Metabolic adaptations to flooding-induced oxygen deficiency and post-anoxia stress in *Rumex* species

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upper left: Rumex thyrsiflorus and Eryngium campestre on a sandy river dune,
   “De Millingerwaard”, the Netherlands
lower right: Vegetation of Rumex palustris and Rumex maritimus along an old
   river arm,
   Waardenburg, the Netherlands

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Chapter 1

General Introduction
Flooding

Flooding is one of the major abiotic stresses that determine the yield of crops and the distribution of wild plant species in many parts of the world (Vartapetian & Jackson, 1997). Rice is the main food source for half of the human population on earth and is cultivated mainly on flooded soils (Maltby, 1991). Many other economically important or food crops are grown in flood prone areas as well. For instance, in Western Australia, two million hectares of wheat are subject to soil flooding and, depending on the rainfall, crop losses can vary between 10% to more than 50% (Dennis \textit{et al.}, 2000). Therefore, flooding stress can both lead to economical losses and scarcity of food. Knowledge of physiological processes that occur in plants during flooding can ultimately be used to improve crops with the aim to give higher yields after flooding (Vartapetian & Jackson, 1997). Apart from an economic or food perspective, knowledge of the impact of floods on plants is important from an ecological viewpoint as well. There is still much to be achieved in the understanding of precisely how wetland ecosystems function and particularly the links between biological processes and environmental characteristics such as flooding (Maltby, 1991). Wetlands cover an estimated 6% of the earth’s land surface and there is increasing evidence of their value in environmental and ecological support. A good notion of the nature of the effects of flooding is an essential requirement for the proper understanding and management of these ecosystems (Maltby, 1991).

The impact of flooding on plants

Excess of water creates a considerable stress to plants, especially when it occurs during the growing season. Flooding implies the transition from a low water level, to high water levels and again back to low water levels. This transitory nature of the water level distinguishes flood-resistant plants from true water plants, which are not adapted to the drought during low water levels. Resistance to fluctuating water levels requires a high degree of phenotypic plasticity, both at morphological and physiological level (Crawford, 1996).

The water layer surrounding the plant during flooding creates a diffusion barrier, which impedes exchange of gases between the plant and its environment approximately 10,000 times (Maberly & Spence, 1989) leading to accumulation or exhaustion of gases in the plant. The most detrimental factor for plants during flooding is a significant decrease in oxygen concentration in plant and soil, which can eventually even lead to complete anoxia (Drew, 1997).
In addition to decreasing the oxygen concentration, complete submergence also inhibits photosynthesis by decreasing light intensity, changing spectral composition (Westlake, 1966; Holmes & Klein, 1987) and reducing availability of CO$_2$ (Maberly & Spence, 1989; Setter et al., 1989; Sand-Jensen & Frost-Christensen, 1999). Apart from oxygen deprivation and hampered photosynthesis, flooding stress includes a variety of other stresses such as hormonal dysfunction and exposure to reducing compounds and other toxins (such as sulphides, reduced metal ions and organic acids (Armstrong et al., 1994; Crawford, 1996). In order to resist flooding, plants have to be adapted to this variety of stresses. However, since oxygen deprivation generally is the major stress for plants during flooding, most adaptations of plants to flooding are aimed at relieving or circumventing the effects of low oxygen concentrations (reviewed by Armstrong et al., 1994; Crawford & Brändle, 1996; Vartapetian & Jackson, 1997).

In addition to oxygen deprivation and hampered photosynthesis during flooding, also the renewed availability of oxygen after oxygen deprivation can cause damage to plants. This so-called post-anoxic injury (Crawford et al., 1994) is caused by the production of highly reactive oxygen radicals (Gutteridge & Halliwell, 1990). A second cause of post-anoxic injury is the oxidation of metabolites that have accumulated during oxygen deprivation leading to toxic products (Armstrong et al., 1994; Crawford & Brändle, 1996), such as the oxidation of ethanol to acetaldehyde (Monk et al., 1987a).

The consequences of oxygen deprivation

Higher plants are obligate aerobic organisms that need molecular oxygen to support respiration and various other life-sustaining oxidations and oxygenation reactions (Vartapetian & Jackson, 1997). If oxygen is not present as terminal electron acceptor oxidative phosphorylation will stop, leading to accumulation of NADH and exhaustion of NAD$^+$ (Fig. 1). Consequently, also the tricarboxylic acid cycle will stop and only glycolysis can continue during anoxia (Kennedy et al., 1992). However, if NAD$^+$ is depleted to a large extent glycolysis will stop as well, so, during submergence NAD$^+$ is limited for respiration in its entirety (Drew, 1997). If only glycolysis is operative during anoxia, the net production of ATP is only 2 moles per mol of glucose instead of up to 36 moles if oxidative phosphorylation works as well (see Fig. 1); accordingly, oxygen deficiency leads to an energy deficit.

Flood levels can range from only waterlogging (soil flooding) to submergence of the whole plant. When only the below ground parts of plants are flooded, aerial
General Introduction

oxygen can enter the plant via the leaves, while this is impossible when also the shoot is submerged. Therefore, waterlogging and complete submergence require (partly) different modes of adaptation to oxygen deficiency. Moreover, one has to keep in mind that there is no common cause of death in flood-intolerant plants and there is no common universal solution to prolonged flooding in tolerant species. Many different mechanisms may be involved in adapting to survive periods of flooding (Van der Sman, 1992). No mechanism on its own is sufficient for ensuring survival. The ability of plants to withstand flooding and the return to non-flooded conditions will depend on an assemblage of properties that may act independently of each other or in combination (Crawford, 1996; Crawford & Brändle, 1996).

**Glycolysis:**

\[
glucose + 2 \text{NAD}^+ + 2 \text{ADP} + 2 \text{Pi} \rightarrow 2 \text{pyruvate} + 2 \text{NADH} + 2 \text{ATP}
\]

**Tricarboxylic acid cycle:**

**Oxidative phosphorylation:**

\[
2 \text{NADH} + 8 \text{NAD}^+ + 2 \text{FADH}_2 + 6 \text{O}_2 + 32 \text{ADP} + 32 \text{Pi} \rightarrow (4+24+4) \text{ATP} + 10 \text{NAD}^+ + 2 \text{FAD} + 6 \text{H}_2\text{O}
\]

*Figure 1:* Simplified overview of the aerobic respiration pathway (after Salisbury & Ross, 1992).
Chapter 1

Adaptations to oxygen deprivation

Three distinct tactics in adaptation to abiotic stresses can be recognised: avoidance, amelioration and tolerance (Fitter & Hay, 1981). The avoidance tactic means that the stress is avoided in the metabolically active plant stage (life history strategy). This tactic often concerns specialised organs such as dormant seeds and perennating organs. Avoidance of flooding stress is realised by an adjusted life cycle so that the plant survives the flooding as a dormant seed (Van der Sman, 1992; Menges & Waller, 1983) or by developmentally passive tolerance of rhizomes (Brändle, 1991; Vartapetian & Jackson, 1997).

Amelioration of abiotic stresses involves modifications of the internal environment to alleviate the stress and allow growth under stress. It seems that organisms employing this tactic are truly physiologically tolerant, but in fact, they survive by protecting the internal metabolic processes from the stress. The amelioration strategy of flooding-induced oxygen deprivation includes 1) shoot elongation so that the water surface is reached and aerial oxygen can enter the plant (Ridge, 1987; Kende et al., 1998; Voesenek & Blom, 1999), 2) formation of aerenchyma (gas channels) in plant tissues to allow diffusion of oxygen to other plant parts (Gaynard & Armstrong, 1987; Laan et al., 1990; Jackson & Armstrong, 1999) and to facilitate the outward diffusion of potentially detrimental gaseous compounds (Visser et al., 1997), 3) formation of aerenchymatous adventitious roots (Justin & Armstrong, 1987; Visser et al., 1996) and 4) underwater photosynthesis resulting in increases in oxygen concentration (Sand-Jensen & Frost-Christensen, 1999; Vervuren et al., 1999; Rijnders et al., 2000) and carbohydrate production (Setter et al., 1989; Laan & Blom, 1990).

Tolerance of stress strictly refers to situations where the physiology and biochemistry of the organism are adapted to operate under the stress. Metabolic adaptations are the main by which tolerance to oxygen deprivation is achieved in plants. These comprise: energy generation without oxygen, maintenance of carbohydrate supply to fuel the energy generating glycolysis, energy conservation through metabolic adjustments, avoidance of cytoplasmic acidification and protection to post-anoxic injury (Davies, 1980; Armstrong et al., 1994; Drew, 1997; Setter et al., 1997). Generation of energy without oxygen is largely achieved through glycolysis. However, since NAD$^+$ is needed for continued operation of glycolysis, it is essential that this cofactor is regenerated from NADH (Drew, 1997).

Fermentation processes such as ethanolic or lactate fermentation are the most important processes by which NADH can be converted to NAD$^+$ during oxygen deficiency (Fig. 2; Kennedy et al., 1992; Perata & Alpi, 1993; Ricard et al., 1994). As mentioned before, the efficacy of energy production by glycolysis and
fermentation is much lower than that of aerobic respiration (compare Fig. 1 and 2). However, some plants are able to accelerate their glycolysis resulting in a higher ATP production per unit of time. This accelerated glycolysis is called the Pasteur effect (Turner, 1951). In contrast, some species diminish their metabolism to minimal levels that just sustain maintenance, but no other processes (anaerobic dormancy; Brändle, 1991; Zhang & Greenway, 1994; Vartapetian & Jackson, 1997).

One common cause of death due to oxygen deficiency is acidification of the cytoplasm (Davies, 1980; Roberts et al., 1984) and it is therefore essential that the cytoplasmic pH is maintained near neutrality. Cytoplasmic acidosis can be prevented by minimising production of organic acids and metabolising or extruding protons (Xia & Roberts, 1996; Saglio et al., 1999; Chang et al., 2000).

Glycolysis:

\[
glucose + 2 \text{NAD}^+ + 2 \text{ADP} + 2 \text{Pi} \rightarrow 2 \text{pyruvate} + 2 \text{NADH} + 2 \text{ATP}
\]

Ethanolic fermentation:

\[
2 \text{pyruvate} \xrightarrow{\text{pyruvate decarboxylase}} 2 \text{acetaldehyde} + 2 \text{CO}_2
\]

\[
2 \text{acetaldehyde} + 2 \text{NADH} \xrightarrow{\text{alcohol dehydrogenase}} 2 \text{ethanol} + 2 \text{NAD}^+
\]

Lactate fermentation:

\[
2 \text{pyruvate} + 2 \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} 2 \text{lactate} + 2 \text{NAD}^+
\]

Figure 2: Main fermentation pathways in plants resulting in regeneration of NAD\(^+\) during oxygen deficiency.

In order to survive flooding including the subsequent reaeration period, plants also have to be adapted to post-anoxic injury. Protection against reactive oxygen species formed upon reaeration is accomplished by the availability or production of antioxidants (such as ascorbate, glutathione or alpha-tocopherol) or enzymes (e.g. catalases, peroxidases or superoxide dismutase) that scavenge the oxygen radicals (Smirnoff, 1995).
Many of these metabolic adaptations require *de novo* synthesis of proteins. These proteins that are only formed during oxygen deficiency are called anaerobic polypeptides or anaerobic proteins (ANPs) and comprise enzymes involved in glycolysis, fermentation and defence against post-anoxic injury (Sachs *et al.*, 1980; Monk *et al.*, 1987b). These ANPs and many adaptations to oxygen deprivation are only induced if some oxygen is present. Plants that are completely deprived of oxygen need a hypoxia pre-treatment (HPT) prior to anoxia for ANP formation (Saglio *et al.*, 1999; Chang *et al.*, 2000). Consequently, HPT induces protection to and prolongs survival of subsequent anoxia (Saglio *et al.*, 1988; Johnson *et al.*, 1989; Ellis & Setter, 1999).

The molecular basis of adaptation to oxygen deprivation has not yet been completely established, but many genes and gene products that are induced by oxygen deficiency have already been identified (e.g. Sachs *et al.*, 1996; Setter *et al.*, 1997; Dennis *et al.*, 2000; Vriezen, 2000). Additionally, it is likely that new techniques in genomics (like DNA microarray analysis) and proteomics (Chang *et al.*, 2000) will facilitate the search for and identification of genes and proteins involved in tolerance to oxygen deprivation. It is clear that plant resistance to oxygen deprivation is a combination of many metabolic and morphological changes. Nevertheless, there is little doubt that energy production through fermentation is the basis for all other adaptive responses in plants under oxygen deficiency and that non-limiting amounts of fermentable carbohydrates are necessary (Perata *et al.*, 1998). Therefore, metabolic adaptations to oxygen deprivation are the subject of this thesis. In general, the array of adaptations to stresses is much wider in wild plant species than in species that have been cultivated already for a long time (Crawford & Brändle, 1996). The more pronounced adaptations found in wild species can lead to a better understanding of resistance to oxygen deprivation in other (both wild and cultivated) plant species. Therefore, the metabolic adaptations of three *Rumex* species that grow in different habitats in Dutch floodplains and that differ in resistance and mode of adaptation to flooding are compared.

**The Dutch river system**

Periodic flooding of river forelands is a common feature of lower reaches of rivers throughout the world (Maltby, 1991). The floodplains of the river Rhine ecosystem in the Netherlands are subjected as well to inundation when the river carries large quantities of water. The floodwater partly originates from melting snow in the mountains of Switzerland and Southern Germany resulting in predictable floods in winter and early spring. Additionally, peak periods in
precipitation in upstream areas cause strongly fluctuating water levels. These rain-fed peaks in water level are highly unpredictable in frequency, duration, height and timing and can occur both in winter and in the growing season of plants (Blom, 1990). The flooding regime determines to a large extent plant community development and patterns of plant zonation in floodplains (Blom, 1999; Casanova & Brock, 2000).

In the Dutch river floodplains a clear plant zonation is found. It turns out that different species from the genus *Rumex* are represented in many different vegetation types present in the floodplains, from strictly terrestrial and never flooded to frequently flooded or amphibious habitats (Blom, 1990; 1999). Since several closely related species occur near to each other but are subjected to a gradient in flooding stress and differ in flooding resistance, the genus *Rumex* is an excellent model to study adaptations to flooding. In this study, three *Rumex* species were selected that grow in different habitats and display different modes of adaptation to oxygen deficiency.

**Rumex species**

*Rumex thyrsiflorus* occurs on seldom-flooded sites like river dunes and dikes and is perennial (Blom, 1990; 1999). In contrast, *R. maritimus* and *R. palustris* inhabit low, frequently flooded mud flats (Hejny, 1960; Tüxen, 1979) and are annual-biennial and biennial-short lived perennial, respectively (Blom 1990; Van der Sman et al., 1993b). This means that *R. thyrsiflorus* avoids the floods by its spatial distribution (high elevation) but would not be able avoid it in time since it is long-lived. In contrast, the other two species are not able to avoid the floods in a spatial sense (low elevation), but especially *R. maritimus* is able to avoid it in time by completing its life cycle between two floods and survive as a dormant seed.

*R. maritimus* and *R. palustris* show a distinct shoot elongation upon flooding (Van der Sman et al., 1993b), while in *R. thyrsiflorus* this elongation response is less pronounced (Banga et al., 1995). Additionally, the development of aerenchyma facilitates diffusion of oxygen in the species from low elevated sites, while in *R. thyrsiflorus* the resistance to diffusion is much higher (Laan et al., 1990). The number of adventitious roots formed during waterlogging and the porosity of lateral and adventitious roots in *R. thyrsiflorus* is many-fold lower than that of *R. maritimus* and *R. palustris* (Laan et al., 1989a; Visser et al., 1996). In sum: apart from avoidance, also the amelioration strategies of *R. maritimus* and *R. palustris* are much more developed than those of *R. thyrsiflorus*. Although it is known that underwater photosynthesis leads to both higher oxygen levels within
the plants (Rijnders et al., 2000) and less decrease in biomass of completely submerged Rumex plants (Laan & Blom, 1990), it is unclear what its influence on survival of these species during submergence is. Moreover, fairly little is known about the true tolerance to oxygen deficiency of these species.

It seems that R. maritimus and R. palustris are equal in all their adaptations to flooding tested so far. However, R. palustris has a longer life cycle, which makes it more likely that this species is exposed to flooding than R. maritimus. Besides this, in R. palustris flowering is more delayed during partial submergence (Van der Sman et al., 1993b) and apparently less resources are allocated to reproductive output and shoot elongation compared to R. maritimus (Van der Sman, 1992). Moreover, R. palustris seems to be more resistant to submergence than R. maritimus (Van der Sman et al., 1993a). Every adaptation has a cost in terms of general fitness (Crawford, 1996). This led Van der Sman (1992) to the hypothesis that there might be a trade-off by which R. palustris may be investing less in avoidance (life cycle and reproductive output) and amelioration (shoot elongation) tactics and probably is more relying on true tolerance by metabolic means.

Aim of this thesis

Energy production is the basis for all other adaptive responses in plants under oxygen deficiency. Moreover, in order to comprehend the distribution of Rumex species along a flooding gradient and to understand how this zonation is caused by differences in adaptations among species, the metabolic adaptations of the species have to be known. Apart from oxygen deprivation, post-anoxic injury can also form a substantial part of the damage caused to plants by flooding (Crawford & Brändle, 1996). Therefore, the aim of this study was to determine the influence of differences in metabolic adaptations to oxygen deficiency and post-anoxic stress on the survival of Rumex species during flooding.

In order to achieve this aim and to test the hypothesis postulated by Van der Sman (1992) that R. palustris might be more tolerant than R. maritimus, these two species were selected. A third species from less flooded habitats and with a lower resistance to flooding, R. thyrsiflorus (Blom, 1990), was incorporated in this study to represent a species with a presumably low tolerance to flooding and a longer life history.

