Plant and Soil **253:** 115–123, 2003.

Submergence-induced petiole elongation in *Rumex palustris* is controlled by developmental stage and storage compounds

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Received 11 June 2002. Accepted in revised form 25 February 2003

Key words: cell walls, petiole elongation, *Rumex palustris*, starch, submergence

Abstract

Submergence stimulates elongation of the leaves of *Rumex palustris* and under laboratory conditions the maximum final leaf length (of plants up to 7 weeks old) was obtained within a 9 day period. This elongation response, mainly determined by petiole elongation, depends on the availability of storage compounds and developmental stage of a leaf. A starch accumulating tap root and mature leaves and petioles were found to supply elongating leaves with substrates for polysaccharide synthesis in expanding cell walls. Changes in the composition of cell wall polysaccharides of elongated petioles suggest a substantial cell wall metabolism during cell extension. Reduced starch levels or removal of mature leaves caused a substantial limitation of submerged leaf growth. From the 5th leaf onward enough reserves were available to perform submerged leaf growth from early developmental stages. Very young petioles had a limited capacity to elongate. In slightly older petioles submergence resulted in the longest final leaf lengths and these values gradually decreased when submergence was started at more mature developmental stages. Submerged leaf growth is mainly a matter of petiole elongation in which cell elongation has a concurrent synthesis of xylem elements in the vascular tissue. Mature petioles still elongated (when submerged) by cell and tissue elongation only: the annular tracheary elements stretched enabling up to 70% petiole elongation.

Abbreviations: DM – dry mass; LMA – leaf mass per unit leaf area

Introduction

Rumex palustris is a common species in flood-prone wetlands of river areas in Central Europe and temperate zones of Asia (Weeda et al., 1985). Floods often lead to complete submergence of grasslands and forest understory habitats. To survive a flooding period this species has developed a 'depth accommodation mechanism' in which enhanced elongation of the submerged shoot restores contact of these tissues with open air (Blom et al., 1996). Enhanced petiole elongation in *Rumex palustris* is mediated by a signal transduction pathway which becomes operative directly after submergence by a steady increase in ethylene levels (Osborne, 1984; Voesenek and Blom, 1999). This growth in response to submergence ceases when contact of leaf blades with the atmosphere is restored.

The first response of *R. palustris* upon ethylene accumulation is a hyponastic response of (mature) petioles. Within a few hours nearly all the leaves of the rosette move to a vertical position and in case none of them reaches the surface, petioles start to elongate. Both hyponastic growth and petiole elongation are regulated by the concerted action of ethylene, auxin, GA, and ABA (Voesenek et al., 2003). The action of these plant hormones results in an increase of cell wall extensibility of petiole cells, probably mediated by the action of expansins (Peeters et al., 2002). All the petioles may show a significantly increased elongation, but the greatest length increase is achieved by

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the youngest petiole, as described by Voesenek and Blom (1989) in a 4 day submergence experiment under laboratory conditions. In these experiments the youngest petiole (of leaf 5) had more than doubled its length. These observations indicate that submergence-induced petiole elongation in *R. palustris* depends on leaf age and/or its developmental stage.

Final leaf length is the prime factor that counts in this 'depth accommodation mechanism'. Under laboratory conditions, the final leaf elongation is not reached within a 4 day submergence period, so prolonged submergence will lead to even longer petioles. Petiole elongation in submerged *R. palustris* plants is predominantly a matter of cell elongation (Voesenek et al., 1990). During normal plant growth elongating cells invest substantial amounts of polysaccharides in their expanding cell walls (Cosgrove, 1999). It may be assumed that the enhanced elongation during submergence also requires substantial amounts of substrates for cell wall synthesis. Recently, Vervuren et al. (1999) showed for *Rumex maritimus* that under water very low rates of photosynthesis occurred at ambient CO_2 concentrations from which hardly any net assimilate production is to be expected. Consistent with these findings Nabben (2001) reported for 3 *Rumex* species that no dry mass was gained during a submergence period of 60 days. Laan and Blom (1990) reported a significant decrease of biomass in shoot and tap root of completely flooded *R. maritimus*. Absence of a net dry mass increase implies that submerged leaf growth and elongation depend on the mobilisation of storage compounds or on the reallocation of resources.

