Regulatory role of auxin in adventitious root formation in two species of *Rumex*, differing in their sensitivity to waterlogging

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Adventitious rooting in *Rumex* plants, in which the root systems were in hypoxic conditions, differed considerably between two species. *R. palustris*, a species from frequently flooded river forelands, developed a large number of adventitious roots during hypoxia, whereas adventitious root formation was poor in *R. thyrsiflorus*, a species from seldom flooded dykes and river dunes. Adventitious rooting could also be evoked in aerated plants of both species by application of auxin (1-naphthaleneacetic acid or indoleacetic acid) to the leaves. The response to auxin was dose-dependent, but even high auxin doses could not stimulate *R. thyrsiflorus* to produce as many adventitious roots as *R. palustris*. Consequently, the difference between the species in the amount of adventitious root formation was probably genetically determined, and not a result of a different response to auxin.

A prerequisite for hypoxia-induced adventitious root formation is the basipetal transport of auxin within the shoot, as specific inhibition of this transport by N-1-naphthylphthalamic acid severely decreased the number of roots in hypoxia-treated plants. It is suggested that hypoxia of the root system causes stagnation of auxin transport in the root system. This can lead to an accumulation of auxin at the base of the shoot rosette, resulting in adventitious root formation.

Key words – Adventitious roots, auxin transport, flooding, hypoxia, IAA, NAA, NPA, *Rumex palustris*, *Rumex thyrsiflorus*.

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Introduction

Adventitious rooting is a characteristic adaptive response of plants to conditions in which the primary root system can not function properly. Waterlogged plants often develop this type of roots, which may assist survival of the stressful situation of oxygen deficiency encountered when there is excessive water in the soil (Jackson and Drew 1984). In most species the flood-induced adventitious roots contain highly porous tissues (Jackson et al. 1985). This aerenchymatous tissue facilitates the diffusion of gases from shoot to roots (oxygen) and vice versa (carbon dioxide, methane, ethylene) and enables plants to grow in hypoxic and anoxic soils (Armstrong 1979, Drew 1992).

However, not all plant species form these new roots when waterlogged, and even within one genus, the ability to develop adventitious roots can differ considerably. An intriguing example is the genus *Rumex*, which includes both flood-resistant and flood-sensitive species (Blom et al. 1990, Voesenek et al. 1993). The flood-resistant species typically produce far more adventitious roots with more aerenchyma than flood-sensitive species (Laan et al. 1989). The number of new roots developing during waterlogged conditions correlates well with the elevation of the habitat of the species in river flood plains characterized by irregular flooding (Blom et al. 1993).

The underlying mechanisms that explain why only some plant species develop large numbers of adventitious roots during waterlogging, are not yet understood. A
similar phenomenon occurs in stem cuttings of plants. Cuttings of many herbaceous plant species develop large numbers of adventitious roots within days or weeks (e.g. Robbins et al. 1985, Bollmark et al. 1988, Liu and Reid 1992) and are consequently easy to propagate. However, cuttings of a considerable number of plant species only root under certain very strict conditions (Geneve 1991, Howard 1994). Both naturally occurring auxins like IAA and indole-3-butyric acid, and synthetic auxins such as 1-NAA and 2,4-dichlorophenoxyacetic acid are able to overcome the inhibition of root formation or shorten the time to the onset of adventitious root development in many plant species (e.g. Jarvis and Shaheed 1986). Nevertheless, although auxin appears to be crucially important in the rooting process, many of the other presently known plant growth substances have also been shown to either stimulate or inhibit rooting of cuttings (Fabijan et al. 1981, Selby et al. 1992).

Also in waterlogged plants a variety of plant growth regulators have been considered to be of crucial importance in the process of adventitious root formation. Again, much attention has focused on the role of auxin in flood-induced adventitious rooting. Phillips suggested already in 1964 that waterlogging might cause a stagnation of auxin transport in the oxygen deficient root system of waterlogged sunflower plants, resulting in the accumulation of auxin at the stem base. This hypothesis was supported by the findings of Wample and Reid (1979), who demonstrated a similar mechanism in sunflower.