From the analysis of flooding characteristics presented in chapter 2, it is clear that complete submergence frequently occurs in the habitats of the selected Rumex species (Nabben et al., 1999). Accordingly, in most experiments described in this thesis plants were submerged completely. In this situation, tolerance mechanisms
are particularly important while amelioration tactics are of secondary importance. The influence of underwater photosynthesis on several aspects of plant performance during complete submergence was tested as well in this present study. Above all other plants organs, especially plant organs buried in the soil are prone to anoxic conditions during flooding. Consequently, rhizomes or tap roots of wetland plants are presumably the only organs that are subject to strictly anaerobic conditions during natural development (Brändle, 1991). Furthermore, tap roots are also the main carbohydrates storage organs and the organs, which survive longest during flooding and from which regrowth of shoots and lateral roots occurs (Armstrong et al., 1994). Therefore, a considerable part of this thesis focuses on tap roots.

Outline of this thesis

In Chapter 2, the flooding characteristics (timing, frequency, duration and number of floods) of the habitats of the three species are described. Furthermore, resistance to complete submergence of the three species was assessed. Plants were submerged both in the light and under complete darkness to establish the influence of underwater photosynthesis on resistance to flooding. In general, young plants have smaller carbohydrate stores than mature plants, so it was tested if the size and developmental stage of plants affected resistance to flooding by differences in the amount of fermentable carbohydrates. The results described in this chapter, show that post-anoxic injury forms a part of the flooding stress experienced by these *Rumex* species.

Chapter 3 describes the *in vitro* activity of enzymes involved in anaerobic regeneration of NAD⁺ during submergence of the species. Since *in vitro* enzyme activity is not an exact measure of the *in vivo* rate of processes, fermentation products were measured as well. The amount of energy available to essential plant processes was assessed by measuring the adenylate energy charge (AEC; a measure of energy status) and the ability to maintain membrane functions such as energy dependent transport of solutes and the capacity to retain energised membranes.

The carbohydrate metabolism of submerged *Rumex* species is analysed in Chapter 4. It was examined if resistance to submergence could be explained by the amount, quality and accessibility of carbohydrates. Again, the effect of underwater photosynthesis was tested, but this time it was determined to what extent it affected carbohydrate content. From the results of this chapter it could also be deduced whether the *Rumex* species displayed a Pasteur effect or, in contrast, a dormancy strategy.
Chapter 1

Survival and growth rate after anoxia followed by reaeration are analysed as well, in Chapter 5. By applying a herbicide that generates reactive oxygen species and measuring survival and growth rate again, the effects of anoxia and those of post-anoxic injury could be partially separated. In this chapter the \textit{in vitro} activity of one of the enzymes involved in defence against reactive oxygen species was also measured and other factors involved in this defence are discussed as well. Since acetaldehyde formed upon reaeration by oxidation of ethanol can possibly be very detrimental for plants, its production in \textit{R. palustris} plants after reaeration was determined.

Finally, Chapter 6 gives a synthesis of the main results of the separate chapters. In addition, it presents a general discussion of the significance of metabolic adaptations in survival of flooding stress by the three \textit{Rumex} species.
Chapter 2

Resistance to complete submergence in *Rumex* species with different life histories: the influence of plant size and light

RHM Nabben, CWPM Blom & LACJ Voesenek
Resistance to complete submergence was tested in three *Rumex* species that occur in the Dutch river forelands. The species differ both in habitat and in life history characteristics. The annual or biennial *R. maritimus* and the biennial or short lived perennial *R. palustris* grow on low elevated, frequently flooded mud flats, while the perennial *R. thyrsiflorus* can be found on dikes and river dunes that are seldom flooded. The flooding characteristics of the habitats of the three species were determined. These data were used to design experiments to determine the survival and biomass development of the three species during submergence and the influence of plant size and light level on these parameters. It was shown that in all three species, plants submerged in the light were much more resistant to flooding than during submergence in the dark. This is most probably due to the generation of oxygen or carbohydrates by underwater photosynthesis. Mature plants of the three species showed higher survival after submergence than juvenile plants, which might be caused by higher carbohydrate levels in the tap roots of mature plants. In addition, the three species clearly differed in survival and biomass development during submergence. *R. thyrsiflorus*, the species, which is least confronted with flooding, is least tolerant to complete submergence. *R. maritimus*, which can avoid the floods by having a short life cycle, is less tolerant to submergence than *R. palustris*, which has to survive the floods as a vegetative plant. It was noted that some plants that survived the flooding period itself, still died in the following period of drained conditions, possibly due to post-anoxic injury.

Introduction

River forelands of the Rhine branches in the Netherlands are flooded more frequently in spring and summer now than in the past. This is due to faster discharge of precipitation, caused by canalisation, cutting of woodlands, urbanisation and drainage of agricultural areas (Blom *et al.*, 1994). Consequently, plants from riparian habitats are flooded more frequently and longer during the growing season. This means that plants have to be adapted to flooding in order to survive in the floodplains. Since the floods are the direct result of rainfall, plants can be flooded at different stages of their life cycle.

The main effect of flooding on plants is a reduced exchange of gases between the plant and its environment, since the diffusion rate of gases in water is approximately 10,000 times slower than in air (Maberly & Spence, 1989). Oxygen deficiency is the main constraint plants have to deal with during flooded
conditions (for reviews see: Armstrong et al., 1994; Crawford & Brändle, 1996; Vartapetian & Jackson, 1997), but in submerging waters a shortage of CO$_2$ and high diffusive impedances may severely curtail photosynthesis (Sand-Jensen & Frost-Christensen, 1999).

From an ecological point of view, the adaptations to stresses can be divided into avoidance, amelioration and tolerance strategies (Fitter & Hay, 1981). With avoidance strategies, the oxygen deficiency in the metabolically active plant stage is avoided, through life history strategies such as survival of the harmful period as a dormant seed. By amelioration strategies the oxygen shortage is alleviated by morphological and anatomical adaptations, for instance the development of aerenchyma in plant tissues or elongation of the shoot so that leaf-air contact is restored (Armstrong et al., 1994). Tolerance strategies allow the plant to tolerate oxygen deficiency at the cellular level and these are mainly metabolic adaptations (Crawford & Brändle, 1996). Anaerobic metabolism enables generation of energy without oxygen as a terminal electron acceptor. The main processes involved are ethanolic and lactate fermentation (Ricard et al., 1994). Since fermentation is much less efficient than aerobic respiration as far as carbohydrate consumption is concerned (Armstrong et al., 1994), carbohydrate reserves are needed to tolerate prolonged flooding (Setter et al., 1987b; Hanhijärvi & Fagerstedt, 1995). In general, young plants have smaller carbohydrate stores than mature plants, so the developmental stage and size of flooded plants might be important in survival of flooding. In submerged tree species, older seedlings survive longer (Siebel & Blom, 1998). We therefore assume that older and larger plants can tolerate submergence for longer periods than younger and smaller plants.

The amount and quality of light also change because of flooding (Maberly & Spence, 1989). In rice fields the light level at a depth of 0.5 m can be as low as 1-16% of the intensity above the water (Setter et al., 1987a). The light level in the river Waal at 0.5 m depth is approximately 40% of that above the water, but during flooding it can drop to 5-10% due to high turbidity (unpublished results PJA Vervuren). Since low light levels reduce underwater photosynthesis (Vervuren et al., 1999), we assume that survival during submergence is also determined by light level.

In river forelands, Rumex species occur in a zonation along the flooding gradient. In this study three Rumex species from different habitats were used: R. palustris and R. maritimus that grow on low, frequently flooded mud flats (Hejny, 1960; Tüxen, 1979) and R. thyrsiflorus that grows on elevated, seldom flooded river dunes and dikes (Blom et al., 1994). These species also show different levels of adaptation to flooding. Both species from low-lying sites can reach the water surface by shoot elongation, while in R. thyrsiflorus the elongation is less pronounced (Banga et al., 1995). Besides this, the species that grow on mud flats
also have a better-developed aerenchyma system resulting in more internal aeration than in *R. thyrsiflorus* (Laan *et al.*, 1990) and they form more adventitious roots that replace the old root system (Visser *et al.*, 1996). These morphological and anatomical adaptations are mainly relevant if the floods are not too deep. However, if deeper floods occur amelioration strategies are not advantageous and plants are dependent on tolerance mechanisms.

The selected species also differ in their life history. *Rumex thyrsiflorus* is a perennial species. *Rumex maritimus* is an annual or biennial species that in principle can complete its life cycle between two floods and survive as a dormant seed, while *R. palustris* is a biennial or short lived perennial that has to survive flooding as a vegetative plant (Van der Sman, *et al.*, 1993b). Vegetative plants of *R. palustris* are more likely to be confronted with flooding than those of *R. maritimus*, and *R. thyrsiflorus* is hardly ever completely submerged, therefore, we expect that these species differ with respect to their tolerance to submergence. Actively growing plants are more susceptible to flooding (Vartapetian & Jackson, 1997) and submergence tolerance is lower when temperatures are higher (Adkins *et al.*, 1990; Siebel & Blom, 1998). Therefore, flooding during the growing season can have a large impact on survival and zonation of these species.

The objectives of this study were 1) To determine in more detail the flooding characteristics of the natural habitats of the selected *Rumex* species. 2) To deduce from this at what plant stage and for how long the plants become submerged under natural field conditions. 3) To compare the submergence tolerance of three *Rumex* species that differ in their life history. 4) To assess what the influence of plant size and light is on the survival of the selected species by completely submerging plants of two size categories under both light and dark conditions.

**Materials and Methods**

*Flooding characteristics*

The flooding characteristics of the habitats in which the three species under study grow, were analysed for the period 1970-1995, by comparing the relative elevation of the habitats of these species along the river Waal (one branch of the Rhine in the Netherlands), with the water level of the river and calculating the timing and duration of the floods. The relative elevation was measured at different sites and normalised to the elevation of one of these locations. Water levels were provided by the Survey Department of the Dutch Ministry of Public Works. Since this article focuses on floods during the growing season and seeds of *Rumex* species germinate (Van der Sman *et al.*, 1993b) and new shoots emerge above
ground level from April onwards, only the data for the period between April 1 and September 30 were analysed. The following parameters were determined: 1) The month in which the water level was below substrate level for the first time (“first exposure”), 2) The month in which the floods took place (“occurrence of floods”), 3) The number of floods in one growing season, 4) The duration of individual floods, 5) The interval between two floods, between uncovering and the first flood or between the last flood and the end of the growing season, 6) The summed duration of all floods in one growing season.

Plant material

Seeds of *Rumex palustris* Sm., *R. maritimus* L. and *R. thyrsiflorus* Fingerh. were collected from natural populations in the river area near Nijmegen, the Netherlands. Seeds were germinated on moist filter paper in Petri dishes, under 12 h light/12 h dark regime of 25/10 °C (PPFD: 25 μmol m⁻² s⁻¹, Philips TL33). After seven days, seedlings were individually transplanted into plastic pots (diameter 14 cm) filled with a mixture of sand and clay (1:1 v/v) and grown in the greenhouse (15-23 °C, 35-55% rh). The soil was watered twice a week and fertilised once a month with nutrient solution (4 mM Ca(NO₃)₂, 2.5 mM K₂SO₄, 1 mM MgSO₄, 1 mM KH₂PO₄, and the micronutrients FeEDTA (30 μM), NaCl (100 μM), H₃BO₃ (50 μM), MnSO₄ (4 μM), ZnSO₄ (4 μM), CuSO₄ (1 μM), H₂MoO₄ (1 μM)). The photoperiod while growing the plants was kept at 12 h by means of 400-W high-pressure sodium lamps, which supplemented normal daylight to a minimal light intensity (PPFD) of 100 μmol m⁻² s⁻¹ at plant level. The short day length was chosen to prevent *R. maritimus* from bolting and flowering (Van der Sman et al., 1992).

Flooding treatments and measurements

The flooding characteristics of the habitats of the *Rumex* species (Table 1) were used to select the plant size and duration of flooding in the submergence experiments. After 35-40 days (juvenile plants: 0.02-0.12 gFW) or 85-110 days (mature plants: 6.0-15.0 gFW) of growth, plants were submerged in large basins (diameter 1.8 m) filled with tap water, either in the light (12 h light/12 h dark regime, PPFD: 75-90 μmol m⁻² s⁻¹ at plant level, temperature: 20 °C) or in complete darkness. No measures were taken to control the oxygen and carbon dioxide levels of the floodwater. Juvenile plants had no tap roots; mature plants
Resistance to submergence

had a distinct tap root at the start of the experiment. The water level was fixed at one metre above the soil surface to avoid restoration of leaf-air contact due to shoot elongation. One set of plants (control plants) was grown under drained conditions (as mentioned above) but with an identical light level as the submerged plants. During the experiment nutrients were added to neither submerged or nor drained plants.

After 14, 35 and 56 days of submergence, 12 plants per species and treatment were taken out of the water. Six plants were harvested and dried (16 h, 105 °C) after which the dry weight of shoots, tap roots and lateral roots was determined. The other six plants were scored for survival based on their physical appearance; plants with green, turgid leaves and green apical buds were judged to be vital. After a regrowth period of 14 days under drained conditions, the survival of these plants was scored again. Plants that did not form new leaves in the regrowth period were considered dead.

The dry weight of the juvenile plants was too small to be measured accurately enough to detect the effects of duration of flooding and light level in the three species. Therefore, these results are not included in this paper and only dry weights of the mature plants will be presented. Plants that were regarded dead are not included in the results for biomass.

**Statistical analysis**

The results were tested by three-way analysis of variance followed by a Tukey test. When required the data were log-transformed. Since the data for dry weight of shoots were, even after transformation, not normally distributed, these results were analysed by Kruskal-Wallis one-way analysis of variance on ranks followed by a Dunn’s test. This means that no interaction effects between variables could be calculated for this parameter. All statistical analyses were performed with the SigmaStat statistical package and according to Sokal & Rohlf (1995).

**Results**

**Flooding characteristics**

*Rumex palustris* and *R. maritimus* occur in a relative narrow zone of one metre (9.50-10.50 metre above sea level), while *R. thyrsiflorus* is found in a wider and higher zone (12.25-14.50 metre above sea level).
In most years germination of seeds and emergence of new shoots of *R. palustris* and *R. maritimus* could take place in April or May, since in these months the sediments were uncovered for the first time after the winter floods (Table 1). The first spring or summer flood came in May to July. This means that very young plants were flooded, but also much older plants were subjected to submergence, especially if plants were flooded that had germinated in one of the previous years. In most years, one to three floods took place that lasted from 1 up to 85 days. The intervals between floods also differed widely and ranged from 5 to 180 days. The summed duration in one season could be as high as 150 days, while in many years the higher parts of the habitat were not flooded at all (Table 1). The depth of individual floods ranged from 0.05-2 metres (data not shown). For the zone in which *R. thyrsiflorus* grows, only the years in which a flood occurred are listed in Table 1. In contrast to the habitat of the other two species, this zone was flooded seldom and only one or two floods occurred per year. These floods lasted up to 14 days and mainly took place in late spring and early summer.

Legend table 1:

*a*: the ranges given apply to the highest and lowest elevated site in a zone, respectively

*b*: first exposure: month in which the water level is below substrate level for the first time (starting from April)

*occurrence of floods*: the month in which the floods took place (after uncovering)

*number of floods*: number of floods in one growing season

*duration of floods*: duration of individual floods

*interval between floods*: period between two floods, between the uncovering and the first flood or between the last flood and the end of the growing season

*summed duration*: summed duration of all floods in the growing season (1/4-30/9)

*c*: NAP: Amsterdam Ordnance Datum; the elevation of the sites is normalised to make a good comparison

*d*: years in which no flood occurred during the growing season are not included
### Table 1: Flooding characteristics during the growing season of the habitat of R. palustris/R. maritimus and R. thyrsiflorus along the river Waal in the period 1970-1995a.

