

## Submergence-Induced Ethylene Synthesis, Entrapment, and Growth in Two Plant Species with Contrasting Flooding Resistances

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Submergence-induced ethylene synthesis and entrapment were studied in two contrasting *Rumex* species, one flood-resistant (*Rumex palustris*) and the other flood-sensitive (*Rumex acetosa*). The application of a photoacoustic method to determine internal ethylene concentrations in submerged plants is discussed. A comparison with an older technique (vacuum extraction) is described. For the first time ethylene production before, during, and after submergence and the endogenous concentration during submergence were continuously measured on a single intact plant without physical perturbation. Both *Rumex* species were characterized by enhanced ethylene concentrations in the shoot after 24 h of submergence. This was not related to enhanced synthesis but to continued production and physical entrapment. In *R. palustris*, high endogenous ethylene levels correlated with enhanced petiole and lamina elongation. No dramatic change in leaf growth rate was observed in submerged *R. acetosa* shoots. After desubmergence both species showed an increase in ethylene production, the response being more pronounced in *R. palustris*. This increase was linked to the enhanced postsubmergence growth rate of leaves of *R. palustris*. Due to the very rapid escape of ethylene out of desubmerged plants to the atmosphere (90% disappeared within 1 min), substantial underestimation of internal ethylene concentrations can be expected using more conventional vacuum extraction techniques.

Enhanced stem or petiole elongation in response to submergence enables aquatic, semi-aquatic, and terrestrial plants to avoid the constraints of oxygen deficiency, toxins, and ion deficiency imposed by the flooded environment. The gaseous growth regulator ethylene plays a crucial role in the stimulation of shoot extension under water (Ku et al., 1970; Musgrave et al., 1972; Métraux and Kende, 1983; Osborne, 1984; Ridge, 1987; Voeselek and Blom, 1989a; Jackson and Pearce, 1991). Presumably, plant tissues produce ethylene continuously. Due to the low diffusion coefficient of ethylene in water under submerged conditions the gas will be entrapped in intercellular gas spaces (Musgrave et al., 1972). The actual amount of physically entrapped ethylene depends on the surface:volume ratio of the tissue (Jackson, 1985), the rate of ethylene oxidation (Beyer, 1984), the depth of the covering water layer (Konings and Jackson, 1979), the flow rate of the water (Ridge, 1987), and the temperature (Jackson, 1985).

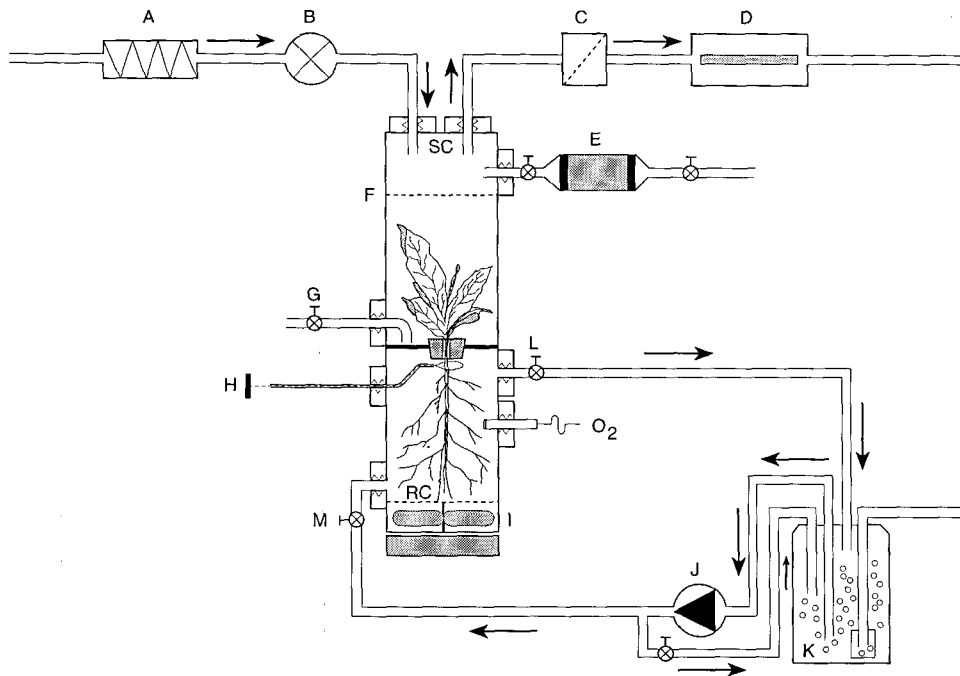
However, in tissues of some plant species physical entrapment is not the only source for increased endogenous concentrations of ethylene. In addition to entrapment, enhanced synthesis contributes to the rise in ethylene levels in flooded deep-water rice and in waterlogged roots of maize and barley (Jackson, 1982; Jackson et al., 1984, 1985; Raskin and Kende, 1984). A decline of the oxygen concentration, which induces enhanced ACC synthase activity, explains the observed increased ethylene production in the submerged stems of deep-water rice (Cohen and Kende, 1987).

The cosmopolitan genus *Rumex*, used as a model to study the hormonal regulation of flooding resistance, is distributed along flooding gradients in river flood plains (Blom et al., 1990; Voeselek et al., 1992b). Rosettes of *Rumex palustris*, a flood-resistant species from frequently flooded parts of the river flood plain, are able to reach the water surface after submergence through enhanced cell elongation induced by ethylene, especially in the youngest petioles. Rosettes of *Rumex acetosa*, a plant from highly elevated, rarely flooded dikes and river levees, do not increase their rate of petiole extension under water (Voeselek and Blom, 1989a; Voeselek et al., 1990). This species difference in petiole growth in response to submergence can be explained by differences in sensitivity to ethylene, as was shown with exogenous ethylene application (Voeselek and Blom, 1989a).

