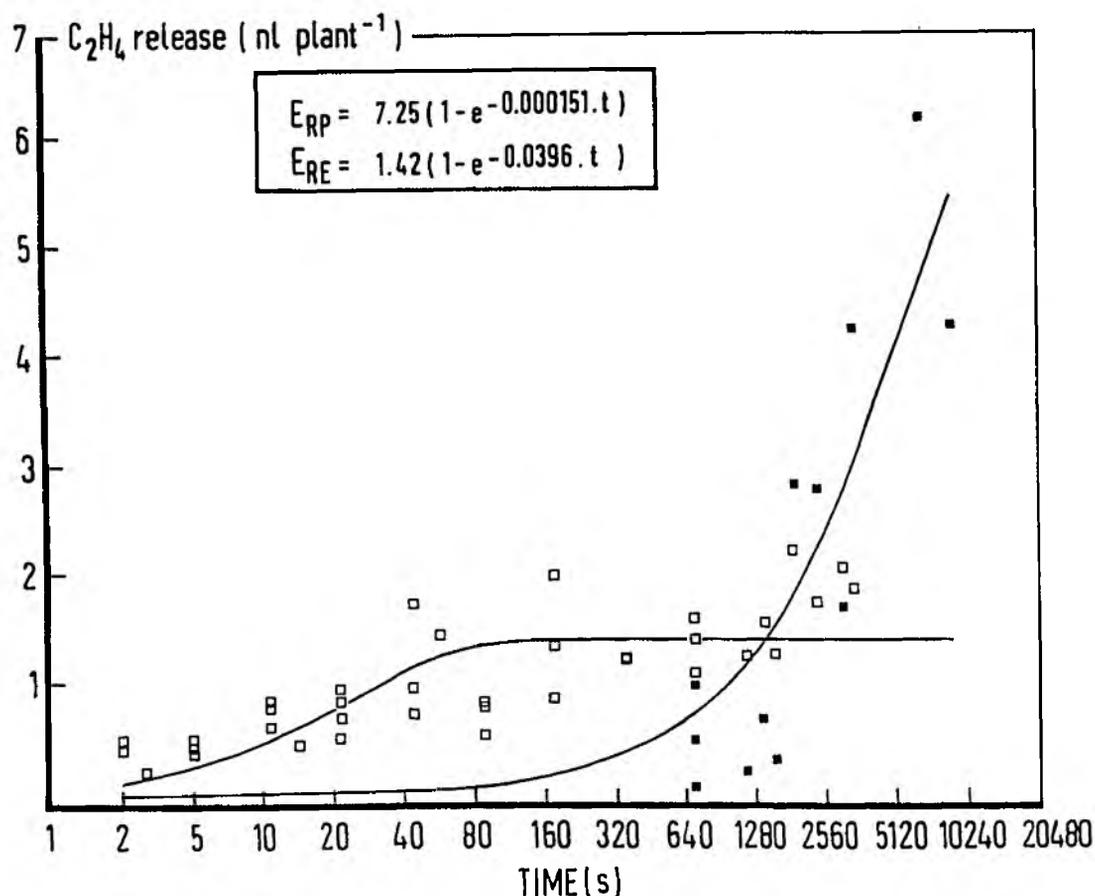
$$E_{RP} = 7.25(1-e^{-0.000151.t})$$
$$E_{RE} = 1.42(1-e^{-0.0396.t})$$

Figure 2. The ethylene release (nL · plant^{-1}) of Rumex palustris plotted against various de-submergence periods (seconds on a log scale) measured with a laser-driven intracavity photoacoustic detection technique in line with a continuous gas-flow system. Ethylene release due to previously entrapped ethylene (E_{RE}) is presented as open squares; ethylene release due to extra production (E_{RP}) above a basic level is plotted as closed squares. A non-linear fitting procedure was used to calculate the included functions for the two sets of ethylene release data.

analyses of ethylene because of insufficient sensitivity (see Bassi and Spencer, 1989).