<table>
<thead>
<tr>
<th>Year</th>
<th>First exposure of floods</th>
<th>Number of floods</th>
<th>Duration of floods (d)</th>
<th>Interval between floods (d)</th>
<th>Summed duration (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1970</td>
<td>Aug-Sep</td>
<td>Aug</td>
<td>0 - 2</td>
<td>0 - 23</td>
<td>6 - 9</td>
</tr>
<tr>
<td>1971</td>
<td>Apr</td>
<td>Jul</td>
<td>1</td>
<td>24 - 32</td>
<td>10 - 86</td>
</tr>
<tr>
<td>1972</td>
<td>Apr-May</td>
<td>May-Aug</td>
<td>0 - 6</td>
<td>0 - 16</td>
<td>7 - 183</td>
</tr>
<tr>
<td>1973</td>
<td>Apr-Jun</td>
<td>May/Jul-Aug</td>
<td>2</td>
<td>7 - 21</td>
<td>10 - 135</td>
</tr>
<tr>
<td>1974</td>
<td>Apr</td>
<td>Jun/Jul</td>
<td>0 - 3</td>
<td>0 - 40</td>
<td>6 - 183</td>
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<tr>
<td>1975</td>
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<td>5 - 45</td>
<td>8 - 55</td>
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<td>0 - 33</td>
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<tr>
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<td>5 - 58</td>
<td>4 - 40</td>
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<tr>
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<td>Jun/Jul</td>
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<td>5 - 44</td>
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<td>0 - 1</td>
<td>0 - 9</td>
<td>7 - 93</td>
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<tr>
<td>1984</td>
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<td>Aug/Sept</td>
<td>2 - 3</td>
<td>5 - 35</td>
<td>5 - 86</td>
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<tr>
<td>1985</td>
<td>Apr-Jul</td>
<td>May/Sept</td>
<td>1 - 3</td>
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<td>1986</td>
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<td>1987</td>
<td>Apr-Sep</td>
<td>May/Jul-Aug</td>
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<td>0 - 22</td>
<td>4 - 45</td>
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<td>1989</td>
<td>May</td>
<td></td>
<td></td>
<td></td>
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<td>1990</td>
<td>Apr</td>
<td>Jun/Jul</td>
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<td>0 - 16</td>
<td>12 - 183</td>
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<tr>
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<td>Apr</td>
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<td>0 - 16</td>
<td>83 - 183</td>
</tr>
<tr>
<td>1992</td>
<td>Apr</td>
<td>May/Jun</td>
<td>0 - 2</td>
<td>0 - 9</td>
<td>11 - 172</td>
</tr>
<tr>
<td>1993</td>
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<td>0 - 1</td>
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<td>57 - 183</td>
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<tr>
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<td>Sep</td>
<td>1</td>
<td>6 - 27</td>
<td>47 - 103</td>
</tr>
<tr>
<td>1995</td>
<td>May-Aug</td>
<td>Sep</td>
<td>2 - 3</td>
<td>8 - 26</td>
<td>5 - 93</td>
</tr>
</tbody>
</table>

**R. palustris / R. maritimus (10.50-9.50 m +NAPc)**

**R. thyrsiflorus (14.50-12.25 m +NAPc)**

<table>
<thead>
<tr>
<th>Year</th>
<th>First exposure of floods</th>
<th>Number of floods</th>
<th>Duration of floods (d)</th>
<th>Interval between floods (d)</th>
<th>Summed duration (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1970</td>
<td>Apr</td>
<td>May</td>
<td>1</td>
<td>3 - 14</td>
<td>3 - 135</td>
</tr>
<tr>
<td>1978</td>
<td>Apr</td>
<td>May</td>
<td>0 - 1</td>
<td>0 - 6</td>
<td>49 - 183</td>
</tr>
<tr>
<td>1980</td>
<td>Apr</td>
<td>Jul</td>
<td>0 - 1</td>
<td>0 - 13</td>
<td>63 - 183</td>
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<tr>
<td>1983</td>
<td>Apr</td>
<td>May</td>
<td>2</td>
<td>4 - 16</td>
<td>9 - 171</td>
</tr>
<tr>
<td>1984</td>
<td>Apr</td>
<td>Jun</td>
<td>0 - 1</td>
<td>0 - 5</td>
<td>61 - 183</td>
</tr>
<tr>
<td>1987</td>
<td>Apr</td>
<td>Jun</td>
<td>0 - 1</td>
<td>0 - 15</td>
<td>79 - 183</td>
</tr>
</tbody>
</table>
Since both very young and mature plants can become flooded and the amount of carbohydrates stored in tap roots can be important in flooding resistance, two plant stages with different tap root sizes were used for the survival experiments. The duration of flooding in the survival experiments was based on the general duration of individual floods in the habitat of *R. thyrsiflorus* and that of the other two species (Table 1).

**Survival**

Juvenile plants that were submerged in the light and all mature plants survived up to 14 days of flooding (Fig. 1). When flooding lasted longer, both juvenile and mature plants that were submerged under light conditions showed higher survival rates than plants submerged in the dark, except for *R. palustris*. Juvenile plants of all three species showed much lower survival than mature plants, and there were clear differences between species, particularly after 56 days of flooding. Half the juvenile plants of *R. palustris* survived this period of flooding in the light, while plants of *R. maritimus* were clearly more affected by submergence, but not as severely as *R. thyrsiflorus* of which half had already died after 35 days of submergence. After 56 days of flooding in the dark no mature plants of *R. palustris* died, while half of the *R. maritimus* plants and all the *R. thyrsiflorus* plants died. When submerged in the light, hardly any mature plants died.

During rinsing of the root system upon harvesting, it became clear that the tap roots are the longest surviving organs during submergence, and that often the leaves seemed quite healthy while the lateral roots had turned grey and black and apparently were dead.

Some plants that looked very healthy at the end of the submergence period were dead after two weeks of regrowth (Table 2). Some leaves clearly lost turgor and wilted or disintegrated within a few hours after the plants were taken out of the water and in some cases chlorotic and necrotic spots appeared. Mortality of juvenile plants in the regrowth period was much higher than that of mature plants; plants submerged under dark conditions also had higher mortality rates than plants flooded in the light (Table 2). There also is a difference between species with respect to mortality in the regrowth period: in *R. palustris* fewer plants died in the regrowth period than in the other two species.
Figure 1: Survival percentages \( (n=6) \) of Rumex species after complete submergence under light (open circles) or dark (closed circles) conditions followed by a regrowth period of 14 days. Juvenile plants: 35-40 days old; mature plants: 85-110 days old.
Table 2: Mortality (%) of Rumex species during 14 days of recovery following complete submergence under light or dark conditions. Juvenile plants: 35-40 days old; mature plants: 85-110 days old at the start of submergence. Number of living plants at the start of the recovery period is shown between parentheses.

<table>
<thead>
<tr>
<th>light treatment</th>
<th>species</th>
<th>submergence period preceding recovery (d)</th>
<th>14</th>
<th>35</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Juvenile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td><em>R. palustris</em></td>
<td>0 (6)</td>
<td>0</td>
<td>0</td>
<td>50 (6)</td>
</tr>
<tr>
<td></td>
<td><em>R. maritimus</em></td>
<td>0 (6)</td>
<td>0</td>
<td>0</td>
<td>50 (2)</td>
</tr>
<tr>
<td></td>
<td><em>R. thyrsiflorus</em></td>
<td>0 (6)</td>
<td>50 (6)</td>
<td>100 (1)</td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td><em>R. palustris</em></td>
<td>67 (6)</td>
<td>100 (2)</td>
<td>-</td>
<td>- (0)</td>
</tr>
<tr>
<td></td>
<td><em>R. maritimus</em></td>
<td>83 (6)</td>
<td>-</td>
<td>(0)</td>
<td>- (0)</td>
</tr>
<tr>
<td></td>
<td><em>R. thyrsiflorus</em></td>
<td>100 (6)</td>
<td>100 (2)</td>
<td>-</td>
<td>- (0)</td>
</tr>
<tr>
<td><strong>Mature</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
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<td>0 (6)</td>
<td>0</td>
<td>0</td>
<td>0 (6)</td>
</tr>
<tr>
<td></td>
<td><em>R. maritimus</em></td>
<td>0 (6)</td>
<td>17 (6)</td>
<td>17 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>R. thyrsiflorus</em></td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td><em>R. palustris</em></td>
<td>0 (6)</td>
<td>0</td>
<td>0</td>
<td>0 (6)</td>
</tr>
<tr>
<td></td>
<td><em>R. maritimus</em></td>
<td>0 (6)</td>
<td>33 (6)</td>
<td>40 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>R. thyrsiflorus</em></td>
<td>0 (6)</td>
<td>17 (6)</td>
<td>100 (1)</td>
<td></td>
</tr>
</tbody>
</table>

**Biomass**

The biomass of all three plant parts (shoots, tap roots and lateral roots) of mature plants differed significantly between the three species (Fig. 2, Table 3), because of both the effect of the submergence treatment, and different initial plant sizes (Fig. 2).

In all three *Rumex* species, shoot biomass declined as a result of submergence (Fig. 2, Table 3), more severely in dark submerged plants than in plants submerged under light conditions. Neither submergence period nor light level affected dry weight of tap roots, even when the three species were analysed separately. Overall there was a significant decrease in lateral root biomass during submergence, but the three species under study differed in their response. In *R. palustris* no effect of duration of submergence on the biomass of lateral roots was found, while in *R. thyrsiflorus*, the lateral root biomass significantly decreased during submergence. In *R. maritimus*, there was only a significant difference in
Resistance to submergence

lateral root biomass between the start of the experiment and after 56 days of submergence (Table 3). Light level did not significantly influence lateral root biomass.

![Graph showing the dry weight of shoots (DWs), tap roots (DWR), and lateral roots (DWL) of Rumex species during submergence in the light (open circles), in the dark (closed circles) or during drained growth (open squares) (Means ± SE, n=6).](image)

**Figure 2:** Dry weight of shoots (DWs), tap roots (DWR) and lateral roots (DWL) of *Rumex* species during submergence in the light (open circles), in the dark (closed circles) or during drained growth (open squares) (Means ± SE, n=6).

The effects of submergence on biomass are the direct result of the submergence treatment and not of other processes influencing growth, as shown by the continued growth of the well drained control plants (Fig. 2).
Table 3: Analysis of variance of dry weight of shoots, tap roots and lateral roots of *Rumex* species during 56 days of submergence in light and dark.


*F*, *H*: statistic; sign. diff.: significant differences

*t*: *R. thyrsiflorus*, *p*: *R. palustris*, *m*: *R. maritimus*; 0,14,35,56: duration of flooding (days); *l*: light, *d*: dark

*: P<0.05, **: P<0.01, ***: P<0.001

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Source of variance</th>
<th>Source of variance</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DW$_S$*(^a)</td>
<td>DW$_{TR}$*(^a)</td>
</tr>
<tr>
<td></td>
<td>df</td>
<td>H</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>28.245***</td>
</tr>
<tr>
<td>T</td>
<td>3</td>
<td>68.231***</td>
</tr>
<tr>
<td>L</td>
<td>2</td>
<td>3.900*</td>
</tr>
<tr>
<td>SxT</td>
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</tr>
<tr>
<td>SxL</td>
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<tr>
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</tr>
<tr>
<td>SxTxE</td>
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</tbody>
</table>

*\(^a\): DW$_S$ was analysed by Kruskal-Wallis analysis of variance on ranks; DW$_{TR}$ and DW$_{LR}$ were analysed by three-way analysis of variance
Discussion

The influence of light

In all three species, the survival of submergence is clearly higher in the light than in the dark (Fig. 1). Most probably, this effect of light on survival is related to photosynthesis under water, which can provide both oxygen (Gaynard & Armstrong, 1987; Setter et al., 1987a; Waters et al., 1989; Voesenek et al., 1993) and carbohydrates (Setter et al., 1989; Clevering et al., 1995). This can lead to better resistance to prolonged flooding, either by a higher and more efficient production of energy by aerobic respiration with photosynthetically derived oxygen or by the provision of readily respirable carbohydrates. The beneficial effects of light on survival of submerged conditions agree with studies on other plant species (Blom et al., 1994; Clevering et al., 1995; He et al., 1999).

The effects of light level during submergence on biomass of mature plants were restricted to the shoot (Fig. 2, Table 3). Although in other species oxygen (Gaynard & Armstrong, 1987) and carbohydrates (Waters et al., 1989) produced by underwater photosynthesis reached the roots, no effects of light on below ground biomass were noted in this study. It was expected that carbohydrates stored in the tap roots would be used when plants are flooded (Setter et al., 1987b). This would lead to a decrease in biomass, especially in the dark, when carbohydrates are more drawn on than in the light. However, the small and non-significant decrease in dry weight of tap roots suggests that there is only very little consumption of stored carbohydrates during submergence. This implies that the plants either are not able to degrade starch (Perata et al., 1998; which is the main storage carbohydrate in Rumex species), have a slow metabolic rate during flooding (Albrecht & Wiedenroth, 1994b), or the carbohydrates needed are allocated from plant parts other than the tap roots. Also in Scirpus maritimus, the amount of carbohydrates in tubers during submergence was independent of light level (Clevering et al., 1995) and no decreases in carbohydrates were found in storage tissues of S. maritimus and Phalaris arundinacea during anaerobic incubation (Barclay & Crawford, 1983).

The influence of plant size

Besides the effects of light level, also plant size clearly influences survival of submergence in these species. Under all experimental conditions juvenile plants died much sooner than mature plants (Fig. 1). The two most important plant characteristics involved in submergence tolerance, apart from underwater
Chapter 2

photosynthesis, are fermentative capacity and the amount of carbohydrates present (Armstrong et al., 1994). In previous studies it was already shown that fermentative capacity during oxygen deficiency in seedlings decreased with age (Andrews et al., 1994; Gudleifsson, 1997), and also in Rumex species this seems to be the case (Van der Sman et al., 1993a; Voesenek et al., 1993). Besides this, a very large portion of the non-structural carbohydrates was used up within one day when juvenile plants of Rumex species were submerged (unpublished results AJ Visser). Hence, probably the difference in survival found in our experiments is not caused by a lower fermentative capacity in juvenile plants, but by very limited amounts of reserve carbohydrates that are used up within a few days, even if the rate of consumption would be slow. Apparently, mature plants do use their carbohydrate stores to continue anaerobic respiration, but the rate is slow, which results in only little decrease in tap root weight. In other terrestrial species, it was shown as well that survival of oxygen deficiency can be determined by their size (Siebel & Blom, 1998) and amount of reserve carbohydrates present (Setter et al., 1987b; Hanhijärvi & Fagerstedt, 1995).

Post-flooding mortality

In a number of cases, Rumex plants that looked quite healthy immediately after submergence nevertheless died in the regrowth period (Table 2). This mortality might be due to desiccation of shoots as a result of either impaired root functioning (Kludze & DeLaune, 1996), damage to meristems or vascular tissues or other direct effects of oxygen deficiency. However, the appearance of chlorotic and necrotic spots and disintegration of seemingly healthy leaves are typical symptoms of what is called post-anoxic injury (Armstrong et al., 1994). This damage might be caused by formation of activated oxygen radicals that lead to lipid peroxidation (Hendry & Brocklebank, 1985), which can cause leakage of membranes followed by desiccation and loss of ions from cells. The formation of phytotoxic compounds as a result of oxidation of anaerobic metabolites, for instance the production of acetaldehyde by oxidation of ethanol (Monk et al., 1987a) might also be involved in post-anoxic injury. It has been shown that oxygen concentrations in the floodwater and in submerged shoots of rice can become very low (Setter et al., 1987a) and there are indications that oxidative stress occurs after submergence in rice (Ushimaru et al., 1992) and maize (Yan et al., 1996), even if the tissues were not completely anoxic during submergence. Moreover, in R. palustris the conversion of ethanol into acetaldehyde actually does take place if plants are exposed to 21% of oxygen after a period of anoxia (te Lintel Hekkert et al., 1998, chapter 5). Therefore, although we cannot rule out
direct effects of oxygen deficiency on post-flooding survival, it is likely that also post-anoxic injury is involved in the death of plants in the regrowth period.

Differences between species

The three species tested clearly differ in their survival of submergence (Fig. 1). The resistance to submergence correlates well with the field distribution of the species. This correlation might even be a causal relation, as was indicated by reciprocal transplantation experiments (Voesenek, 1990). *R. thyrsiflorus* grows in the highest parts of the floodplain and is flooded at maximum for 14 days (Table 1). Since the floods in this zone occur in late spring at the earliest, only larger plants are flooded and they are well able to survive a flooding period of 14 days, even without underwater photosynthesis (Fig. 1). In general, both the CO$_2$ concentrations (He et al., 1999) and the light intensity in flooded river forelands are low; therefore, underwater photosynthesis will be severely restricted. Since *R. thyrsiflorus* is dependent on underwater photosynthesis to survive prolonged periods of flooding (Fig. 1), this species cannot be found in the lower zone, where the floods last rather long (Table 1). Besides this, *R. thyrsiflorus* is a perennial species that cannot avoid the floods by completing its life cycle between two floods or ameliorate the stress.

In the zone in which the other two species grow, floods occur already early in the growing season (Table 1), so also young plants are flooded. Juvenile plants can only survive floods that last a few weeks (Fig. 1). Mature plants have a much better survival of flooding and even after 8 weeks of flooding in the dark, at least fifty percent of the plants are still alive.

*R. maritimus* is rather tolerant to flooding, but if the floods last too long and underwater photosynthesis is impeded, the plants die. In addition to amelioration of the oxygen deficiency and metabolic tolerance, this species can maintain itself at low-lying sites in the floodplain by having a short life cycle and survive the floods as a dormant seed. The large intervals between floods in some years (Table 1) are long enough to enable flowering (Van der Sman et al., 1993b).

*R. palustris* clearly is most tolerant to complete submergence, and is capable of surviving long lasting floods, even without underwater photosynthesis (Fig. 1). It is also the only species in which root biomass did not decrease during submergence (Fig. 2, Table 3). Most probably, the tolerance to oxygen deficiency is achieved by fermentation processes (Ricard et al., 1994). *R. palustris* is not able to avoid the floods (Van der Sman et al., 1992) and vegetative plants are confronted with flooding in most years (Table 1). Therefore, this species is dependent on tolerance and amelioration strategies.
In riverine plants there is a trade-off between competitive and stress tolerance abilities (Carter & Grace, 1990; Hills et al., 1994). *R. thyrsiflorus* is a species that is able to compete with tall, fast growing grasses on high elevated sites (Menges & Waller, 1983) but is not tolerant to flooding. In contrast, the two more tolerant species are weak competitors and will be outcompeted at higher lying sites. In summary, we found that the submergence tolerance of the three *Rumex* species tested agrees well with their field distribution and life history. The survival of complete submergence in these species is improved by high light levels, probably as a result of underwater photosynthesis. In addition, the size of the plants is important in this respect, presumably because of the carbohydrate stores in tap roots of mature plants.
Chapter 3

Fermentation, energy charge and net solute uptake in oxygen deficient *Rumex* species differing in flooding resistance

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Abstract

Three *Rumex* species occurring in a natural flooding gradient were compared with respect to their capacity to regenerate NAD⁺ during oxygen deficiency. To assess this capacity, the *in vitro* activities of enzymes involved in ethanolic and lactate fermentation and nitrate reduction were measured as well as the *in vivo* production of ethanol and lactate. Furthermore, the concentration of adenylates, the adenylate energy charge (AEC) and the net exchange of K⁺, Cl⁻, glucose, and acid equivalents were determined. During complete submergence in the light, virtually no PDC, ADH, or LDH enzyme activity was found in the shoots of three *Rumex* species, while there was clear increase in activity of these enzymes in lateral roots and tap roots. This suggests a higher oxygen status in the shoot compared to the below ground plant parts. In all three species, enzyme activities, adenylate energy charge (AEC) and the total amount of adenylates were highest in the tap roots. Compared to ethanolic fermentation, lactate fermentation and nitrate reduction seemed to play a minor role in acclimation to oxygen deficiency in *Rumex* species. The ethanolic fermentation rate was highest in the two most flooding resistant species, whereas fermentative capacity was low in the species more sensitive to flooding. The increase in enzyme activity in lateral roots and the AEC and the total amount of adenylates in below ground parts of the three species accords with their difference in flooding resistance. The net uptake of K⁺, Cl⁻, and glucose and the net extrusion of acid equivalents were clearly inhibited by anoxic conditions in all three species. However, the limited loss of solutes during anoxia and the resumption of ion uptake after reaeration suggest that membrane integrity of tap root cells of these species was retained during ten days of anoxia, if exogenous glucose was provided.

