An Ethylene-Mediated Increase in Sensitivity to Auxin Induces Adventitious Root Formation in Flooded *Rumex palustris* Sm.[1]

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The hormonal regulation of adventitious root formation induced by flooding of the root system was investigated in the wetland species *Rumex palustris* Sm. Adventitious root development at the base of the shoot is an important adaptation to flooded conditions and takes place soon after the onset of flooding. Decreases in either endogenous auxin or ethylene concentrations induced by application of inhibitors of either auxin transport or ethylene biosynthesis reduced the number of adventitious roots formed by flooded plants, suggesting an involvement of these hormones in the rooting process. The rooting response during flooding was preceded by increased endogenous ethylene concentrations in the root system. The endogenous auxin concentration did not change during flooding-induced rooting, but a continuous basipetal transport of auxin from the shoot to the rooting zone appeared to be essential in maintaining stable auxin concentrations. These results suggest that the higher ethylene concentration in soil-flooded plants increases the sensitivity of the root-forming tissues to endogenous indoleacetic acid, thus initiating the formation of adventitious roots.

Flooding causes many changes in the hormone physiology of plants. For instance, transport of auxin from shoots to roots may be inhibited by soil flooding, resulting in accumulation of auxin at the base of the shoot (Phillips, 1964; Wample and Reid, 1979). Other hormones such as ethylene in hypoxic roots are produced in larger amounts during flooding (Voesenek et al., 1990; Brailsford et al., 1993). In wetland plants, the change in hormonal status of the flooded plant is followed by a number of responses that alleviate the negative effects of flooding on plant growth. The mechanisms that underlie these adaptations to flooding have been explained in terms of changes in hormone concentrations or sensitivity to a hormone (see reviews by Reid and Bradford, 1984; Jackson, 1990; Voesenek et al., 1992; Blom et al., 1994). However, for one major adaptation to flooding, i.e. adventitious root formation, persuasive evidence for a hormone-mediated regulation is still lacking.

Adventitious roots can contain air channels connected to the shoot that enable shoot-to-root diffusion of air. The formation of these roots is a prerequisite for long-term survival of many wetland plants under flooded, oxygen-deficient soil conditions (Justin and Armstrong, 1987; Visser et al., 1996a). The induction of adventitious roots has been attributed to several plant hormones (Drew et al., 1979; Wample and Reid, 1979); two are important for the rooting response in species from the genus *Rumex*. We have shown that application of either auxin or ethylene induces formation of adventitious roots in *Rumex palustris* and *Rumex thyrsiflorus* plants and that inhibition of auxin transport from the shoot to the rooting zone decreases the number of roots induced by flooding (Visser et al., 1995). Recently, it was demonstrated that inhibition of ethylene synthesis in roots also led to a decline in root formation under flooded conditions (Visser et al., 1996b). From these experiments, we may conclude that a sufficiently high concentration of each of these hormones is essential for the flooding-induced formation of adventitious roots. It is well known that auxin can increase the rate of ethylene biosynthesis (Imaseki et al., 1977; Riov and Yang, 1989; Kelly and Bradford, 1990), whereas ethylene may affect auxin transport positively (Musgrave and Walters, 1973) or negatively (Beyer and Morgan, 1970; Suttle, 1988) or influence auxin perception (Bertell et al., 1990; Liu and Reid, 1992). The aim of the present study was to establish the individual roles of auxin and ethylene and the ways in which these hormones interact in the process of flooding-induced adventitious root formation. Intact plants of *R. palustris* Sm. were used for these experiments, because this wetland species readily forms large numbers of adventitious roots when flooded (Visser et al., 1996b).