However, very little is known about the endogenous ethylene concentration in either species during submergence. In most other plant species, internal concentrations are approximately 1 to 2 nL mL<sup>-1</sup> (Musgrave et al., 1972; Malone, 1983; Métraux and Kende, 1983; Raskin and Kende, 1984). A much higher concentration of 6 nL mL<sup>-1</sup> was described for *Ranunculus sceleratus* submerged for 24 h (Samarakoon and Horton, 1984). Jackson et al. (1987) also reported relatively high internal ethylene concentrations (2.2–11.8 nL mL<sup>-1</sup>) in shoots of rice plants (*Oryza sativa* L. cv IR42). The rice plant parts used for ethylene determination were never exposed to air between excision and extraction, which may explain these high levels (M.B. Jackson, personal communication).

The aim of this study was to determine submergence-induced ethylene synthesis and entrapment in two *Rumex* species with contrasting flood resistance. Until recently, most techniques for measuring ethylene production depended on head space analyses, whereas ethylene entrapment in submerged plant tissue was measured predominantly with vac-

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**Figure 1.** Schematic overview of the experimental set-up, which includes a two-compartment cuvette and a laser-driven photoacoustic ethylene detector. A, Catalyst to dissociate hydrocarbons in carbon dioxide and water; B, flow controller; C, KOH scrubber to remove carbon dioxide; D, photoacoustic cell; E, Ethersorb scrubber; F, water level during submergence; G, L, and M, gate clips; H, stainless-steel tube containing a stainless-steel wire to separate root from shoot; I, magnetic stirrer and a flea; J, Eheim rotary pump; K, aerated nutrient solution; RC, root compartment; SC, shoot compartment. The set-up consists of cuvettes with two compartments in which intact plants were placed with their roots in the root compartment (RC; volume 370 mL) and their shoots in the shoot compartment (SC; volume 500 mL). The root-shoot junction was sealed gas-tight with a hollow rubber stopper filled with clay pushed into a conical opening in the plate separating SC and RC. In the root compartment a well-aerated nutrient solution was circulated with a rotary pump (flow rate,  $2.1 \text{ L h}^{-1}$ ) (Eheim type: 1016) (J). The shoot compartment was flushed with air at  $2 \text{ L h}^{-1}$  after passing a catalyst (A) to remove traces of hydrocarbons. Before entering the photoacoustic cell (D), the air was flushed through a KOH-based scrubber (C) to remove carbon dioxide.

uum extraction methods. Both methods can induce artifacts related to physical perturbation, changes in gas composition, and escape of ethylene. Additionally, real-time monitoring to follow fast changes in ethylene production were, until now, seldom included in studies on ethylene production rates in plants under stress. Therefore, our experiments were performed with intact plants in a flow-through system in line with a laser-driven intracavity photoacoustic ethylene detector (Harren et al., 1990; Voeselek et al., 1990; Van der Sman et al., 1991). The objectives were to determine (a) the internal ethylene concentration in the shoots of both species during submergence and compare it with vacuum extraction measurements; (b) the ethylene production level before, during, and after submergence; (c) how rapidly ethylene escapes into the atmosphere after desubmergence; and (d) whether petiole elongation during submergence is related to endogenous ethylene concentration, taking into account the observed differences in the sensitivity of these species to ethylene.

## MATERIALS AND METHODS

### Ethylene Concentration, Production, and Release

Achenes of *Rumex palustris* Sm. and *Rumex acetosa* L. were collected from flood plain populations and sown in pots filled

with black polyethylene granules (Stamylan Low Density, DSM, Geleen, The Netherlands). Germination occurred within 3 to 4 d under a temperature and light regime (Philips TL 8W/33) of  $25/10^\circ\text{C}$  (12 h day/12 h night) and a PFD of  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Thereafter, the seedlings were transferred to the growth chamber in which the experiments were conducted and were grown for a further 7 d on the polyethylene granules at a day temperature of 26 to  $28^\circ\text{C}$  (16 h; PFD,  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; Philips TLD 36W/84) and a night temperature of 21 to  $23^\circ\text{C}$ . At the age of 10 to 11 d the seedlings were transferred to aerated hydroponics and grown for approximately 12 to 17 d. By this time, *R. palustris* had developed five leaves and *R. acetosa* had developed four leaves.

The experimental set-up is presented and described in Figure 1. Information on the photoacoustic detection of ethylene can be found in Voeselek et al. (1992b) and Harren et al. (1990). Experiments with two-compartment cuvettes (Fig. 1) were conducted at a temperature of 26 to  $28^\circ\text{C}$  and under continuous illumination at a PFD of  $75 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Philips TLD 36W/84).

To determine both the internal ethylene concentration of submerged *Rumex* shoots and the ethylene production levels before, during, and after submergence, three two-compartment cuvettes, two with one plant each and one empty

reference cuvette, were installed. Three computer-controlled valves connected each cuvette in turn with the photoacoustic cell. When not in line with the photoacoustic cell, the air in a cuvette was vented into the atmosphere. After transfer to the cuvettes the plants were allowed to acclimatize for 24 h. Thereafter, the shoot of one of the plants was completely submerged (height water column, 10 cm). The nutrient solution in the root compartment was allowed to become stagnant by closing gate clips L and M (Fig. 1). The root system depleted the oxygen in the nutrient solution almost completely within 20 to 25 h (data not shown). After an inundation period of 24 h, the water level was lowered by opening gate clip G and E. Outside air, passing a scrubber filled with Ethysorb (Stay Fresh, Ltd., London, UK) (Fig. 1E) to remove ethylene (efficiency > 95%) entered the shoot compartment to replace the water. As soon as all the water had drained (after approximately 2 min), gate clips G and E (Fig. 1) were closed and the controlled air flow was reinstated. The root compartment kept its stagnant character to mimic waterlogged conditions. In the second cuvette, the plant was not flooded and the nutrient solution was continuously circulated. For each species this experiment was repeated three to four times; representative data are shown.