Introduction

Poor aeration leading to oxygen shortage is one of the major abiotic stresses that determine distribution of terrestrial vascular plant species and the success of some crops world wide (Vartapetian & Jackson, 1997). One important event leading to poor aeration and subsequently to oxygen deficiency is flooding of terrestrial habitats along rivers (Drew, 1990; Armstrong *et al.*, 1994; Blom, 1999). Plants can display several adaptations to flooding that lead to an improved oxygen supply (Armstrong *et al.*, 1994; Vartapetian & Jackson, 1997), such as enhanced shoot elongation (Kende *et al.*, 1998; Voesenek & Blom, 1999), formation of aerenchyma (Jackson & Armstrong, 1999) and development of adventitious roots (Visser *et al.*, 1996). However, these adaptations are only useful if plants are able
to reach the water surface. If contact with the air is not established, plants have to deal with low oxygen concentrations at the cellular level. Without oxygen, oxidative phosphorylation stops due to the lack of a terminal electron acceptor and consequently only limited amounts of ATP can be produced (Drew, 1990). The generalised biochemical basis for tolerance of oxygen deficiency includes (i) maintenance of glycolysis for generation of ATP and (ii) regeneration of NAD\(^+\) from NADH. Regeneration of NAD\(^+\) is crucial, since it is an essential substrate in glycolysis (Drew, 1997).

Two important pathways by which NAD\(^+\) regeneration takes place in many plant species during oxygen deficiency are ethanolic and lactate fermentation (Perata & Alpi, 1993; Ricard et al., 1994). The enzyme pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate into acetaldehyde, from which ethanol is formed. The latter step, which oxidises NADH to NAD\(^+\), is catalysed by alcohol dehydrogenase (ADH). Lactate is produced from pyruvate by the action of lactate dehydrogenase (LDH), which regenerates NAD\(^+\) as well.

Apart from ethanolic and lactate fermentation, also nitrate reduction by nitrate reductase (NR) can regenerate NAD\(^+\) during oxygen deficient conditions in some species (Bertani et al., 1987) and may therefore supplement fermentation in sustaining glycolysis and energy production. In some studies beneficial effects of nitrate and nitrate reductase activity on anoxic functioning have been found (Kemp & Small, 1993; Reggiani et al., 1993; Fan et al., 1997; Oberson et al., 1999; Vartapetian & Polyakova, 1999), while in others no effects were noted (Saglio et al., 1988; Botrel & Kaiser, 1997). However, most of these studies used only short-term experiments (up to one day). Consequently, the role of nitrate reduction in regeneration of NAD\(^+\) during oxygen deficiency in plant cells is still a matter of debate and little is known about nitrate reduction during oxygen deficiency lasting more than one day, a situation which often occurs in natural floodplains (Nabben et al., 1999; chapter 2).

An elegant way to assess the effect of anaerobic metabolism on the energy status of oxygen deficient plants is by evaluation of the adenylate energy charge (AEC). The AEC appears to be correlated with metabolic activity under hypoxia (Pradet & Raymond, 1983). However, sometimes the AEC remains high but is dominated by a small population of surviving cells, while the plants as a whole do suffer from stress and their vitality declines. In these cases, the total amount of adenylates may give a better estimate of the vitality of the tissues (Saglio et al., 1988).

Energy deficit will eventually lead to a lower activity of H\(^+\)-ATPases, resulting in a slower uptake rate of solutes (Drew, 1990). Besides this, a decreased ATP synthesis can cause depolarisation of the cell membranes, which leads to net efflux of cations. Therefore, the ability of tissues to maintain membrane functions
such as energy dependent solute uptake and retention of accumulated solutes can also give an indication of the amount of energy allocated to these processes (Zhang et al., 1992; Gibbs et al., 1998).

Previous studies have not examined fermentation rate, AEC and solute transport during oxygen deficiency in the same series of experiments. Besides this, most studies that focus on one of these aspects were performed with crop species, which in general have a lower resistance to oxygen deficiency than wild species from wet habitats (Crawford & Brändle, 1996). In this paper, we present a more complete set of data on tolerance to oxygen deficiency of three closely related species, which differ in ecological niche and resistance to submergence. Additionally, the NR activity was measured in tissues of plants after up to seven days of submergence and therefore, the contribution of nitrate reduction to regeneration of NAD$^+$ during oxygen stress lasting more than one day was assessed.

Three *Rumex* species from different habitats in river floodplains were compared with respect to their capacity to regenerate NAD$^+$ during oxygen deficiency. *R. palustris* Sm. and *R. maritimus* L. grow on frequently flooded sites, while *R. thyrsiflorus* Fingerh. is seldom flooded since it grows on higher elevated sites. Although *R. palustris* and *R. maritimus* inhabit the same locations, they differ in resistance to complete submergence, with *R. palustris* being the most resistant. The higher survival of *R. palustris* when submerged in complete darkness implies that this species is better able to tolerate oxygen deficiency at the cellular level than *R. maritimus*. *R. thyrsiflorus*, which is least confronted with flooding, shows the poorest survival during submergence of the three species, probably because it is least adapted to oxygen deficiency at the cellular level (Nabben et al., 1999; chapter 2).

To assess the differences in metabolic adaptations to submergence-induced oxygen deficiency in these species, we conducted two types of experiments. We measured the *in vitro* enzyme activities of PDC, ADH, LDH, and NR as well as the concentration of adenylates and the AEC in submerged plants. In a separate experiment we determined both the *in vivo* production of ethanol and lactate and the net exchange of K$^+$, Cl$^-$, glucose, and acid equivalents in tap root slices during anoxia. In this way we were able to assess the capacity to regenerate NAD$^+$ and to estimate the energy status and the integrity and permeability of membranes during oxygen deprivation.
Chapter 3

Materials and Methods

Plant growth and treatments for enzyme, AEC and adenylate measurements

Seeds of *Rumex palustris* Sm., *R. maritimus* L. and *R. thyrsiflorus* Fingerh. were collected from natural populations in the river Rhine area near Nijmegen, the Netherlands. Seeds were sown on polyethylene grains (Lacqtene Low Density, Elf Atochem, France) that were soaked in nutrient solution containing (in mM): 2 Ca(NO₃)₂, 1.25 K₂SO₄, 0.5 MgSO₄, 0.5 KH₂PO₄ and the micronutrients (in µM): 15 FeEDTA, 50 NaCl, 25 H₃BO₃, 2 MnSO₄, 2 ZnSO₄, 0.5 CuSO₄ and 0.5 H₂MoO₄; pH=5.8. After one week of germination (12 h, 20 µmol m⁻² s⁻¹ (PPFD), Philips TL33, 27 °C; 12 h dark, 10 °C), the seedlings were transferred to a climate room (16 h, 120 µmol m⁻² s⁻¹ (PPFD), Philips TL84, 22 °C; 8 h dark, 20 °C; relative humidity 50%) and two weeks later uniform seedlings were transferred to hydroponic culture (same conditions as before). Each hydroponic flow-through unit consisted of three 20 l PVC containers connected to a 30 l aeration vessel (120 l air h⁻¹), nutrient solution circulated at a rate of 60 l h⁻¹ per container (Visser et al., 1996). Six plants were mounted in a PVC lid that was placed on top of each container. Nutrient solutions were replaced every two weeks, which prevented nutrient deficiency.

After three (*R. palustris* and *R. maritimus*) or four weeks (*R. thyrsiflorus*) of growth on hydroponics, the lids with plants were transferred to 20 l containers with stagnant deoxygenated agar solution (0.1 % (w/v) in nutrient solution), in order to mimic the gas composition of a flooded soil (Wiengweera et al., 1997). The lids were fixed to the containers, and the containers with plants were completely submerged in tap water (pH=7.8, 0.05 mM CO₂, 20 °C) in 250 l tanks. The leaves were prevented from reaching the water surface by wire gauze that was mounted just below the water surface. Non-submerged plants that were placed on 20 l containers filled with continuously aerated fresh nutrient solution served as controls. The enzyme activities were measured for plants grown in a separate experiment from that in which the adenylates and AEC were determined. The light intensity at plant level of both submerged and control plants was 115 µmol m⁻² s⁻¹ (PPFD, Philips TL84, 16 h light, 8 h dark) in the experiment in which the enzyme activities were measured, and 50 µmol m⁻² s⁻¹ (PPFD, Philips TL84, 16 h light, 8 h dark) in the AEC experiment. These light levels are in the range of light intensities in flooded forelands of the river Rhine (unpublished results PJA Vervuren) and they are above the light compensation point for underwater photosynthesis in *Rumex* species (derived from Vervuren et al., 1999; Rijnders et al., 2000). On days 0, 1, 2, 4 and 7 of the treatments plants were harvested for enzyme determinations, while the plants used for the determination
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of AEC were harvested on days 0, 7, 14 and 21 after the start of flooding (flooded plants: n=6; control plants: n=3 for both experiments). To minimise the effects of diurnal rhythms on the parameters measured, plants were always harvested seven hours after the start of the light period. Plants were divided into shoots, tap roots and lateral roots and after dissection, which took less than one minute, the samples were frozen in liquid nitrogen. This means that oxygen deficient tissues were reaerated for a short period following submergence. Although we aware that adenylate levels can change rapidly after transfer to aerated conditions (Pradet & Raymond, 1983), we haven chosen to test the ecologically relevant situation of submergence thereby accepting the problems of reaeration. Because of the size of the plants it was not possible to transfer them to an anaerobic workbench without exposing them to air. Exposure to air during dissection is likely to have increased the AEC, so that values presented here may be an overestimate of the energy status of tissues.

The samples used for enzyme determinations were stored at -20 °C and the samples used for AEC determination were lyophilised (Virtis Bench Top 3) and stored at -80 °C until analysis.

**Extraction and analysis of in vitro enzyme activity**

Plant material was homogenised in a cooled mortar with 4 to 8 ml of ice-cold extraction buffer (0.05 M K$_2$HPO$_4$, 0.01 M KH$_2$PO$_4$, 1 mM Na$_2$EDTA, 5 mM dithiothreitol, 0.5% (w/v) bovine serum albumin, 2% (w/v) polyvinylpyrrolidone (pH=7.5) per gram of fresh weight. The homogenate was centrifuged (20 min at 1400g, 4 °C) and the supernatant was used for determination of enzyme activities.

The *in vitro* activity of PDC, ADH, and LDH were determined according to Bergmeyer (1974) by recording the maximum rate of decrease in NADH concentration at 20 oC by monitoring A$_{340}$ with a spectrophotometer. In all cases, enzyme activities were correlated proportionally to the volume used.

PDC (EC 4.1.1.1) activity was determined in 2.2 ml of an assay mixture containing: 0.2 M Na-citrate (pH 6.0), 0.1 mM NADH, 45 IU ADH, 1 mM KCN, 8 mM Na-pyruvate and 300 µl supernatant. Since this is an unusual method to extract and analyse PDC, we extracted and analysed also some samples of different species and tissues according to Zhang & Greenway (1994), with thiaminepyrophosphate (TPP) and Mg$^{2+}$ included in the extraction and assay buffer (pH 6.0). The results obtained without TPP and Mg$^{2+}$ in extraction and assay buffer were 70 to 80% of those obtained with TPP and Mg$^{2+}$ included. Results presented in all the figures and those of recovery measurements are from
samples extracted and assayed with the first mentioned buffers and the values were not adjusted for the difference between buffers.

ADH (EC 1.1.1.1) activity was determined in the acetaldehyde to ethanol direction, with 2.5 ml assay mixture containing: phosphate buffer (0.05 M K$_2$HPO$_4$ and 0.01 M KH$_2$PO$_4$, pH 7.5), 0.1 mM NADH, 3 mM acetaldehyde and 300 µl supernatant.

LDH (EC 1.1.1.27) activity was determined in an assay mixture containing: 2.5 ml phosphate buffer (pH 7.5), 0.1 mM NADH, 1 mM Na-pyruvate and 200 µl supernatant.

The in vitro NR (EC 1.6.6.1) activity was determined in 0.7 ml of a reaction mixture containing: phosphate buffer (pH=7.5), 15 mM KNO$_3$, 0.3 mM NADH and 200 µl supernatant. After 20 minutes incubation at 30 °C, the reaction was stopped by addition of 100 µl 1 M zinc acetate. Then, 1 ml sulphanilamide (10 mg l$^{-1}$ in 3 M HCl) and 1 ml 0.01 % (w/v) (N-(1-naphthyl)ethylenediaminedichloride) were added and the mixture was incubated for 20 minutes and then centrifuged (10 min at 1400g, 4 °C). Nitrite formed was determined against a calibration curve of 0 to 200 µM NaNO$_2$ by measuring A$_{540}$ (modified after Hageman & Reed, 1980). Since the extraction buffer contains EDTA and no Mg$^{2+}$ was added during the assay, most probably all enzyme was present in the active form and the in vitro NR activity measured represents the maximum activity (Kaiser & Huber, 1997).

The recovery of pure enzymes added before extraction was tested in plants of all three species submerged for seven days and extracts of shoots, tap roots and lateral roots were analysed (n=3 for each combination of species and plant part). The overall recoveries were 96 ± 3%, 117 ± 5%, 86 ± 6% and 81 ± 5%, for PDC, ADH, LDH and NR, respectively (means ± SE, n=27). No significant differences in recovery among species or plant parts were observed.

**Extraction and estimation of adenine nucleotides**

Since the largest responses to flooding occurred in the below ground plant parts (see Results), only tap roots and lateral roots were analysed for their adenylate content. 100 mg lyophilised root material was homogenised in a cooled mortar with 4 ml ice-cold 10% (w/v) HClO$_4$. The homogenate was neutralised with KOH and after addition of 20 mg ml$^{-1}$ polyvinylpolypyrrolidone, the extract was stored on ice and mixed intermittently. After 20 minutes, the extract was centrifuged (20 min at 5000g, 4 °C) and the supernatant was used for determination of the nucleotides. The method described by Mendelssohn & McKee (1981) was used to determine the concentration of nucleotides, apart from the HEPES-buffer, which
was replaced by Tris buffer (100 mM Tris(hydroxymethyl)aminomethane, 50 mM MgSO$_4$, 0.5 mM Na$_2$EDTA, pH=7.4) to maximise the yield. Briefly, adenine nucleotides were determined using the ATP-dependent light yielding reaction of the firefly-lantern luciferin-luciferase complex (Sigma FLE-50) with a LKB 1250 Luminometer. ATP was determined directly, while ADP and AMP were converted enzymatically to ATP by pyruvate kinase (EC 2.7.1.40) and myokinase (EC 2.7.4.3), respectively. The ATP concentration was determined against a calibration curve of 0.1 to 10 µM ATP. The recovery of AMP added after extraction was tested in all three species submerged for 21 days and did not differ significantly among species or between tap roots and lateral roots and the overall recovery was 81 ± 1% (means ± SE, n=24). AEC was calculated as (([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP])) (Pradet & Raymond, 1983).

Plant growth and treatments for determination of fermentation products and net uptake of solutes

The same seed lots were used and germination and plant growth were as reported in a previous publication (Nabben et al., 1999; chapter 2). After 7 months of growth, roots were washed out of the potting mix and shoot and lateral roots were removed. Tap roots (5 to 7 mm diameter) were surface sterilised (5 min. in 0.5% (w/v) NaClO), cut in slices (0.6 to 0.8 mm thick), and washed two times with 0.5 mM CaSO$_4$. Three hundred tap root slices were aged (20 °C) for two days in 800 ml of aerated 0.5 mM CaSO$_4$ and subsequently for three more days in 800 ml of aerated incubation buffer (pH=6.5) containing (in mM): 0.5 CaSO$_4$, 0.5 KCl, 0.5 K-MES (2-[N-morpholino]ethanesulfonic acid), 10 glucose, 10 mg l$^{-1}$ carbenicillin, at 20 °C (modified after Zhang et al., 1992). The addition of K$^+$, Cl$^-$ and glucose ensured that the concentrations of these solutes in the tissues were relatively high at the start of the treatments. The incubation buffer was aerated vigorously with sterile compressed air and was replaced regularly. All glassware and solutions were sterile and manipulation of the tap root slices was done in a sterilised cabinet. After five days of ageing the respiration of the tap root slices had stabilised (own unpublished results) indicating that stress responses had faded away. After ageing, the tap root slices were pre-treated hypoxically for 24 hours by bubbling the incubation buffer with a sterile mixture of air and nitrogen (final concentration 1.0% (v/v) O$_2$). A hypoxia pre-treatment increases anoxia tolerance and rate of alcoholic fermentation in several species (Saglio et al., 1988; Waters et al., 1991; Zhang & Greenway, 1994) and reduces loss of solutes during anoxia (Greenway et al., 1992). Besides this, it resembles the change in oxygen concentration that occurs upon flooding more than does sudden imposition of
anoxia (anoxic shock). After the hypoxia pre-treatment, the slices were distributed over six serum bottles per species; each bottle contained 25 ml incubation buffer (14 slices per bottle, approximately 0.4 g FW). Three bottles were incubated in a normal air environment while the other three bottles, which had been pre-flushed with high purity nitrogen gas to remove oxygen, were incubated in an anaerobic workbench (<10 ppm O₂; model 1029 Forma Scientific Anaerobic System, Ohio, USA). The stoppered bottles were incubated (20 °C) on a rotary shaker (100 rpm) and the headspace was flushed regularly to keep the oxygen concentration at the desired level. After 10 days of incubation, the anoxic tissues were transferred back to aerated conditions for three days. After 1, 3, 6, 10 days anoxia and 3 days reaeration samples of incubation medium were taken and the pH was measured, after which the incubation medium was replaced by fresh, sterile medium. No signs of increased microbial activity were noted during the whole experiment. The concentrations of solutes did not drop below 0.35 mM, 0.20 mM and 5.6 mM for K⁺, Cl⁻ and glucose respectively. The medium samples were stored at −20 °C for subsequent chemical analyses. Tissues were harvested at the end of the experiment after 13 days.