In the present study, an attempt has been made to unravel the importance of auxin transport and action in adventitious root formation of waterlogged plants. To achieve this, two Rumex species, which have contrasting abilities to develop adventitious roots during waterlogging, were compared. One of the species, R. palustris Sm., inhabits regularly flooded sites in river forelands and develops large numbers of adventitious roots when flooded, while R. thrysiflorus Finger., which only grows on rarely flooded river dunes and dykes, produces much fewer adventitious roots (Blom et al. 1994). Our aim was to determine (1) whether auxin is involved in this rooting process, and if so, (2) whether differences in adventitious root formation between the two species can be attributed to a different response to auxin or to contrasting genetically determined capacities to form adventitious roots. Finally, (3) experiments were performed to establish the importance of auxin transport in the event of adventitious root formation in Rumex.

Abbreviations – 1-NAA, 1-naphthylacetic acid; 2-NAA, 2-naphthylacetic acid; NPA, N-1-naphthylphthalamic acid.

Materials and methods

Plant growth

Seeds of R. palustris and R. thrysiflorus were collected from the forelands of the river Waal near Nijmegen (The Netherlands) and sown in flat plastic trays, half-filled with black polyethylene grains (Lacqisene Low Density grains, Elf Atochem, France). The seeds were soaked with nutrient solution containing 2 mM Ca(NO₃)₂, 1.25 mM K₂SO₄, 0.5 mM MgSO₄, 0.5 mM KH₂PO₄ and micronutrients: FeEDTA (90 μM), NaCl (50 μM), H₃BO₃ (25 μM), MnSO₄ (2 μM), ZnSO₄ (2 μM), CuSO₄ (0.5 μM) and H₂MoO₄ (0.5 μM). The tray was covered with a glass plate and incubated in a climate room [16 h 20 μmol m⁻² s⁻¹ PPFD (Philips TL33), 27°C; 8 h dark, 10°C] for one week. Thereafter, the seedlings were placed in a growth chamber with 16 h 120 μmol m⁻² s⁻¹ PPFD (Philips TL84), 22°C and 8 h dark, 20°C (relative humidity 50%), and transferred to hydroponic culture after two or three weeks.

Each hydroponic flow-through unit consisted of three 20-l containers connected with a 30-l aeration vessel (120 l air h⁻¹), through which nutrient solution circulated at a rate of 60 l h⁻¹ container⁻¹. Six plants per container were grown for one or two weeks before treatments were started (conditions as described for the growth chamber).

Hypoxic treatment

Plants were transferred from the aerated hydroponic culture to a 20-l container with unstirred agar solution [0.1% (w/w), high gel strength; nutrient concentrations as described above], through which nitrogen gas had been bubbled for at least 16 h prior to the transfer. Control plants were placed on containers containing nutrient solution, aerated continuously at a rate of 60 l air h⁻¹.

Treatment with growth substances

IAA, 1-NAA, 2-NAA (Merck) and NPA (prepared by the Dept of Organic Chemistry, Univ. of Nijmegen, The Netherlands, at a purity of 99%) were dissolved in a small volume of ethanol, diluted with water and then sprayed with a spray gun onto the leaves of the plants. Volumes larger than 1 ml per plant were supplied in two successive treatments with a 2 h interval. Control plants were sprayed with similar amounts of ethanol in water [ca 0.2% (v/v)].

In an additional experiment, IAA was applied in 100 μl lanolin at the base of the shoot rosette. Control plants were treated with lanolin without IAA.