MATERIALS AND METHODS

Plant Growth

Seeds of *Rumex palustris* Sm. were collected from a river foreland and sown in flat trays filled with polyethylene grains (Lacqtene Low Density, Elf Atochem, Balan, France) and nutrient solution (2 mM Ca[NO₃]₂, 1.25 mM K₂SO₄, 0.5

Abbreviations: AVG, L-α-(2-aminoethoxyvinyl)-Gly; NPA, N-1-naphthylphthalamic acid.

mM $MgSO_4$, 0.5 mM KH_2PO_4, and the micronutrients FeEDTA [90 μM], NaCl [50 μM], H_3BO_3 [25 μM], $MnSO_4$ [2 μM], $ZnSO_4$ [2 μM], $CuSO_4$ [0.5 μM], and H_2MoO_4 [0.5 μM]). After germination of the seeds under a 16-h day (20 μmol m^{-2} s^{-1} PPFD [Philips TL33; 27°C]) and an 8-h night (10°C) for 1 week, the trays were placed in a climate room (16-h day, 120 μmol m^{-2} s^{-1} PPFD [Philips TL84]; 8-h night; 22°C) for 1 more week. Uniform seedlings were then transferred to polystyrene rafts that floated on nutrient solution (concentrations as above) in 20-L containers (six to eight plants per raft). The nutrient solution was well aerated by flushing air through bubble stones at a rate of approximately 60 L h^{-1}.

Hypoxia and Ethylene Treatment

Waterlogging (soil flooding) was simulated by transferring hydroponically grown plants from nutrient solution to an unstirred agar solution (0.1%, w/v; nutrient concentrations as in the nutrient solution), which was first deoxygenated by vigorously flushing with nitrogen gas. Oxygen concentrations in such an agar solution varied between 0.4 and 1.0 mg L^{-1} 2 d after the onset of the experiment.

For ethylene treatments, plants were transferred to containers in which the nutrient solution was flushed with various concentrations of ethylene. These concentrations were obtained by blending 50 μL L^{-1} ethylene (Hoekloos, Dieren, The Netherlands) with air, using gas blenders (model E55N3, Bronkhorst High Tech, Ruurlo, The Netherlands). Ethylene concentration in the nutrient solution was examined by introducing small volumes of ambient air under Petri dishes at the bottom of each container. After these air pockets were allowed to reach equilibrium with the gas concentrations in the nutrient solution, gas samples were drawn and analyzed on a gas chromatograph (model 437A, Chrompack, Bergen op Zoom, The Netherlands; 1.2 m $\times$ 2 mm Haysep QS-column). The ethylene concentration in these samples was at all times within 5% of the ethylene concentration bubbled through the solution.

1-NAA and inhibitors of auxin transport (NPA; Visser et al., 1995) or ethylene biosynthesis (AVG; Yang and Hoffman, 1984) were applied by brushing the shoots of the plants with an aqueous solution of these substances. About 0.01% (v/v) of the detergent Triton X-100 was added to prevent formation of drops on the leaves.

Control plants were included in each experiment. These plants were transferred to fresh nutrient solution at the beginning of the experiment, grown under well-aerated conditions and, when appropriate, brushed with a 0.01% (v/v) Triton X-100 solution.

The number of adventitious roots was counted 7 d after the onset of treatment. Adventitious roots were easily distinguishable from the original lateral root system because of their large diameter and white, unbranched appearance. All experiments were performed at least twice.

Measurements of Auxin-Induced Ethylene Production

Four-week-old plants of R. palustris were transferred to 0.6-L glass cuvettes. One-half the volume of the cuvettes

was filled with glass beads and nutrient solution so that the roots were immersed. Either water or 250 μmol of AVG, dissolved in 0.3 mL of water, was brushed on the leaves of each plant (as described above), and the plants were allowed to acclimatize for 24 h. Then, each AVG-pretreated plant was brushed with 25 nmol of 1-NAA (dissolved in 0.3 mL water), whereas the water-pretreated plants were brushed with either water or 25 nmol of 1-NAA (dissolved in 0.3 mL water). Measurements of ethylene production started after the cuvettes were closed using a lid with an inlet and an outlet. The inlet was connected to a stream of ethylene-free air (flow rate 1 L h^{-1}). Ethylene concentration in the outflowing air was measured every 100 min during 70 h by means of photoacoustic spectroscopy (Voesenek et al., 1990; detection limit 0.05 nL ethylene h^{-1}). Measurements on an empty cuvette were used for calibration. After 3 d, the cuvettes were opened and the fresh weights of the shoots and the root systems were determined. This experiment was performed twice, each time with two plants per treatment.

