Ethylene Production and Petiole Growth in *Rumex* Plants Induced by Soil Waterlogging

The Application of a Continuous Flow System and a Laser Driven Intracavity Photoacoustic Detection System

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ABSTRACT

Petiole growth of *Rumex acetosa* L., *Rumex crispus* L., and *Rumex palustris* Sm. in response to soil waterlogging was studied in relation to production of the gaseous plant hormone ethylene. Ethylene production was monitored in a flow-through system and a recently developed laser driven photoacoustic detection system, which allowed ethylene measurements as low as 6 picoliters per liter. *R. acetosa* showed a two-fold increase in ethylene production correlated with a slight enhancement of the growth of the petiole that developed during the waterlogging treatment. Both *R. crispus* and *R. palustris* showed a strong petiole elongation of existing as well as newly formed petioles, which was correlated with a 20-fold increase in ethylene production after approximately 7 days. Increased rates of ethylene production in *R. palustris* were related to a strong increase in 1-aminocyclopropane-1-carboxylic acid (ACC) concentration and a slight, but detectable, increase in ethylene forming enzyme activity. In *R. acetosa* on the other hand, only a very small increase in ACC concentration was observed. Changes in ethylene production in *Rumex* are strongly correlated with variation in ACC content and ethylene forming enzyme activity. The interaction between ethylene production/internal concentration and ethylene sensitivity of the three *Rumex* species is discussed in relation to their field location in a flooding gradient and their differential resistance toward waterlogging and submergence.

Temporary flooding of riparian habitats along river sides is a worldwide phenomenon, which may interfere with agricultural functions and nature conservation (11). Comparative plant physiological research improves insight into adaptive traits of plants to floods and provides important knowledge for the management of these areas.

The genus *Rumex* is cosmopolitan with various species occurring in Dutch river areas. *Rumex crispus* L. and *Rumex palustris* Sm. are two species from the lower, frequently flooded parts of river areas. The formation of new aerenchymatous roots is an important mode for these species to survive the anaerobic soil conditions during a flooding period (12,

22). However, the development of these new roots and the diffusion of oxygen from the shoot to the root tips can take place only when the shoot is not totally submerged. The flooding resistant *R. crispus* and *R. palustris* have developed a mechanism to ensure leaf-atmosphere contact during flooding periods; accumulation of the gaseous hormone ethylene in the shoot mediates an enhanced elongation of petioles under submerged conditions (20, 21).

Rumex acetosa L. occurs mainly on elevated and seldomly flooded areas such as dikes and river levees. This species is not resistant to flooding conditions, it develops only a very restricted number of new roots (22), and submersion and high exogenous ethylene concentrations are unable to induce enhanced petiole growth (20, 21). The interaction between the internal ethylene concentration and the ethylene sensitivity of *Rumex* plants under completely submerged conditions corresponds fairly well with their distribution in a flooding gradient in the field (20).

Previous studies (6, 17, 22) and field observations indicated that ethylene is also involved in the growth of *Rumex* plants under waterlogged conditions (= flooding of the roots only). Especially, *R. crispus* and *R. palustris* showed the typical *Rumex* ethylene reaction (20) in response to soil waterlogging, an erected rosette stature, an enhanced elongation of petioles, and, to a lesser extent, laminae (21). Additional evidence for the important role of ethylene in the growth of partially submerged *Rumex* plants comes from the observation that enhanced growth of completely submerged petioles and stems continues until a large part of the shoot is above the water surface. To guarantee long-term survival under flooded conditions, it is essential that a large part of the *Rumex* shoot reaches the water surface (18). Trapped ethylene in the shoot rapidly dissipates into the atmosphere as soon as one or more leaves reach the water surface (14). It is therefore assumed that an increased ethylene production might be of major importance in explaining the growth responses under waterlogged and partially submerged conditions.

Increased rates of ethylene production in relation to soil waterlogging have already been described for other plant species (7–9, 16). Bradford and Yang (2–4) showed for tomato

plants that ACC,[1] the immediate precursor of ethylene, accumulates in flooded roots and is subsequently transported to the shoot via xylem vessels. In the shoot ethylene is synthesized from the transported ACC, a conversion catalyzed by EFE (26, 27).