Plant parameters

Depending on the type of experiment, the number of roots from each plant was counted 7 or 11 days after application of the growth substances or after the onset of the hypoxia treatment. Dry weights of adventitious roots, primary roots, tap root and shoot were determined after drying for 24 h at 105°C.
Time after transfer to agar (days)

80 -
60 -
40 -
20 -
0  

Time after application of 1-NAA (days)

80 -
60 -
40 -
20 -
0

Fig. 1. Adventitious root formation in *R. palustris* (open symbols) and *R. thyrsiflorus* (closed symbols) after (A) transferring the plants from aerated hydroponic culture to hypoxic (0.1%, w/w) agar solution or (B) spraying the leaves with 10 nmol (○, ●) or 100 nmol (△, ▲) 1-NAA per shoot when treatments began. At that time *R. palustris* plants were 5 1/2 weeks or 4 weeks old and *R. thyrsiflorus* plants were 4 weeks or 4 weeks old in (A) and (B), respectively; n = 6 (A) or 3 (B); bars represent SE.

**Radiolabelling**

Petioles were sampled from young, full-grown leaves of *R. palustris* or *R. thyrsiflorus* and cut into 15-mm segments. NPA-treated petioles were wrapped in tissue paper soaked with 100 μM NPA (adjusted to pH 5.6 with KOH) 24 h before cutting; control petioles in this experiment were treated similarly with tissue paper soaked in water.

Experiments were started by placing an agar cube (1%, w/v; 5 x 5 x 3 mm) containing ca 300 Bq 1-[3H]-NAA (donor; specific activity 185 TBq mol⁻¹) generously supplied by the Dept of Experimental Plant Science, Univ. of Nijmegen, The Netherlands and manufactured by Amersham, Slough, UK) on one end of the petiole cutting, and an agar cube without auxin (receiver) on the opposite side. After 24 h incubation (22°C, relative humidity 100%, dark) the cuttings were divided into 3 or 4 equal parts, which were extracted twice with methanol for at least 6 h at 20°C. The extracts were dried and counted in 4.5 ml Lumagel (Lumac LSC) with a Wallac 1410 Liquid Scintillation Counter; results were corrected for quenching. Recovery of the label was at least 98%.

For determination of the amount of free 1-NAA, petiole cuttings of *R. palustris* were incubated with a donor and a receiver block according to the method described above (donor containing 1.1 kBq 1-[3H]-NAA). After 24 h, petioles were extracted twice with methanol overnight at ~20°C. Extracts were dried under nitrogen at 40°C, redissolved in 50 μl methanol and chromatographed according to Smulders et al. (1990). The position of 1-[3H]-NAA was found by cochromatography of this compound in a separate lane. The silica gel was scraped off and counted in 4.5 ml Lumagel. The amount of free 1-NAA was expressed as percentage of the total label of the extract.

**Results**

**Hypoxia and auxin treatments**

The first adventitious roots developed in both species within 2 days of hypoxia of the root system (Fig. 1A). After 6 to 10 days, the number of roots formed by *R. palustris* was 4 to 5 times higher than in *R. thyrsiflorus*. The majority of adventitious roots developed at the most basal part of the shoot, and at the upper 2 cm of the tap root. All adventitious roots were thick and unbranched for at least the first 15 cm behind the root apex; adventitious roots of *R. palustris* were generally thicker than those of *R. thyrsiflorus* (data not shown).

**Fig. 2.** Adventitious root formation after spraying leaves of 4-week-old *R. palustris* plants with different concentrations of 1-NAA (1.5 ml per plant). The number of roots was counted 7 days after treatment; n = 10; bars represent SE; curve drawn by sigmoidal best fit.
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Spraying the shoot of aerobically grown plants with 1-NAA closely mimicked the results of the hypoxia treated plants (Fig. 1B). However, after auxin treatment the adventitious roots tended to arise more scattered along the tap root instead of being localized almost exclusively at the shoot base and upper part of the tap root. No difference was observed between the 10 and 100 nmol 1-NAA treatments in the rooting response of either species.

Root initiation by 1-NAA was strongly dose-dependent, as is shown for *R. palustris* in Fig. 2. The maximum response in this experiment was obtained at 25 nmol 1-NAA per shoot, whereas at 15 nmol hardly any roots developed. It should be noted that replicate experiments showed some variation in the amount of 1-NAA that caused the maximum response. This depended primarily on the size of the shoot; however, in all replicate experiments auxin doses that caused no effect and those that caused the maximum response were invariably within the same order of magnitude. The higher auxin doses had a typical effect on shoot morphology; leaves curled downwards and petioles elongated within two days after spraying.