Measurements of Free IAA Concentrations

Five-week-old plants of R. palustris were transferred to containers filled with either fresh nutrient solution (control) or deoxygenated agar (as described above). A third group of plants was pretreated with 150 nmol of NPA (in 0.3 mL of water) per shoot 24 h before transfer to agar. At various times, plants were harvested and samples of the root-forming zone (i.e. the apical 5 mm of the tap root) were quickly frozen in liquid nitrogen and stored at -80°C for a maximum of 6 weeks. One group of plants from each treatment (control, agar, and NPA/agar) was harvested after 7 d to determine the number of adventitious roots formed.

Purification and quantification of free IAA were performed essentially as described by Chen et al. (1988). About 100 to 300 mg fresh weight of tissue (rooting zones of two plants) were ground in liquid nitrogen, and approximately 5 mL of extraction buffer (35% [w/v] 0.2 M imidazole, pH 7, 65% [v/v] propanol) was added to the plant material, together with 150 ng of [$^{13}C_6$]IAA as an internal reference. After 3.3 kBq of [^{3}H]IAA (specific activity 962 GBq $mmol^{-1}$; Amersham) was added to the sample to trace the fraction containing the free IAA during purification, the homogenate was allowed to equilibrate at 4°C for 1 h. The sample was then centrifuged at 1500g for 10 min and the supernatant was collected. The pellet was taken up twice in another 1 mL of extraction buffer and centrifuged. Subsequently, the supernatants were pooled and the propanol in the sample was evaporated in a rotating evaporator (Rotavapor R110, Büchni, Flawil, Switzerland) at 45°C. The remaining sample was then applied to an NH_2 column (model 456 SPE, J.T. Baker) that had been washed successively with 5 mL of hexane, 5 mL of acetonitrile, and twice with 5 mL of distilled water and preconditioned with 5 mL of 0.2 M imidazole (pH 7) and two lots of 5 mL of water. After the extract had passed through the column, the column was washed successively with 5 mL of hexane, ethyl acetate, acetonitrile, and methanol before free IAA was

eluted with 3 mL of 5% (v/v) acetic acid in methanol. The sample was evaporated until near dryness, taken up in 120 μL 50% (v/v) methanol, injected on an HPLC column (Phenomenex Ultracarb 30 5-micron ODS reverse-phase column [Torrance, CA] connected to a Waters M45 HPLC-pump; pump rate 1 mL min^{-1}) and eluted with 1% (v/v) acetic acid in 25% (v/v) methanol. The radioactive fractions were pooled, dried in a warm sand bath under nitrogen gas, taken up in 150 μL of methanol, and methylated with approximately 1.5 mL of diluted diazomethane (Cohen, 1984) for 5 min. The sample was dried under nitrogen, taken up in 20 μL of ethyl acetate, and quantified by GC-selected ion monitoring MS, using a gas chromatograph (Hewlett-Packard 5890 series II) connected to a mass-selective detector (Hewlett-Packard 5971a) equipped with a 15-m column (model DB-1701, J&W, Folsom, CA), as described by Michalczuk et al. (1992). Extraction, purification, and quantification were repeated for three separate samples of tissue taken at each time interval per treatment.

RESULTS

Transferring *R. palustris* plants from aerated nutrient solution to deoxygenated agar resulted in the formation of a large number of adventitious roots (Fig. 1). Application of either a high dose of auxin (100 nmol of 1-NAA in 0.3 mL of water per shoot; Fig. 1A) or a high concentration of ethylene (2.5 μL L^{-1}; Fig. 1B) caused a very similar response in well aerated plants. When a relatively low dose
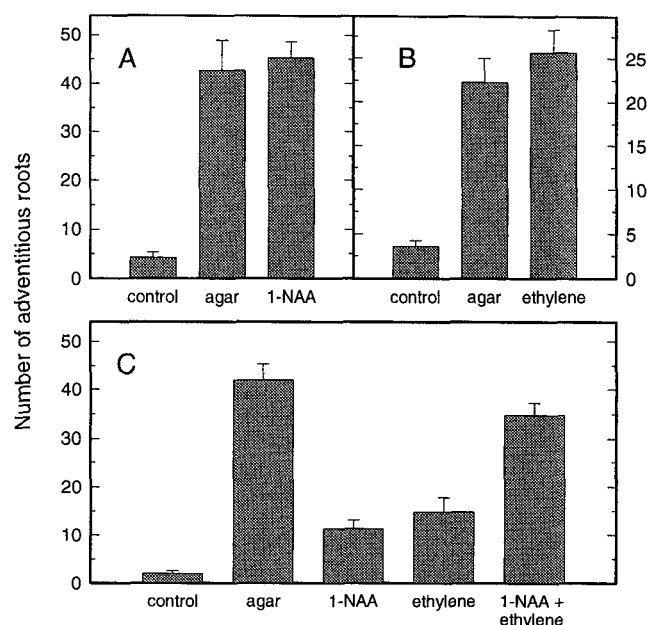

