

# Ethylene Sensitivity and Response Sensor Expression in Petioles of *Rumex* Species at Low O<sub>2</sub> and High CO<sub>2</sub> Concentrations

Laurentius A.C.J. Voeselek\*, Wim H. Vriezen, Marret J.E. Smekens<sup>1</sup>, Francis H.M. Huitink, Gerard M. Bögemann, and Cornelis W.P.M. Blom

Department of Ecology and Department of Experimental Botany, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

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*Rumex palustris*, a flooding-tolerant plant, elongates its petioles in response to complete submergence. This response can be partly mimicked by enhanced ethylene levels and low O<sub>2</sub> concentrations. High levels of CO<sub>2</sub> do not markedly affect petiole elongation in *R. palustris*. Experiments with ethylene synthesis and action inhibitors demonstrate that treatment with low O<sub>2</sub> concentrations enhances petiole extension by shifting sensitivity to ethylene without changing the rate of ethylene production. The expression level of the *R. palustris* gene coding for the putative ethylene receptor (*RP-ERS1*) is up-regulated by 3% O<sub>2</sub> and increases after 20 min of exposure to a low concentration of O<sub>2</sub>, thus preceding the first significant increase in elongation observable after 40 to 50 min. In the flooding-sensitive species *Rumex acetosa*, submergence results in a different response pattern: petiole growth of the submerged plants is the same as for control plants. Exposure of *R. acetosa* to enhanced ethylene levels strongly inhibits petiole growth. This inhibitory effect of ethylene on *R. acetosa* can be reduced by both low levels of O<sub>2</sub> and/or high concentrations of CO<sub>2</sub>.

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Some aquatic and amphibious plant species respond to complete submergence with stimulated elongation of shoot organs. This adaptive reaction allows the survival of these plants in habitats with sustained high water levels by re-establishing contact with the aerial environment (Van der Sman et al., 1991, 1993). The O<sub>2</sub> status of the roots improves as shoots emerge from the water (Armstrong et al., 1994). At the same time, root-growth-inhibiting amounts of the gaseous plant hormone ethylene that had accumulated in flooded roots can be vented off (Visser et al., 1997).

Ethylene plays a key role in stimulated shoot elongation in many wetland plants; however, other plant hormones and gases also affect this submergence response (Ridge, 1987; Voeselek and Blom, 1997). Apart from ethylene, stimulated shoot elongation also depends on the presence of auxin (Horton and Samarakoon, 1982) or GAs (Raskin and Kende, 1984a). Studies of the effect of gases other than ethylene on stimulated shoot elongation showed that the promotive action of O<sub>2</sub> and CO<sub>2</sub> on elongation operated

independently of each other and of ethylene (Raskin and Kende, 1983; Pearce et al., 1992; Nishizawa and Suge, 1995). Others, however, demonstrated an effect of O<sub>2</sub> and CO<sub>2</sub> on the tissue sensitivity toward ethylene (Suge and Kusanagi, 1975; Horton, 1991). In internodes of deepwater rice low levels of O<sub>2</sub> stimulated elongation via an increased production rate of ethylene (Raskin and Kende, 1984b; Cohen and Kende, 1987).

The genus *Rumex* is used as a model system to explain the distribution patterns of plants in river floodplains, which are characterized by regular floods even in the growing season (Blom and Voeselek, 1996). Rosettes of wetland *Rumex* sp., such as *Rumex palustris*, accommodate to complete submergence by stimulated petiole elongation. This petiole response requires ethylene and GA (Voeselek and Blom, 1989; Rijnders et al., 1997). Continued ethylene production and physical entrapment of the gas causes a 100-fold increase in the endogenous ethylene concentration in *R. palustris* within 24 h of submergence (Voeselek et al., 1993a; Banga et al., 1996b). However, an enriched ethylene atmosphere could only mimic 80% of the submergence-induced petiole growth in *R. palustris*.

Submergence of the flooding-intolerant species *Rumex acetosa* resulted in a completely different petiole response. In this species petiole growth of the submerged plants was the same as that of control plants in air (Voeselek and Blom, 1989). Submergence, however, induced entrapment of ethylene to similar levels as in *R. palustris* (Voeselek et al., 1993a). Exposure of *R. acetosa* to elevated concentrations of ethylene resulted in the classical response of terrestrial plants: a reduction of elongation growth (Voeselek and Blom, 1989; Voeselek et al., 1996). We hypothesize that the discrepancies between submergence experiments and ethylene exposures in both *Rumex* sp. are related to the atmospheric concentrations of O<sub>2</sub> and/or CO<sub>2</sub> used during the ethylene-exposure experiments.

While testing this hypothesis we found that low O<sub>2</sub> concentrations stimulated petiole elongation in the flooding-tolerant *R. palustris* and that this response was ethylene dependent. Low concentrations of O<sub>2</sub> sensitized the petiole tissue to ethylene, and this increase in respon-

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<sup>1</sup> Present address: Department of Plant Population Biology, Netherlands Institute of Ecology, P.O. Box 40, 6666 ZG Heteren, The Netherlands.

\* Corresponding author; e-mail rensvo@sci.kun.nl; fax 31-24-365-2409.

Abbreviations: AVG, L- $\alpha$ -(2-aminoethoxyvinyl)-Gly; NBD, 2,5-norbornadiene.

siveness was preceded by an increase in the expression level of a gene coding for the putative *R. palustris* ethylene receptor. Ethylene inhibited petiole elongation in the flooding-intolerant *R. acetosa*. This effect of ethylene could be counteracted by low concentrations of O<sub>2</sub> and high concentrations of CO<sub>2</sub>.