This paper describes the results of a comparative study on the growth of petioles, ethylene production, ACC levels, and EFE activity in three *Rumex* species, from habitats with different flooding regimes, in response to soil waterlogging. ACC concentrations and EFE activity were measured to quantify the ethylene biosynthesis in order to gain insight into changes in ethylene production in response to environmental stimuli. A flow-through system (to avoid interference of CO_2, O_2, autoinhibition, and autocatalyses [24]) connected with a recently developed laser driven intracavity photoacoustic detection system was used to monitor the ethylene production of *Rumex* plants.

MATERIAL AND METHODS

Plant Material and Growth Experiments

Characteristics of *Rumex acetosa* L., *Rumex crispus* L., and *Rumex palustris* Sm. and their natural habitats in Dutch river areas were described in Voesenek and Blom (19-21). Plants used for all experiments had an age of 26 d and were grown from achenes mixtures (collected in 1987 and 1988) under conditions described for laboratory experiments in Voesenek and Blom (20).

To study the effect of soil waterlogging on petiole growth, 20 plants per species were placed in plastic trays (50 × 30 cm) filled with a water layer of 6 cm (1 cm above the pots) for 9 d. Petiole growth was measured nearly every other day and compared with 20 control plants. Inhibitors of ethylene synthesis (AVG: 0.1 mM) and ethylene action (AgNO₃: 1 μM) were used in another waterlogging experiment to elucidate the involvement of ethylene in petiole growth. A fine brush was used to spread the inhibitors over the leaf and petiole surface of 7 to 10 plants per species per treatment (a 0.1% [v/v] Tween solution was used to ensure optimal flow-out of the inhibitors). This procedure was repeated every day; AVG brushing started 1 d before waterlogging. Previous experiments demonstrated that the inhibitor concentrations used were nontoxic. The experiment was conducted in the aforementioned plastic trays and lasted 8 d. Petiole growth was compared with a waterlogging treatment without inhibitors and a well-drained control treatment.

In all growth and inhibitor experiments responses of two petioles per plant were recorded: (a) the youngest petiole that was already initiated before the onset of an experiment; (b) the first petiole that developed entirely during treatment.

The plants were carefully handled to avoid stress-induced ethylene production. Both experiments were conducted in the same growth chamber in which the plants were raised. Differences between treatment means were analyzed with Bonferroni *t*-tests after an analysis of variance.

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[1] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; EFE, ethylene forming enzyme.

Ethylene Measurements with a Laser Driven Intracavity Photoacoustic Detection System

Ethylene production was measured in a laser driven photoacoustic detection system in line with a flow-through system (Fig. 1). A mechanically chopped CO_2 laser beam is directed into a photoacoustic cell containing an ethylene sample, which absorbs at the emission frequencies of the laser (9-11 μm wavelength). The ethylene molecules are excited by the laser beam from the ground state into the rotational level of a higher vibrational state. Deexcitation processes redistribute the energy. In general this deexcitation or relaxation takes place through collisions with other molecules present in the sample. This causes an increase in kinetic energy of the gas molecules and hence also of gas temperature. In a closed vessel (the photoacoustic cell) the increase in temperature leads to an increase in pressure. When the radiation source is modulated at audio frequency, the generated pressure fluctuations can be detected by a microphone. In order to maximize the microphone signal, the chopper was tuned to the resonance frequency of the photoacoustic cell, creating an acoustic 'standing-wave' inside the resonator (length: 100 mm; diameter: 6 mm) of the photoacoustic cell. To increase the sensitivity even more, the experiments were performed in a recently developed setup, in which the photoacoustic cell was placed inside the laser cavity. The increased sensitivity, due to the high intracavity laser power (100 W), allowed trace detection of ethylene as low as 6 picoliters per liter (6). To monitor ethylene production of *Rumex* plants, induced by soil waterlogging, two plants, aged 26 d (for germination and growth conditions see Voesenek and Blom [20]), were selected and carefully transported to the laser-equipment. They were separately placed in glass cuvettes (volume 0.2 L), in which they were allowed to acclimatize for 24 h. Both cuvettes were continuously flushed with air (flow rate: 0.9 L/h) which was, from the outlet port, directed into the photoacoustic cell. Before entering this cell the air flowed through a scrubber filled with KOH grains to remove water and CO_2. Hereafter, one of the cuvettes was slowly filled with tap water up to a height of 1 cm above the upper pot edge. The ethylene production of plants in both cuvettes (control and experiment) was monitored for 8 d.