Figure 1. Number of adventitious roots per plant formed by 4-week-old *R. palustris* plants 7 d after transferring the plants to either fresh, well-aerated nutrient solution (control) or an unstirred, deoxygenated agar solution. Treatments were a high dose of 1-NAA (100 nmol in 0.3 mL of water per shoot, A), a high concentration of ethylene (2.5 μL L^{-1}, B), or a combined suboptimal dose of 1-NAA (25 nmol in 0.3 mL of water per shoot) and a suboptimal concentration of ethylene (1.0 μL L^{-1}, C); *n* = 6 (A and C) or 16 (B); error bars indicate SES.
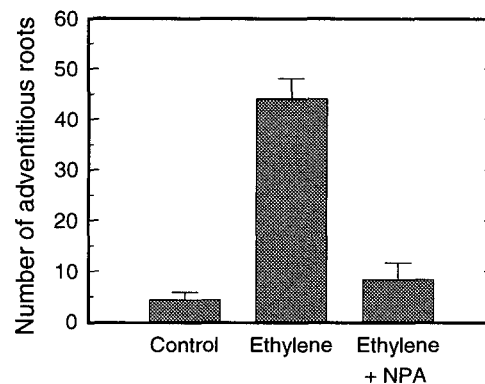

Figure 2. Number of adventitious roots per plant formed by 5-week-old *R. palustris* plants 7 d after transferring the plants to fresh, well-aerated nutrient solution bubbled with either air (control) or ethylene (5 μL L^{-1}) and with or without NPA pretreatment (250 nmol in 0.3 mL of water per shoot); *n* = 8; error bars indicate SES.

or concentration of these two hormones was applied (25 nmol of 1-NAA in 0.3 mL of water per shoot or 1.0 μL L^{-1} ethylene; Fig. 1C), only a limited number of roots were induced. Simultaneous application of a low auxin dose and a low ethylene concentration almost fully restored the maximum rooting response. The maximum number of adventitious roots (induced by stagnant agar, high ethylene concentrations, or high auxin doses) varied somewhat between replicate experiments, but the relative difference between treatments was not affected by this variation (data not shown).

To separate the effects of auxin and ethylene, experiments were conducted using inhibitors of polar auxin transport (NPA) or ethylene biosynthesis (AVG). The ethylene-stimulated formation of adventitious roots was almost completely counteracted by pretreatment of the shoot of the plants with NPA (Fig. 2). Unfortunately, in our experimental system it was technically not possible to reverse the effect of NPA by the application of auxin because the rosette form of *R. palustris* (and, thus, the lack of a stem) prevented application of IAA to a more basal part of the stem than NPA. However, application of NPA did not influence root growth or shoot appearance, and we conclude that at the concentrations applied the inhibiting effect is probably specific to auxin transport (Visser et al., 1995). Treatment of *R. palustris* plants with 1-NAA almost immediately resulted in a strong increase in the ethylene production rate (Fig. 3). This peak in ethylene release was much higher than the stress ethylene peak that control plants produced upon brushing with water and declined only gradually with time. Pretreatment of plants with AVG reduced regular ethylene production dramatically within a few hours and fully prevented the auxin-induced increase of ethylene biosynthesis (Fig. 3). Auxin-induced formation of adventitious roots, however, was not negatively affected by this pretreatment (Fig. 4). These experiments demonstrate that auxin can induce adventitious roots independently of ethylene, whereas the root-inducing effect of ethylene is mediated by auxin.