## MATERIALS AND METHODS

### Plant Material and Gas Treatments

Experiments were performed with small seedlings of *Rumex acetosa* and *Rumex palustris* that had four or five leaves. Plants were grown in climate-controlled rooms from seeds that were collected locally from field populations. Unless otherwise stated, plants were raised under the temperature and light conditions described by Voeselek and Blom (1989).

The impact of low O<sub>2</sub> (3%) and high CO<sub>2</sub> (5%) concentrations on petiole elongation of *R. acetosa* and *R. palustris* in the presence and absence of ethylene (5  $\mu\text{L L}^{-1}$ ) was studied. The appropriate gas mixture was flushed continuously (flow rate, 10 L h<sup>-1</sup>) through airtight desiccators with a volume of approximately 10.8 L. The inlet tube bubbled the gas mixture through a thin layer of water at the bottom of the desiccator to maintain a high RH. Seedlings in 70-mL pots filled with a mixture of sand and potting compost (1:1, v/v) were placed in the desiccators and exposed during the gas experiment to a PPFD of 100 to 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (daylength, 16 h) (TLD 36W/84 bulbs, Philips, Eindhoven, The Netherlands).

The gas treatment experiment lasted 96 h, with nine replicates used per desiccator and per gas mixture. Before and after the experiment the length of the youngest petiole was measured. Three gases, ethylene (0 versus 5  $\mu\text{L L}^{-1}$ ), O<sub>2</sub> (3 versus 21%), and CO<sub>2</sub> (0.03 versus 5%), were used, resulting in a total of eight treatments. The effects of these gas treatments were compared with the elongation occurring in plants belonging to the same batch ( $n = 9$ ) that had been submerged in tap water for 96 h under the same light regime as the gas experiment.

To produce O<sub>2</sub> concentration-response curves for both species, the soil was gently washed from the seedling roots and the plants were placed individually in 40-mL glass vials filled with tap water. The seedlings were kept in position with a rubber stopper that clamped the root-shoot junction just above the water level. The seedlings and vials were prepared the day before the experiment started to avoid interference of handling-induced ethylene production. The next day vials containing the seedlings were placed in desiccators and the required O<sub>2</sub> concentration was installed with gas blenders (HI-TEC model E55N3, Bronkhorst, Ruurlo, The Netherlands). The gas inlet and outlet were then closed and the O<sub>2</sub> concentration was checked at least twice a day with a gas chromatograph (Chrompack CP-9000, Bergen op Zoom, The Netherlands) fitted with a column-switching valve connected to a Porapak QS column (length, 150 cm), a Molsieve 5A column (length, 200 cm), and a TCD 903 detector (Chrompack).

These O<sub>2</sub> measurements were used to calculate a mean O<sub>2</sub> concentration that was plotted against petiole growth. Experiments lasted 48 h and were performed with eight replicates, and the petiole length of the youngest leaf was measured just before and after the start of the experiment. This O<sub>2</sub> experiment was performed in a growth chamber with a temperature of 20°C and a PPFD of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (daylength, 16 h).

### Ethylene Inhibitors

To answer the question of whether the low-concentration-O<sub>2</sub>-induced stimulation of petiole growth in *R. palustris* requires the presence of ethylene, inhibitors of ethylene synthesis and action were applied under low-concentration-O<sub>2</sub> conditions. Seedlings of *R. palustris* were prepared as mentioned for the O<sub>2</sub> concentration-response experiment. In this experiment 10-mL vials filled with glass beads and 6 mL of a 5 mM potassium phosphate buffer, pH 6.0, were used. Ethylene biosynthesis was inhibited by 0.2 mM AVG, and ethylene action was inhibited by 5000  $\mu\text{L L}^{-1}$  NBD. AVG was applied to the buffer solution in the vials at least 16 h prior to the start of the experiment; NBD was injected into the desiccators just before the onset of the experiments. Both AVG and NBD were applied to seedlings ( $n = 8$ ) exposed to 21 and 3% O<sub>2</sub>. The specificities of AVG and NBD were tested by applying 1 mM ACC and 10  $\mu\text{L L}^{-1}$  ethylene, respectively. This experiment was performed in a growth chamber with a temperature of 20°C and a PPFD of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (daylength, 16 h).

### Ethylene Production and Leaf Growth

The effect of a low concentration of O<sub>2</sub> on the ethylene production rate and leaf growth of *R. palustris* was monitored simultaneously on the same individual plants. To achieve this, a special cuvette (Voeselek et al., 1997) was connected to a flow-through system that ventilated the shoot compartment at a rate of 2 L h<sup>-1</sup> with ethylene-free air. The roots were adjusted in a separate compartment in which a well-aerated nutrient solution circulated. The ethylene concentration was measured every 1 min in the out-flowing air of the shoot compartment with laser-driven photoacoustic spectroscopy (Harren et al., 1990; Voeselek et al., 1997). The ethylene concentration was multiplied by the applied flow rate to calculate ethylene production (nL h<sup>-1</sup> shoot<sup>-1</sup>). Growth of the youngest leaf (petiole and leaf blade) was measured with a linear-variable displacement transducer (type ST 2000, Schlumberger Industries, Bognor Regis, UK). A stainless steel wire with a diameter of 0.1 mm connecting the leaf tip to the transducer entered the plant cuvette through a "water seal" filled with a saturated solution of ammonium sulfate.