The effectivity of AVG in inhibiting ethylene synthesis in *Rumex* plants, was tested in an additional experiment with the intracavity laser configuration. During this experiment the ethylene production in three, instead of the usual two, cuvettes was monitored. One of them was used as a reference for zero ethylene production. The second cuvette contained a *Rumex* plant which was waterlogged after an acclimatization period of 24 h (brushed with water 12 and 24 h before the onset of the waterlogging treatment), whereas the third contained a plant which was waterlogged and had received a pretreatment with AVG (brushed with AVG (0.1 mM) in a Tween solution 12 and 24 h before the onset of the waterlogging treatment). The waterlogging treatment lasted 24 h. The experiments were conducted at a constant temperature in the sample cuvettes of 25 ± 1°C. All experiments in which the ethylene production of *Rumex* plants was monitored were performed at a continuous photosynthetic photon flux density of 50 μEm⁻²S⁻¹. These continuous light conditions might give
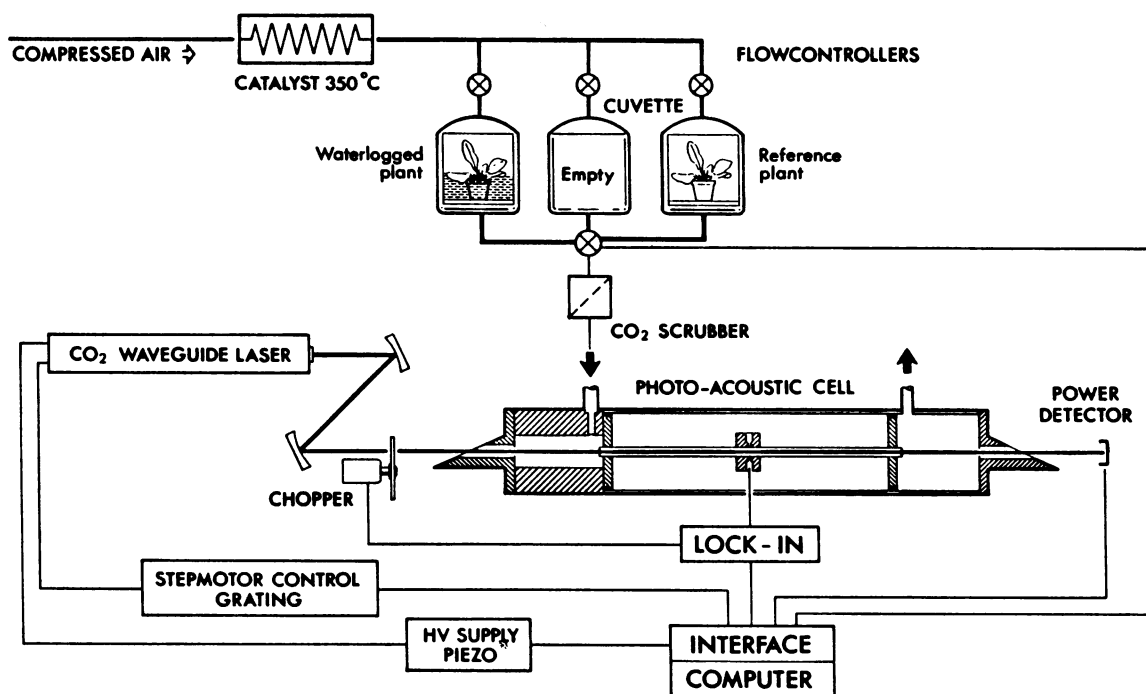
Figure 1. The experimental set-up of a flow through system in line with a laser-driven photoacoustic detection system to monitor ethylene production of *Rumex* plants. The actual measurements were performed with a setup, in which the photoacoustic cell was placed inside the laser cavity (intracavity).

insight into the control mechanisms of fluctuations in the ethylene production rate. All experiments were at least once repeated; representative data are presented.