With the growth conditions employed in this study *R. palustris* developed a tap root at the time leaf 5 appeared. This storage organ is rapidly filled with starch and might serve as a substantial source for assimilates during submergence. The appearance of a tap root seemed to us an interesting developmental stage to study the elongation capacity of a petiole in relation to availability of storage compounds. In this study we investigate to which extent submergence-induced maximum leaf elongation depends on: (i) leaf number, (ii) developmental stage of the petiole at the start of submergence, (iii) availability of storage compounds and (iv) synthesis of cell wall polysaccharides.

Materials and methods

Plant growth

Seeds of *Rumex palustris* Sm. (collected from natural populations in the river area near Nijmegen, The Netherlands) germinated on a floating layer of black polyethylene grains at a 12 h light/12 h dark regime of 25/10 °C (70 μE m^{-2} s^{-1}). After 10 days, seedlings with a root length of 20–40 mm were transferred to 60 mL pots containing a mixture of sand and potting compost (1:2, v:v) enriched with 19.2 mg MgO per pot. The pots were supplied with one sample of 24 mL nutrient solution containing 7.5 m*M* $(NH_4)_2SO_4$, 15 m*M* KH_2PO_4, 15 m*M* KNO_3, 86.3 μM Fe-EDTA , 4.27 μM $MnSO_4$, 1.82 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 42.67 μM H_3BO_3, 0.53 μM Na_2MoO_4. Pots with seedlings were placed on a 4 mm thick moist mat (continuously saturated with water) in a controlled environment (16 h light, photosynthetic photon flux density at mean plant height 190–210 μE m^{-2} s^{-1}, temperature 20 °C, relative humidity 70%). Under these conditions the blade of leaf 5 appeared 21 days after potting.

When leaf 5 had emerged from its protective sheath sets of 6 plants were submerged in glass cuvettes with a 0.5 m water column in a controlled environment with the same light and temperature regime. Elongation of leaf 5, 6, 7 was marked daily on the glass cuvettes. In all experiments the start of submergence was marked as day 0. At the harvest, plants were dissected, lengths of leaves and petioles were measured with a ruler and individual leaves of the main shoot, tap root, lateral roots and lateral shoots were dried for 48 h at 70 °C. All experiments were carried out twice, results of a typical experiment are presented in this paper.

Red light treatment

This pre-treatment was used to deplete stored carbohydrates prior to a subsequent submergence experiment. During 2 days, plants were continuously illuminated in a controlled environment (20 °C, 70% RH) with red light only (10 μE m^{-2} s^{-1}) from one red fluorescent tube (Philips 40W;15, λ_{max} 665 nm). This treatment mimics darkness (no net photosynthesis), but prevents enhanced shoot elongation which normally occurs in prolonged darkness (etiolation).

Starch detection

Occurrence of starch was detected with the lugol reagent (0.5% KI + 0.1% I_2) in slices of tap root (5 mm below the first leaf) and cross sections of petioles. The initial pellet obtained during cell wall isolation contains cell wall polymers as well as starch. Levels of starch were calculated from the difference in glucose levels measured in hydrolysates from de-starched and from *non* de-starched cell wall preparations.

Tissue clearing and staining

Longitudinal 0.3–0.5 mm thick slices were stored in 10% NaOH for at least 1 day, washed in water and transferred to 70% ethanol. The tissue was stained with 1% safranin G in 50% ethanol for 1 minute, washed in 50% ethanol to remove excess dye and viewed in water. Micrographs were made with a green filter to enhance contrast. Cross sections were stained with phloroglucinol reagent for lignin detection.

Cell wall isolation and analysis

Procedures described by Fry (1988) were slightly changed. Experiments were carried out *in duplo.* Samples from 2 petioles were combined and homogenised in 1.5% SDS solution containing 3 m*M* sodium metabisulphite with an all glass Potter tube at 900 rpm. The homogenate was centrifuged in a table top centrifuge and the obtained clear supernatant was discarded. The pellet was washed in water to eliminate the remaining SDS and subsequently extracted with phenol:acetic acid: water (2:1:1) for 1 h. After washing with water the obtained cell wall preparation was extracted with ethanol 70%, partitioned in two equal fractions of which one was de-starched with 90% DMSO. The cell wall preparations were hydrolysed in 2 *M* trifluoroacetic acid for 1 h at 120 °C, which decomposed the occurring pectins, hemicelluloses and starch. After cooling, the hydrolysate was mixed with an appropriate amount of inositol (internal standard) and centrifuged in a table top centrifuge. The residue (mainly cellulose, as its hydrolysate was found to consist of over 99.5% glucose) was hydrolysed for 40 h at 30 °C with 1% purified driselase (Sigma) in a 1% pyridine/acetic acid buffer (pH 4.7) containing 0.05% $\beta\beta\beta$-trichloro-tert-butyl-alcoho l (Fluka) and an appropriate amount of inositol (internal standard) as described by Fry (1988). Aliquots of both supernatants were dried under a gentle stream of N_2 at 40 °C and silylated as described by Sweely et al. (1963). The obtained TMS derivatives were analysed with a HP5890 gaschromatograph equipped with a FID detector and a 25 m WCOT CP-Sil 5B column (Chrompack), temperature programmed from 130 to 210 °C at 4 ° min^{-1}.