**Chemical analyses**

The concentration of K⁺ in the medium was measured with a Technicon Flame Photometer IV (Technicon Autoanalyser Methodology (TAM): N-20b), while the concentration of Cl⁻ was determined colorimetrically with a Technicon Autoanalyser II (TAM: Industrial Methodology 11). Glucose was measured enzymatically according to Guglielminetti et al. (1995a). Tap root slices (0.4 g FW) were ground in a cooled mortar in 5 ml ice-cold 1 N HClO₄. After neutralisation with K₂CO₃, the samples were centrifuged (10 min at 1400g, 4 °C). The samples of incubation medium and tissue extracts were analysed for ethanol and lactate content according to Bernt & Gutmann and Gutmann & Wahlefeld in Bergmeyer (1974). Recovery of added ethanol was 92.3 ± 1.9% and 98.9 ± 0.7% and recovery of lactate was 99.9 ± 0.9% and 98.1 ± 0.4%, for medium and tissues respectively (means ± SE, n=18). The exchange of acid equivalents was estimated from the pH change and the Kₐ of MES by using the Henderson-Hasselbalch equation (Price & Dwek, 1982). The resulting data are the average rates of net solute exchange over the time interval (1 to 4 days) between two sampling times. In the figures, these average data are plotted on the time point of sampling.
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Statistical analyses

After rank transformation of the data on enzyme activities, the results for enzyme activity, AEC and amount of adenylates were tested by two-way analysis of variance (species x time) followed by a Tukey test. The results for fermentation products and net exchange of solutes were tested by two-way repeated measures analysis of variance (treatment x time and species x time). All analyses were performed with the SigmaStat statistical package and according to Sokal & Rohlf (1995).

Results

Enzyme activities

To estimate the contribution of ethanolic and lactate fermentation and nitrate reduction in regeneration of NAD$^+$ during submergence-induced oxygen deficiency, the in vitro activities of enzymes involved in these processes were studied. The in vitro enzyme activities in all parts of control plants of the three species did not change appreciably during the course of the experiment, except for the NR activity, which decreased slightly (results not shown). In the shoots of submerged plants, hardly any change in the activity of all enzymes tested was detected (results not shown). However, a clear increase in activity of all enzymes except nitrate reductase occurred in the below ground parts of R. palustris and R. maritimus (Fig. 1).

In R. palustris and R. maritimus the PDC activity of the tap roots increased significantly during seven days of submergence and reached a stable level after four days (Fig. 1a). In R. thyrsiflorus, however, the PDC activity stayed at a constant and low level. The ADH activity of the tap roots increased significantly in all three species, but the increase in R. maritimus was larger than in the other two species (Fig. 1c). Both the PDC and the ADH activity in lateral roots of the three species increased significantly during submergence (Figs. 1b and d) with the largest increases in R. palustris. In all plant parts, the PDC activity was always several folds lower than the ADH activity (compare Figs. 1a and b with c and d).

The LDH activity of tap roots and lateral roots of R. maritimus increased considerably (Figs. 1e and f). In R. palustris the LDH activity increased moderately in the below ground parts, while in R. thyrsiflorus the activity stayed approximately at the same level (lateral roots) or even decreased slightly (tap roots) after submergence.
Figure 1: In vitro enzyme activity of PDC (a and b), ADH (c and d), LDH (e and f) and NR (g and h) of tap roots (a, c, e and g) and lateral roots (b, d, f and h) of submerged plants of Rumex palustris (circles), R. maritimus (triangles), and R. thyrsiflorus (squares). (Means ± SE, n=6).
The \textit{in vitro} enzyme activity of PDC, ADH, and LDH in all plant parts attained after four to seven days of complete submergence were the maximum levels reached; after ten days of submergence the enzyme activities started to decrease slightly until day 21 (own unpublished results).

The NR activity in all plant parts declined slowly but significantly during submergence and no differences among species were observed (Figs. 1g and h). The NR activity of submerged plants was similar to that of control plants (data not shown).

\textbf{AEC and total amount of adenine nucleotides}

We determined the energy status of lateral and tap roots after submergence, by measuring the AEC and the total amount of adenylates. The AEC of control plants of all three species stayed at a constantly high level of 0.85 to 0.9. The total amount of adenylates in control plants of \textit{R. maritimus} and \textit{R. thyrsiflorus} also remained constant whereas it increased slightly in control \textit{R. palustris} plants (results not shown).

The AEC in the tap roots of each of the three species during submergence remained at a high level of approximately 0.85 (Fig. 2a). On the other hand, in lateral roots of \textit{R. maritimus} and \textit{R. thyrsiflorus} the AEC declined strongly at 14 days of submergence to values less than 0.65 and only in \textit{R. palustris} did the AEC of the lateral roots stay at the initial level (Fig. 2b). In contrast to the AEC, the total amount of adenylates in tap roots of \textit{R. maritimus} and \textit{R. thyrsiflorus} declined markedly during submergence, while this value increased in tap roots of \textit{R. palustris} after 14 days (Fig. 2c). In lateral roots of all three species, the amount of adenine nucleotides declined markedly (Fig. 2d). However, in \textit{R. palustris} this decrease began later than in \textit{R. thyrsiflorus}, while the initial decline was even more severe in \textit{R. maritimus}. This resulted in significantly higher adenylate levels in \textit{R. palustris} than in the other species during the entire period of submergence. The levels in \textit{R. thyrsiflorus} were significantly higher than those of \textit{R. maritimus} after one and two weeks of submergence.

\textbf{Fermentation products}

Since the \textit{in vitro} enzyme activities do not give a measure of the \textit{in vivo} fermentation rate, the production rates of ethanol and lactate by anoxic tap root slices were measured. The amount of ethanol present in both aerated and anoxic tap root slices at the end of the experiment was very low and negligible compared
to the amount accumulated during the whole experiment in the incubation medium, as concentrations in tissues and medium were approximately equal (results not shown).

Figure 2: Adenylate energy charge (AEC; a and b) and total adenine nucleotides (c and d) of tap roots (a and c) and lateral roots (b and d) of submerged plants of Rumex palustris (circles), R. maritimus (triangles), and R. thyrsiflorus (squares) after a reaeration period of less than one minute. (Means ± SE, n=6). Adenine nucleotide concentrations are given as the percentage of the time zero value for each plant part. One hundred percent corresponds to: R. palustris: 1.08 and 2.60; R. maritimus: 0.55 and 0.85; R. thyrsiflorus: 0.26 and 0.56 μmol g⁻¹ dry weight for tap roots and lateral roots respectively.
Figure 3: Ethanol production by aerated (closed circles) or anoxic (open circles) tap root slices of Rumex palustris (a), R. maritimus (b), and R. thyrsiflorus (c) (Means ± SE, n=3; the data represent the average rate of the preceding time interval). The hatched area represents the reaeration period.
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The ethanol production by anoxic tap root slices of all three species was much higher than that of aerated tissues (Fig. 3). The presence of a low production of ethanol in aerated tissues was probably caused by induction of fermentation in the hypoxic period preceding the start of the measurements or by the presence of an anoxic core in the tap roots (Zhang & Greenway, 1994). In the anoxic tissues, *R. maritimus* showed a somewhat higher production than *R. palustris*, while that of *R. thyrsiflorus* was clearly the lowest. In all three species, the anoxic ethanol production declined with time and decreased to control levels upon reaeration. In both tap root slices and the medium only very low and therefore negligible amounts of lactate were measured (results not shown).

**Net exchange of solutes**

To assess whether or not tap root slices of the three species were capable of active solute transport and retention of accumulated solutes during anoxia, we determined the net uptake or loss of K⁺, Cl⁻, glucose and acid equivalents between tissues and medium. The net uptake patterns of K⁺ and Cl⁻ were very similar (Fig. 4). There was a clear net uptake of these ions by aerated tissues, at least for the first days of treatment. In *R. palustris* net uptake was relatively slow and constant, while in *R. maritimus* the net uptake of both K⁺ and Cl⁻ declined with time resulting in net loss at 6 or 13 days of incubation. The net uptake of both K⁺ and Cl⁻ collapsed in tap root slices of *R. thyrsiflorus* during the first days of aerated incubation resulting in a steady net loss from day 6 onwards. In contrast to aerated tissues, there was hardly any uptake of K⁺ and Cl⁻ in anoxic tap root slices of the three species. After one day of anoxia, there was even a small net loss of K⁺ and Cl⁻ in *R. palustris*. Upon reaeration, all three species showed clear net uptake of both K⁺ and Cl⁻. Glucose net uptake of both aerated and anoxic tap root slices of all three species declined during the experiment (Fig. 5). The glucose net uptake of *R. thyrsiflorus* was slower than that of the other two species and with time it decreased to a greater extent in *R. palustris* compared to *R. maritimus*. There was no difference in uptake between aerated and anoxic tap root slices of *R. palustris*, while in *R. maritimus* glucose net uptake was higher in aerated tissues than in anoxic tissues during the first three days. Only at ten days was a significantly higher glucose net uptake noted in aerated tap root slices of *R. thyrsiflorus* compared to anoxic slices.

In aerated *R. palustris* tissues, the net loss of acid equivalents declined slowly, while it increased in *R. maritimus* and rose dramatically in *R. thyrsiflorus* (Fig. 6).
In anoxic tap root slices of all three species it was rather constant and low compared to aerated slices and it increased when the tissues were reaerated.

Figure 4: $K^+$ (a-c) and $Cl^-$ (d-f) net uptake or loss by aerated (closed circles) or anoxic (open circles) root slices of Rumex palustris (a and d), R. maritimus (b and e), and R. thyrsiflorus (c and f) (Means ± SE, n=3; the data represent the average rate of the preceding time interval). The hatched area represents the reaeration period.
Figure 5: Glucose net uptake by aerated (closed circles) or anoxic (open circles) tap root slices of Rumex palustris (a), R. maritimus (b), and R. thyrsiflorus (c) (Means ± SE, n=3; the data represent the average rate of the preceding time interval). The hatched area represents the reaeration period.
Figure 6: Net extrusion of acid equivalents by aerated (closed circles) or anoxic (open circles) tap root slices of Rumex palustris (a), R. maritimus (b), and R. thyrsiflorus (c) (Means ± SE, n=3; the data represent the average rate of the preceding time interval). The hatched area represents the reaeration period.
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Discussion

Significance of nitrate reduction

The *in vitro* NR activity during seven days of submergence was clearly lower than that of the other NAD\(^+\) regenerating pathways (Fig. 1). In addition, the *in vitro* NR activity was at least two times lower than the *in vivo* production rate of ethanol in anoxic tap root slices (compare Figs 1 and 3). Generally, *in vitro* rates overestimate the *in vivo* enzyme activity, especially in the case where NR was probably completely activated during the assay (see Materials and Methods), a condition that is unlikely to occur *in vivo* (Kaiser & Huber, 1997). Therefore, the *in vivo* rate of nitrate reduction in anoxic tap roots of *Rumex* species was most probably several folds lower than the *in vivo* rate of ethanolic fermentation. Moreover, in contrast to anoxic rice coleoptiles (Reggiani et al., 1993), the NR activity in submerged *Rumex* tissues decreased with time and was similar in submerged and control plants. Ethanolic and lactate fermentation and nitrate reduction all oxidise NADH in equimolar amounts. Therefore, it is likely that the contribution of nitrate reduction in regeneration of NAD\(^+\) in completely submerged *Rumex* species is limited, especially after four to seven days of submergence. This could be expected, since the nitrate concentration in soils drops severely upon flooding (Engelaar et al., 1991). In anaerobically germinating rice seeds, nitrate is translocated from the seed to the growing coleoptile (Reggiani et al., 1993). However, although also *Rumex* species can contain considerable amounts of nitrate (Gebauer et al., 1984), this source will probably also be exhausted within a few days of flooding (Kemp & Small, 1993; Reggiani et al., 1993).

If NR and ADH would compete for NADH, as has been suggested by some authors, addition of nitrate to oxygen deficient plants could lead to lower ADH activities. However, exogenous supply of 8 mM nitrate to roots of *R. maritimus* and *R. thyrsiflorus* did not lead to a lower *in vitro* ADH activity after three days of oxygen deficiency, compared to roots without exogenous nitrate (Laan, 1990), as was also found in maize root tips (Saglio et al., 1988). Consequently, the presumably low availability of nitrate in flooded soils and the low *in vitro* enzyme activity of NR in tissues of submerged *Rumex* species suggest that nitrate is not an important alternative metabolic sink for reducing power during long-term complete submergence of *Rumex* species.
Ethanolic and lactate fermentation

In all plant parts, the in vitro PDC activity during submergence was much lower than the ADH activity (compare Figs. 1a and b with c and d). This is in agreement with previous studies, which indicated that the reaction catalysed by PDC is the rate-limiting step in ethanolic fermentation (Waters et al., 1991; Bucher et al., 1994; Zhang & Greenway, 1994, Quimio et al., 2000). The rapid conversion of acetaldehyde into ethanol by ADH is beneficial to cells since it prevents accumulation of this phytotoxic intermediate.

The low in vitro activities of PDC, ADH and LDH in shoots of all three species was probably due to the oxygen status of the submerged leaves. It is likely that underwater photosynthesis (Rijnders et al., 2000) or oxygen dissolved in the floodwater maintained an internal oxygen concentration high enough to allow aerobic respiration to continue. In contrast to shoots, activities of PDC, ADH and LDH did increase in root tissues of the three species. To the best of our knowledge this is the first time that it is shown experimentally that fermentation enzymes are not induced in submerged shoot tissues.

In below ground parts of *R. maritimus* the in vitro LDH activity had also strongly increased (Figs. 1e and f). However, this did not result in appreciable amounts of lactate in tap root slices or bathing medium during anoxia. It is possible that there was lactate produced in the tissues during the anoxic period, which was re-oxidised to pyruvate in the three days of normoxia following anoxia (Sweetlove et al., 2000). However, it is likely that if a lot of lactate was produced, a part of it would be excreted (Rivoal & Hanson, 1993; Sweetlove et al., 2000), but we found hardly any lactate in the bathing medium. Although we can not completely exclude that lactate was formed in oxygen deficient tap roots of *R. maritimus* and that the accumulation of lactic acid causes the reduced resistance to flooding of this species compared to *R. palustris*, it is unlikely that there was a large lactate production in anoxic tissues of any of the *Rumex* species. Also in other species a high in vitro LDH activity did not lead to high lactate production (Good & Muench, 1993; Rivoal & Hanson, 1994). It is still not clear what the function of a potential for high LDH activity not resulting in actual lactate production might be.

The absence of lactate fermentation is in contrast with studies on some anoxia sensitive species (e.g. Joly & Brändle, 1995; Germain et al., 1997) and species from the genus Limonium (Rivoal & Hanson, 1993). Furthermore, it accords with the generally accepted idea that species tolerant to oxygen deprivation show little lactate production during prolonged oxygen deficiency (Armstrong et al., 1994; Joly & Brändle, 1995; Vartapetian & Jackson, 1997). In contrast to lactate, there were relatively high rates of production of ethanol in the three species during
anoxia (Fig. 3). Accordingly, ethanolic fermentation appears to be the main pathway for NAD⁺ regeneration in these *Rumex* species as it is in many other species (Perata & Alpi, 1993; Ricard et al., 1994).

The rate of ethanol production declined in all three species during anoxia (Fig. 3). However, this was not due to inhibition by high external ethanol concentrations, as these remained below 5 mM and tests showed that ethanol at this concentration did not reduce ethanol production rates (own unpublished results). A decrease in ethanol production was also found for anoxic beetroot leading to the hypothesis that down regulation of glycolysis may be an acclimation to reduced ATP requirements during anoxia (Zhang & Greenway, 1994). Down regulation of glycolysis will result in conservation of substrate for continued energy supply during prolonged oxygen deficiency. Glucose net uptake by tap root slices decreased during anoxia (Fig. 5), in agreement with similar reductions found in rice coleoptiles and aged beetroot (Zhang & Greenway, 1995). In the tap root slices this decline was similar to the decline in ethanol production (compare Figs 5 and 3). The ratio of ethanol production to glucose net uptake was approximately 2:1 during the whole time course. This implies that sugar reserves were maintained during anoxia, so the decline in ethanolic fermentation was not due to a lack of substrates. Besides this, it is tempting to conclude that mainly the (readily fermentable) glucose that was taken up or already present in the tissues, was converted into ethanol and that other carbohydrates stored in the tissues were hardly used.