The photoacoustic ethylene detector measures a concentration ( $\text{nL L}^{-1}$ ). To calculate ethylene production ( $\text{nL h}^{-1} \text{g}^{-1}$  dry weight) this concentration must be multiplied by the gas flow rate ( $\text{L h}^{-1}$ ) and divided by the shoot dry weight (g). Desubmergence of *Rumex* shoots results in release of entrapped ethylene. This release is characterized by a typical time course of response as it passes through the detector, i.e. a steep rise followed by an exponential decay curve. The slope of this last curve is determined by the volume of the flow-through system and the flow rate. The area under this peak corresponds to the amount of ethylene released. The internal ethylene concentration in the shoot just before desubmergence can be calculated as follows: area under peak ( $\text{nL h}^{-1} \text{g}^{-1}$  dry weight  $\times$  h) is multiplied by the total dry weight (g) of the plant. The total amount of released ethylene (nL) and the internal gas volume of the shoot, obtained by the pycnometer method (Jensen et al., 1969), were used to calculate the endogenous ethylene concentration. If a plant produces large amounts of ethylene during the decay-curve period the slope will be less steep. This might lead to an overestimation of released ethylene. Correction is possible by fitting the real slope for release into the graph (see Voesenek et al., 1992a). This slope was experimentally derived by injecting ethylene into the flow-through system. The decay constant ( $-0.0349$ ) thus derived was identical to a decay constant of ethylene released from a shoot desubmergence in pure  $\text{N}_2$  (no ethylene production).

### Kinetics of Ethylene Release

To determine how rapidly ethylene escapes into the atmosphere after desubmergence, intact *R. palustris* plants were transferred to a one-compartment cuvette (volume 600 mL) connected to a continuous flow system ( $2 \text{ L h}^{-1}$ ) and a photoacoustic ethylene detector. After 24 h of acclimatization (PPFD,  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; Philips TLD 36W/84; 24–25°C), the plants were completely submerged in tap water (24–26°C)

for 24 h. The position of the plant in the cuvettes could be changed vertically over a height of 14 cm by sliding a stainless-steel rod, connected vertically, through a water-tight seal to the underside of the plant container. After 24 h of submergence plants were moved upward until the whole shoot was desubmerged. The plants were pulled down and submerged again after the following periods of emergence: 2 to 3, 5, 11, 15, 22, 45, 60, 90, 180, 360, 720, 1200, 1440, 1620, 1980, 2400, 3180, and 3600 s. One individual plant was used for one desubmergence period only, whereas every desubmergence treatment was repeated one to four times. Again, an experimentally derived decay curve was fitted, starting from the top of the release peak downward. The area under the instantaneous rise and decay curve corresponds to the amount of ethylene released after the desubmergence treatment. This ethylene release ( $\text{nL plant}^{-1}$ ) was plotted on a graph against exposure times to the atmosphere. A nonlinear regression procedure (procedure nlin; SAS Institute, Inc., 1985) was used to fit a function through the data. Division of the ethylene release by the mean internal gas volume of the shoots ( $0.349 \text{ mL}$ ;  $n = 36$ ) gave the ethylene concentration in the shoot. The decline of the endogenous ethylene concentration with time in *R. palustris* after desubmergence was modeled as a negative exponential function, using the equation

$$E_t = E_o \cdot e^{-kt}$$

in which  $E_t$  = the internal ethylene concentration ( $\text{nL mL}^{-1}$ ) after  $t$  seconds of desubmergence,  $E_o$  = the internal ethylene concentration ( $\text{nL mL}^{-1}$ ) just before desubmergence,  $k$  = the ethylene release rate constant for *R. palustris* (derived with the nonlinear regression procedure mentioned above), and  $t$  = time in seconds.

The ethylene release rate constant for *R. acetosa* was estimated with data on the total number of stomata and the internal gas volume of this species. The stomata were counted in five representative regions on both sides of all laminae and petioles. Based on leaf area data, this information was converted to the total number of stomata per plant. Means and SE values were calculated for four plants.

### Internal Ethylene Concentration and Vacuum Extraction

The internal ethylene concentration in shoots of *R. acetosa* and *R. palustris* that were completely submerged (in tap water of 22°C) for 24 h was also determined with the vacuum extraction technique described by Beyer and Morgan (1970) and was compared with ethylene concentrations in nonsubmerged control plants. The application of this method to *Rumex* and the germination and growth conditions of the plants are described in detail by Voesenek and Blom (1989a). The vacuum method was refined to limit the plant-atmosphere contact and the subsequent loss of endogenous ethylene to the atmosphere. The various methods of extraction are described in detail in the legend to Table I. The PPFD was  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Philips TLD 36W/84) at 23 to 25°C. Gas samples (0.8–1.0 mL) were collected with gas-tight syringes and injected directly into a Chrompack Packard gas chromatograph model 438A with a Poropack Q column (length, 100 cm) at 60°C packed to  $0.34 \text{ g cm}^{-3}$ .

**Table 1.** Endogenous ethylene concentrations measured by vacuum extraction

The internal ethylene concentration (nL mL<sup>-1</sup>) in shoots of *R. acetosa* and *R. palustris* after 24 h of submergence as determined with the vacuum extraction method. A, Data of Voeselek and Blom (1989). B, A refined vacuum method in a light/dark regime (16/8 h): excision of the shoot was performed under water, and submergence in Tween 20 and excision of petioles and leaflets was omitted completely to avoid extra handling and possible wound-ethylene formation. C, Treatment identical to B, but now under continuous illumination. D, A refined vacuum method with ice-cold (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution in a light/dark regime. We assume that ethylene production under these conditions is negligible (see Schierle and Schwark, 1988).