To establish whether a change in auxin concentration triggered flooding-induced adventitious root formation in
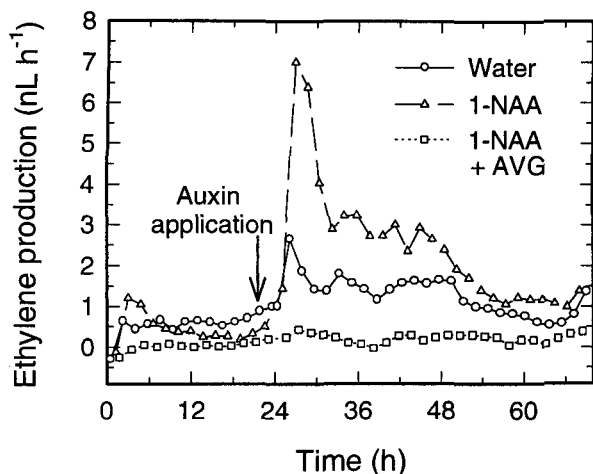
Visser et al.

Figure 3. Ethylene production (nL h^{-1}) of individual 4-week-old *R. palustris* plants brushed with water, 1-NAA (250 nmol in 0.3 mL of water per shoot), or 1-NAA (250 nmol in 0.3 mL water per shoot) and AVG (25 nmol in 0.3 mL water per shoot). Representative lines chosen from four plants per treatment are shown.
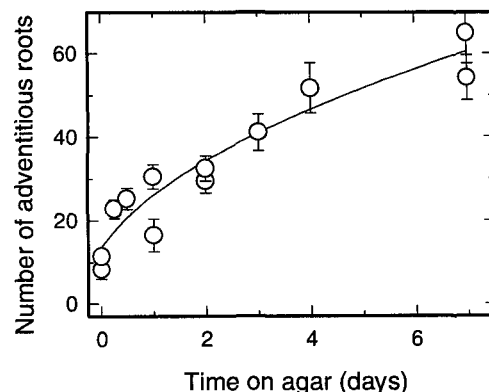
R. palustris, we quantified the levels of free IAA in the rooting zone during the time interval in which these roots were irreversibly induced. When *R. palustris* plants were transferred to hypoxic, stagnant conditions and then transferred back to aerated conditions, the formation of many adventitious roots was attained by 3 to 4 d of oxygen deficiency (Fig. 5). Even 6 h of hypoxia resulted in an increase in the number of adventitious roots formed during the subsequent period on aerated nutrient solution. Induction of the rooting process thus occurs shortly after the onset of flooding, and auxin concentrations in the root-forming zone were therefore determined during these first hours of hypoxia. In nontreated *R. palustris* plants, the IAA concentration remained between 50 and 70 ng free IAA g^{-1} fresh weight (Fig. 6). Plants transferred to deoxygenated agar also showed little variation in the level of auxin, and, surprisingly, the concentration did not differ from that of

Figure 4. Number of adventitious roots per plant formed by 4-week-old *R. palustris* plants 7 d after transferring the plants to fresh, well-aerated nutrient solution and brushing the shoots with either water (control) or 1-NAA (250 nmol in 0.3 mL of water per shoot) and without AVG pretreatment (250 nmol in 0.3 mL of water per shoot); *n* = 6; error bars indicate SES.

Figure 5. Number of adventitious roots per plant formed by 5-week-old *R. palustris* plants placed on an unstirred, deoxygenated agar solution for various times and subsequently transferred to aerated nutrient solution. Roots were counted 7 d after the onset of the treatment; *n* = 6; error bars indicate SES.

control plants. As expected, rooting was almost absent in control plants, whereas plants treated with agar had formed large numbers of adventitious roots 7 d after the onset of hypoxia (Table I).