Starch estimation

TFA hydrolysates of cell wall preparations which had been de-starched with 90% DMSO had a lower sugar content compared with those which were not de-starched. This reduction was mainly due to glucose (> 96%) , which is considered to be derived from starch.

Results

Length of elongation period

In this study, *R. palustris* was submerged at various developmental stages of leaf 5 and the elongation of this leaf was monitored until its elongation ceased. Results in Figure 1 show that this flooding-induced leaf elongation lasted for a 9-day period at the most. The older the leaf at the start of submergence, the shorter its elongation period: in flooded conditions, a mature leaf 5 elongated only for 3 days. Maximum submergence-induced lengths of leaf 5 were obtained when this leaf had reached 75% of its final length at the start of submergence. But after its normal, aerial growth had stopped a 60% leaf elongation within a subsequent 4 day submergence period still occurred. Similar profiles were obtained with leaf 6 and 7 (Figure 1). Their maximum leaf lengths were obtained when submergence was started during early growth and gradually ceased at leaf maturation. When plants were flooded when leaf 6 or 7 emerged from their protective sheaths the period of elongation of these leaves did not exceed 10 days and this period also gradually decreased to 4 days at the most for their mature state (data not shown).

Absence of net carbon gain

The carbon balance of submerged plants was estimated by dry mass measurements. Plants were submerged for 10 days at 2-day intervals from the appearance of leaf 5 (= day 0) and dry mass at the start and end of the treatment were measured. In this 10-day submergence period, the total plant dry mass did