ACC Determinations and EFE Activity

ACC was determined in *R. acetosa* and *R. palustris*; complete shoots (0.5–1.5 g fresh weight) were ground in liquid nitrogen and extracted with 80% ethanol, in two heating cycles at 70°C. The ethanolic supernatants were combined and concentrated in vacuo at 45°C. The dry residue was redissolved in 2 mL water and lipids and other materials were reextracted by partitioning with a 4 mL dichloromethane phase. The ACC content in the aqueous upper layer was determined according to Lizada and Yang (13) with only minor modifications to get optimal conversion of ACC to ethylene ($\approx$70%). All ACC determinations were performed with internal standards to determine the conversion efficiency of ACC into ethylene (ACC was purchased from Sigma). All determinations were repeated twice; calculated means ($n = 3$) are presented. The ethylene analyses were conducted with a Chrompack 438A gas chromatograph, equipped with a flame ionization detector and a stainless steel column (2 mm $\times$ 1 m) filled with Porapak Q 100–120 mesh (temperature: 60°C; carrier gas: N_2).

EFE activity in all three *Rumex* species was determined by measuring the ethylene production of all leaves excised from one plant, loaded with saturated levels of ACC. The saturating ACC concentration was determined in a previous experiment by monitoring ACC to ethylene conversion of leaves incu-

bated in 1, 2, 3, and 5 mM ACC during several time intervals. No increase in conversion was observed beyond 1 mM and after a loading period of 60 min. The actual measurements were performed in capped 40 mL serum vials with a 60 min loading period (20°C) and a 3 mM ACC concentration. Four replicates were used for each determination; ethylene analyses were conducted as mentioned before.

RESULTS

Two types of growth responses of petioles to soil waterlogging can be distinguished in the studied *Rumex* species (Fig. 2). The first response type, observed in *R. crispus* and *R. palustris*, was characterized by a strongly stimulated elongation of already developed petioles as well as new ones. In addition the emergence of new petioles is also accelerated by the waterlogging treatment. *R. acetosa* showed the second type of response in which no growth enhancement of the existing petioles occurred, whereas the newly developed petiole showed a small, but significant, increase in length after soil waterlogging.

These two types of growth responses correlated well with two distinct patterns in ethylene production induced by soil waterlogging (Fig. 3). A few hours (1–2 h) after the onset of the flooding treatment, the ethylene production increased in both *R. crispus* and *R. palustris*. This increase, reaching its highest production rate after 5 to 6 h (9–10 times control production), was followed by a sharp decrease of the production. The period hereafter was characterized by a gradual increase of the ethylene production. Although temperature
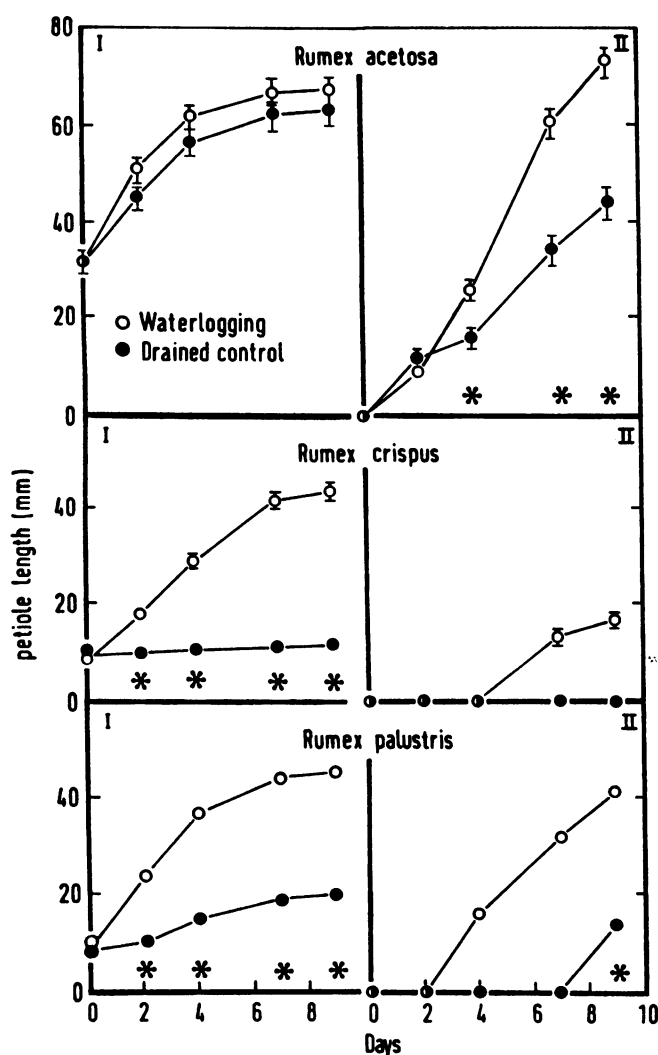
Figure 2. The growth of petioles ($n = 20$; $\pm$ 1 SE) of R. acetosa, R. crispus, and R. palustris respectively, in response to soil waterlogging and drained soil conditions (controls): I. The youngest petiole which was already initiated before the onset of the experiment; II. the first petiole which developed entirely during treatment conditions (* significantly different from control petiole length: Bonferroni t-test (P < 0.05)).