It appears that during the first 2 d of flooding there is no change in the IAA concentration in the rooting zone that could signal the onset of adventitious root formation. Nevertheless, a certain level of IAA seems to be required for maximum adventitious root formation, since plants in which the shoot was pretreated with NPA demonstrated a decrease in the endogenous IAA concentration during the first 24 h of hypoxia (Fig. 6), followed by a strongly reduced formation of adventitious roots (Table I).
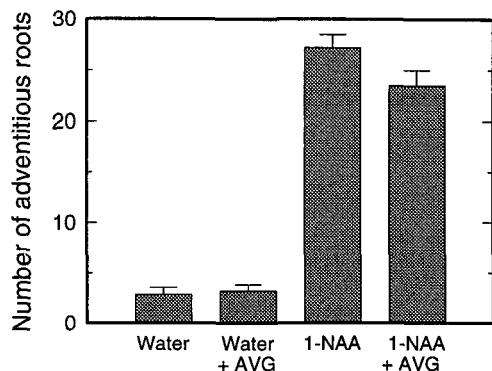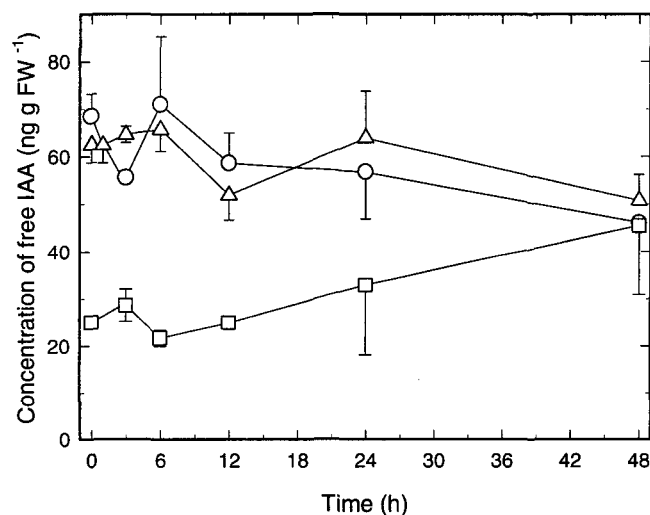

Figure 6. Time course of the concentration of free IAA (ng g^{-1} fresh weight) in the rooting zone of 5-week-old *R. palustris* plants transferred to either fresh, well-aerated nutrient solution (control; ○) or unstirred, deoxygenated agar without (△) or with (□) NPA pretreatment (150 nmol in 0.3 mL of water per shoot); *n* = 3 (each sample from two plants); error bars indicate SE values. FW, Fresh weight.

Table I. *Effect of NPA on flooding-induced adventitious root formation*

Number of adventitious roots per plant formed by 5-week-old *R. palustris* plants 7 d after transferring the plants to fresh, well-aerated nutrient solution or to an unstirred, deoxygenated agar solution with or without NPA pretreatment (150 nmol in 0.3 mL of water per shoot); n = 6; means ± SEs.

Treatment	No. of Adventitious Roots
Control	0.2 ± 0.2
Deoxygenated agar	81.0 ± 5.9
Deoxygenated agar + NPA pretreatment	52.2 ± 13.1

DISCUSSION

Flooding the soil strongly induces the formation of adventitious roots in the wetland species *R. palustris* (Visser et al., 1996a). The same response was observed in plants grown on a deoxygenated agar solution or treated with either ethylene or auxin (Fig. 1, A and B). Suboptimal doses or concentrations of either hormone resulted in the formation of fewer adventitious roots. However, when suboptimal amounts of ethylene and auxin were supplied simultaneously, a maximum response was obtained (Fig. 1C). An explanation for such similar effects of auxin and ethylene is the strongly enhancing effect auxin may have on ethylene production (Dubucq et al., 1978; Riov and Yang, 1989; Kelly and Bradford, 1990). We found an increased production of ethylene in auxin-treated plants (Fig. 3), but, in contrast to findings by Riov and Yang (1989), such plants continued to form adventitious roots when ethylene biosynthesis was almost completely inhibited by application of AVG (Fig. 4). Thus, it seems that, although the two hormones can each enhance rooting, auxin possesses activity that is largely independent of ethylene. Consequently, we anticipated that an accumulation of endogenous auxin in the root-forming zone during flooded conditions may be the key factor regulating adventitious root formation. This theory, first proposed by Kramer (1951) and later supported by Phillips (1964) and Wample and Reid (1979), corresponds with studies of nonflooding-related adventitious root formation in cuttings, in which increased IAA concentrations were found in the basal, root-forming part of the shoot (Maldiney et al., 1986; Blakesley, 1994). In *R. palustris*, however, free IAA did not accumulate in the rooting zone (Fig. 6). Therefore, adventitious root formation in *R. palustris* during flooding is not induced by a change in auxin concentration.