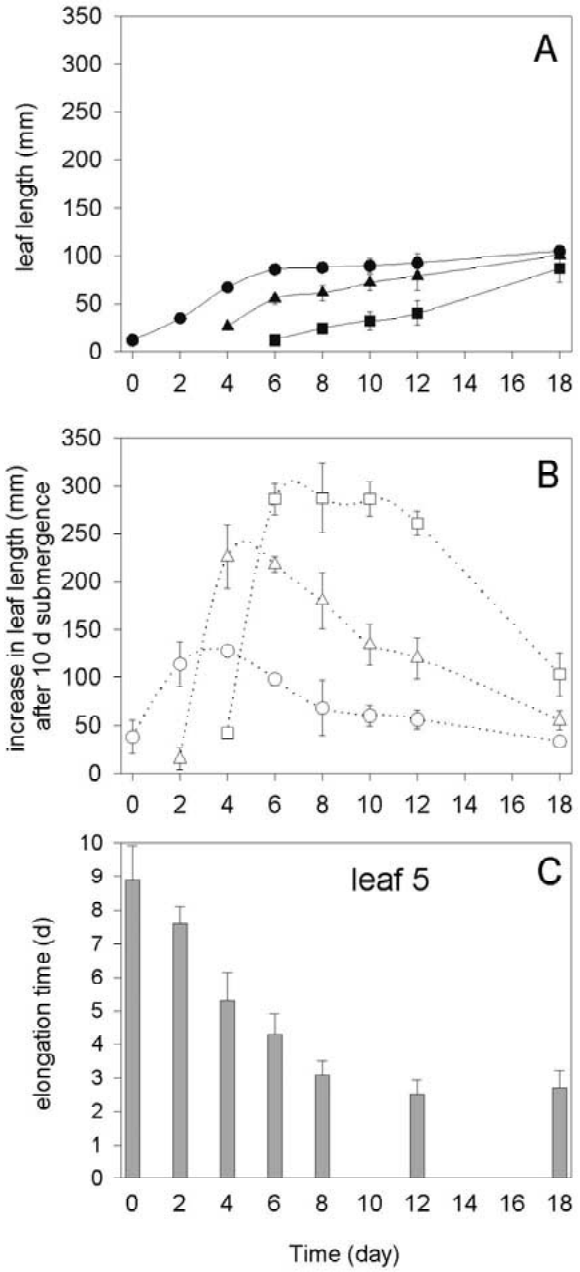

Figure 1. (A) Growth (control) of leaf 5 (●), 6 (▲) and 7 (■) of *Rumex palustris*. At day 0 leaf 5 appeared from its protective sheath ($n = 6$; ± SE). (B) Impact of developmental stage of leaf 5 (○), 6 (△) and 7 (□) at the start of submergence upon their final leaf length after submergence ($n = 6$; ± SE). Open symbols represent final length after a 10 day submergence period: e.g. leaf 6 with a length of 65 mm at day 8 (Figure 1A) had performed a submergence-induced elongation of 180 mm at day 18 ($n = 6$; ± SE). (C) Impact of developmental stage of leaf 5 on duration of submergence-induced leaf elongation ($n = 6$; ± SE).

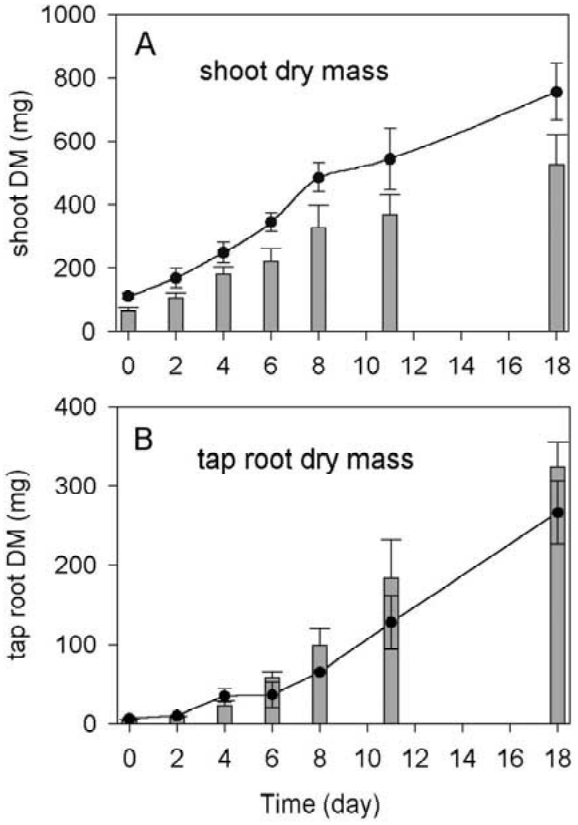

Figure 2. Increase in shoot (A) and tap root dry mass (B) during plant growth under control conditions (closed symbols) and the corresponding change after a 10 day submergence period (bars); ($n = 6$, ± SE).

not increase (Table 1), in all cases even a slight reduction in dry mass was measured. It may be concluded that a positive carbon balance no longer exists during submergence and that elongation and growth of leaves probably depend on utilization and/or mobilization of reserves.

Tap root development

When leaf 5 appeared from its protective sheath the tap root started to develop and within 2 days starch was detected in this storage organ. This accumulation continued during normal growth and occurred simultaneously with an increase in root diameter (data not shown). In the early stages of tap root formation this starch disappeared (as judged by lugolstaining) during a 10-day submergence period, in which leaf 5 and 6 rapidly elongated. Submerged at day 0, the occurring 1.1–1.9 mg starch in the taproot completely disappeared and the tissue showed a negative lugol test.

