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The contrasting role of auxin in submergence-induced petiole elongation in two species from frequently flooded wetlands

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The involvement of auxin in the submergence-induced petiole elongation has been investigated in *Rumex palustris* and *Ranunculus sceleratus*. Both wetland species are capable of enhanced petiole elongation upon submergence or treatment with exogenous ethylene (5 μl l⁻¹). Treatment of intact *Rumex palustris* plants with 1-naphthalene acetic acid (NAA) at 10⁻⁴ M enhanced petiole elongation, while treatment with N-1-naphthylphthalamic acid (NPA) had no effect on petiole elongation. The elongation response after NAA or NPA treatment was comparable for plants in both submerged and drained conditions. Pre-ageing of detached petioles of *Rumex palustris* for 3 h in light or in dark conditions had no effect on the submergence-induced elongation. In comparison to intact plants, detached petioles of *Rumex palustris*, with or without lamina, did not show significant differences in responsiveness to IAA between drained or submerged conditions. This was in contrast to *Ranunculus sceleratus* where submergence caused a clear increase in responsiveness towards IAA. Removal of the lamina, the putative source of auxin, or treatment with NPA did not hinder the submergence-induced elongation of detached *Rumex palustris* petioles, but severely inhibited elongation of detached *Ranunculus sceleratus* petioles. This inhibition could be restored by application of NAA, suggesting the specific involvement of auxin in the submergence response of *Ranunculus sceleratus*. It is concluded that, in contrast to *Ranunculus sceleratus*, auxin is probably not involved in the submergence-induced petiole elongation of *Rumex palustris*.

Key words – Auxin, ethylene, *Ranunculus sceleratus*, *Rumex palustris*, submergence.

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Introduction

One of the adaptative features of plants to submergence is the stimulated elongation of petioles, stems or inter-nodes, enabling plant structures to emerge from the water. This elongation is causally related to increased levels of endogenous ethylene found under submerged conditions (Ku et al. 1970, Musgrave et al. 1972, Voesenek et al. 1993) and leads to a relief of oxygen deficits, thus ensuring survival and sexual reproduction (Van der Sman et al. 1991, Voesenek and Van der Veen 1994). Enhanced petiole elongation and its relation to ethylene has been well described for the flooding resistant rosette plant *Rumex palustris*, occurring at low-elevation, frequently-flooded sites in river floodplains (Blom et al. 1994).

However, the mechanism by which ethylene stimulates shoot elongation in *Rumex palustris* is still largely unknown. For a number of aquatic and semi-aquatic species (*Regnellidium diphylhum*, Walters and Osborne 1979; *Ranunculus sceleratus*, Horton and Samarakoon 1982; *Nymphoides peltata*, Malone and Ridge 1983) the presence of auxin has been shown to be prerequisite for optimal submergence-induced elongation. The similarity between *Rumex palustris* and *Ranunculus sceleratus* in submergence response, growth form (both are dicotyledonous rosette species) and habitat (frequently flooded sites) led to the assumption that auxin would also play a role in *Rumex palustris*. Therefore, the overall aim of this study was to determine whether auxin was also involved in submergence-induced petiole growth of...
Rumex palustris. In order to compare and validate our results, both Rumex palustris and Ranunculus sceleratus were used in similar experiments, since for the latter species auxin involvement has been clearly demonstrated (Musgrave and Walters 1973, Cookson and Osborne 1978, Horton and Samarakoone 1982, Smulders and Horton 1991). The main objectives were to determine whether (1) the submergence-induced petiole elongation of Rumex palustris can be arrested by application of an auxin transport inhibitor, ageing of petiole tissue or removal of the lamina, (2) submergence results in a change in petiole responsiveness of Rumex palustris towards auxin, and (3) the involvement of auxin in the submergence response of Rumex palustris and Ranunculus sceleratus is comparable.

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

Materials and methods

Chemicals

Indoleacetic acid (IAA) and 1-naphthaleneacetic acid (NAA) were purchased from Sigma Chemical Company. N-1-naphthylphthalamic acid (NPA) was synthesized in the Dept of Organic Biochemistry at the Univ. of Nijmegen. IAA, NAA and NPA were first dissolved in a few drops of ethanol (96%). Controls received an equal amount of ethanol. Ethylene (Hoekloos, mixture in medical air) was applied at a growth saturating concentration of 5 μl l⁻¹ (Voessen and Blom 1989).