Our results do not exclude the possibility that flooding and the subsequent hypoxic conditions and ethylene accumulation affect auxin transport or metabolism in the roots. Slowing of the polar, energy-dependent transport of auxin was proposed as the main cause of the flooding-induced accumulation of auxin at the base of the shoot (Kramer, 1951; Visser et al., 1995) and subsequent adventitious root formation. In *R. palustris*, either hypoxic conditions do not develop in the rooting zone because of internal aeration via aerenchymatous tissues (Laan et al., 1990; Visser et al.,

1996a) or accumulation of IAA is prevented by a high metabolism and/or conjugation rate of free IAA.

The concentration of free IAA in control and rooting plants of *R. palustris* was high (approximately 60 ng g^{-1} fresh weight; Fig. 6) compared with the auxin concentration in maize roots (25 ng free IAA g^{-1} fresh weight; Ribaud and Pilet, 1994) and carrot roots (3.5 ng free IAA g^{-1} fresh weight; Guivarc'h et al., 1993). Such high concentrations are usually found only in shoots (Nordström and Eliasson, 1991) or roots of "rooty" mutants (King, 1994), and it is probably the proximity of the shoot apex (located within approximately 1 cm of the rooting zone) of the rosette-shaped *R. palustris* plant that causes these constitutively high levels of IAA. Despite the high concentration of endogenous auxin in nonflooded plants, the levels were not sufficiently high to induce adventitious root formation in *R. palustris*, since no constitutive adventitious root system was observed in this species. Only exceptionally high concentrations of auxin, obtained by applying a large dose of 1-NAA to the shoot, were able to induce adventitious root formation under normally aerated conditions (Fig. 1A; Visser et al., 1995). Endogenous auxin probably never reaches such high levels.

Still, the rooting process during flooding is not independent of endogenous auxin. Root formation appears to require a certain level of free IAA, because low endogenous concentrations in NPA-pretreated plants (Fig. 6) were followed by a decrease in the number of adventitious roots formed upon agar treatment (Table I). Therefore, a second factor must be involved that increases the sensitivity of the root-forming tissue to auxin. Our results show that this factor may be the large increase in ethylene concentration (up to 2 μL L^{-1}) that occurs in waterlogged root systems of *R. palustris* (Visser et al., 1996b). Ethylene alone could not evoke the rooting response, because ethylene-induced formation of adventitious roots was suppressed by NPA pretreatment (Fig. 2). Thus, ethylene treatment of nonflooded plants only resulted in the formation of adventitious roots (Figs. 1B and 2) when combined with a sufficiently high endogenous concentration of free IAA (such as is found in plants not pretreated with NPA; Fig. 6).

Our results are comparable to those of Liu and Reid (1992), who studied the rooting response of sunflower hypocotyl cuttings. In their system, too, auxin was the primary regulator in the induction of adventitious roots, and ethylene-stimulated rooting was mediated by a change in the plant's sensitivity to auxin. However, in their experiments, ethylene was applied to the cuttings to stimulate rooting, whereas in our case accumulation of endogenous ethylene was an intrinsic factor in the flooding event.

In conclusion, we reason from our results that the formation of adventitious roots in flooded *R. palustris* plants is preceded by a rapid and large increase in the endogenous ethylene concentration, whereas the endogenous auxin concentration does not change. The high ethylene concentration, which is caused mainly by the physical entrapment of ethylene in the submerged roots, sensitizes the root-forming tissue to auxin. This increased sensitivity to the

constitutively high concentrations of endogenous free IAA subsequently induces the formation of adventitious roots, which is necessary for the survival of flooded *Rumex* plants.

ACKNOWLEDGMENTS

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