(Table I). However, in petioles of R. crispus that emerged during the course of the experiment and all the petioles of R. palustris, this growth inhibition was not complete, suggesting a partial inhibition of the ethylene biosynthesis (AVG) and/ or action (AgNO₃) or the involvement of other factors besides ethylene. Figure 4 shows that AVG treatment during the first 24 h of waterlogging led to an ethylene production below or near the normal control level (ethylene production of the plants not treated with AVG before onset of waterlogging). This indicates that the remaining ethylene production can not be responsible for the enhanced petiole growth in AVG treated waterlogged plants of R. crispus and R. palustris. Both R. crispus and R. palustris showed a relatively high rate of ethylene production in plants that were only brushed with water in order to function as controls (Fig. 4; −AVG). These increased production levels, probably due to handling (brush-
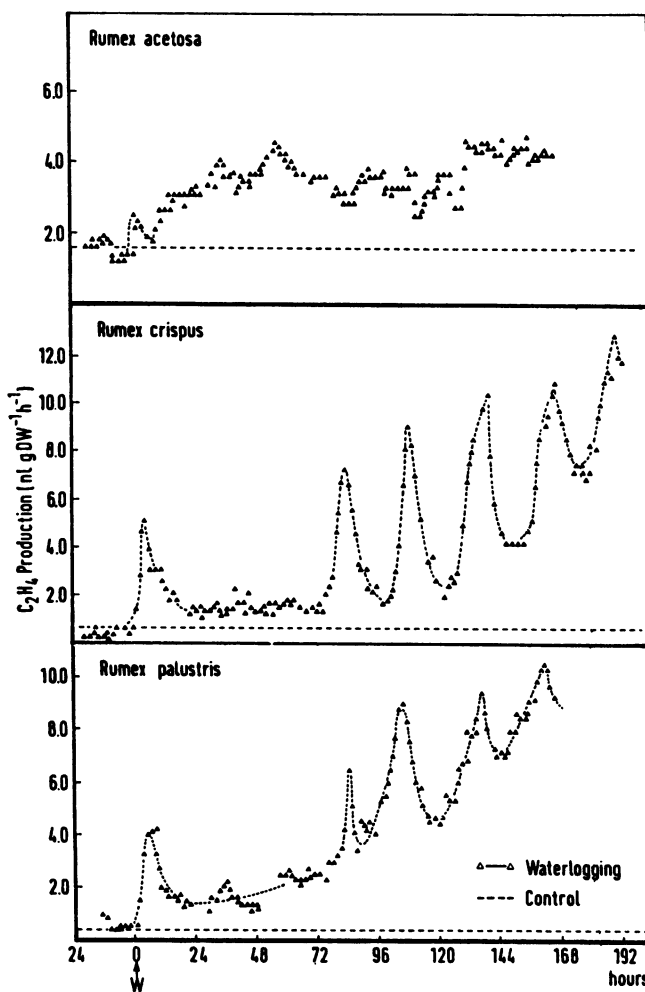

Figure 3. The ethylene production of R. acetosa, R. crispus, and R. palustris in response to soil waterlogging, monitored in a continuous flow system in line with a laser driven intracavity photoacoustic detection system. The ethylene production of a well drained control plant is presented as a mean production level. The arrow indicates the onset of the waterlogging treatment. Shoot dry weight was determined at the end of an experiment.

and light conditions were constant, an apparent circadian rhythm in the production rate was observed. At the end of the experiment a 20-fold increase in ethylene production was observed in the waterlogged plants. A rather different response was observed in R. acetosa. This species showed only a low peak immediately after waterlogging (1.5 times control production), followed by a slightly enhanced ethylene production in the course of time (approximately two times the control production). A circadian rhythm as found in both other species was not detected. Noteworthy is the relatively high ethylene production of R. acetosa under control conditions when compared with both other species.