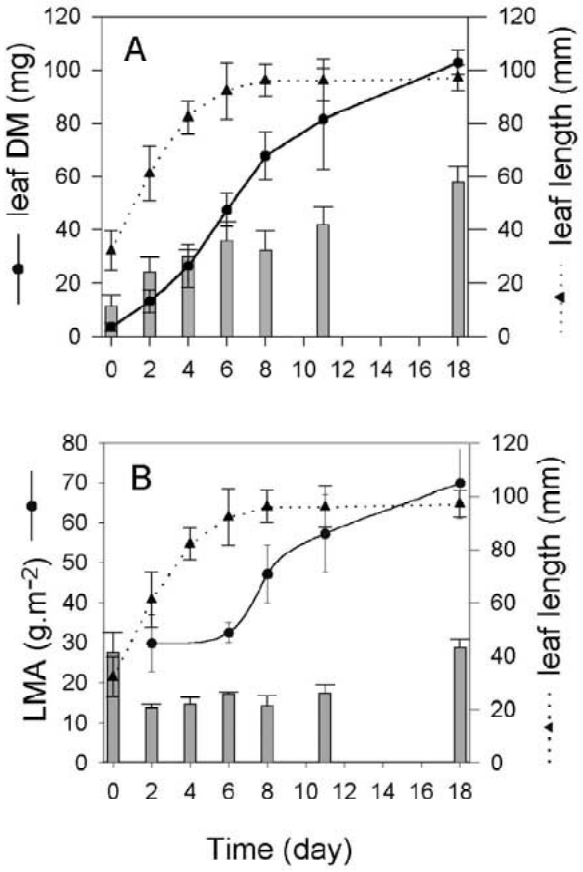

Figure 3. (A) Impact of developmental stage (at the start of submergence) on dry mass of leaf 5 of *R. palustris* after a 10 day submergence period. (●): control growth; bars represent the corresponding leaf dry weight after 10 days submergence ($n = 6$; ± SE). The length increase of leaf 5 (▲ is included for comparison. (B) Impact of developmental stage (at the start of submergence) on LMA of leaf 5 of *R. palustris* after a 10 day submergence period. (●): control growth; bars represent the corresponding LMA after 10 days submergence ($n = 6$; ± SE). The length increase of leaf 5 (▲ is included for comparison.

When submerged at day 2 the starch levels in the tap root dropped from 2.4–3.1 mg to 0.2–0.7 mg per tap root. Submergence at day 4 showed a partial depletion of starch in the cross-sections, but no significant changes in starch levels were measured. Submergence at later stages did not cause any partial starch depletion in the root tissue as judged by the lugol staining. Apparently a 10-day submergence period is then too short to exhaust this storage organ.

Reallocation of storage compounds

Submergence caused a loss in dry mass of the shoot (Figure 2). Although most of the leaves elongated,

Table 1. Plant dry weight of *R. palustris* at various developmental stages (days) during control growth and corresponding change after a 10 day submergence period. At day 0 leaf 5 just emerged from its protective sheath ($n = 6$, ± SE)

Day	Plant dry mass (mg)	
	before submergence	change after submergence
0	151 ± 14.1	−44 ± 27.4
2	248 ± 42.0	−75 ± 49.1
4	346 ± 53.5	−58 ± 54.4
6	508 ± 49.6	−114 ± 64.0
8	730 ± 79.6	−194 ± 157.4
11	825 ± 176.6	−130 ± 231.9
18	1193 ± 181.0	−158 ± 226.7

50–30% of the shoot dry mass had disappeared after a 10 day submergence period. Concurrently, the tap root maintained its dry mass and even gained weight when submergence was started at day 6 (the appearance of leaf 8) or later. The loss of dry matter in the shoot occurred mainly in older, mature leaves. Results presented in Figure 3, show that this loss of dry matter became apparent as soon as the increase of leaf length ceased. A fully grown leaf 5 lost up to 50% of its dry weight in a 10 day submergence period. It was mainly the leaf blade that lost dry matter, indicated by the decline of leaf mass per unit leaf area (LMA) from 30–68 $g.m^{-2}$ to 15–30 $g.m^{-2}$ (Figure 3B). It may be assumed that the majority of this leafy dry matter is translocated to the tap root and to the young, elongating leaves.

Dependence on storage compounds

When *R. palustris* plants were transferred to continuous red light (when leaf 6 appeared from its sheath and the tap root just had stored up to 4 mg of starch), their growth did not change apparently. Two days of continuous red light had hardly any effect on the usual length increase of the growing leaves 5 and 6 (Table 2), but depleted the starch levels in the tap root completely and reduced the dry mass of the shoot by 21% (data not shown). The lugol staining of root slices was negative and presence of starch in this tissue could not be detected using the analytical procedures as described in the 'Materials and methods'. A subsequent 10 day submergence period elongated leaf 5 and 6 by about 100%, but these data

are small compared with control plants: 120 and 500% elongation for leaf 5 and 6, respectively. Although the reduction in elongation of leaf 5 is not significant, leaf 6 was significantly reduced in elongation. In addition, leaf 7 and 8 did not appear while in the control plants both leaves reached an average length of 150 and 30 mm, respectively. Similar reductions in submergence-induced leaf elongation (from leaf 5 onward) were obtained when two, three or four leaves were removed (Table 3). Apparently, mature leaves do supply substantial amounts of dry mass to young, elongating leaves during submergence.