*Correlation between ethanolic fermentation, energy status and flooding resistance*

In tap roots of both *R. palustris* and *R. maritimus* and in lateral roots of *R. palustris* there was a clear increase in *in vitro* PDC and ADH activity (Figs. 1a-d) and ethanol production (Fig. 3), which rates are comparable to earlier studies of other species (Waters et al., 1991; Good & Muench, 1993; Zhang & Greenway, 1994). Although the rate of ethanol production in anoxic tap roots of *R. maritimus* was equal or even higher (Fig. 3) than in *R. palustris*, there was a steeper decrease in total adenylates (Figs. 2c) during submergence in the former species. Besides this, the *in vitro* enzyme activity of PDC and ADH (Fig. 1b and d), the AEC and the total amount of adenylates (Fig. 2 b and d) in lateral roots of *R. palustris* were higher than in those of *R. maritimus*. This reflects the higher resistance to complete submergence of *R. palustris* (Nabben et al., 1999; chapter 2). It is possible that the measured AEC gives an overestimation of the actual AEC during submergence since tissues were exposed to normoxic conditions for a short period.
before killing the tissues and measurement of the adenylates (Pradet & Raymond, 1983). However, the AEC will be at least a measure of the damage done to the tissues during submergence combined with the capacity to restore the AEC within one minute of normoxia. Therefore, the present data for AEC and total amount of adenylates still give an estimate of the vitality of the tissues at the moment of desubmergence (VanToai et al., 1995). Moreover, the small standard errors for the AEC and adenylates determinations (Fig. 2) show that all replicates responded in the same manner to submergence and a short reaeration period.

Both the enzyme activities involved in fermentation (Figs. 1) and the production of ethanol (Fig. 3) of *R. thyrsiflorus* were lower than that of the other two species. However, the rate of ethanol production in anoxic tap root slices of *R. thyrsiflorus* was higher than could be expected from the *in vitro* PDC activity. This may be related to a higher *in vivo* PDC activity (Zhang & Greenway, 1994) and consequently of ethanolic fermentation by exogenous glucose (Waters et al., 1991). Possibly, *R. thyrsiflorus* has carbohydrate stores that are exhausted fast or that are not used efficiently during anoxia. Exogenously supplied glucose may overcome these problems and can sustain prolonged fermentation (Zhang & Greenway, 1994; Perata et al., 1998). Theoretically, the low *in vitro* enzyme activity could mean that aerobic respiration was still functioning in the below ground parts during submergence. However, the oxygen supply by underwater photosynthesis and internal aeration (Laan et al., 1989b) of *R. thyrsiflorus* most probably are not more efficient than that of the other two species. Moreover, the AEC in lateral roots (Fig. 2b) and the amount of adenylates in lateral roots and tap roots (Figs. 2c and d) had declined substantially after 7 days of submergence. Therefore, it seems highly unlikely that *R. thyrsiflorus* was performing aerobic respiration in the below ground parts when completely submerged. Accordingly, the lower fermentation rate in submerged *R. thyrsiflorus* presumably contributes to the decrease in viability of this species during oxygen deficiency (Fig. 2) and its only moderate resistance to submergence (Nabben et al., 1999; chapter 2).

The tap roots survive longer than the other organs during complete submergence (Nabben et al., 1999; chapter 2). This is reflected in the high *in vitro* PDC activity (and therewith a high capacity for ethanolic fermentation) in *R. palustris* and *R. maritimus* (Figs. 1a-d), the maintenance of AEC levels similar to aerobic controls (Fig. 2a) and the high amounts of adenylates (Fig. 2c). Since the tap roots are also the location of the main carbohydrate reserves and new root and shoot primordia (Armstrong et al., 1994), this is the organ from which regeneration occurs after the floods have receded (Nabben et al., 1999; chapter 2). The rate of ethanolic fermentation and therewith the ability to maintain the tap roots vital may therefore contribute to the differences in survival during submergence among the three species.
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Exchange of solutes during anoxia

The very low net uptake of ions in aerated tap root slices of *R. palustris* and the gradual decrease in ion net uptake in aerated tissues of *R. maritimus* (Fig. 4) might be caused by a saturation of the internal ion content. In this situation, the gross influx and efflux are approximately equal, leading to no net exchange of ions. However, the fast decrease in ion net uptake and the very high net loss of acid equivalents (Fig. 6) in aerated tap root slices of *R. thyrsiflorus* (and to a lesser extent also in *R. maritimus*) suggest that the membranes of these tissues become leaky. This may be due to enhanced senescence as indicated by the brown discoloration of the tap root slices after ten days of aerated incubation (probably by oxidation catalysed by polyphenol oxidase; results not shown), indicating deterioration of the tissues. Apparently, senescence is slowed down under oxygen deprivation since these phenomena do not occur under anoxia. In anoxic rice coleoptiles senescence also appeared to be slower compared to aerated tissues (Ishizawa et al., 1999), while in potato tubers several aspects of the wound response were inhibited by hypoxia (Butler et al., 1990). This was mainly caused by a decrease in synthesis of RNA and proteins involved in the wounding response. Perhaps, also processes involved in senescence of tap root slices of *R. thyrsiflorus* were inhibited by oxygen deficiency or energy deficit, although this cannot be proven by the current experiments. One important factor causing senescence (also in *Rumex* species, Banga et al., 1997) is ethylene. Therefore, the absence of oxygen and therewith the absence of ethylene production (Armstrong et al., 1994) and oxidative stress, which could result in lipid peroxidation (Crawford & Brändle, 1996), can also explain the slower rate of senescence in anoxic tap root slices compared to aerated slices.

The external ion concentrations used in this study (K⁺: 1 mM and Cl⁻: 0.5 mM) are within the range in which ion uptake is dominated by energy dependent high-affinity transport (Läuchli & Epstein, 1971; Maathuis & Sanders, 1996). The active uptake of K⁺ and Cl⁻ and the net loss of acid equivalents were much lower under anoxic than under aerated conditions (Figs 4 and 6). This can be caused by a decreased availability of energy for active solute exchange during oxygen deficiency or down regulation of this process (Zhang & Greenway, 1995). The rate of net K⁺ loss in tap root slices of all three *Rumex* species during anoxia was lower than that in hypoxic wheat roots (Buwalda et al., 1988; Greenway et al., 1992) and anoxic beet root storage tissue (Zhang et al., 1992). The very small loss of Cl⁻ in anoxic *Rumex* tissues is comparable to the loss in anoxic beet root tissue (Zhang et al., 1992, Zhang & Greenway, 1995) and maize roots (Gibbs et al., 1998); only anoxic rice coleoptiles are able to take up more Cl⁻ during anoxia.
Fermentation, energy charge and solute uptake

(Zhang & Greenway, 1995). Accordingly, tap roots of Rumex species are very tolerant to oxygen deprivation. Anoxic wheat roots lost soluble sugars during anoxia (Greenway et al., 1992), while in rice coleoptiles and beetroot storage tissue, the reduction in glucose uptake during anoxia compared to aerated conditions amounted 35% and 60% respectively (Zhang & Greenway, 1995). In tap root slices of the tested Rumex species, the difference between anoxic and aerated tissues is much smaller (Fig. 5). Although it is not sure that sugar uptake always requires energy (Xia & Saglio, 1990) the continued uptake of glucose in anoxic tap root slices of Rumex species and the small difference with aerated slices indicate as well that some energy is directed to membrane associated processes during anoxia. Moreover, the absence of extensive losses of solutes during anoxia and the resumption of active net uptake of both K⁺ and Cl⁻ and the net loss of acid equivalents upon reaeration show that no irreversible damage to the membranes has occurred and that membranes can be re-energised after ten days of anoxia (Zhang et al., 1992).

Supply of glucose during ageing and anoxic incubation can increase tolerance (Drew, 1990; Waters et al., 1991; VanToai et al., 1995) and can reduce the loss of ions during anoxia because of increased ethanolic fermentation (Zhang et al., 1992; Zhang & Greenway, 1994). Therefore, the exchange of solutes during anoxia in this study most probably gives an overestimation of the intrinsic anoxia tolerance of intact plants. On the other hand, since the tap root slices of all three species still maintain their membrane integrity even after ten days of complete anoxia, these tissues are considered quite tolerant to anoxia. In a previous study (Nabben et al., 1999; chapter 2) it was shown that the resistance to anoxia of these three species differed considerably, while in this study no differences in resistance were apparent. It is very well possible that R. thyrsiflorus is less able to mobilise carbohydrates to fuel fermentation, but that this was not noted in the present study because of exogenous sugar supply. It is also conceivable that differences in resistance would have occurred if the experiments had lasted longer.

Conclusions

The integration of measurements of fermentation rate, AEC and solute transport on the same three plant species enabled us to assess the importance of different metabolic pathways in these species. Furthermore, these results could explain the differences in flooding resistance of these species. Nitrate reduction and lactate fermentation were not important in regeneration of NAD⁺ in submerged Rumex species, although in vitro LDH activity had increased markedly. Regeneration of NAD⁺ was most probably accomplished by ethanolic fermentation. In submerged
shoots, induction of fermentation was virtually absent, presumably because the oxygen supply in the leaves was large enough to sustain aerobic respiration. The production of ethanol, the AEC and the total amounts of adenylates were higher in \textit{R. palustris} than in \textit{R. maritimus}, which accords with the difference in submergence tolerance. Fermentation rate was low in \textit{R. thyrsiflorus} which probably led to a lower energy status and therefore, to a lower resistance of oxygen deficiency. In all three species, the tap roots seemed to have a higher fermentation capacity than lateral roots or shoots, which led to a higher energy status and consequently to longer survival during oxygen deficiency. The retention of accumulated ions and the continued uptake of glucose during anoxia and the resumption of ion net uptake after reaeration indicate that in all three species the membrane integrity was maintained during up to ten days of anoxia which classifies them as rather resistant to flooding.
Chapter 4

Neither quantity nor quality of carbohydrates determine resistance to submergence in *Rumex* species

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Abstract

In this paper the carbohydrate metabolism during complete submergence of three Rumex species from different habitats in river floodplains (viz. R. palustris, R. maritimus and R. thyrsiflorus) is described. Submergence leads to oxygen deprivation and consequently, inefficient fermentation processes are needed for energy generation. We hypothesised that differences in resistance to submergence among the three species are caused by differences in the amount, accessibility, or quality of carbohydrates, and that underwater photosynthesis influences these parameters by increasing the availability of carbohydrates and oxygen. Plants were grown drained or submerged in the light or in complete darkness. Dry weight of shoots and tap roots and the non-structural carbohydrate content of tap roots were determined. All three species were able to degrade reserve carbohydrates and metabolise the resulting hexoses during flooding. Submergence resulted in a higher net carbohydrate use, probably because of a decrease in photosynthesis under water or an increased use of carbohydrates for shoot elongation. Submergence in the light led to a reduction in the depletion rate of carbohydrates compared to dark conditions, probably by means of underwater photosynthesis. No Pasteur effect occurred in any of the species during submergence. In contrast, these species appeared to down-regulate their metabolism, thereby minimising carbohydrate use. During submergence fructans were apparently mainly used for fuelling fermentation and might have a role in enabling rapid regrowth after the floods have receded. Neither quality nor quantity of carbohydrates could explain the differences in flooding resistance and field distribution of these species satisfactorily.

Introduction

Terrestrial plants that occur in river floodplains have to cope with flooding during periods with a high discharge due to melting snow or heavy rainfall (Blom et al., 1996). The severity of the flooding stress can vary from soil flooding (waterlogging) to complete submergence and from flooding periods lasting only a few days to several weeks (Nabben et al., 1999; chapter 2). An important effect of submergence is impedance of gas exchange between the plant and its environment, leading to changes of gas concentrations inside the plant. The most detrimental factor for plant growth during flooding is a decreased availability of oxygen, leading to slower respiration and hence less generation of energy (Vartapetian & Jackson, 1997). Other important limitations for plant growth during submergence are a decreased availability of CO₂ and a considerable change
in light level and spectral composition limiting photosynthesis (Setter et al., 1987b; 1989). Accordingly, to survive submergence, plants have to be adapted to the constraints imposed by flooding stress. If floods are deep the effects of morphological adaptations such as shoot elongation and aerenchyma formation, leading to improved availability of oxygen and carbon dioxide (Armstrong et al., 1994; Vartapetian & Jackson, 1997; Jackson & Armstrong, 1999), are limited. Under such circumstances, plants have to find ways to generate energy without oxygen. Ethanolic and lactate fermentation are the most important ways of accomplishing this (Ricard et al., 1994). However, the efficacy of ATP production by these processes is much lower than that of aerobic respiration (2 and 32 moles of ATP per mole of glucose respectively). In some plants the energy deficiency is alleviated by accelerating glycolysis, which is known as the Pasteur-effect (Armstrong et al., 1994). Obviously, acceleration of glycolysis leads to a faster depletion of sugars, which can result in exhaustion of carbohydrates. Therefore, physiological traits important for submergence tolerance include availability of carbohydrates, high rates of fermentation, and energy conservation (Armstrong et al., 1994; Crawford & Brändle, 1996; Setter et al., 1997). Usually, the amount of readily fermentable carbohydrates in plants is limited and therefore, more complex storage carbohydrates have to be used. However, if storage carbohydrates cannot be broken down during oxygen deficiency (like in some cereal seeds, Perata et al., 1992; Guglielminetti et al., 1995b), the survival time of oxygen deprivation will be short. The importance of carbohydrates is further demonstrated by the increased survival and energy status of excised tissues during anoxia when these are supplied with exogenous sugars (reviewed by Vartapetian & Jackson, 1997) and the anoxic germination of wheat that only occurs after addition of glucose or sucrose (Perata et al., 1992). Besides fuelling fermentation, carbohydrates are assumed to play a role in fast resumption of growth upon re-aeration (Albrecht et al., 1993; Setter et al., 1987b; 1997).

The total non-structural carbohydrate content (TNC content) in roots of waterlogged plants generally increases due to a decreased growth rate of the roots while photosynthesis continues (Setter et al., 1987b; Albrecht, 1993; 1997). In several species a substantial part of this increase is formed by fructans. In contrast to starch synthesis, biosynthesis of fructans does not require phosphorylated nucleotides such as UTP and ATP (Nelson & Spollen, 1987; Albrecht et al., 1997), and therefore, fructan synthesis is energetically more favourable than starch production. Besides this, fructans are an easily accessible source of carbohydrates for rapid growth after the floods have receded (Albrecht et al., 1997).
Most studies on carbohydrate metabolism in relation to flooding stress focused on soil-flooded conditions. However, plants in floodplains often face the more severe stress of total submergence. Submergence can affect photosynthesis considerably and underwater photosynthesis increases both oxygen levels within plants (Rijnders et al., 2000) and biomass and carbohydrate content of plants (Setter et al., 1989; Laan & Blom, 1990). During submergence soluble sugar and starch contents generally decrease, because carbohydrate production by photosynthesis decreases more than carbohydrate consumption (Setter et al., 1987b). However, no papers are currently available that describe changes in fructan content during complete submergence, while this was extensively studied in waterlogged plants by Albrecht et al. (1993; 1997).

In this study we used three Rumex species whose field distribution (Blom et al., 1996) correlates with their resistance to complete submergence. R. palustris Sm. is the most resistant one, R. maritimus L. occupies an intermediate position, whereas R. thyrsiflorus Fingerh. is most sensitive to complete submergence. Survival of submergence in these three species is improved by underwater photosynthesis (Nabben et al., 1999; chapter 2). We hypothesise that the differences in resistance among the three species and the influence of underwater photosynthesis are caused by differences in the amount, accessibility, or quality of carbohydrates.

To test this hypothesis experiments were performed according to the following research questions: i) What is the influence of submergence and underwater photosynthesis on non-structural carbohydrate levels in tap roots of these three Rumex species and ii) Can the differences in carbohydrate metabolism explain their resistance to submergence and field distribution?

Materials and Methods

Plant material

Seeds of Rumex palustris Sm., R. maritimus L. and R. thyrsiflorus Fingerh. were collected from natural populations in the river area near Nijmegen, the Netherlands. Seeds were germinated on moist filter paper in Petri dishes, under 12 h light/12 h dark regime of 25/10 °C (PPFD: 25 μmol m⁻² s⁻¹, Philips TL33). After seven days, seedlings were individually transplanted into plastic pots (diameter 14 cm) filled with a mixture of sand and clay (1:1 v/v) and grown in the greenhouse (15-23 °C, 35-55% rh). Tap water was given twice a week to reach field capacity. Nutrient solution (4 mM Ca(NO₃)₂, 2.5 mM K₂SO₄, 1 mM MgSO₄, 1 mM KH₂PO₄, and the micronutrients FeEDTA (30 μM), NaCl (100 μM), H₃BO₃ (50
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\[\text{MnSO}_4 \ (4 \ \mu\text{M}), \ \text{ZnSO}_4 \ (4 \ \mu\text{M}), \ \text{CuSO}_4 \ (1 \ \mu\text{M}), \ \text{H}_2\text{MoO}_4 \ (1 \ \mu\text{M})\]

was applied once a month instead of tap water. The plants were grown in winter and the photoperiod while growing the plants was kept at 12 h by means of 400-W high-pressure sodium lamps, which supplemented normal daylight to a minimal light intensity (PPFD) of 100 \(\mu\text{mol m}^{-2}\ \text{s}^{-1}\) at plant level. The short day length was chosen to prevent \textit{R. maritimus} from bolting and flowering (Van der Sman \textit{et al.}, 1992).