Condition	Source or Treatment	<i>R. acetosa</i>		<i>R. palustris</i>	
		n	Mean ± SE	n	Mean ± SE
Controls	A	2	0.23 ± 0.02	2	0.27 ± 0.10
	B	8	0.17 ± 0.03	17	0.26 ± 0.03
	C				
	D	5	0.07 ± 0.02	5	0.05 ± 0.03
Submerged	A	2	0.51 ± 0.05	2	0.36 ± 0
	B	10	0.46 ± 0.06	21	1.40 ± 0.10
	C	2	0.33 ± 0.01	2	1.57 ± 0.25
	D	5	0.25 ± 0.11	5	0.22 ± 0.03

## Leaf Growth

Petiole growth in *R. palustris* and *R. acetosa* was continuously measured with linear variable displacement transducers (Schlumberger Industries; type ST 2000). Plants were grown under conditions described by Voeselek and Blom (1989a). The growth rate of the youngest leaf (laminae + petiole) (*R. acetosa*, leaf 4; *R. palustris*, leaf 5) was measured before, during, and after a submergence period of 24 h. Desubmergence was always followed by soil waterlogging to mimic the experimental conditions of the experiments with the two-compartment cuvettes. At the start of the experiments the youngest petioles had a length of 15 to 20 mm. The experiments were conducted at a temperature of 26 to 28°C and under continuous illumination at a PPFD of 75 μmol m<sup>-2</sup> s<sup>-1</sup> (Philips TLD 36W/84). The results were compared with non-submerged controls. Representative data are presented.

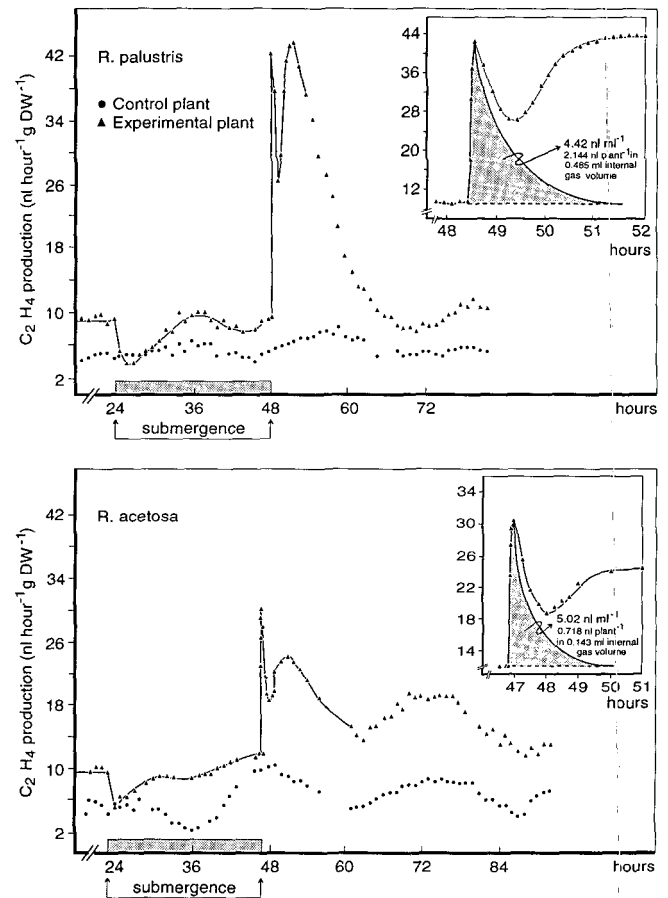
## RESULTS

### Ethylene Entrapment and Production

The ethylene production of *R. palustris* and *R. acetosa* before, during, and after a submergence period of 24 h measured continuously on a single shoot of a whole plant without physical perturbations is given in Figure 2. Neither species showed any difference in ethylene production level (controls, 2–10 nL g<sup>-1</sup> dry weight h<sup>-1</sup>) under nonsubmerged conditions. Submergence induced a sharp decline in ethylene release, followed by a more gradual increase due to diffusion

through the water layer. After equilibrium between ethylene in the plant and the water layer was attained (approximately 12 h after submergence), the ethylene release from a cuvette containing a submerged plant can be interpreted as ethylene production and thus can be compared with the level before submergence. Submerged shoots of both species showed no increase in ethylene production rate within a 24-h submergence period compared to presubmergence.

Desubmergence resulted in characteristic patterns of ethylene release; the absolute values differed between the species. This pattern consisted of a rapid first peak reaching its maximum after 13 (*R. palustris*) or 18 (*R. acetosa*) min, followed by a more gradual peak with its maximum after 3 (*R. palustris*) or 4 (*R. acetosa*) h.



**Figure 2.** Production and release of ethylene from shoots of *R. acetosa* and *R. palustris* measured in two-compartment cuvettes with laser-driven photoacoustic spectroscopy before, during, and after a 24-h submergence period (shaded horizontal bar). During submergence the nutrient solution in the root compartment became stagnant. This root environment was maintained after desubmergence to mimic a lowering of the water level from total submergence to soil waterlogging. The insets represent the release of ethylene induced by desubmergence. A negative exponential release curve, derived from an injection of ethylene in the flow-through system, was fitted onto the peak in ethylene evolution. The shaded area under this peak corresponds to the total amount of released ethylene. The remainder is new production.

To increase insight into the origin of ethylene release peaks after desubmergence, an additional experiment was performed during which shoots of *R. palustris* were submerged for 24 h. However, desubmergence was now performed in a pure  $N_2$  (oxygen concentration  $< 10 \text{ nL mL}^{-1}$ ) atmosphere, which blocks the conversion of ACC to ethylene (Yang and Hoffman, 1984). This experiment was conducted with the two-compartment cuvettes. The design of the experiment was identical to the desubmergence experiment in air, with the exception that the replacement of water by outside air during desubmergence was changed to a replacement by pure  $N_2$  coming from a balloon filled with  $N_2$  connected to inlet E (Fig. 1). The results of this experiment show that the second peak did not appear when *R. palustris* was desubmerged in a  $N_2$  atmosphere (Fig. 3). However, this peak can be induced later by allowing air to enter the cuvette at any chosen time. We conclude that the first peak represents release of the ethylene entrapped during the submergence period and the second peak represents ethylene production after desubmergence. Therefore, it is possible to calculate the internal shoot ethylene concentration just before desubmergence from the area under the first peak after correction for new production with the fitted release curve (Fig. 2, insets). Although *R. palustris* showed a higher accumulation of ethylene compared with *R. acetosa*, the concentrations in the plant were nearly equal in both species due to the larger internal gas volume of *R. palustris*. *R. acetosa* also showed a higher variability in internal ethylene concentrations after 24 h of submergence ( $1.60\text{--}5.89 \text{ nL mL}^{-1}$ ) than *R. palustris* ( $3.46\text{--}4.42 \text{ nL mL}^{-1}$ ).