Adventitious root formation was restricted to treatments with 1-NAA. In an experiment where 4-week-old *R. palustris* plants were sprayed with either water, 1-NAA or an isomer without auxin action, 2-NAA (250 nmol 1-NAA and 2-NAA per plant; n = 6), the number of adventitious roots per plant 11 days after treatment was 0.8 (SE ± 0.5), 27.7 (SE ± 3.7) and 0.2 (SE ± 0.2), respectively.

Application of IAA to 4-week-old *R. palustris* plants as a foliar spray caused leaf growth responses (i.e. elongation and downward curling) similar to those produced by 1-NAA, but failed to stimulate adventitious root formation. The number of roots was only comparable to plants treated with 1-NAA and hypoxia, when 10 μmol IAA in 0.1 ml lanolin was supplied directly to the site where adventitious roots normally appear, i.e. the base of the shoot rosette. Seven days after this treatment the number of adventitious roots was 21.0 (SE ± 1.8), com-

Fig. 3. Distribution of label in 1.5 cm petiole cuttings of *R. palustris* (A) and *R. thyrsiflorus* (B) 24 h after supplying 140 Bq 1-[14]H]-NAA to the apical side of the cutting; n = 10; bars represent SE.

Fig. 4. Distribution of label in 1.5 cm petiole cuttings of *R. palustris* 24 h after supplying 300 Bq 1-[14]H]-NAA to (A) the apical or (B) basal side of the cutting; n = 10; bars represent SE.
Twenty-four hours after 1-[\(^{3}H\)]-NAA was supplied to the apical end of \textit{R. palustris} or \textit{R. thyrsiflorus} petioles, a significant amount of radioactivity had been transported to the middle and basal parts of the petiole. The amount of label transported did not differ between the two species (Fig. 3). Transport of label in the reverse direction was almost non-existent in \textit{R. palustris} when radioactive auxin was given to the basal end of the petiole (Fig. 4). Analysis of the contribution of unmetabolized 1-NAA to the total amount of radioactivity revealed that 24 h after application of 1-[\(^{3}H\)]-NAA to the apical end of petioles of \textit{R. palustris} still 17.5\% (\(se \pm 8.8\)) of the label consisted of free 1-NAA (\(n = 4\)).

Figure 5 shows that transport of label from apical to basal parts could be effectively inhibited by pretreatment of the \textit{R. palustris} petioles with NPA. Although 4-week-old \textit{R. palustris} plants normally developed a large number of adventitious roots when grown in hypoxic agar, a severe reduction in adventitious root formation was observed when NPA was supplied to the leaves before hypoxic treatment of the roots. The number of adventitious roots 10 days after transfer to hypoxic agar decreased from 41.0 (\(se \pm 6.7\)) in untreated plants to 12.2 (\(se \pm 1.7\)) in plants which were sprayed with 27 nmol NPA per shoot (\(n = 6\)).

**Discussion**

The two \textit{Rumex} species studied showed a clear difference in root formation (Fig. 1A). \textit{R. thyrsiflorus}, being sensitive to flooding, produced only a small number of adventitious laterals (see also Laan et al. 1989). In contrast, the flood-tolerant \textit{R. palustris} developed a large number of adventitious roots, which was noted earlier but not quantified by Voesenek et al. (1989). A very similar response was shown for the closely related species \textit{R. maritimus} (Laan et al. 1989). The adventitious roots of \textit{R. palustris} were thicker than adventitious roots of \textit{R. thyrsiflorus} and laterals of the primary root system, due to highly aerenchymatous cortical structures (cf. Laan et al. 1989).