Ethylene production and growth were measured in a light and dark regime of 16 and 8 h, respectively. The experiments were performed in a controlled environment with a temperature of 20°C and a PPFD inside the cuvette of approximately 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (daylength, 16 h). During the first 48 h of an experiment the shoots were exposed to a 21% O<sub>2</sub> concentration. Hereafter, the O<sub>2</sub> level

in the flowing air passing the shoot was changed to 3% without opening the cuvette or handling the plant. In a separate experiment AVG was added to the nutrient solution of the root compartment to give a concentration of 0.05 mM at the onset of the experiment.

### Ethylene Sensitivity

To test whether subambient O<sub>2</sub> concentrations induce a shift in the ethylene sensitivity of *R. palustris*, defined as the petiole growth response to applied ethylene, a concentration-response experiment was carried out at 21 and 3% O<sub>2</sub>. Seedlings of *R. palustris* were prepared as in the ethylene-inhibitor experiment. To avoid interference of endogenously produced ethylene, AVG was added at least 16 h prior to the start of the experiment to the root solution to achieve a concentration of 0.2 mM. Ethylene concentration treatments were applied in desiccators filled with either 3 or 21% O<sub>2</sub> by injecting pure ethylene to obtain the required concentrations. Ethylene was checked at least twice a day with a gas chromatograph (Chrompack model 437A, Bergen op Zoom) with a Hayesep N column (length, 100 cm; Chrompack) and a flame ionization detector; O<sub>2</sub> and CO<sub>2</sub> were checked with the gas chromatograph.

In daylight photosynthesis reduced the CO<sub>2</sub> concentrations significantly within a few hours. A separate experiment with a flow-through system demonstrated that this strong reduction did not affect the ethylene sensitivity of the petioles (data not shown). To obtain a concentration-response curve, the mean ethylene concentration calculated from the ethylene checks was plotted against the length increase of the youngest petiole after 48 h. The experiment was performed with seven replicates per ethylene concentration in a growth chamber with a temperature of 20°C and a PPFD of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (daylength, 16 h).

### Kinetics of Low-Concentration-O<sub>2</sub>-Induced Growth Stimulation and Ethylene-Response Sensor Expression

Two experiments were carried out to compare the kinetics of the low-concentration-O<sub>2</sub>-induced stimulation of petiole growth with the kinetics of the expression of the ethylene-response sensor gene (*RP-ERS1*) in *R. palustris*. Seedlings were prepared as described for the O<sub>2</sub> concentration-response curves.

For the first experiment one individual plant was placed in a glass cuvette with approximately 600 mL. The leaf tip of the youngest full-grown leaf (leaf number 4) was connected to a linear displacement transducer to monitor elongation continuously. The stainless steel wire (0.1 mm) joining the leaf tip with the transducer entered the top of the cuvette via a 0.2-mm hole. After a few hours of growth in a 21% O<sub>2</sub> gas mixture, a mixture containing 3% O<sub>2</sub>, 0.03% CO<sub>2</sub>, and 97% N<sub>2</sub> was flushed into the cuvette at a rate of 20 L h<sup>-1</sup>. During the shift in gas composition an extra gas outlet was created in the cuvette by opening a small valve. Gas was sampled every 2 min with a syringe and injected into the gas chromatograph to quantify the change in O<sub>2</sub> concentration. Within 5 min after the onset of the switch

the O<sub>2</sub> concentration had declined to 3% (data not shown). After 10 min the 3% O<sub>2</sub> gas flow was reduced to 1.5 L h<sup>-1</sup>.

In the second experiment seven desiccators were filled with 15 seedlings each and flushed with air before closure. Hereafter, the desiccators were placed in an airtight glove box (model 1029, Forma Scientific, Marietta, OH) with a gas-blender (model 0152, Brooks Instruments, Veenendaal, The Netherlands)-installed gas mixture of 2% O<sub>2</sub> and 98% N<sub>2</sub>. The experiment started with the opening of the seven desiccators by lifting their tops. The gas mixtures from the desiccators diffused into the glove box and resulted in a very fast change to a new equilibrium concentration of 3% O<sub>2</sub>. With the aid of a lock, desiccators were removed from the glove box after 0, 20, 40, 60, 120, 240, and 360 min. Within 3 min the youngest full-grown petiole of 15 plants was cut from the shoot (total fresh weight, approximately 200 mg) and frozen in liquid N<sub>2</sub>. This petiole tissue was used for RNA extraction.

*RP-ERS1* was isolated with an *ETR1* probe from *Arabidopsis thaliana* from a cDNA library derived from the two youngest leaves (petioles and leaf blades) of *R. palustris* plants that had been submerged for 24 h (Vriezen et al., 1997).

Total RNA was isolated by homogenizing 200 mg of tissue from the youngest full-grown petiole in 2 mL of extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1% SDS, 0.1 M NaCl, 50 mM  $\beta$ -mercaptoethanol, and 1% Tri-isopropyl-naphthalene sodium salt [Kodak]) (Goldberg et al., 1981) and an equal volume of phenol (Tris- and EDTA-saturated, pH 8.0) with a 10-mL glass-Teflon homogenizer. After the homogenized mixture was transferred to a centrifuge tube and incubated for 10 min at 60°C, 2 mL of chloroform:isoamyl alcohol (49:1) was added and vortexed for 20 s; it was then centrifuged at 3000g at 4°C for 20 min and the aqueous phase was extracted with 2 mL of phenol:chloroform:isoamyl alcohol (50:49:1) until no interface remained. RNA/DNA was precipitated with ethanol (1 h, -20°C) and separated with an additional 2 M LiCl precipitation overnight at 4°C.