To study whether, in *R. palustris*, entrapped root ethylene contributes to the ethylene release of the shoot after desubmergence, an additional experiment was performed in the same two-compartment cuvettes. During this experiment, the whole root was excised from the shoot just before desubmergence. This was accomplished with the aid of a small stainless-steel tube (inner diameter, 1 mm) through which a stainless-steel wire (diameter, 0.2 mm) was led (Fig. 1H). This wire was bent around the root-shoot junction and a short pull at the back end of the wire cut off the root system without opening the cuvette. Thereafter, the water level in the shoot compartment was lowered. The results indicate that plants with ( $2.38 \pm 0.34 \text{ nL mL}^{-1}$ ;  $n = 3$ ) and without ( $2.57 \pm 0.52 \text{ nL mL}^{-1}$ ;  $n = 4$ ) roots showed no significant differences in internal ethylene concentrations. Thus, it can be concluded that the rapidly released ethylene (peak 1) escapes exclusively from the shoot.

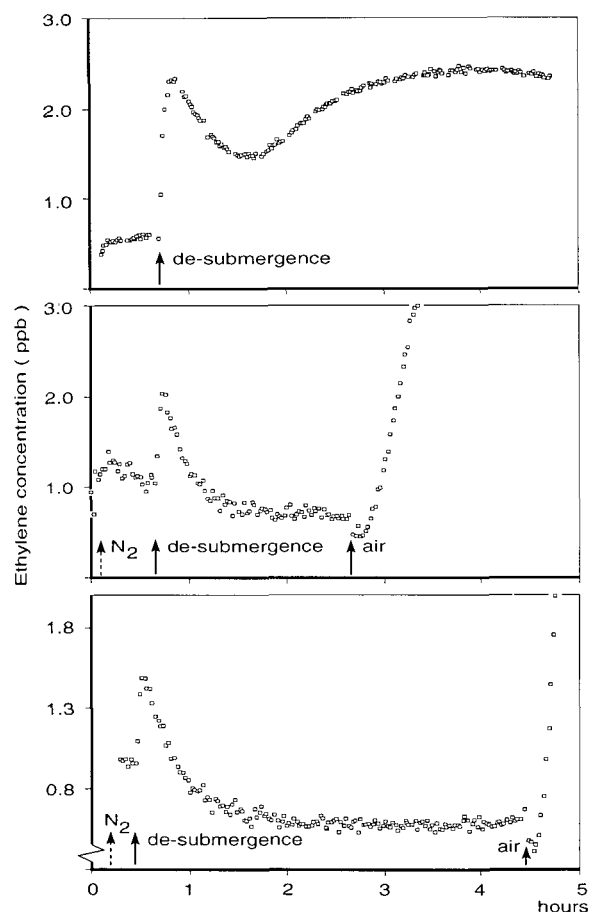
The area under the second, more gradual peak, representing a temporary increase in ethylene synthesis, is approximately twice as high in *R. palustris* than in *R. acetosa* (Fig. 2). After this typical pattern of ethylene release and production, the ethylene production falls back and remains more or less constant at a level similar to the presubmergence production (*R. palustris*) or at a level higher than the control plants (*R. acetosa*).

The internal ethylene concentrations as determined with the vacuum method indicate that in both species submergence results in higher overall concentrations (Table I) than in drained controls, but the concentrations are low compared

with values obtained by photoacoustic detection (Fig. 2). At room temperature, most submerged shoots of *R. palustris* contained higher internal ethylene concentrations than submerged shoots of *R. acetosa*. In submerged *R. palustris*, the refined technique resulted in detection of higher endogenous ethylene concentrations. Different light regimes hardly affected ethylene levels in either species. The use of an ice-cold ammonium sulfate solution dramatically reduced the internal ethylene concentrations in both control and submerged plants of *R. palustris*; no differences were found between submerged plants of the two species.

### Kinetics of Ethylene Release

Entrapped ethylene diffuses very rapidly out of desubmerged *R. palustris* shoots; 90% of the accumulated ethylene escapes within 58 s (Fig. 4). The fitted exponential regression (ethylene release =  $1.42 [1 - e^{-0.0396t}]$ ) yielded an asymptote



**Figure 3.** The release of shoot ethylene (expressed as ethylene concentration at the photoacoustic detector) of *R. palustris* after desubmergence (first solid arrow) in an air atmosphere (upper graph) and in a pure  $N_2$  atmosphere (middle and lower graphs). The air flow through the cuvettes was changed to  $N_2$  approximately 30 min before desubmergence (dashed arrow). The second solid arrow indicates the timing of the switch from the  $N_2$  atmosphere back to the air atmosphere.

or maximum ethylene release of  $1.42 \pm 0.09 \text{ nL plant}^{-1}$ . Divided by the mean absolute internal gas volume of the *R. palustris* shoots ( $0.349 \pm 0.067 \text{ mL}$ ), this results in a mean endogenous ethylene concentration of  $4.07 \text{ nL mL}^{-1}$  just before desubmergence. The decrease of the internal ethylene concentration with time in *R. palustris* can thus be expressed by the following equation:  $E_t = 4.07 e^{-0.0396t}$  (Fig. 4, inset).