Petiole elongation

Submergence-induced leaf elongation in *R. palustris* is mainly a matter of petiole elongation. This was studied in detail for leaf 5 (Figure 4.) Highest values of petiole elongation were obtained during early leaf growth and this value gradually decreased with development. When leaf 5 just had obtained its final length, a 100% petiole elongation by submergence was still possible, while the length of the blade increased marginally. Petiole elongation slowly decreased to about 70% in the succeeding 10 days.

By the time leaf 5 had reached its final length, the vascular system in the petiole was also fully developed. This full-grown petiole was found to contain 7 vascular bundles in which altogether 50 lignified xylem vessels in a cross section were present. These xylem vessels were found to contain annular and spiral vessels only and these trachean elements are extensible to a certain extent upon submergence. When leaf 5 was submerged at a very early developmental stage, the few occurring (proto-xylem) elements in the 1–2 mm long petiole were torn apart during the rapid petiole elongation (Figure 4a). In the strongly elongated petioles the annular and spiral tracheary elements were not extended. The distance in between the mutual rings and spirals matched those of normal grown petioles, containing tightly stacked spirals. Apparently the occurring synthesis of xylem elements during normal petiole development was enhanced to cope with the rapid tissue expansion. When a full grown, mature petiole was submerged, the tissue still showed a 70% length increase. The spirals in these tracheary elements were less tightly stacked and the distance in between the individual spirals had increased to the thickness of an individual spiral. The successive rings and spirals had been pulled a little apart and this extensibility enabled the petiole to elongate by 70%. Apparently, synthesis of secondary xylem elements no longer occurred in a mature petiole. This elongation was not limited by a shortage of assimilates since this petiole still contained substantial amounts of starch after the 10 day submergence period.

Cell wall synthesis and composition

The submergence-induced maximum elongation of petiole 5 had a concurrent substantial increase in cell wall polysaccharides per petiole (Figure 5). This increase in cell wall polysaccharides gradually decreased as petioles were submerged at later developmental stages. As long as the petiole was able to double its length upon submergence, the elongated petiole was found to contain more cell wall polysaccharides. The elongated cell walls were found to contain relatively more cellulose: the younger the petiole at the start of submergence, the more cellulose was found in the elongated tissue (Figure 5). Apparently, the final cell wall composition of a submergence-induced elongated petiole also depends on the developmental stage of the leaf at the start of submergence.

Discussion

The maximum elongation of petioles of *R. palustris* in a 10 day submergence period depends on leaf number, developmental stage at the start of submergence and availability of storage compounds. Devoid of aerial carbon dioxide the growth of submerged *Rumex palustris* depends mainly on storage compounds and reallocation of dry matter. In general, mature leaves do contain substantial amounts of transitory starch in leaf blade and petiole as described by Beck and Ziegler (1989). The tap root, which appears during the expansion of leaf 5, rapidly fills with starch grains under the growth conditions employed in this study. Enhanced petiole elongation and new growth of more mature petioles during submergence is also a matter of cell wall synthesis from storage compounds (Figure 5). Mature leaves and petioles contain their own transitory starch to elongate their petioles upon submergence. Young, growing leaves that have not yet made the transition from sink to source depend on ‘exogenous’ starch supplies: primarily from starch in the tap root and in addition a direct import of dry matter from mature leaves cannot be excluded. A similar submergence-induced depletion of starch and concurrent translocation of dry mass has been observed

Table 2. Leaf length of *R. palustris* after a 2-day illumination with red light and corresponding lengths after a 10 day submergence period ($n = 6$; ± SE)

Leaf number	Leaf length (mm)		Leaf length (mm) + 10 days submergence	
	− red light (control)	+ red light	− red light (control)	+ red light
5	78.7 ± 5.8	90.3 ± 4.9	170.7 ± 33.9	152.3 ± 8.1
6	41.3 ± 15.6	29.7 ± 4.2	238.0 ± 23.3	77.2 ± 12.7
7	–	–	155.5 ± 106.0	–
8	–	–	32.5 ± 26.4	–

Table 3. Impact of removal of leaf 1–4 on the final length of leaf 5, 6 and 7 after a 10-day submergence period. Leaves (1–4) were removed (–) just before submergence ($n = 4$, ± SE)