Flooding treatments and measurements

After 12 or 14 weeks of growth (\textit{R. palustris} and \textit{R. maritimus/R. thyrsiflorus} respectively), plants were submerged in large basins (diameter 1.8 m) filled with tap water (pH=7.8, 0.05 mM CO\(_2\)). To study the effect of underwater photosynthesis, plants were submerged either in the light (12 h light/12 h dark regime, PPFD: at least 90 \(\mu\text{mol m}^{-2}\ \text{s}^{-1}\) at plant level, temperature: 20 \(^{\circ}\text{C}\)) or in complete darkness. The water level was fixed at one metre above the soil surface to avoid restoration of leaf-air contact due to shoot elongation. Additionally, two sets of plants were grown under drained conditions with identical light (control treatment) or dark conditions to the submerged plants. During the experiment no nutrients were added to either submerged or drained plants.

After 0, 7, 14 and 21 days of treatment, six plants per species and treatment were harvested. The roots were washed out of the potting mix and plants were divided in shoots and tap roots, while the lateral roots were discarded. The shoots were dried (16 h, 105 \(^{\circ}\text{C}\)) after which shoot dry weight was determined. Upon harvest, tap roots were rapidly frozen in liquid nitrogen, lyophilised (Virtis Bench Top 3), subsequently weighed and stored at –80 \(^{\circ}\text{C}\) until analysis.

Carbohydrate analysis

Since the tap root is the main carbohydrates store in these species and this is also the organ, which survives longest during submergence and from, which regrowth of shoots and lateral roots occurs (Armstrong \textit{et al.}, 1994), we focussed our study on this organ. Lyophilised tap roots of four plants per species and treatment were ground and non-structural carbohydrates were extracted. Samples of 40 mg plant material were extracted twice in 5 and 2.5 ml 80\% ethanol at 25 \(^{\circ}\text{C}\) for 10 min to give the soluble sugar fraction. The fructan fraction was obtained by extracting the residue in 5 ml water at 60 \(^{\circ}\text{C}\) for 30 min followed by a wash with 2.5 ml water. Starch was extracted out of the remaining residue by acid hydrolysis with 6
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ml 2.7 M HCl at 100 °C for 1 h followed by neutralisation with NaOH. The residue was washed once more with 2.5 ml water (modified after Visser, 1997). After extraction the soluble sugar fraction was made up to 25 ml, while the other two fractions were made up to 100 ml.

Glucose, fructose and sucrose contents were determined by a coupled enzymatic assay method (Guglielminetti et al., 1995a). The ethanol present in the extract did not influence the enzymatic conversion (results not shown). The total amount of soluble sugars analysed in some samples by the Anthrone-method gave the same results (not shown) as the analysis of separate sugars by the enzymatic method. This proves that contamination of the soluble carbohydrate pool with fructans with a low degree of polymerisation was negligible. Fructans and starch were measured using the anthrone method (Yemm & Willis, 1954). Recovery of sucrose added before analysis was tested for all three species after 21 days of all treatments. No differences among species or treatments were found and the overall recovery amounted (in %): 99.1 ± 1.0, 107.3 ± 2.2 and 102.1 ± 2.3 for the soluble sugars, fructans and starch assay respectively (means ± SE, n=24).

The initial contents of soluble sugars, fructans and starch were: 10%, 8% and 43% in R. palustris; 10%, 28% and 24% in R. maritimus; 6%, 16% and 32% in R. thyrsiflorus, respectively (carbohydrates as percent of dry weight). This means that as much as 54-62% of the dry weight of the tap roots consisted of carbohydrates. Therefore, non-structural carbohydrate contents were expressed on a structural dry weight basis (SDW: dry weight minus weight of total non-structural carbohydrates (TNC)) to avoid errors associated with simultaneous changes in carbohydrate content and dry weight (Chatterton et al., 1987).

Statistical analyses

All results were tested per species by two-way analysis of variance (treatment x time) followed by a Tukey test. Statistical analyses were performed with the SigmaStat statistical package and according to Sokal & Rohlf (1995).

Results

Biomass

In all three Rumex species dry weight of shoots (DWS) of plants kept in the dark decreased, while the DWS of plants grown in the light did not change appreciably (Fig. 1a-c). At both light levels, no significant differences in DWS were noted.
between flooded and drained plants. No clear differences in the time course of DWS among the three species occurred.

Only in plants that were grown drained in the light (control plants), there was an increase in dry weight of the tap roots, while this parameter did not change significantly with time in the other treatments (Fig. 1d-f). No differences among the three species in response to the treatments were noted. It is, however, noteworthy that the size of the tap roots of *R. thyrsiflorus* was much smaller than that of the other two species.

![Figure 1: Dry weight of shoot (DWS; a-c) and tap root (DWT; d-f) of drained (circles) and submerged (squares) plants of Rumex palustris (a, d), R. maritimus (b, e), and R. thyrsiflorus (c, f) in the light (open symbols) and in the dark (closed symbols) (Means ± SE, n=6).](image-url)
Figure 2: Glucose (a-c), fructose (d-f) and sucrose (g-i) content (g g\(^{-1}\) SDW) of tap roots of drained (circles) and submerged (squares) plants of Rumex palustris (a, d, g), R. maritimus (b, e, h), and R. thyrsiflorus (c, f, i) in the light (open symbols) and in the dark (closed symbols) (Means ± SE, n=4).
Figure 3: Fructans (a-c) and starch (d-f) content (g g$^{-1}$ SDW) and starch:fructan ratio (g-i) of tap roots of drained (circles) and submerged (squares) plants of Rumex palustris (a, d, g), R. maritimus (b, e, h), and R. thyrsiflorus (c, f, i) in the light (open symbols) and in the dark (closed symbols) (Means ± SE, n=4).
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**Soluble sugars**

There were no significant effects of the four treatments on either glucose or fructose content of the three species (Fig. 2a-f) but the initial contents of both glucose and fructose in *R. maritimus* were approximately two times higher than in the other two species. In contrast to the hexoses, the sucrose content in all three *Rumex* species decreased considerably in all four treatments (Fig. 2g-i). The decrease in the sucrose content of control plants was significantly smaller than that of plants that were submerged in the dark. Besides this, plants that were grown in the dark showed the strongest decrease in sucrose content in the first week, while plants grown in the light decreased in sucrose content mainly in the second week of treatment. The relative decrease in sucrose content in tap roots of *R. maritimus* (Fig. 2h) was much smaller in all treatments than that of the other two species. Additionally, the initial sucrose content in tap roots of *R. thyrsiflorus* (Fig. 2i) was much lower than that of the other two species. The initial sucrose content of the tap roots of the three species was several folds higher than that of the hexoses. Therefore, the time course of the sum of soluble sugars was almost identical to that of sucrose (results not shown).

**Fructans**

The fructan content of tap roots of *R. palustris* increased after one or two weeks in all treatments, while it had decreased again to initial values by the end of the experiment after three weeks (Fig. 3a), showing no significant differences among treatments. In *R. maritimus* the fructan content was higher in control plants than in the other treatments (Fig. 3b). Moreover, the fructan content in plants of *R. maritimus* and *R. thyrsiflorus* kept in the dark decreased gradually but significantly during the experiment, while in plants kept in the light no significant differences in time were found (Fig. 3b, c). It should be noted that the initial fructan content of tap roots of *R. maritimus* was two to four times higher than that of *R. thyrsiflorus* and *R. palustris* respectively.

**Starch**

The initial starch content of tap roots of *R. palustris* was almost twice as high as in the other species, and it decreased as a result of all four treatments (Fig. 3d). However, the decrease in control plants was much smaller than that in the other
treatments, which were all equal. Tap roots of control plants of *R. maritimus* showed an increase in starch content by the end of the experiment (Fig. 3e). In contrast, the other treatments resulted in a decreasing starch content that was similar in the three treatments. In *R. thyrsiflorus* plants grown in the light the starch content in the tap roots was significantly higher over the whole experiment than in plants grown in the dark (Fig. 3f). This was due to a constant (drained) or only slightly decreasing (submerged) starch content in plants in the light, while this parameter decreased steeply in dark grown plants. The decrease in starch content in all three species mainly occurred in the first or in some cases in the second week, while in the third week the starch content remained approximately the same.

**Starch:fructan ratio**

The starch:fructan ratio gives immediate insight in the change in concentration of these two compounds relative to each other. This ratio in tap roots of *R. palustris* and *R. thyrsiflorus* decreased in all treatments, except in control plants of *R. thyrsiflorus* (Fig. 3g, i). Moreover, also in the control plants of *R. palustris* the decrease in the starch:fructan ratio was smaller than in plants kept in the dark. This means that *R. palustris* and *R. thyrsiflorus* preferentially consumed starch instead of fructans. Besides this, the initially high ratio in *R. palustris* and the somewhat lower ratio in *R. thyrsiflorus* indicate that these species predominantly stored starch instead of fructans. In *R. maritimus* there was a temporary decrease in the starch:fructan ratio after one and two weeks (Fig. 3h), and no differences among treatments were found. In contrast to the other two species, the starch:fructan ratio in this species was close to one during the whole experiment, which means that *R. maritimus* stored and used equal amounts of starch and fructans during carbohydrate metabolism.

**Total non-structural carbohydrates**

The development of the total non-structural carbohydrate (TNC) content roughly followed that of the starch content, since this fraction contributes most to the total pool of carbohydrates or changed the most during the experiment (compare Fig. 2 and 3). Accordingly, the TNC content of tap roots of *R. palustris* declined equally in all treatments except for the plants that were grown drained in the light (control plants). This treatment resulted in a constant TNC content during the whole experiment (Fig. 4a). The TNC content of tap roots of control plants of *R.*
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*maritimus* increased in the course of the experiment, while it declined in the other treatments (Fig. 4b). The TNC content of tap roots of *R. thyrsiflorus* plants grown in the light stayed constant in drained plants and decreased after two weeks of submergence (Fig. 4c). However, in plants treated in the dark the TNC levels were significantly lower, due to a strong decrease starting already in the first week of treatment.

![Figure 4: Total non-structural carbohydrates content (g g⁻¹ SDW) of tap roots of drained (circles) and submerged (squares) plants of Rumex palustris (a), R. maritimus (b), and R. thyrsiflorus (c) in the light (open symbols) and in the dark (closed symbols) (Means ± SE, n=4).](image)

**Discussion**

Influence of submergence and underwater photosynthesis on carbohydrate metabolism

The biomass of the shoots of all three species decreased significantly if plants were kept in the dark and no differences were noted between submerged and drained plants (Fig. 1). Changes in biomass will be largely caused by changes in carbohydrate content. The processes determining changes in carbohydrate content are photosynthesis (increase), metabolism (decrease) and allocation. Since photosynthesis was absent during darkness, the decrease in DWS must have been
caused by either consumption of carbohydrates in the shoot or by export of carbohydrates to the below ground parts or by a combination of both. Both phloem loading and unloading can be either energy dependent (apoplastic) or energy independent (symplastic, via plasmodesmata) and in some plants these processes can take place during anoxia (Kühn et al., 1999 and references therein). Therefore, it is very well possible that transport of sucrose to the tap roots has occurred during submergence. In contrast to plants submerged in the dark, underwater photosynthesis was apparently sufficient to maintain shoot biomass in plants that were submerged in the light.

In contrast to what was expected, the sucrose (Fig. 2g-i) and starch content (Fig. 3d-f; and therewith the TNC content, Fig. 4) initially decreased in tap roots of plants that were grown drained in the light (control treatment). This was probably caused by the transfer of the plants from the relatively high light levels at which they were grown to the lower light levels in the tanks in which the treatments took place. Lower light levels would lead to a diminished photosynthetic rate and, consequently, to lower carbohydrate levels. However, the decrease in content of all fractions was lower in the control treatment than in the other treatments. The sucrose, fructan and starch content (and therewith the TNC content) also decreased during both submergence and darkness. This means that also under submerged conditions these Rumex species were capable of breakdown of reserve carbohydrates, a feature of plants tolerant to oxygen deficiency (Perata et al., 1992; Guglielminetti et al., 1995b; Arpagaus & Brändle, 2000). The decrease in sucrose content in the Rumex plants indicates that enzymes needed for channelling sugars to glycolysis were present during submergence (Perata et al., 1996) or that sucrose was allocated to other plant parts. Moreover, the fructose and glucose produced by fructan or starch breakdown were not recovered in the tap roots (Fig. 2a-f). This means that these hexoses soluble sugars were either respired in the tap roots or allocated to other organs after conversion to sucrose, which is the form in which carbohydrates are generally transported between plant parts (Kühn et al., 1999).

In the light the decrease in carbohydrates either occurred later (sucrose; Fig. 2g-i) or was less pronounced (fructans and starch; Fig. 3a-f) than in the dark. This means that (underwater) photosynthesis was important in providing carbohydrates or led to a more efficient (aerobic) metabolism due to oxygen production, resulting in conservation of carbohydrates (Setter et al., 1989; Laan & Blom, 1990). This confirms the experiments with the closely related species R. crispus, in which starch content of the tap root was two times lower after five weeks of submergence in the dark than in the light (Laan & Blom, 1990). Additionally, during oxygen deficiency in ice-encased cereals less ethanol was produced and less oxygen was taken up when plants were incubated in the light than in the dark.
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(Andrews, 1988). This indicates that photosynthesis leads to higher cellular oxygen levels.

Submergence only affected carbohydrate contents in the light, but not in complete darkness (Figs 2-4). Moreover, in plants submerged in the dark or grown drained hardly any shoot elongation took place (results not shown; Laan & Blom, 1990), while plants of *R. palustris* and *R. maritimus* showed a clear elongation of petioles and laminae when submerged in the light (results not shown). Therefore, the effect of submergence in the light might have been caused by a decreased photosynthetic rate during submergence (lower availability of CO₂; Setter *et al.*, 1989) or by carbohydrates and energy invested in shoot elongation (Setter *et al.*, 1987b; 1997) or a combination of both.

The similar rate of carbohydrate consumption in the absence of photosynthesis during dark conditions indicates that a Pasteur effect was absent during submergence in these species, which is in agreement with earlier studies on *R. maritimus* and *R. thyrsiflorus* (Laan, 1990). This accords as well with studies on other wetland plant rhizomes, in which a Pasteur effect was either absent or only present during the first few days of oxygen deprivation (reviewed by Armstrong *et al.*, 1994). Wetland plants may also slow down the metabolic activity of tissues and enter a quasi-dormant state (Vartapetian & Jackson, 1997). This depresses the demands of ATP, thereby saving carbohydrate reserves and reducing the formation of possibly harmful anaerobic end products (Albrecht & Wiedenroth, 1994b; Armstrong *et al.*, 1994). The studied *Rumex* species also seem to employ this “dormancy strategy” since all three species down-regulate ethanolic fermentation in tap roots as *in vitro* activities of enzymes involved in ethanolic fermentation decline after ten days of flooding (own unpublished results) and ethanol production decreases during anoxia (chapter 3). Consequently, the rate of carbohydrate consumption decreased after one or two weeks of submergence (Fig. 4).

**Possible functions of fructans during submergence**

Several functions of fructans apart from storage carbohydrates have been proposed especially during stressful conditions, such as a role in low temperature and drought resistance (Pollock & Cairns, 1991; Hendry, 1993). It is, however, not clear if fructans also have functions during low oxygen stress. The decrease in fructan content in *R. maritimus* and *R. thyrsiflorus* (Fig. 3a, c) shows that fructans can indeed be used during oxygen deficient conditions, probably mainly for fuelling fermentation. In the flooding tolerant *Iris pseudacorus* fructans comprise a large part of the carbohydrates present. TNC content in this species decreased
during anoxia, indicating that fructans were used, although the content was not determined (Hanhijärvi & Fagerstedt, 1995). In defoliated ryegrass, fructans were degraded if the sucrose content decreased below a certain level (Morvan-Bertrand et al., 1999), which was similar to the results of our study (compare Figs 2g-i and 3a-c). Fructans are a source of carbohydrates that is more easily accessible than starch (Pollock & Cairns, 1991; Albrecht et al., 1994). Therefore, apart from supplying fermentable carbohydrates during flooding, fructans might also be important for rapid regrowth after the floods have receded (Albrecht et al., 1994; Morvan-Bertrand et al., 1999). Besides being a source of fermentable sugars and a carbohydrate source used for regrowth after reaeration, a third role for fructans in submerged plants might be to serve as osmoticum during rapid growth by cell inflation (Nelson & Spollen, 1987; Hendry, 1993). Besides R. palustris, especially R. maritimus depends on fast shoot elongation for long-term survival of submerged conditions (Laan & Blom, 1990; Van der Sman et al., 1991; Blom et al., 1996). The latter species also has the highest fructan content in the tap roots (Fig. 3). However, for both species the consumption of fructans did not differ between plants submerged in the light and in the dark, while elongation only occurred in the light (results not shown). This lack of correlation makes it very unlikely that fructans play an important role in shoot elongation during flooding of Rumex species.

*Differences between species related to their field distribution*

All three species were able to use both soluble sugars and reserve carbohydrates during flooding, which means that both glycolytic and amylolytic enzymes were active during submergence. In none of the studied species, the carbohydrate stores were exhausted by the end of the experiment (Fig. 4). In many studies, death of plants during oxygen deprivation occurred before the exhaustion of fermentable substrate (reviewed by Ricard et al., 1994) and must have been caused by other factors. Also in these Rumex species it is doubtful that carbohydrate depletion is the reason for differences in survival of submergence. It is true that tap roots of R. thyrsiflorus, the least flooding-tolerant species, contained less sucrose (Fig. 2g-i) and TNC (Fig. 4) than the other two species, but this is probably related to its much smaller plant size (Fig. 1d-f). It is conceivable that larger plants of R. thyrsiflorus contain equal amounts of soluble sugars as the other two species of the same size. In some species carbohydrate depletion rate is dependent on tap root or tuber size, while in others it is not (Clevering et al., 1995 and references therein). Therefore, it is unclear if the smaller tap root size would influence the reaction to submergence or darkness. However, survival of submergence by
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*Rumex* species is clearly related to plant size (Nabben *et al.*, 1999; chapter 2) and regrowth after flooding probably is faster in plants with large reserve organs than in plants with small ones.