Both AVG and AgNO₃ inhibited the growth of petioles capable of enhanced elongation in response to waterlogging

Table I. *Length of Petioles of Rumex after a 8-d Exposure to Several Treatments*

I, The youngest petiole present at the onset of the experiment; II, the first petiole that developed entirely during treatment conditions.

Species		Control	Waterlogging		
			No inhibitor	AVG	AgNO₃
			mmᵃ		
Rumex acetosa	I	51.4 ± 3.9a	55.9 ± 3.9a	52.2 ± 3.8a	54.4 ± 3.2a
	II	26.7 ± 4.0a	44.7 ± 5.0b	29.6 ± 3.8ab	30.3 ± 3.9ab
Rumex crispus	I	22.0 ± 2.5a	35.6 ± 2.5b	25.4 ± 2.2a	26.0 ± 1.4a
	II	—ᵇ	19.3 ± 1.6a	12.3 ± 1.4b	12.0 ± 0.9b
Rumex palustris	I	10.8 ± 0.7a	24.7 ± 1.0d	18.3 ± 0.8c	14.8 ± 0.5b
	II	8.3 ± 0.7a	22.6 ± 1.2c	12.2 ± 0.6b	10.8 ± 0.5ab

ᵃ Means ± SE; $n = 7$ to 10. Means with the same letter in a row are not significantly different by the Bonferroni t-test ($P < 0.05$).   ᵇ Petiole too small to measure.
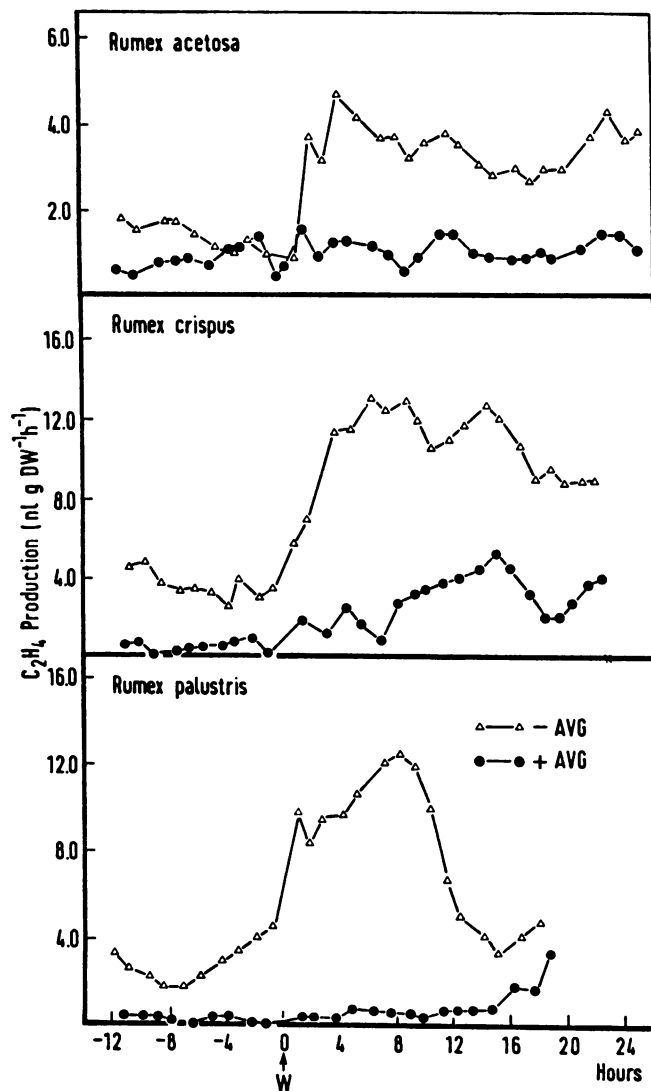

Figure 4. The ethylene production of *R. acetosa, R. crispus,* and *R. palustris* in response to soil waterlogging with (+AVG) and without (−AVG) AVG pretreatment. The arrow indicates the onset of the waterlogging treatment. These data were obtained by means of the same laser-equipment as in Figure 3. Shoot dry weight was determined at the end of an experiment.

ing procedure) 12 and 24 h before the onset of the waterlogging treatment, are additional evidence that *R. crispus* and *R. palustris,* in contrast to *R. acetosa,* strongly increase their ethylene production in response to various kinds of stress.