Total RNA was separated on a 1% agarose gel containing 0.4 M formaldehyde and 0.1  $\mu\text{g mL}^{-1}$  ethidium bromide. After electrophoresis the gel was inspected by UV exposure and photographed to check the amounts of RNA present in each lane. RNA was transferred overnight to a nylon membrane by capillary transfer in 20 $\times$  SSC. RNA was fixed to a Hybond-N nylon membrane according to the directions of the manufacturer (Amersham). The membrane was prehybridized for 4 h and hybridized overnight at 62°C with a solution containing 6 $\times$  SSC, 5 $\times$  Denhardt's reagent, 0.1% SDS, and 100  $\mu\text{g mL}^{-1}$  denatured, fragmented salmon sperm DNA. Probes were labeled in low-melting-point agarose with [ $\alpha$ -<sup>32</sup>P]dATP by the random-priming method (Church and Gilbert, 1984). Hereafter, the membrane was washed two times in 2 $\times$  SSC plus 0.1% SDS at 65°C for 15 min each and two times in 0.2 $\times$  SSC plus 0.1% SDS for 15 min.

### Statistical Analysis

The gas treatment experiment was performed twice. The two similar data sets were pooled before analyses. This

total data set was analyzed with a three-way analysis of variance (SAS Institute, 1989) per species to determine the main effects and interactions between gases (ethylene, O<sub>2</sub>, and CO<sub>2</sub>). Since the initial length of the petiole can have an effect on the elongation response, we included initial petiole length as a co-variate in the analysis of variance.

A nonlinear regression method (SAS Institute, 1989) calculated ethylene concentration-response curves for both O<sub>2</sub> concentrations. The response (*R*) at a given ethylene concentration (*H*) is described by the following equation (Peng and Weyers, 1994):

$$R = R_{\min} + \{[R_{\max} - R_{\min}] \times [H]^p\} / \{[H]_{50}^p + [H]^p\} \quad (1)$$

where *R*<sub>min</sub> is the minimum response, *R*<sub>max</sub> is the maximum response, [*H*]<sub>50</sub> is the hormone concentration giving a response of 0.5 × (*R*<sub>max</sub> − *R*<sub>min</sub>) + [*R*<sub>min</sub>], and *p* is the interaction of the Hill coefficient.

General assumptions for this model were formulated by Weyers et al. (1987). More specifically, in our experiments it is assumed that *p* equals 1 (Banga et al., 1996a). It was emphasized by Weyers et al. (1987) that *R* should be the initial rate of response to avoid (a) the consequences of feedback effects that may affect the rate of response, (b) the interference of unknown factors that might limit the overall response, and (c) a possible change in the transduction process during the experiment. In an attempt to measure the initial growth response of the youngest leaf of *R. palustris* exposed to various exogenous ethylene concentrations with linear displacement transducers, it was concluded that the initial response depended not only on the applied ethylene concentration but also strongly on the stage of development of a leaf and the growth rate before the addition of ethylene (data not shown). Based on these experiments we decided to use petiole growth over a span of 48 h as a response unit, since in this relatively long span of ethylene exposure the effects of stage of development and growth rate before ethylene exposure are negligible.

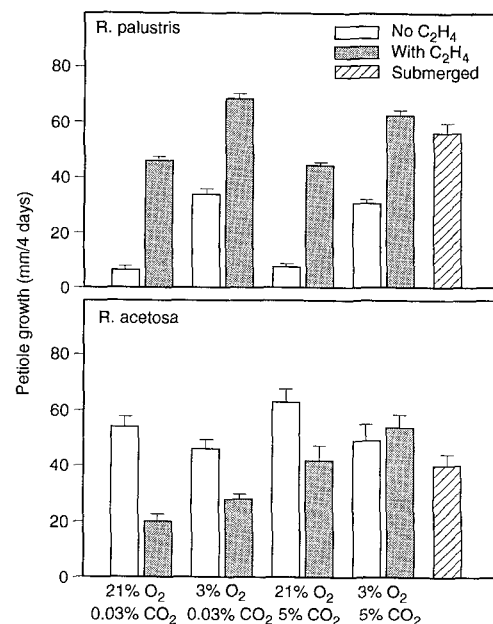
The kinetics of 3% O<sub>2</sub>-induced petiole elongation was analyzed with analysis of variance (SAS Institute, 1989) for each time, with O<sub>2</sub> treatment (3 versus 21%) as the main effect. Comparison of means was performed with pairwise *t* tests to obtain LSD.

## RESULTS

### Low O<sub>2</sub> and High CO<sub>2</sub>

The effects of various combinations of ethylene, O<sub>2</sub>, CO<sub>2</sub>, and submergence on petiole growth of *R. palustris* and *R. acetosa* are presented in Figure 1. The three-way analysis of variance per species is given in Table I.

High ethylene and low O<sub>2</sub> concentrations significantly stimulated petiole elongation in *R. palustris*, whereas high levels of CO<sub>2</sub> reduced petiole growth slightly (Fig. 1). However, in contrast to the ethylene and O<sub>2</sub> effects, only a very limited amount of variance could be explained by CO<sub>2</sub> (Table I). The increase in petiole growth of *R. palustris* upon exposure to a low O<sub>2</sub> concentration was larger in the absence than in the presence of ethylene (Fig. 1). In the statistical analysis this is expressed as a significant interac-



**Figure 1.** Elongation of the youngest petiole (*n* = 18; means ± SE) of *R. palustris* and *R. acetosa* in response to different gas mixtures and submergence.

tion between O<sub>2</sub> and ethylene (Table I). High ethylene levels with ambient concentrations of O<sub>2</sub> and CO<sub>2</sub> mimicked only 80% of the submergence response in *R. palustris*. However, when ethylene was added with low levels of O<sub>2</sub> petiole growth was more strongly stimulated than under submerged conditions (Fig. 1).