Plant material

Seeds (achenes) of Rumex palustris, collected in river floodplains, were germinated on moistened filter paper in Petri dishes for 10 days in a germination cabinet (16-h photoperiod, day/night temperature of 25/10°C, photosynthetic photon flux density, PPFD, of 30 μmol m⁻² s⁻¹ from fluorescent lamps, Philips TL 8W/33). Seedlings were planted in plastic pots (volume 60 ml) filled with a mixture of sand and potting compost (1:1, v/v). Seedlings of Ranunculus sceleratus were planted in plastic pots (volume 150 ml) filled with the same substrate. Both plant species were grown for 4–5 weeks in a growth chamber (16-h photoperiod, day/night temperature of 21/15°C, PPFD of 95 μmol m⁻² s⁻¹ from fluorescent lamps, Philips TLD 58W/84). Plants were selected for experiments on the basis of homogeneity of developmental stage of the 5th leaf.

Submergence and ethylene response

Intact Rumex palustris and Ranunculus sceleratus plants were submerged in 200-l tanks filled with tapwater (22°C) placed in the greenhouse (16-h photoperiod, day/night temperature of 22/16 ± 2°C) with additional illumination (150 μmol m⁻² s⁻¹ at water surface). The plants were approximately 50 cm below the water surface. Exogenous ethylene was applied in an air-tight growth cabinet, located in the greenhouse, at ambient temperature and with additional illumination (80 μmol m⁻² s⁻¹ from fluorescent lamps, Philips TL 8W/33, 16-h photoperiod, day/night temperature of 23/17 ± 2°C). Enough pure ethylene was initially injected into the cabinet to give a final concentration of 5 μl l⁻¹ ethylene in air. Subsequently, ethylene at a concentration of 5 μl l⁻¹ in air was then flushed through the system at a rate of 20 l h⁻¹. Drained plants were maintained in the greenhouse. Petiole elongation was measured after 48 h in leaves of three different developmental stages: (1) the youngest leaf with still unfurled lamina, (2) an intermediate stage with partly unfurled lamina, and (3) an old leaf with fully-expanded lamina.

Auxin and NPA dose response experiments

Intact Rumex palustris plants were submerged in 10 mM K-phosphate buffer (pH 6.0, tapwater) or in buffered NAA (10⁻⁵–10⁻⁴ M) or NPA (10⁻⁵–10⁻⁴ M) solutions in 20-l glass containers. Drained plants were sprayed until runoff once every 24 h with the same solutions used for submergence. Both submerged and drained plants were kept in the growth chamber mentioned in Plant material. Elongation of petioles of leaves in the intermediate stage was measured 48 h after the onset of experiments with a ruler to the nearest 0.5 mm.

Experiments with detached petioles

Ranunculus sceleratus petioles were detached according to Samarakoone and Horton (1983) by removing a leaf with fully expanded lamina and cutting off the petiole at 25 mm from the lamina. For drained conditions of detached petioles with lamina attached, petioles were inserted through Parafilm, spread over a 100-nil beaker, into the test solution, thus leaving the lamina in air (cf. Musgrave and Walters 1973). Petioles without lamina were floated as another drained control. For submerged conditions, petioles (with or without lamina) were weighted by gently clasping the petiole in a latex serum cap and submerged in 1 l of test solution in a 1-l glass beaker.

For Rumex palustris a different method was followed. Preliminary experiments showed that floating or partly submerged petioles also displayed enhanced elongation and that removal of the root system had no effect on the enhanced petiole elongation at submergence (results not shown). Detached Rumex palustris petioles were obtained by removal of the leaves 1–4 (petiole and lamina), leaf 6 and the apex. The root system was cut off approximately 10 mm below the rosette node and laterals on the remaining part of the tap root were removed. This left the 5th petiole and lamina attached to the rosette node and ca 10 mm tap root. The tap root remainders of the plantlets were put in latex serum caps. These serum caps were mounted in a PVC plate and placed in a 1-l
glass beaker. The beaker was filled to root level for drained experimental conditions (ca 200 ml) or with 1 l of test solution for submergence.

For *Rumex palustris*, ageing of detached petioles without lamina was performed by positioning the petioles upright with the root remainder in distilled water in 1-l glass containers covered with plastic foil or wrapped in aluminium foil for dark treatment. For *Ranunculus sceleratus*, petioles were floated on distilled water. In all experiments with detached petioles, with or without lamina attached, plant material was aged for 3 h.

Elongation of petiole segments of detached *Rumex palustris* and *Ranunculus sceleratus* petioles was measured by making marks with waterproof ink every 2 mm. Petioles were either kept drained or submerged in K-phosphate buffer (10 mM, pH 6.0) with and without lamina. Segment length was measured again after 48 h with a ruler to the nearest 0.5 mm.