The experimentally determined ethylene release rate constant of *R. palustris* ( $-0.0396$ ) and data on total number of stomata and internal gas volumes were used to estimate the release rate constant of *R. acetosa*. *R. palustris* plants have a higher number of stomata than plants of *R. acetosa*, which is mainly due to the higher leaf area in *R. palustris* (Table II). It is assumed that diffusion of ethylene occurs predominantly via the stomata, that the release rate of ethylene after desubmergence is determined by the number of stomata per volume of internal gas space, and that there exist or develop no differences in stomatal dimensions and degree of closure between the species. *R. acetosa* has more stomata per volume of internal gas space. Consequently, ethylene may be released slightly faster in *R. acetosa* (Table II).

From the ethylene release rate constants and the ethylene concentration just before desubmergence ( $E_0$ ), it is possible to estimate the internal ethylene concentration for individual *R. palustris* and *R. acetosa* plants as time passes after desubmergence (Fig. 5). Because the ethylene release rate constants and half-times for both species were nearly equal (Table II), most of the observed variation (Fig. 5) can be attributed to the variability in endogenous ethylene levels in *R. acetosa* after 24 h of submergence. Figure 5 further indicates that a few seconds of desubmergence before vacuum extraction will diminish the differences in endogenous ethylene concentrations between individuals and species.

### Leaf Growth

The fifth leaf of *R. palustris* control plants showed a gradual length increase during the experiment (Fig. 6); its growth rate

**Table II.** Summary of parameters related to the ethylene release rate in two *Rumex* species

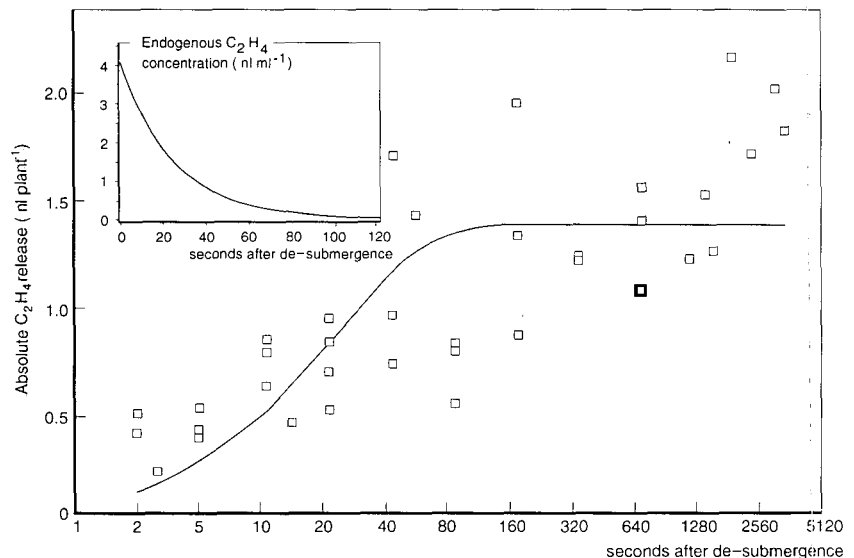
The two-sided leaf area, the total number of stomata on lamina and petioles, the internal gas volume, the number of stomata per volume of internal gas space, the ethylene release rate constant, and the half-time of ethylene release after desubmergence of plants of *R. acetosa* and *R. palustris* at the growth stage used in all experiments.

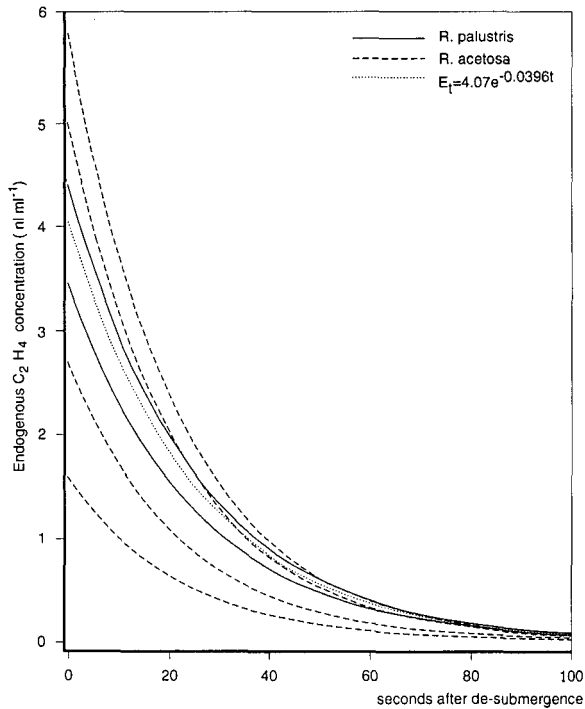
	<i>R. palustris</i>	<i>R. acetosa</i>
Leaf area (cm <sup>2</sup> )	68.3 ± 6.6	32.7 ± 2.7
Number of stomata	198,116 ± 25,796	84,963 ± 10,933
Internal gas volume (mL)	0.349 ± 0.067	0.132 ± 0.010
Number of stomata per volume of internal gas space	567,668	643,659
Ethylene release rate constant	-0.0396	-0.0449
Half-time (s)	18	15

varied between 0.3 and 0.5 mm h<sup>-1</sup>. Submergence induced in *R. palustris* a transient increase in growth rate (length increase 1 mm in 10 min). Thereafter, the fifth leaf gradually increased its growth rate compared with control plants after a lag phase of approximately 60 min. In all growth experiments with *R. palustris* ( $n = 19$ ) the lag phase varied between 60 and 150 min (data not shown). The maximum growth rate of 1.4 to 1.7 mm h<sup>-1</sup> was reached within 3 h after submergence. Desubmergence also induced a short-term decline in growth. The postsubmergence growth rate (0.6–0.8 mm h<sup>-1</sup>) was intermediate between the growth rates under submerged and control conditions.