A high ethylene concentration inhibited petiole growth in *R. acetosa*, whereas a promotion of petiole growth was observed during exposure to a high CO<sub>2</sub> concentration (Fig. 1; Table I). The ethylene-induced inhibition of petiole growth in *R. acetosa* was reduced in the presence of low O<sub>2</sub> and/or high CO<sub>2</sub> concentrations (Fig. 1). In the statistical analysis this is expressed as significant interactions between ethylene and O<sub>2</sub> and between ethylene and CO<sub>2</sub> (Table I). High concentrations of ethylene applied to *R. acetosa* in a gas mixture with ambient O<sub>2</sub> and CO<sub>2</sub> levels inhibited petiole growth much more strongly than submergence. The best mimic of the submergence effect was exposure to 5 μL L<sup>-1</sup> ethylene, 5% CO<sub>2</sub>, and 21% O<sub>2</sub> (Fig. 1).

The O<sub>2</sub> concentration-response curve of *R. palustris* showed a stimulation of petiole growth in the subambient concentration range between 2 and 11% O<sub>2</sub> (Fig. 2). Petiole growth in *R. acetosa* decreased with the decline in the O<sub>2</sub> concentration. Another marked difference between the two species was the very slow petiole growth of *R. acetosa* when exposed to 0.3% O<sub>2</sub>. At the same O<sub>2</sub> concentration *R. palustris* had a growth rate identical to that observed at 21% O<sub>2</sub>.

### Ethylene Dependence of the Low-O<sub>2</sub>-Concentration-Induced Elongation

The ethylene-biosynthesis inhibitor AVG inhibited petiole growth in *R. palustris* plants exposed to 21 and 3% O<sub>2</sub> (Fig. 3). At both O<sub>2</sub> concentrations AVG treatment resulted

**Table 1.** Analysis of variance of gas treatments

Three-way analysis of variance of petiole growth of *R. palustris* and *R. acetosa* (type III MS) exposed for 4 d to various combinations of ethylene (E), oxygen (O), and carbon dioxide (C). Initial petiole length is included for both species as a co-variate. F, Statistic; MS, mean square.

Source of Variance	<i>R. palustris</i>			<i>R. acetosa</i>		
	df	MS	F	df	MS	F
Initial length	1	22.5	0.5	1	7990.8	32.0 <sup>a</sup>
E	1	44,929.4	1062.3 <sup>a</sup>	1	9932.4	39.8 <sup>a</sup>
O	1	19,054.9	450.5 <sup>a</sup>	1	20.5	0.1
C	1	211.5	5.0 <sup>c</sup>	1	7553.2	30.2 <sup>a</sup>
E × O	1	227.4	3.4 <sup>c</sup>	1	3964.6	15.9 <sup>a</sup>
E × C	1	60.7	1.4	1	2828.5	11.3 <sup>b</sup>
O × C	1	121.1	2.9	1	8.2	0
E × O × C	1	0.2	0	1	126.7	0.5
Error	134	42.3		135	249.8	

<sup>a</sup> P < 0.001. <sup>b</sup> P < 0.01. <sup>c</sup> P < 0.05.

in a similar amount of remaining growth. The addition of ACC, the immediate precursor of ethylene in its biosynthetic pathway, with AVG restored the original 3% induced stimulation of petiole growth completely.

The ethylene action inhibitor NBD did not inhibit petiole growth at the ambient O<sub>2</sub> concentration but reduced the increase in petiole growth at 3% O<sub>2</sub> completely (Fig. 3). Simultaneous addition of NBD and ethylene restored the original 3% growth response partially.

These inhibitor studies demonstrate that in *R. palustris* subambient O<sub>2</sub> concentrations stimulate petiole growth indirectly via the plant hormone ethylene.

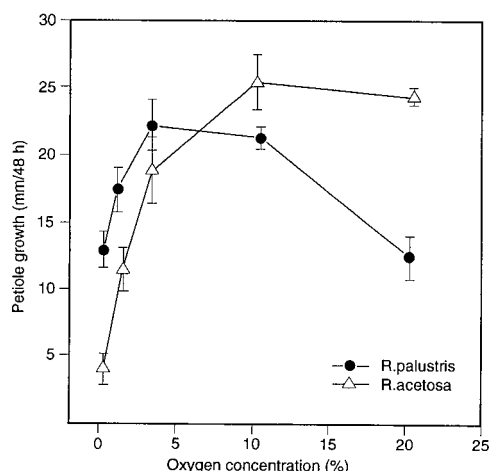
### Ethylene Production and Sensitivity

The ethylene release from shoots of *R. palustris* showed a clear day-night rhythm, especially during exposure to air. The highest release was observed during the day (Fig. 4A). A slightly higher growth rate of the youngest leaf (petiole and leaf blade) was observed during darkness, especially during the first night. The shift to 3% O<sub>2</sub> did not result in

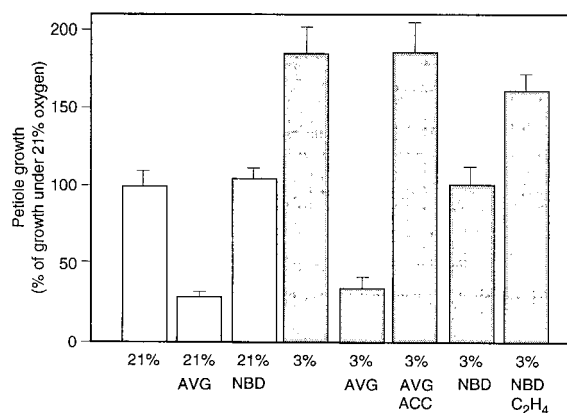
an increase in ethylene release during the light period. However, 3% O<sub>2</sub> did stimulate ethylene release during darkness, especially in the second night. Leaf growth was strongly stimulated shortly after the switch to 3% O<sub>2</sub>. The circadian rhythm in the rate of leaf growth was reversed compared with the 21% O<sub>2</sub> period; the fastest growth was observed during the day (Fig. 4A).