For other experiments with detached petioles, test solutions were 10⁻²–10⁻⁵ M IAA in 10 mM K-phosphate buffer (pH 6.0) and 10⁻⁴ M NAA, 10⁻⁵ M NPA or a combination of NAA/NPA in 10 mM K-phosphate buffer (pH 6.0). Ethylene (5 μl l⁻¹) was applied continuously in a flow-through system at 1 h⁻¹ in gas-tight 2-l containers with an in- and outlet port and a sealed port for gas analysis. Unless stated otherwise, all experiments lasted 48 h. Petiole length was measured with a ruler to the nearest 0.5 mm at the beginning and end of the experiments.

**Statistics**

For each experiment 10–12 replicates were used unless stated otherwise. Experiments were repeated at least once. Representative data are shown. Differences between the means of the treatments were assessed with Tukey and LSD multiple comparison procedures after ANOVA.

**Results**

**Submergence and ethylene**

Submergence and exposure to 5 μl l⁻¹ ethylene significantly enhanced elongation for nearly all developmental stages of both species (Fig. 1). In *Rumex palustris*, treatment with ethylene partly mimicked the submergence response. In *Ranunculus sceleratus* petiole elongation of all three developmental stages was significantly higher upon ethylene treatment compared to submergence (Fig. 1). In both species petiole elongation upon submergence was most pronounced in leaves at the intermediate developmental stage. Therefore, this developmental stage was used in subsequent experiments.

**Effect of NAA and NPA on petiole elongation of intact plants**

Intact *Rumex palustris* plants were submerged in buffered aqueous solutions of 10⁻⁶–10⁻⁴ M NAA while drained plants were sprayed daily with an identical range of NAA concentrations. There was no significant petiole elongation response to auxin at 10⁻⁶ and 10⁻⁵ M NAA over a 48-h period (Fig. 2A). Elongation was significantly stimulated by 10⁻⁴ M NAA and this effect was comparable in both submerged and drained conditions.

In *Rumex palustris* plants, application of the auxin transport inhibitor NPA did not affect growth at concentrations of 10⁻⁷ to 10⁻⁴ M, whether sprayed on drained plants or for plants submerged in NPA solution (Fig. 2B). Extra pretreatment with NPA 3 h before submergence in a NPA solution of the same concentration produced comparable results as shown in Fig. 2B (data not shown).

However, it can not be excluded that high endogenous auxin levels interfered with exogenous auxin application and concealed these results. Therefore, in subsequent experiments petioles were detached and aged for 3 h in an attempt to minimize endogenous auxin levels (cf. Malone and Ridge 1983).

**Effect of IAA, NAA and NPA on elongation of detached petioles**

Detachment of *Rumex palustris* petioles reduced the response towards ethylene and submergence when compared in both submerged and drained conditions, submergence and treatment with 5 μl l⁻¹ ethylene. Young leaves had furled lamina, lamina of intermediate leaves were expanding and lamina of old leaves were fully expanded. Experiment lasted for 48 h. Different letters indicate significant difference of means (n=10; +SE) within leaf stages (ANOVA, P<0.05).
Fig. 2. Effect of NAA (A) and NPA (B) on petiole elongation of submerged and drained intact *Rumex palustris* plants (n=10; ±SE) over a 48-h period. Plants were either sprayed with NAA or NPA in 10 mM P-buffer (pH 6.0) or submerged in the same solutions. All solutions, including controls contained 0.1% ethanol. Spraying was repeated after 24 h. Bars represent SE for each treatment (P<0.05).

These results showed a significant increase in petiole elongation when the plants were submerged. Nevertheless, a significant growth stimulation upon submergence and ethylene treatment was noticed, permitting the use of detached petioles as a model system.

Detached *Rumex palustris* petioles without lamina were aged for 3 h in light and in dark by standing petioles with the root in water. Ageing was found not to have a significant effect on petiole elongation of this species over a 48-h period at either submerged or drained conditions (Tab. 2). Floating of the petioles on distilled water or age-times of 1 or 6 h produced similar results (data not shown), which could indicate that the submergence response is not depending on metabolites (e.g. auxin produced outside the petiole).