De-submergence of R. palustris results in a rapid release of ethylene; more than 90% of the entrapped ethylene is released within 1 min. This initial release is followed by a more gradual increase of the production, reaching a peak after 3 h. The origin of both ethylene peaks is different. The entrapped ethylene results from the balance between ethylene production and release to the water environment shortly before de-submergence, whereas the ethylene of the second peak is probably produced entirely during the de-submergence period.

The surface area under the entrapment peak corresponds to the total amount of ethylene released upon de-submergence. Dividing this amount by the internal volume of gas space in the shoot (0.349 mL) allows an estimate of the internal concentration of ethylene within the plant to be calculated (4.07 nl · mL^{-1}). This value exceeds the values obtained by conventional vacuum extraction methods (Beyer and Morgan, 1970) we have used on this same material (0.36–2.47

nl · mL^{-1}). Thus we conclude that the second peak, with its much larger surface area and thus ethylene concentration can never be attributed to a sort of delayed release of entrapped ethylene. Additional evidence comes from submergence experiments with AVG (aminoethoxyvinylglycine), an inhibitor of ethylene biosynthesis at the ACC (1-aminocyclopropane-1-carboxylic acid) synthase step (Yang and Hoffman, 1984). These experiments were performed with a related species: *R. maritimus* which has the same distribution in flooding gradients in riparian habitats. A similar pattern of ethylene release after de-submergence was observed in this species. When submerged in an AVG solution (0.1 mol · m^{-3}) instead of water, de-submergence resulted in a reduced first peak and complete absence of the second peak (Van der Sman *et al.*, 1991). Since the plants were not pretreated with AVG before submergence it probably took some time for the ethylene production under water to be completely inhibited. The produced ethylene was entrapped and released later after de-submergence, whereas extra ethylene production during de-submergence was prevented by the now optimally inhibiting AVG. As far as we know this is one of the first papers presenting experimental evidence for the hypothesis formulated by Musgrave and co-workers (1972) that entrapped ethylene in submerged plants rapidly dissipates into the atmosphere after plant parts protrude the water surface. In an indirect way our results also showed actual accumulation of ethylene during a 24 h submergence period. This result was obtained without the risk of artifacts caused by excision of plant parts and vacuum extraction procedures.

Flooding resistant *Rumex* species increase their ethylene production during submergence (Van der Sman *et al.*, 1991). Such an increase was also observed in flooded deep water rice (Kende *et al.*, 1984; Raskin and Kende, 1984). Low partial pressures of oxygen, which accompany shoot submergence, induce an enhancement of ACC-synthase, which catalyses the conversion of S-adenosyl-methionine (SAM) to ACC (Cohen and Kende, 1987). The increased activity of the key regulatory enzyme in ethylene biosynthesis, ACC synthase, leads, in deep water rice, to an enhancement of ethylene synthesis. We assume that such a mechanism might also play a role in the increased ethylene production rate of *R. palustris* under water. It is, however, also possible that not all the ACC produced, is actually converted to ethylene due to inhibition of conversion to ethylene in certain root zones (root-tips, tap-root) because of a lack of oxygen. As soon as the leaf-atmosphere contact is restored, the *Rumex* root system becomes oxygenated due to the process of internal aeration (Laan *et al.*, 1990). Consequently more ACC can then be converted to ethylene. This could explain the extra ethylene production (second peak) observed within a few hours after de-submergence.

Functionally this increased ethylene production might be related to the observed continued growth after a leaf tip has reached the water surface. The amount of shoot tissue above the water is, in *R. maritimus*, positively related to the amount of internal aeration (Laan *et al.*, 1990). The resulting improvement in energy status of plants due to increased aerobic root respiration explains the

positive correlation between the amount of shoot tissue above the water surface and survival/reproduction (Voesenek, 1990; Van der Sman *et al.*, 1991).

Although *Rumex* plants can photosynthesize under water (Voesenek, 1990; Laan and Blom, 1990), the limited supply of carbon under these conditions is probably rate limiting (Setter *et al.*, 1987). Ethylene mediated shoot growth to reposition a substantial part of the shoot above the water surface, where CO_2 is abundant, can therefore not only be seen as a mechanism that restores aerobic root respiration, but it may play an important role in increasing the leaf area used for aerial photosynthesis.

Acknowledgements

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