The addition of AVG reduced the release of ethylene in *R. palustris* by approximately 30 and 40% during the day and night, respectively, but did not change the circadian character of the ethylene release (Fig. 4). The low O<sub>2</sub> level only stimulated the ethylene release slightly during the second night of exposure. Leaf growth under 21 and 3% O<sub>2</sub> was dramatically reduced in the AVG-treated plant, although a slight stimulation of leaf growth was observed shortly after the onset of the 3% O<sub>2</sub> treatment (Fig. 4B).

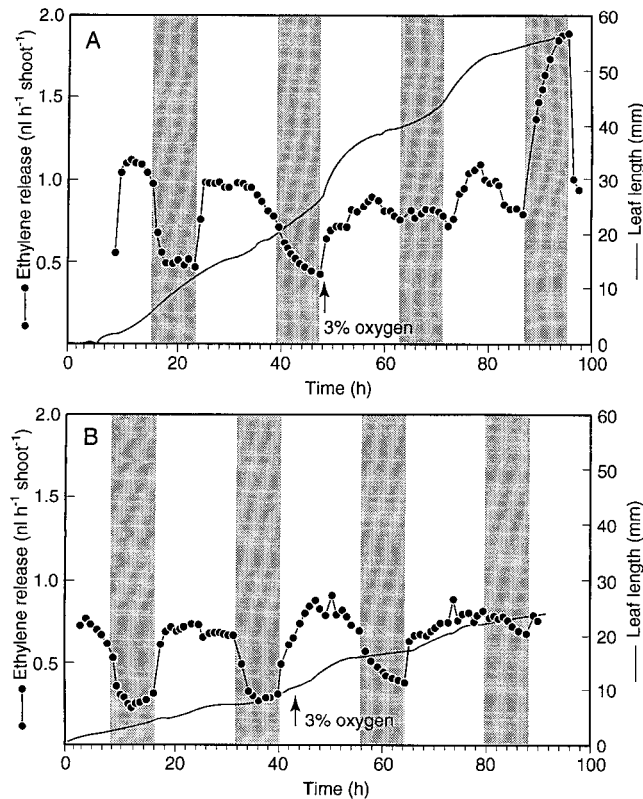
The ethylene concentration-response curves of *R. palustris* during exposure to 21 and 3% O<sub>2</sub> are presented in Figure 5. The [H]<sub>50</sub> of 0.26 ± 0.12 μL L<sup>-1</sup> ethylene during



**Figure 2.** Elongation of the youngest petiole ( $n = 8$ ; means  $\pm$  SE) of *R. palustris* and *R. acetosa* in response to a range of O<sub>2</sub> concentrations.



**Figure 3.** Elongation of the youngest petiole ( $n = 8$ ; means  $\pm$  SE) of *R. palustris* in an atmosphere containing 21 or 3% O<sub>2</sub> in the presence and absence of the ethylene biosynthesis inhibitor AVG (0.2 mM) and the ethylene action inhibitor NBD (5000 μL L<sup>-1</sup>). The specificities of AVG and NBD were tested by applying 1 mM ACC and 10 μL L<sup>-1</sup> ethylene, respectively. The petiole growth under 21% O<sub>2</sub> was set at 100% and had an absolute value of 5.9 ± 1.0 mm.



**Figure 4.** Ethylene release and elongation of the youngest leaf monitored simultaneously on the same *R. palustris* plant in the absence (A) and presence (B) of 0.05 mM AVG, the ethylene biosynthesis inhibitor. The first 2 days and nights of ethylene release and growth were measured on plants exposed to 21% O<sub>2</sub>; afterward, the O<sub>2</sub> level was decreased to 3%. Ethylene release was measured almost continuously with photoacoustic spectroscopy, whereas leaf growth was measured continuously with linear displacement transducers. The detailed experimental design of this experiment was described by Voeselek et al. (1996). Gray bars represent 8-h dark periods.

21% O<sub>2</sub> decreased to  $0.04 \pm 0.02 \mu\text{L L}^{-1}$  ethylene during exposure to 3% O<sub>2</sub>. During 48 h of treatment with a low concentration of O<sub>2</sub> no significant change in the maximum petiole growth ( $R_{\text{max}}$ ) was observed.

In conclusion, 3% O<sub>2</sub> induced in *R. palustris* a fast increase in petiole growth that was not related to changes in the production level of ethylene but was related to at least a 6-fold increase in the ethylene sensitivity, expressed as  $[H]_{50}$ , of the youngest petiole.

#### Kinetics of Elongation and Ethylene Response Sensor Expression

The transducer data were used to calculate growth rates of the youngest full-grown leaf (petiole and leaf blade) over intervals of 10 min (Fig. 6A). The growth rate increased significantly after 40 to 50 min of exposure to 3% O<sub>2</sub>. The maximum growth rate was reached after 80 to 90 min.