To further analyze petiole elongation in *Rumex palustris* and *Ranunculus sceleratus*, 6 segments were marked on detached petioles from top to base to establish which part of the petiole elongates most. In drained petioles, each segment elongated more or less to the same extent in both species (Fig. 3). Upon submergence, the elongation of *Rumex palustris* segments remained more or less equal, while *Ranunculus sceleratus* segments closest to the lamina elongated significantly more than basal segments. Removal of the lamina did not affect this pattern in petiole elongation for either species, but drastically reduced overall elongation in *Ranunculus sceleratus*. This in contrast to *Rumex palustris*, where petiole elongation response only was slightly reduced (Fig. 3).

Detached petioles of *Rumex palustris* were less responsive to applied IAA than those of *Ranunculus sceleratus* (Fig. 4). Submergence increased the responsive-
petioles with lamina also suppressed petiole elongation significantly. This growth reduction could be overruled by application of $10^{-5} \text{M} \text{NAA}$, thus showing the specific involvement of auxin in the submergence response of *Ranunculus sceleratus*.

**Discussion**

*Rumex palustris* and *Ranunculus sceleratus* are both dicotyledonous rosette plants occurring in river floodplains at frequently flooded sites. Both species are adapted to flooding by their ability to initiate rapid petiole elongation, especially those of the younger leaves. This elongation response can be mimicked to a large extent by exogenous application of ethylene and these results are in agreement with earlier reports (Musgrave and Walters 1973, Ridge 1985, Voesenek and Blom 1989).

While the morphological response upon submergence is similar for *Rumex palustris* and *Ranunculus sceleratus*, the results of the present study demonstrate that the involvement of auxin clearly differs in these two species. In *Rumex palustris* the responsiveness of petioles to exogenously applied auxin did not differ between drained or submerged conditions and the response was

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Fig. 4. Effect of IAA on elongation growth of detached *Rumex palustris* and *Ranunculus sceleratus* petioles with and without lamina. Petioles were incubated for 48 h in 10 mM P-buffer (pH 6.0) with 0 to $10^{-5} \text{M} \text{IAA}$. All solutions, including controls, contained 0.1% ethanol. IAA was applied as submergence medium and as spray for drained *Rumex palustris*. Initial petiole length was trimmed to 25 mm for *Ranunculus sceleratus* and 23 mm ($\pm$0.4) for *Rumex palustris*. Points represent the means ($\pm$SE) of 10–12 petioles. Bars represent LSD for each treatment ($P<0.05$). ANOVA showed significant interaction ($P<0.05$) for submergence and drained treatment with IAA only for *Ranunculus sceleratus*, either with or without lamina.

To investigate the importance of auxin transport, detached petioles of both species were submerged in either NPA, NAA or a combination of NPA and NAA (Fig. 5). Application of $10^{-6} \text{M} \text{IAA}$ brought back the response of *Ranunculus sceleratus* to those levels found with attached lamina (Fig. 4). A significant interaction (ANOVA, $P<0.05$) for submergence treatment with IAA, indicating a change in responsiveness to IAA upon submergence, was only found for *Ranunculus sceleratus*, either with or without lamina.

To determine the involvement of auxin in the submergence response, detached petioles of both species were submerged in either NPA, NAA or a combination of NPA and NAA (Fig. 5). Application of $10^{-5} \text{M} \text{NPA}$, $10^{-5} \text{M} \text{NAA}$ or a combination of NPA and NAA to detached *Rumex palustris* petioles led to a small but significant increase in elongation compared to controls. Removal of the lamina of detached *Rumex palustris* petioles reduced the overall elongation response but had no effect on the response to applied NPA and NAA. As shown before, removal of the lamina of *Ranunculus sceleratus* reduced the elongation of detached petioles. NPA applied to *Ranunculus sceleratus*
generally small (Figs 2 and 4). Petioles of *Ranunculus sceleratus* were found to be more responsive in general to exogenous auxin, either when drained or submerged (Figs 4 and 5 this report; Samarakoon and Horton 1983, Smulders and Horton 1991), and responsiveness was further enhanced by submerged conditions. The small elongation response towards auxin in *Rumex palustris* could be explained by the assumption that (1) the applied exogenous auxin does not reach auxin responsive cells, (2) sufficient endogenous auxin is present or (3) the tissue is not very sensitive in this respect for auxin. Floating of petioles on $10^{-4}$ M buffered NAA or IAA solution caused a 10- and 25-fold increase, respectively, in ethylene production compared to the control (results not shown). Furthermore, submergence of petioles in NAA or IAA caused a swelling of the petiole tissue that showed a strong linear relationship with the applied NAA or IAA concentration (results not shown). Both phenomena demonstrate that auxin is perceived by the petiole tissue even at low exogenous concentrations. Since intact plants and detached petioles were completely submerged in NAA or IAA solution, assumption (1) can probably be neglected. For assumption (2) no definite conclusion could be made without determination of endogenous auxin. Although, in an effort to deplete petiole tissue of endogenous hormones, petioles were aged in both light and dark without significant effect on submergence response (Tab. 2), rejecting assumption (2) would be disputable. Consequently, regarding elongation, *Rumex palustris* petioles are either less susceptible towards auxin than *Ranunculus sceleratus* petioles or enough active auxin remains in the tissue after detachment and ageing.