Leaf number	Treatment	Leaf length (mm)		
		5	6	7
	+ leaf 1, 2, 3, 4 (control)	159 ± 16.9	215 ± 17.9	74 ± 43.3
	− leaf 1, 2, 3, 4	122 ± 10.5	66 ± 22.4	–
	− leaf 1, 2, 3	124 ± 17.6	72 ± 48.9	
	− leaf 1, 2	161 ± 23.0	86 ± 61.9	
	− leaf 3, 4	139 ± 19.0	80 ± 55.0	

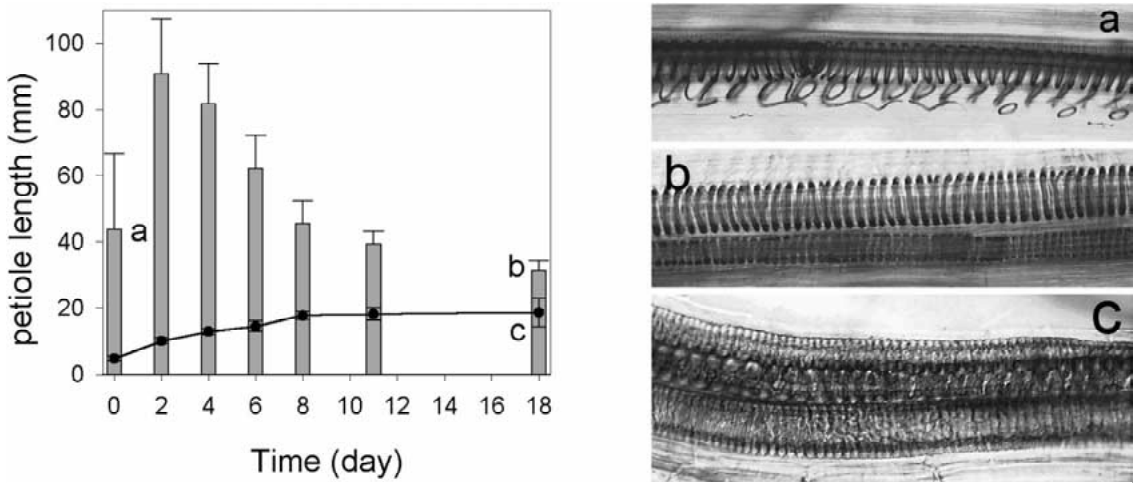

Figure 4. Elongation of petiole 5 of *R. palustris* after a 10 day submergence period and the xylem elements of the vascular tissue at day 0 and 18 ($n = 6$; ± SE). (●): control petiole growth; bars: elongated petiole length after 10 day submergence. a, b and c: xylem elements from the main vascular bundle (corresponding harvesting times marked in the graph). Note the stretched trachean spirals in a and b (submerged) compared with the tightly stacked ones in the control (c) at day 18.

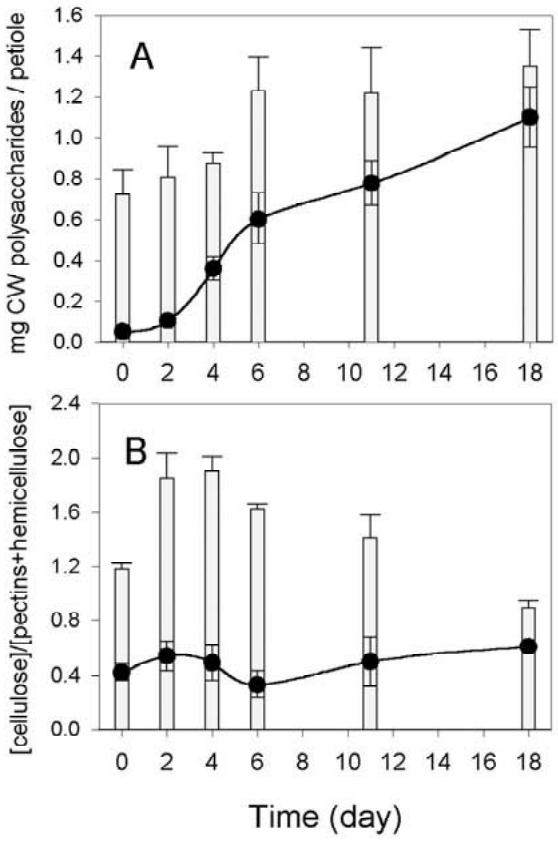

Figure 5. (**A**) Cell wall polysaccharides per petiole during control growth (●) and after a 10 day submergence period (white bar) ($n = 2$; ± SE). Data correspond with the petioles in Figure 4. (B) Corresponding change in the cellulose / hemicellulose + pectin ratio by 10 day submergence period of petiole 5. Control petioles (●); submerged petioles: bars ($n = 2$; ± SE).

in deepwater rice (Raskin and Kende, 1984; Sauter, 2000). Although deepwater rice has no tap root, the cells in the internodal elongation zones of this plant elongate at the expense of storage compounds during flooding.