Since *R. crispus* and *R. palustris* showed nearly equal changes in petiole growth and ethylene production rates in response to soil waterlogging, ACC levels were only determined in *R. palustris* and compared to those of *R. acetosa.* In both species the ACC level in the shoot increased within a few hours after the onset of the treatment; the by far largest increase, followed by a decrease, was observed in *R. palustris* (Fig. 5). The changes in ACC levels correlated well with the observed differences in ethylene production between *R. acetosa* and *R. palustris* during the first 24 h of waterlogging (Fig. 3). A two- to sixfold increase above the basic ACC level was observed in *R. palustris* after 24 to 128 h of soil waterlogging (data not shown). No distinct circadian rhythm was present. In the same period the ACC concentration in shoots of waterlogged plants of *R. acetosa* hardly exceeded the control levels (data not shown). Waterlogging induced an increase in EFE activity, especially in *R. crispus* and *R. palustris* (Fig. 6). This enhanced EFE activity in both species was still evident after 156 h of waterlogging (data not shown). The large standard errors hamper interpretation of the EFE data of *R. acetosa,* but they show also a trend toward an increased activity in this *Rumex* species. No circadian rhythm was observed in the EFE activity of the three species. The relatively high level of EFE activity and the slightly higher ACC levels in control plants of *R. acetosa* are positively correlated with its higher basal ethylene production (Fig. 3).

DISCUSSION

The intracavity photoacoustic detection setup allowed a unique continuous monitoring of ethylene production of small individual plants. The actual measured concentrations in the photoacoustic cell ranged from 0.05 to 1.0 nL/L in the course of our experiments. Changes in ethylene production rates of individual intact plants in response to soil waterlogging were detected with great accuracy and without interference of changes in O₂ and CO₂ concentrations and artifacts introduced by excision of petioles.

It can be concluded that ethylene plays a central role in the regulation of petiole growth in *Rumex* species in response to
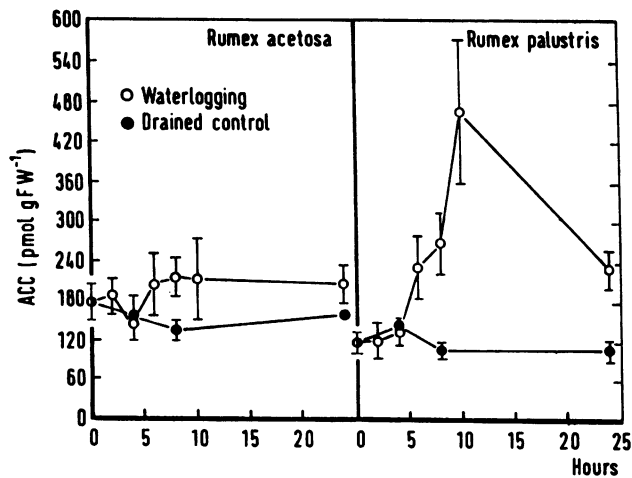
Figure 5. The ACC-concentration in shoots of (n = 3; ± 1 se) of *R. acetosa* and *R. palustris* in response to soil waterlogging.

soil waterlogging. Since the ethylene production was monitored on one single plant, direct comparison with mean ACC and EFE data (measured on several plants) demands careful interpretation. Taking this in account our experimental evidence shows that in *Rumex* plants ethylene is synthesized via the pathway described by Adams and Yang (1): methionine→ *S*-adenosyl methionine→ACC→ethylene. Waterlogging stimulates ethylene production in *Rumex* plants via increases in ACC levels and EFE activity. Although a significant increase in EFE activity was observed within a few hours after flooding, the formation of ACC is probably the rate-limiting step since application of ACC during the waterlogging treatment always led to an increased ethylene production (EFE experiment). Stress-induced synthesis of ACC-synthase and increased EFE activities have already been described for other plant species (for reviews, see refs. 7, 8, 27, and 28). The observation that waterlogging actually induces an immediate peak in ethylene production of *R. crispus* and *R. palustris*, which is followed by a more substantial increase only after approximately 2 d

is of special interest. This early ethylene peak may have initiated the increase in production that follows later. A similar process was suggested to take place during stress-induced senescence in flowers (24, 25). In tomato plants, ACC was shown to be translocated from the roots to the shoot within a few hours after the roots were flooded (2). The early ethylene peak observed in our experiments may therefore be the result of ACC translocation from the roots, although translocation of ethylene from roots to shoot can not be ruled out. This will be a subject of future research.