The *RP-ERS1* mRNA in the youngest full-grown petiole of *R. palustris* is positively regulated by low O<sub>2</sub> concentrations (Fig. 6B). *RP-ERS1* mRNA was barely detectable dur-

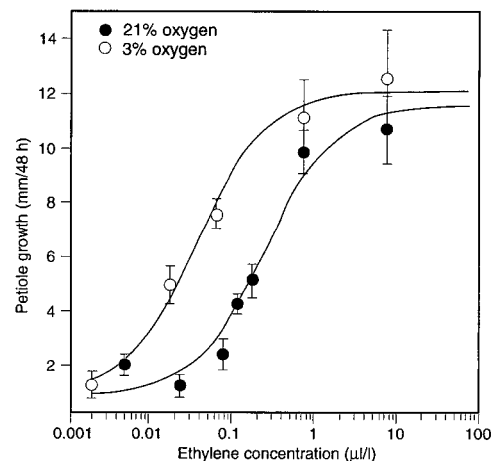
ing growth under 21% O<sub>2</sub> but rapidly accumulated after exposure to 3% O<sub>2</sub>. This increase was transient, with maximum expression after 2 h of 3% O<sub>2</sub> treatment. The first increase in *RP-ERS1* mRNA was observed after 20 min, thus preceding the first significant increase in the growth rate of the youngest full-grown petiole (Fig. 6).

#### DISCUSSION

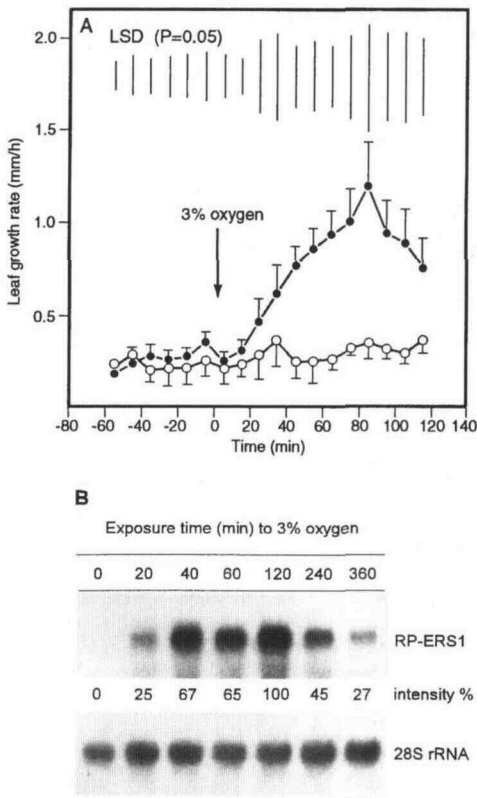
Enhanced levels of the gaseous plant hormone ethylene and low O<sub>2</sub> levels both stimulate petiole elongation in the wetland plant *R. palustris*. Both gases are required to mimic the petiole response induced by submergence. High levels of CO<sub>2</sub> have no effect on petiole growth in *R. palustris* (Fig. 1).

A stimulation of elongation growth by reduced O<sub>2</sub> concentrations is also observed in coleoptiles of rice (*Oryza sativa*) (Raskin and Kende, 1983; Satler and Kende, 1985; Horton, 1991; Pearce and Jackson, 1991), internodes of deepwater rice (*O. sativa* cv Habiganj Aman II) (Raskin and Kende, 1984b), and stems of *Potamogeton pectinatus* (Summers and Jackson, 1993). In the first two examples and in *R. palustris* simultaneous addition of low O<sub>2</sub> and ethylene concentrations resulted in even faster elongation. In *R. palustris* fast growth and thus a quick restoration of contact with the aerial environment is most important under O<sub>2</sub>-limiting conditions. This occurs under natural conditions when plants are submerged in turbid water and, therefore, produces little photosynthetic O<sub>2</sub>.

Under less-turbid conditions photosynthetically derived O<sub>2</sub> improves not only the O<sub>2</sub> status of the whole plant (Laan and Blom, 1990; Voeselek et al., 1993b) but also increases its chances of survival considerably (Laan and Blom, 1990; Blom et al., 1994). A lower petiole elongation



**Figure 5.** Elongation of the youngest petiole of *R. palustris* in an atmosphere with 3 or 21% O<sub>2</sub> in response to a range of ethylene concentrations. Plants were pretreated with 0.2 mM AVG to avoid interference of endogenously produced ethylene. The concentration-response data were fitted with a nonlinear regression method according to an equation described by Peng and Weyers (1994). Both lines were highly significant with respect to the individual data points (21% O<sub>2</sub>:  $F_{3,46} = 152.15^{***}$ ,  $r^2 = 0.91$ ; 3% O<sub>2</sub>:  $F_{3,32} = 90.88^{***}$ ,  $r^2 = 0.90$ ).



**Figure 6.** A, Growth rate ( $n = 5-6$ ; means  $\pm$  SE) during 10-min intervals of the youngest full-grown leaves of *R. palustris* with (●) and without (○) a switch to 3% O<sub>2</sub>. Within 5 min after the switch the O<sub>2</sub> concentration was decreased to 3%. LSD values ( $P = 0.05$ ) per time point are included in the graph. B, Expression of the *RP-ERS1* gene in the youngest full-grown petiole of *R. palustris* exposed to 3% O<sub>2</sub>. The blot was reprobbed with 28S rRNA as a control for the loaded amount of total RNA. The intensity percentages represent the *RP-ERS1* transcript level relative to the highest signal detected and corrected for the loaded amount of total RNA.

rate driven only by ethylene under these conditions would be less harmful in terms of survival.