Low amounts of assimilates will limit the development and elongation of leaves that are submerged at early developmental stages. Due to a lack of starch in the main root at the time leaf 4 is about to emerge from its protective sheath, this leaf can hardly elongate under water. Leaf 5 is the first leaf which can elongate (during its sink stage) at the expense of starch in the tap root, but limited amounts from this source (starch was depleted) may reduce elongation when submerged at early developmental stages. When leaf 6 is about to emerge from its protective sheath and has started its growth, the tap root contains ample supplies of starch for the submergence-induced maximum obtainable elongation of its petiole. In a very early developmental stage, when growth has not yet started and the sink strength is rather low, the submergence-induced leaf elongation is very modest (day 2 in Figure 1B).

When growing leaves are submerged after their transition from sink to source, the capacity to elongate under water decreases. Normal growth of a petiole is a matter of cell elongation with a simultaneous development of various tissues. A variety of factors may be involved in this reduction of obtainable final length. Changes in cell wall polysaccharides of developing green bean pods (Stolle-Smits et al., 1999), developing leaves of *Holcus lanatus* (Groeneveld and Bergkotte, 1998) and *Phaseolus vulgaris* (Arribas et al., 1991), hypocotyls of *Lactuca sativa* (Katsu and Kamiska, 1983), different patterns of cell wall development in a variety of tissues in as shown in alfalfa stems by Engels and Jung (1998), esterification patterns of pectic polysaccharides of carrot and tobacco (McCann and Roberts, 1994), and feruloylation of hemicelluloses of *Oryza sativa* coleoptiles (Tan et al., 1991), all these phenomena may act as rate limiting in cell wall extensibility. Bret-Harte and Talbot (1993) demonstrated a different structure of the epidermal outer wall of pea stems and the mechanical loosening of its polylamellate structure might control the rate of auxin induced expansion of the stem. All these features relate to a more rigid cell wall which may occur in the older *Rumex* petioles and can hamper submergence-induced elongation. As cell walls gradually build up rigidity during maturation, the final petiole length in submerged growth will depend on the developmental stage at the start of submergence. We distinguish three important stages in leaf development determining the final leaf (petiole) length upon submergence: (i) a very young leaf, which growth is still very low and hence does not yet act as a sink, shows a very modest elongation upon submergence, (ii) a growing leaf, which has not yet made the switch from sink to source, obtains the longest length under water, (iii) a mature leaf which encounters limiting factors in tissue elongation when submerged. Once leaf growth has ceased, cell shape is locked in place in which extensins might be a component of this locking mechanism (Cosgrove, 1999). However, submergence induces elongation of mature petioles in *Rumex* and apparently breaks this locking mechanism. Although this elongation lasted only 3 days in our experiments, a 70% expansion of a mature petiole is easily obtained. Concurrently, the composition of the cell walls changes and the trac-

heary elements of the vascular tissue are stretched. Our results indicate that as far as the tracheary elements are concerned, no more secondary walls (spiral or annular elements) are deposited in the submerged-induced elongation of mature petioles.

Summarizing, it may be concluded that the submergence-induced petiole elongation of *R. palustris* depends on developmental stage and the presence of storage compounds which in turn are used for cell wall synthesis. In our experiments with a rather limited nutrient supply a tap root was developed when the 5th leaf appeared and this organ rapidly accumulated starch. With ample starch supplies during submergence young leaves of the rosette of field grown *R. palustris* can fully develop into leaves with elongated petioles which easily accommodate depths of about half a meter.

Acknowledgements

The authors thank WHC Huibers for his excellent technical assistance, Dr. TL Pons and Dr. AJM Peeters for their valuable comments on an earlier version of this paper and the Dutch Science Foundation (NWO) for the PIONIER grant (800.84.470) to LACJV.

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