The fourth leaf of control plants of *R. acetosa* grew much faster than control plants of *R. palustris*; the growth rate ranged from 0.5 to 1.3 mm h<sup>-1</sup>. As in *R. palustris*, submergence induced a transient increase in growth rate (absolute length increase 3–5 mm in 10 min). The growth rate of *R. acetosa* during a 24-h submergence period varied consider-

**Figure 4.** Ethylene release of *R. palustris* plotted against various desubmergence periods (seconds on a logarithmic scale) measured with laser-driven photoacoustic spectroscopy. A nonlinear fitting procedure was used and revealed the following equation: ethylene release =  $1.42 (1 - e^{-0.0396t})$ . Divided by the mean absolute internal gas volume of the *R. palustris* plants (0.349 mL), this gives a mean endogenous ethylene concentration just before desubmergence of  $4.07 \text{ nL mL}^{-1}$ . The decline of the internal ethylene concentration in time (inset) can be expressed by the following equation:  $E_t = 4.07 e^{-0.0396t}$ .





**Figure 5.** Decline in endogenous ethylene concentration of *R. acetosa* and *R. palustris* in response to desubmergence. Data from two individual *R. palustris* plants and the general function for this species ( $E_t = 4.07 e^{-0.0396t}$ ) are plotted. The ethylene release rate constant for *R. acetosa* ( $-0.0449$ ) was estimated from the number of stomata per volume of internal gas space (see Table II).

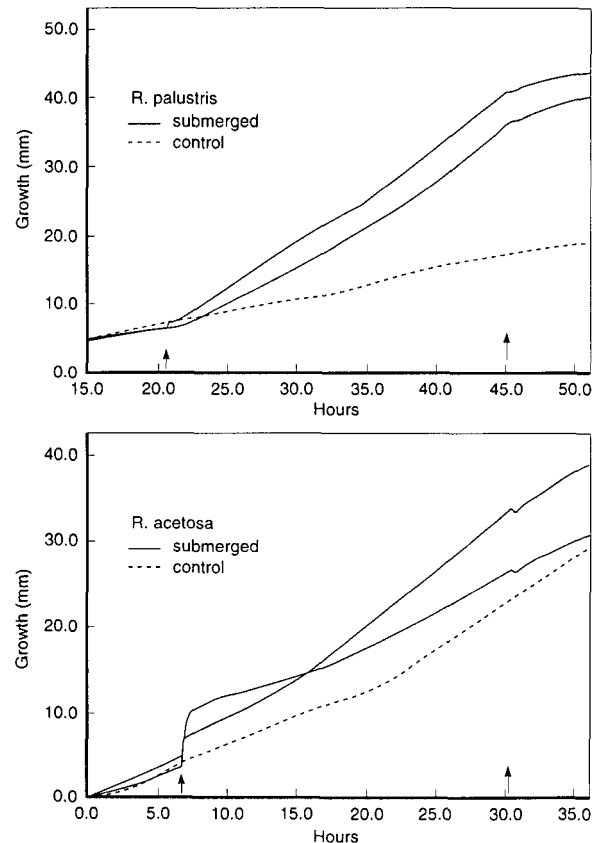
ably. Some individuals showed a slightly lower growth rate than controls, whereas others demonstrated a slightly enhanced growth rate; two extremes are shown (Fig. 6). Desubmergence induced a short-term growth decline, which was followed by a growth rate that was similar to that before desubmergence.

## DISCUSSION

The endogenous ethylene concentration in submerged shoots of two *Rumex* species with contrasting flooding resistances was quantified by two techniques, i.e. vacuum extraction followed by gas chromatographic ethylene detection and photoacoustic ethylene detection after desubmergence of intact plants in a flow-through system. The two methods yielded considerably different estimates of endogenous concentrations (Table I, Figs. 2 and 5). The photoacoustic technique indicated higher ethylene concentrations and small differences between the two species. The use of a flow-through system in combination with highly specific photoacoustic detection (Voeselek et al., 1992b) completely prevents the escape of ethylene from the plant and/or the inclusion of atmospheric ethylene. This is the only technique so far reported by which ethylene concentrations in submerged plants can be determined without the physical perturbations caused by cutting and handling. The low ethylene concentrations obtained with the vacuum technique can be explained by a substantial loss of ethylene before a shoot is

transferred to the collection flask prior to the extraction procedure. Indeed, the kinetic experiments indicate that ethylene escapes very fast into the atmosphere when a plant desubmerges: 90% escapes within 1 min (Fig. 4). However, vacuum extraction caused only a very short exposure of the shoot to the atmosphere (5–8 s) (Voeselek and Blom, 1989a). An internal shoot ethylene concentration in *R. palustris* of  $2.74 \text{ nL mL}^{-1}$  is to be expected if we assume a 10-s exposure time to an unstirred atmosphere. However, the vacuum extraction data were much lower and can be explained only by stimulated ethylene loss into the water layer when a shoot, after decapitation, is moved through the water to the surface. In addition, stimulated ethylene diffusion into the atmosphere may occur upon transfer into the collection flask. Movement in both water and air will decrease the boundary layer resistance around the shoot and thus stimulate diffusion.

When comparing the results of the vacuum extraction procedure using ice-cold  $(\text{NH}_4)_2\text{SO}_4$  solution with those using noncooled solutions it is obvious that ethylene production during the vacuum extraction can cause significant errors (Table I). This may explain the overall higher ethylene con-



**Figure 6.** Leaf (petiole + lamina) growth of *R. acetosa* and *R. palustris* measured with linear variable displacement transducers. The first arrow indicates the onset of the submergence treatment; the second arrow indicates the start of desubmergence. During this last treatment plants were desubmerged to soil waterlogging level. Two submerged and one control plant are presented per species.

centrations in submerged *R. palustris* shoots extracted at room temperature. The higher postflood production peak in *R. palustris* (Fig. 2) will induce overall higher concentrations.