Both low O<sub>2</sub> and high CO<sub>2</sub> concentrations counteracted the inhibitory effect of ethylene on petiole growth of *R. acetosa*. When applied together their effects were additive (Fig. 1). Comparable results were gained with pea (*Pisum sativum*) seedlings (Burg and Burg, 1967; Beyer, 1979), in which both low O<sub>2</sub> and high CO<sub>2</sub> concentrations were thought to interfere with ethylene action via their effect on the ethylene receptor site. Sanders et al. (1990) demonstrated that ethylene binding in rice seedlings is not influenced by CO<sub>2</sub> and O<sub>2</sub>. The cyclo-olefin NBD, however, did inhibit ethylene binding and stimulation of growth by ethylene. It is concluded that for rice seedlings, and probably also for petioles of *R. acetosa*, high CO<sub>2</sub> and low O<sub>2</sub> concentrations act independently of ethylene responses and thus do not modulate elongation by altering ethylene receptor activity (Sanders et al., 1990). Complete submergence of *R. acetosa* only slightly inhibited petiole growth. However, ethylene in combination with ambient levels of O<sub>2</sub> and CO<sub>2</sub> more strongly inhibited petiole growth. The

results presented in Figure 1 show that this difference in response between submergence and ethylene application in *R. acetosa* can easily be explained by variations in the endogenous levels of CO<sub>2</sub> and O<sub>2</sub>.

The simultaneous measurement of ethylene evolution and growth of the youngest leaf allowed us to compare the changes in ethylene release directly with petiole growth. After the switch to 3% O<sub>2</sub> no change in ethylene release was observed during the day. However, in darkness 3% O<sub>2</sub> induced an increase in ethylene release compared with the release levels under ambient O<sub>2</sub> concentrations (Fig. 4A). Photosynthetic activity during the day probably increased the endogenous O<sub>2</sub> concentration in leaf blades and petioles to levels higher than 3%. Darkness, however, probably resulted in endogenous concentrations of approximately 3%. We conclude that in shoots of *R. palustris* O<sub>2</sub> concentrations of approximately 3% stimulate ethylene production. Such a stimulation of low O<sub>2</sub> levels was earlier observed in roots and stems of other plants (Jackson, 1982; Metraux and Kende, 1983; Jackson et al., 1984; Brailsford et al., 1993). In tomato (*Lycopersicon esculentum*) roots this increase in ethylene production in response to low O<sub>2</sub> levels is explained by an increased expression of an ACC synthase gene, *LE-ACS3* (Olson et al., 1995).

Because the highest growth rates of leaves exposed to 3% O<sub>2</sub> were observed during the day, we conclude that the low-concentration-O<sub>2</sub>-induced stimulation of petiole elongation in *R. palustris* is not related to an upsurge of the ethylene production rate.

Low concentrations of O<sub>2</sub> exert their stimulating effect on the petiole elongation of *R. palustris* primarily via a sensitization of the petiole tissue to ethylene (Figs. 3 and 5). This shift in sensitivity is expressed by a reduction of the  $[H]_{50}$ . The concentration-response curve did not demonstrate an increase in  $R_{max}$ , although such a shift was expected based on the results shown in Figure 1. This difference can be explained by the short exposure (48 h) to low O<sub>2</sub> and ethylene concentrations in the concentration-response experiment compared with the exposure time in the gas-treatment experiment (96 h).

The kinetics of low-concentration-O<sub>2</sub>-stimulated growth and the expression of the *RP-ERS1* gene clearly demonstrated that *RP-ERS1* mRNA accumulated before a significant increase in growth was observed. Although only transcription of the gene coding for an ethylene response sensor is described, this observation indicates that increases in ethylene sensitivity might simply be controlled by an up-regulation of the number of receptor molecules. During ripening, tomatoes increase their responsiveness to ethylene dramatically. This, too, is accompanied by an increased expression of the *Never-ripe* (*NR*) gene, a homolog of the Arabidopsis gene coding for a putative ethylene receptor (*ERS1*) (Wilkinson et al., 1995).

*RP-ERS1* has a high homology with the Arabidopsis *ERS* and with the tomato *NR* gene (Vriezen et al., 1997). In contrast to *ETR1*, another Arabidopsis gene coding for an ethylene receptor, all of these genes share the lack of a response regulator domain, and *RP-ERS1* and *NR* are the only putative ethylene receptor genes so far described that



are developmentally and environmentally regulated (Wilkinson et al., 1995; Vriezen et al., 1997).

It is not yet clear how the increase in *NR* mRNA and *tETR* mRNA during fruit ripening, flower senescence, and flower abscission after ethylene exposure (Wilkinson et al., 1995; Payton et al., 1996) and the accumulation of *RP-ERS1* mRNA during flooding and exposure to high levels of ethylene and/or low concentrations of O<sub>2</sub> (Vriezen et al., 1997) fit into one of the models of ethylene receptor functioning. This model assumes that the ethylene receptor protein is constitutively active in the absence of ethylene and negatively regulates the transduction chain by activating the CTR1 protein. However, in the presence of ethylene both the ethylene receptor and the negative regulator CTR1 are turned off, consequently evoking ethylene responses. Ethylene changes the receptor-signaling activity from "on" to "off." In this model an increase in sensitivity can only be achieved by a reduction in the number of receptor molecules (Chang and Meyerowitz, 1995; Bleecker and Schaller, 1996; Chang, 1996).

In summary, we found that ethylene and a low O<sub>2</sub> concentration stimulated petiole elongation in the flooding-tolerant plant, *R. palustris*. The response to a low O<sub>2</sub> concentration was ethylene dependent and operated via a sensitization of the petiole tissue to ethylene. This increase in ethylene responsiveness was preceded by an increase in the expression level of a gene coding for the putative *R. palustris* ethylene receptor (*RP-ERS1*). In contrast, ethylene inhibited petiole elongation in the flooding-intolerant *R. acetosa*. This effect of ethylene could be counteracted by low O<sub>2</sub> and high CO<sub>2</sub> concentrations.

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