The circadian fluctuations in ethylene production rates of *R. crispus* and *R. palustris* are probably endogenously controlled since they continued under conditions of constant light and temperature. The acquaintance with such a rhythm is a prerequisite for studying ethylene production in a time course. Endogenous circadian rhythms in ethylene production are also known for other plant species (5, 10, 15).

The studied *Rumex* species strongly differ in their interaction between ethylene production/internal concentration and ethylene sensitivity. The differential responses fit well into the habitat selection of the species. The growth of petioles of *R. acetosa* is inhibited by high exogenous ethylene concentrations which is demonstrated by a dose response curve (see ref. 20). High endogenous concentrations in this species are probably prevented under waterlogged conditions since the ethylene production only slightly increases (Fig. 3). Low momentary internal ethylene concentrations under these conditions stimulate growth of newly developed petioles only slightly (Fig. 2). Submergence of the whole plant, however, results in entrapment of ethylene and therefore in relatively high and for *R. acetosa* inhibitory ethylene levels (23). The response of *R. acetosa* to endogenous ethylene levels correlate with its occurrence in seldomly submerged parts of the river area and in moist hayfields with high ground water levels.

Both *R. crispus* and *R. palustris* are characterized by enhanced petiole growth in response to high exogenous ethylene concentrations (20). If we assume that elongated petioles and an erected rosette stature are both adaptive traits under partially submerged conditions, a strong enhancement of the ethylene production under these conditions must be favora-
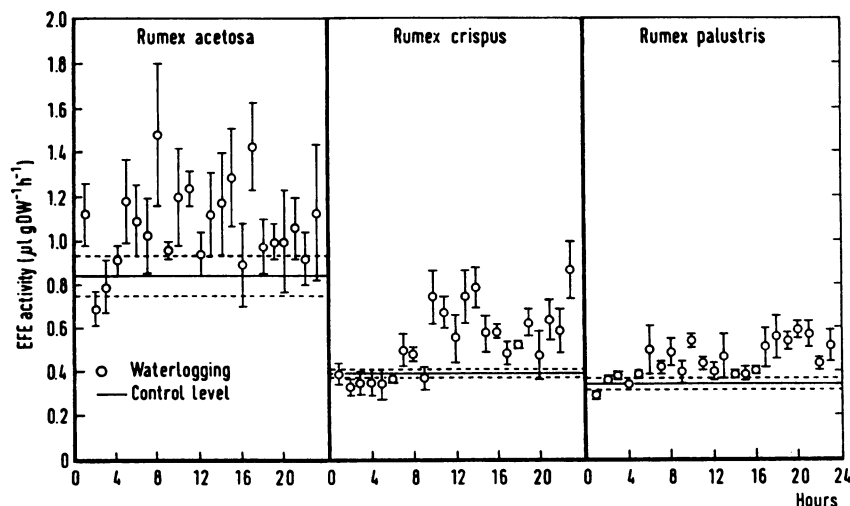

Figure 6. The EFE activity in shoots (n = 4; ± 1 se) of *R. acetosa, R. crispus,* and *R. palustris* in response to soil waterlogging. The EFE activity of well drained control plants (n = 16–20) is presented as a mean level (±1 se).

ble. Both traits are probably functionally related to an increase of the photosynthetic area and a maximal diffusion of air to the oxygen deficient roots, if a large part of the shoot protrudes from the raised water level. Probably due to ethylene entrapment, an even greater petiole elongation of *R. crispus* and *R. palustris* was observed under submerged conditions (21). *R. palustris* and, to a somewhat lesser extent, *R. crispus* are found in habitats with frequent long-lasting floods even in the growing season.

ACKNOWLEDGMENTS

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