The standard method to determine the influence of a treatment (e.g. submergence) on the ethylene production rate is head space analysis of treated and untreated plants or excised parts (see Métraux and Kende, 1983; Khan et al., 1987; Ridge, 1987). The application of this technique to study the impact of submergence on ethylene production of *Rumex* shoots would have resulted in misleading conclusions. Due to the fast-occurring (Voeselek et al., 1992b) postflood ethylene production peak, both species would be characterized as species that increase production rates in response to submergence. The photoacoustic measurements showed, however, that no such increase in shoot ethylene biosynthesis occurred under submerged conditions. This stresses the importance of real-time measurements of ethylene evolution without the use of accumulation techniques. Therefore, ethylene production rates of submerged plant tissues obtained by head space analysis should be treated with caution. Our results on ethylene production rates under water agree with findings in *Regnellidium diphyllum* and *Nymphoides peltata* (Ridge, 1987). Deep-water rice, however, shows an enhancement of ethylene synthesis in response to submergence (Rasikin and Kende, 1984). Because in this system plants (stem sections) were only partially submerged, outward diffusion of entrapped ethylene might remain high. We think that increased concentrations could then be achieved only when the production levels increase.

For *Rumex* species growing in frequently flooded river sites the shoot elongation response is crucial in maintaining or reestablishing air contact and, consequently, in surviving irregular floods (Van der Sman, 1992; Voeselek et al., 1992b). The positive correlation between surface area of the shoot that protrudes from the water surface and plant fitness can be explained mechanistically by the importance of atmospheric oxygen for root respiration. When a larger part of the shoot emerges from the water, the influx of oxygen will increase (Laan and Blom, 1990). Shoots of *R. palustris* exhibit accumulation of ethylene and stimulated leaf growth when surrounded by water. The increase in ethylene concentration is realized by continued production and physical entrapment (Fig. 2), which leads to a sufficiently high endogenous ethylene concentration to saturate the petiole growth response. Previous work showed that in this species petiole elongation in response to exogenous ethylene saturates at a concentration of 5 nL mL<sup>-1</sup> (Voeselek and Blom, 1989a). Thus, for *R. palustris* it is not necessary to invest in stimulated ethylene biosynthesis to achieve this level of enrichment under water. Increased production levels, as in deep-water rice, can gain in importance when outward diffusion of entrapped ethylene remains high due to partial emergence.

*R. palustris* shows short-term transient changes in growth rate in response to both submergence and desubmergence (Fig. 6). Identical transient changes were observed when the soil of control plants was watered (data not shown). These rapid effects of submergence, desubmergence, and normal watering are probably related to perturbations of the water status of the plant (Stünzi and Kende, 1989) and have also

been described for leaf elongation in maize (Hsiao et al., 1970). In *R. palustris*, the lag phase between submergence and the onset of enhanced elongation (60–150 min) is comparable with that of *N. peltata* (100–110 min; Funke and Bartels, 1937), but shorter than in *R. diphyllum* (200 min; Ridge, 1987) and deep-water rice (180–220 min; Rose-John and Kende, 1985). Both survival and reproduction of *R. palustris* are positively correlated with the amount of shoot tissue that emerges from the water (Van der Sman et al., 1991). When a plant is desubmerged, and thus an important diffusion resistance is removed, endogenous ethylene concentrations in shoot tissue and leaf growth rates drop quickly. Under these conditions, enhanced endogenous ethylene concentrations and high growth rates can be achieved only by a significant increase in ethylene production. This phenomenon is demonstrated in *R. palustris* and is linked to the high growth rate after desubmergence when compared with control plants. In conclusion, the postsubmergence production increase may be interpreted as an adaptive mechanism to ensure relatively high leaf extension rates above water to ensure adequate entry of oxygen and carbon dioxide.

Shoots of *R. acetosa*, as a representative of species poorly adapted to flooding, also accumulate ethylene under water. However, this species shows no strong stimulation of leaf elongation in response to submergence. As in *R. palustris*, the increase in the endogenous ethylene level is achieved by continued production and physical entrapment. *R. acetosa* showed a higher variability in endogenous concentrations between individuals than *R. palustris*. This variation is also reflected in leaf growth under water: some individuals showed slightly stimulated growth, whereas others demonstrated inhibited growth. In this short-term submergence (24 h) experiment the leaf growth rate of *R. acetosa* (submerged or nonsubmerged) is just as high as for the submerged *R. palustris*. However, in long-term (4 d) experiments the growth rate of *R. acetosa* (submerged or nonsubmerged) declines after 40 h to levels of 0.05 to 0.4 mm h<sup>-1</sup> (the leaves have reached their maximum length), whereas submerged *R. palustris* plants continue to grow during this same period at much higher rates (0.5–0.8 mm h<sup>-1</sup>) (Voeselek and Blom, 1989b). There is evidence that growth reduction of *R. acetosa* petioles in response to high ethylene concentrations can be relieved when ethylene (5 nL mL<sup>-1</sup>) is applied in combination with subambient oxygen partial pressures (3 kPa) and elevated carbon dioxide partial pressures (5 kPa) (data not shown). This means that the ethylene response of petioles of *R. acetosa* will depend strongly on the balance between respiration and photosynthesis under water. *R. acetosa* shows only a small enhanced ethylene production after desubmergence; a higher production would probably be a wasteful investment with respect to tissue responsiveness.

The following main conclusions can be drawn. (a) Both *R. palustris* (flood-resistant) and *R. acetosa* (flood-sensitive) demonstrate enhanced endogenous shoot ethylene concentrations in response to a 24-h submergence period. (b) This does not represent enhanced ethylene production levels under water, but continued production (5–10 nL h<sup>-1</sup> g<sup>-1</sup> dry weight) and physical entrapment. However, both species showed an increase in ethylene production after desubmergence, with this increase being much more pronounced in *R. palustris*. (c)



Accumulated ethylene escapes very rapidly out of desubmerged plants; 90% disappears within 1 min. Differences in release rates and half-times between both *Rumex* species were very small and probably without physiological significance. (d) *R. palustris* is characterized by enhanced leaf elongation in response to submergence, whereas *R. acetosa* demonstrates no dramatic change in leaf growth rate when it is submerged. This behavior reflects a greater responsiveness of *R. palustris* than *R. acetosa* toward ethylene.

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