Auxin, in our case 1-NAA or IAA, was able to mimic the effects of hypoxia on adventitious root formation (Fig. 1B). The number of roots after the auxin treatment was comparable in both species to the number developed during the hypoxic treatment. Even very high auxin doses (100 nmol per plant) could not further increase adventitious rooting in \textit{R. thyrsiflorus} (Fig. 1B), which suggests a difference in the root forming capacity between the species rather than a difference in sensitivity to auxin. The limiting factor, relating to this capacity, may be the maximum number of root primordia that can be formed in \textit{R. thyrsiflorus}. This species has a much lower overall relative growth rate than \textit{R. palustris}. Therefore, if the number of primordia is related to e.g. the number of leaves (or nodes; Barlow 1994), \textit{R. palustris} plants should develop far more primordia than \textit{R. thyrsiflorus} plants of the same age.

The specificity of the auxin effect was illustrated by the treatment of \textit{R. palustris} with 2-NAA. This compound has a structure highly similar to 1-NAA, but has no auxin action (Smulders et al. 1989), and consequently did not produce a rooting response. \textit{R. palustris} also did not develop adventitious roots when IAA was supplied to the plants either via the shoots as a foliar spray or via the roots dissolved in nutrient solution. Adventitious rooting could only be induced by high concentrations of IAA, applied as close as possible to the rooting zone. These levels exceeded physiological concentrations largely and, therefore, we presume that most of the free acid of applied auxin did not reach the site of action, i.e. the location of adventitious root initiation. The bulk of applied IAA was probably degraded and conjugated into
derivatives with no auxin action. High exogenous concentrations of free IAA can be reduced very effectively by plant tissues (Eliasson 1972, Yang et al. 1993) and even in the nutrient solution on agar (Bhatnagar and Tillberg 1982).

A variety of developmental processes in plants have been related to auxin transport and the ensuing auxin gradients set up in plant tissues. Auxin transport in herbaceous plants can occur through the parenchyma cells of the cortex, but also vascular transport has been found (Eliasson 1972). Auxin transport in isolated petioles did not differ between R. palustris and R. thyrsiflorus (Fig. 3) and proved to be strongly polar: application of tritiated 1-NAA to the apical part of R. palustris petioles resulted in a significant transport of label in a basipetal direction, whereas 1-NAA given to the basal side was not transported at all (Fig. 4). Furthermore, auxin transport in R. palustris was very susceptible to NPA (Fig. 5), which is a very specific inhibitor of the auxin transport carrier (Thomson et al. 1973). These results, however, do not preclude that in intact plants auxin is transported through the vascular system as well, since both xylem and phloem transport are not functioning in petiole explants.

At the end of our experiments, a large part of the label was no longer present in the tissue as free auxin. However, Fig. 5B shows that diffusion plays only a minor role in the distribution of the label in the petiole, so we can assume that the distribution patterns shown in Figs 3–5 were the result of transport of the free auxin. In general, auxin carrier transport is considered to be highly specific and not to transport auxin metabolites.

Adventitious rooting in flooded plants has been related to an inhibition of auxin transport in the plant (Kramer 1951). After the onset of waterlogging, oxygen concentrations in the roots can drop to very low values (Jackson and Drew 1984) and auxin transport in the roots is likely to be severely limited. Several studies have reported an accumulation of auxin at the stem base when root systems were in waterlogged media (Phillips 1964, Wample and Reid 1979). Also in cuttings adventitious root formation has been related to auxin accumulation in the rooting zone (Maldiney et al. 1986, Blakesley 1994).

Our results indicate that auxin transport from auxin-producing shoot parts to the base of the shoot is required for adventitious root formation. Application of NPA to the shoot prevented auxin transport to the shoot base, thus severely reducing hyphoxia-induced rooting.

In conclusion, our results have demonstrated that (1) auxin is a major contributing factor in regulating adventitious rooting during waterlogging of Rumex species. (2) Adventitious rooting differs between these species, with R. palustris always developing more adventitious roots than R. thyrsiflorus. This is not due to a difference in the sensitivity to auxin, but rather to a difference in the capacity of the species to develop adventitious roots. (3) Finally, auxin transport appears to be the key factor in the rooting response, since the stimulation of adventitious root formation by hyphoxia can be prevented by simulta-

neous application of an auxin transport inhibitor to the shoot.

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