More important, in *Rumex palustris*, the inhibition of auxin transport with NPA did not cause a reduction in elongation in either intact plants or detached petioles. NPA has been shown previously to be active in inhibiting polar auxin transport in *Rumex palustris*: next to petiole elongation, submergence or flooding also initiates the formation of adventitious roots, a process that is auxin mediated and arrested by application of NPA (Visser et al. 1995). Like NPA, removal of the lamina of detached petioles, the putative source of auxin, had no effect on petiole elongation under submerged conditions. In contrast, elongation in *Ranunculus sceleratus* during submergence was significantly inhibited by application of auxin transport inhibitors (Fig. 5; Horton and Samarakoon 1982) or by removal of the lamina (Fig. 5; Samarakoon and Horton 1983). This inhibiting effect on elongation could be counteracted by application of $10^{-6}$ M IAA or $10^{-5}$ M NAA, showing that the inhibition is auxin-specific and demonstrating the obligatory presence of auxin in submergence elicited petiole elongation. Similar results were reported for *Regnellidium diphyllum* (Walters and Osborne 1979) and *Nymphoides peltata* (Malone and Ridge 1983).

Under drained conditions, the growth of petiole segments was found to be evenly distributed over the petiole for both *Rumex palustris* and *Ranunculus sceleratus*. If auxin is involved in this growth pattern, two explanations seem possible: (1) a classical longitudinal gradient in auxin content exists (high in tip, low at basal parts, Sánchez-Bravo et al. 1993) with an opposing gradient in responsiveness (low at tip, high at basal part, as mentioned in Histon et al. 1992) resulting in a similar growth rate along the petiole, or (2) both auxin content and tissue responsiveness are evenly distributed over the petiole. Upon submergence the growth pattern did not change for *Rumex palustris*, unlike in *Ranunculus sceleratus*, where segmental growth declined along the apical-basal gradient. A comparable gradient was found for epidermal cell length in submerged or ethylene treated *Ranunculus repens* petioles (Ridge 1985). It is difficult to explain this discrepancy due to several, mutually influencing factors like variations in endogenous auxin levels between different organs (high in young leaves, low in older leaves or vice versa; Ueda et al. 1991 and references therein) or possible changes in auxin transport velocity or auxin responsiveness. For *Ranunculus sceleratus* it was shown that treatment with $50 \mu l 1^{-1} \text{ethylene}$ increased auxin transport velocity significantly (Musgrave and Walters 1973). This would mean that basal petiole parts receive more auxin and thus should show an enhanced growth compared to the apical petiole segments. However, our results show an opposing growth pattern even after removal of the lamina. Therefore, we hypothesise that in *Ranunculus sceleratus* submergence or ethylene causes an increase in responsiveness towards endogenous auxin of especially the apical segments and that a similar mechanism is lacking in *Rumex palustris*.

Together our results indicate that, in contrast to *Ranunculus sceleratus*, involvement of auxin in the submergence response of *Rumex palustris* is at least uncertain. This would be, as far as we know the first time an auxin independent ethylene-mediated elongation is reported for a terrestrial, dicotyledonous plant.

Noteworthy is the phenomenon of enhanced petiole elongation of *Rumex palustris* at floating or partially submerged conditions, contrasted to *Ranunculus sceleratus*. *Rumex palustris* shares this feature with deep water rice. Over the past ten years, Kende and co-workers showed that growth of deepwater rice under submerged conditions is elicited by gibberellin (GA) via an ethylene mediated change in responsiveness towards GA (Raskin and Kende 1984) and an enhanced endogenous GA concentration (Hoffmann-Benning and Kende 1992). Further experiments will show whether or not GA is involved in the submergence response of *Rumex palustris*.

In conclusion, it is shown that auxin does not play a crucial role in the submergence response of *Rumex palustris*, while in *Ranunculus sceleratus* previous findings have been confirmed and extended with respect to the elongation pattern down the petiole. Therefore, with respect to auxin, the hormonal mechanism of submergence-induced petiole elongation in *Rumex palustris*
appears to be different from that in *Ranunculus sceleratus*.

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