One would further expect that the species with the lowest fermentation rate would also have the lowest rate of carbohydrate depletion. Therefore, it is striking that the rates of carbohydrate depletion were more or less equal among the three species, while the fermentation rate in tap roots of *R. thyrsiflorus* during anoxia is much lower than that of the other two species (chapter 3).

Apart from quantity, also quality of carbohydrates might play a role in flooding resistance (Albrecht *et al.*, 1997). Only in *R. maritimus* and *R. thyrsiflorus* the fructan content decreased as a result of submergence or darkness (Fig. 3a-c), while in *R. palustris* it increased even temporarily. It is very unlikely that a species that stores fructans would be unable to degrade them. Therefore, it is possible that *R. palustris* only uses fructans if starch reserves are consumed to a large extent. Although *R. thyrsiflorus* consumed fructans, its starch consumption was much more pronounced (Fig. 3) and only *R. maritimus* stored and consumed starch and fructans in equal amounts. Since enzymatic breakdown to hexoses of both starch and fructans (Nelson & Spollen, 1987) does not require energy, there seems to be no advantage in terms of energy costs or yield of using either form of polysaccharide. Only if starch would be degraded by starch phosphorylase instead of amylase, energy could be saved (Perata *et al.*, 1998). The difference in main storage carbohydrate (fructan versus starch) also was not an explanation for the difference in resistance to oxygen deprivation between *Iris pseudacorus* and *I. germanica* (Hanhijärvi & Fagerstedt, 1995).

In summary, all three species seemed to be able to degrade reserve carbohydrates and to metabolise the resulting hexoses during submergence, which makes them rather tolerant to flooding. The higher net carbohydrate use resulting from submergence was probably caused by either a decreased photosynthetic rate or by an increased consumption of carbohydrates for shoot elongation. Underwater photosynthesis led to a reduction in depletion rate of carbohydrates. No Pasteur effect occurred in any of the species, but instead these species appeared to minimise carbohydrate use by down-regulating their metabolism. The role of fructans during flooding seemed to be restricted to fuelling fermentation and possibly supplying carbohydrates for regrowth after the floods have receded. No indications were found that fructans were important as osmoticum in elongating shoots. Neither quantity nor quality of carbohydrate stores could satisfactorily explain the differences in flooding resistance or the field distribution of these *Rumex* species.
Chapter 5

Post-anoxic injury in flooding resistant and more sensitive *Rumex* species

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Abstract

Reaeration after flooding-induced oxygen deficiency can lead to the formation of reactive oxygen species (ROS) and the oxidation of metabolites that have accumulated during oxygen deprivation. Both processes can lead to serious damage to plants, known as post-anoxic injury, which can form a substantial part of flooding stress experienced by plants. In this paper, three species that differ in resistance to flooding were compared with respect to their resistance to post-anoxia stress. Survival of root tips and growth rates of whole plants were measured after anoxia followed by reaeration and after treatment with paraquat, a herbicide generating oxygen radicals. It is discussed why growth was a better measure for resistance to these stresses than survival of roots. Growth rates decreased after anoxia followed by reaeration, with *R. palustris* (the most flooding resistant species) being least affected, followed by *R. maritimus*, while *R. thyrsiflorus* (the most flooding sensitive species) suffered most. However, the growth rate of *R. thyrsiflorus* was less affected by paraquat treatment than that of the other species. It was deduced that survival of anoxia and post-anoxia of these species was mainly determined by their resistance to anoxia and to lesser extent by resistance to oxidative stress. The activity of superoxide dismutase (SOD), an enzyme involved in detoxification of ROS, decreased in *R. thyrsiflorus* during anoxia and reaeration, while it did not change in *R. palustris*. Apart from SOD, also non-enzymatic antioxidants (probably including polyphenols) appeared to be important in scavenging of superoxide in these *Rumex* species. Production of toxic acetaldehyde by ethanol oxidation occurred in *R. palustris* upon aeration after prolonged anoxia and it is discussed how this can influence survival of flooding.

Introduction

Reactive oxygen species (ROS), like the superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen, are produced in plants during many stress conditions (Bowler *et al.*, 1992; 1994). Examples of stresses leading to the formation of ROS are photosynthetic cells exposed to high light levels, high salinity, drought, wounding, low temperature, UV-radiation and pollutants such as ozone, sulphur dioxide and nitrogen oxides (Smirnoff, 1995; Noctor & Foyer, 1998). The formation of ROS under such stressful conditions might be in excess of antioxidant scavenging capacity, thus creating oxidative stress (Yu & Rengel, 1999). Plants can acclimate to oxidative stress by increasing the expression of the antioxidant system (Smirnoff, 1995). This consists of 1) radical scavenging
enzymes (e.g. superoxide dismutase, catalase and peroxidases), 2) natural antioxidants (e.g. ascorbic acid, glutathione and \( \alpha \)-tocopherol), and 3) enzymes regenerating the active form of antioxidants (e.g. ascorbate reductases and glutathione reductase).

Another situation that leads to oxidative stress in plants is the re-exposure to oxygen after a period of oxygen deprivation (Gutteridge & Halliwell, 1990), causing post-anoxic injury (Hunter et al., 1983; Crawford et al., 1994). Post-anoxic injury has been reported for a wide variety of plant species and organs such as \textit{Iris} rhizomes (Hunter et al., 1983), soybean seedlings (VanToai & Bolles, 1991), rice seedlings (Ushimaru et al., 1999), wheat roots (Albrecht & Wiedenroth, 1994a), \textit{Senecio} and \textit{Myosotis} species (Biemelt et al., 1996) and other wetland plants (Monk et al., 1987a). Reacreation after oxygen deprivation can lead to increased peroxidation of proteins, nucleic acids and lipids (Scandalios, 1993; Crawford et al., 1994). This peroxidation is caused by a higher production of ROS and/or to weakened enzymatic or non-enzymatic detoxification systems (VanToai & Bolles, 1991; Ushimaru et al., 1999). Therefore, the maintenance of the antioxidative system during oxygen deprivation may be fairly important for protection against post-anoxic injury. As could be expected there is a clear negative correlation between lipid peroxidation and anoxia tolerance in many species, which is probably linked to membrane stability (Blokhina et al., 1999). A common situation leading to oxygen deprivation and re-exposure to higher oxygen concentrations is flooding (Armstrong et al., 1994; Vartapetian & Jackson, 1997). Flooding can lead to oxidative stress through: 1) disturbed photosynthesis; 2) accumulation of Fe in plants, which facilitates formation of ROS through the \( \text{Fe}^{2+} \) catalysed Haber-Weiss reaction; 3) uncontrolled production of ROS by the superoxide generating enzyme xanthine oxidase; 4) spontaneous oxidation of reduced compounds accumulated during anoxia (Smirnoff, 1995; Yu & Rengel, 1999 and references therein).

Numerous studies show that oxidative stress can be mimicked very well by the application of the herbicide methyl viologen (MV or paraquat). In the presence of light, paraquat accepts electrons originating in photosystem I and donates the electrons to oxygen, thus forming the superoxide anion. Paraquat can also accept electrons originating in the electron transport chain (Matters & Scandalios, 1986). It is a freely translocated cation that is mainly transported in xylem tissue (Purba et al., 1995). Application of paraquat to the roots can therefore lead to increased concentrations in the whole plant. Superoxide generated by paraquat, or hydrogen peroxide or hydroxyl radicals formed from it (Gutteridge & Halliwell, 1990), cause its deleterious effects and not paraquat itself (Hassan & Fridovich, 1978) and superoxide scavenging antioxidants are therefore involved in paraquat resistance.
The antioxidative enzyme superoxide dismutase (SOD) converts the superoxide anion to hydrogen peroxide. Superoxide and hydrogen peroxide are not harmful at physiological concentrations, but their toxicity in vivo arises from their metal ion-dependent (mainly Fe$^{2+}$) conversion into hydroxyl radicals (Haber-Weiss reaction; Gutteridge & Halliwell, 1990; Bowler et al., 1994). Hydroxyl radicals are among the most reactive species known to chemistry (Bowler et al., 1992) and because hydroxyl radicals themselves are far too reactive to be easily removed, defence mechanisms are needed to prevent their formation (Bowler et al., 1994). Since SOD determines the concentration of both superoxide and hydrogen peroxide it is likely to be central in the defence mechanism (Bowler et al., 1992).

SOD gene expression is increased when environmental conditions cause increased ROS formation (Smirnoff, 1995; Ushimaru et al., 1999) and resistant cultivars or species exhibit greater activities of SOD and/or lower levels of ROS relative to the sensitive genotypes exposed to the same level of different abiotic stresses (Yu & Rengel, 1999 and references therein). Formation of superoxide is also enhanced after reaeration of oxygen-deprived tissues (Monk et al., 1987b, VanToai & Bolles, 1991). Thus, an increase in SOD activity during flooding would be of advantage to the plant considering the fact that temporal oxygen deficiency and subsequent reaeration are representative of the field situation. Moreover, the effect of reaeration following a period of oxygen deficiency may be potentially more damaging than oxygen deficiency itself (Ushimaru et al., 1999; Yu & Rengel, 1999). In some species SOD activity is increased during oxygen deprivation (Monk et al., 1987b; VanToai & Bolles, 1991; Yu & Rengel, 1999; Biemelt et al., 2000).

Apart from the formation of ROS, another cause of post-anoxic injury can be the oxidation of metabolites that have accumulated during oxygen deficiency (Armstrong et al., 1994; Crawford & Brändle, 1996). One of the most drastic effects is post-anoxic oxidation of ethanol leading to a surge of acetaldehyde production (Monk et al., 1987a; Zuckerman et al., 1997). Like other aldehydes, acetaldehyde is a highly reactive and long-lived molecule that may have a variety of negative effects on biological systems. Aldehydes cause their effects by reacting with cellular nucleophiles, including proteins and nucleic acids (Op den Camp & Kuhlemeier, 1997).

In this study we used species from the genus Rumex from different habitats in river floodplains. Because of their different habitat, these species are naturally exposed to different flooding regimes (Blom et al., 1994). R. thyrsiflorus grows on high elevated and, therefore, seldom-flooded sites like river dunes and dikes. R. palustris and R. maritimus grow on low elevated mud flats that are frequently flooded. Previous studies showed that these species also differ in their resistance to flooding. R. palustris clearly is the most resistant to complete submergence,
while *R. maritimus* is somewhat more sensitive. As was expected, *R. thyrsiflorus* had the worst performance of the three species during complete submergence. That study also indicated that post-anoxic injury did occur after the flooding period had ended and that the species seemed to differ in their resistance to post-anoxic injury in the same way as they differ in flooding resistance (Nabben et al., 1999; chapter 2).

The aim of the current study was to elucidate the causes of differences in resistance to post-anoxic injury in these three species. To this end, we treated plants anoxically and assessed their resistance by determining their survival and growth rate after reaeration. To separate the effects of anoxia and those of post-anoxia stress, we subjected the plants to oxidative stress without a preceding anoxia period. Since application of paraquat is the simplest model available to study the effect of oxidative stress on plants (Bowler et al., 1992), we treated the three species with this herbicide and again monitored their survival and growth rate after treatment. We expected that the order of species in resistance to oxidative stress was similar to that of the overall resistance to submergence. Because SOD seems to play a central role in defence against oxidative stress (Bowler et al., 1992), we measured the *in vitro* SOD activity in *R. palustris* and *R. thyrsiflorus* (the most and the least flooding resistant *Rumex* species) after increasing periods of anoxia. Since ethanol fermentation is the main catabolic pathway during oxygen deficiency in these species (chapter 3) we assumed that the production of toxic acetaldehyde by ethanol oxidation upon reaeration could contribute to the post-anoxic injury displayed by these species. To test this assumption we measured the acetaldehyde production by shoots and roots of *R. palustris* during the shift to normoxic conditions after prolonged anoxia with a photo-acoustic detection technique.

**Material and Methods**

*Growth conditions*

Seeds of *Rumex palustris* Sm., *R. maritimus* L. and *R. thyrsiflorus* Fingerh. were collected from natural populations in the river Rhine area near Nijmegen, the Netherlands. They were sown on polyethylene grains (Lacqtene Low Density, Elf Atochem, France) that were soaked in nutrient solution containing (in mM): 2 Ca(NO$_3$)$_2$, 1.25 K$_2$SO$_4$, 0.5 MgSO$_4$, 0.5 KH$_2$PO$_4$ and the micronutrients (in µM): 15 FeEDTA, 50 NaCl, 25 H$_3$BO$_3$, 2 MnSO$_4$, 2 ZnSO$_4$, 0.5 CuSO$_4$ and 0.5 H$_2$MoO$_4$; pH 5.8. After one week of germination (12 h, 20 µmol m$^{-2}$ s$^{-1}$ (PPFD), Philips TL33, 27 °C; 12 h dark, 10 °C), the seedlings were transferred to a climate
room (16 h, 120 μmol m\(^{-2}\) s\(^{-1}\) (PPFD), Philips TL84, 22 °C; 8 h dark, 20 °C; relative humidity 50%). For the acetaldehyde production experiment uniform seedlings were transferred to hydroponic culture three weeks after germination (same growth conditions as before). Each hydroponic flow-through unit consisted of three 20 l PVC containers connected to a 30 l aeration vessel (120 l air h\(^{-1}\)), nutrient solution circulated at a rate of 60 l h\(^{-1}\) per container (Visser et al., 1996). Six plants were mounted in a PVC lid that was placed on top of each container. Nutrient solutions were replaced every two weeks to prevent nutrient deficiency.

Anoxia treatment

Containers filled with nutrient solution were deoxygenated by flushing with pure N\(_2\) gas. Floaters in which the seedlings were mounted were placed on the containers, which were subsequently incubated anoxically (<10 ppm O\(_2\), complete darkness, 20 °C) for 0, 3, 5, 6, 7 or 8 hours in an anaerobic workbench (model 1029 Forma Scientific Anaerobic System, Ohio, USA). After treatment, survival of roots and growth rate of the seedlings were determined.

Paraquat treatment

The roots of seedlings were immersed in methyl viologen solution (MV or paraquat, Sigma M-2254) in 0.2 M KH\(_2\)PO\(_4\) (pH 7.2) in the dark at room temperature, whereas contact of the leaves with the solution was avoided (method modified after Matters & Scandalios, 1986). Pilot experiments indicated that 10\(^{-4}\) M and 10\(^{-5}\) M paraquat were the most suitable concentrations for the survival and the growth experiment respectively (results not shown). After 0, 0.5, 1, 1.5, 2, 4, 5 or 6 hours of paraquat treatment, the roots were rinsed with tap water. Like in the anoxia experiment, survival of roots and growth rate of the seedlings were determined after the treatment.

Survival of roots

To assess the survival of the seedlings, 10 seedlings (22 days old) per duration and species were either treated anoxically or with paraquat. These experiments were conducted four times independently. Following anoxia, the plants were incubated in air in the dark for 30 minutes before the survival was assessed to allow post-anoxic injury to occur, while plants treated with paraquat were tested.
right away. Survival of the seedlings after the treatments was estimated by incubating the roots for 90 minutes in the dark in a solution of 3 mM iodonitrotetrazolium chloride (Tetrazolium Red, TZ, Fluka, 58030) in 10 mM KH$_2$PO$_4$ (pH 7.0; after VanToai & Bolles, 1991). Dehydrogenases reduce TZ, which leads to a shift in colour of the roots from white to red (Moore, 1973). Plants with root tips that did not change colour were considered dead. The survival data were fitted with a non-linear regression function according to the Weibull survivorship curve (Brown & Rothery, 1993):

\[ p = e^{-\ln(2)(\frac{t}{c})^b} \]  

(Equation 1)

- \( p \): proportion of individuals surviving
- \( t \): time of treatment
- \( c \): duration of treatment at which 50% of the plants survive (LT$_{50}$)
- \( b \): shape parameter

**Relative growth rate**

Five seedlings (24 days old) per species and duration were weighed before the experiment and treated anoxically or with paraquat. After the treatments the plants were transferred to fresh nutrient solution. After 8 days of growth (conditions as mentioned above) the plants were weighed again. The relative growth rate (RGR: \( \frac{(\ln(FW_{end})-\ln(FW_{start}))}{t_{end}-t_{start}} \)) of the treated plants was expressed as percentage of the RGR of untreated plants. These experiments were also conducted four times independently.

**SOD assay**

Seedlings of *R. palustris* and *R. thyrsiflorus* (24 days old) were incubated for 0, 3, 5 and 8 days under anoxia and grown aerobically for two more days (conditions as above). This two-day reaeration period was incorporated to increase the SOD activity, compared to before reaeration (Yu & Rengel, 1999). With a cork borer discs (\( \varnothing \) 5 mm) were cut from the oldest leaves of these seedlings. Three replicate samples of each 14 discs (ca. 0.15 g FW) were homogenised on ice in 1.5 ml phosphate buffer (50 mM KH$_2$PO$_4$, pH 7.0) and 1% (w/v) polyvinylpolypyrrolidone (PVPP) with a Potter glass homogenisor (1000 rpm)
and the crude extract was centrifuged (15 min at 20,000g, 4 °C; after Del Longo et al., 1993). SOD enzymes were purified by application of 1 ml of supernatant and 1.5 ml of phosphate buffer on a Sephadex G-25 column (PD-10 Pharmacia, Uppsala Sweden), which was washed in advance with 25 ml phosphate buffer. The enzymes were eluted with 1.4 ml phosphate buffer, snap frozen in liquid nitrogen and stored at −20 °C until the next day for analysis.

The SOD activity was determined by measuring the inhibition of the photochemical reduction of p-nitro blue tetrazolium (NBT) to blue formazan (Beauchamp & Fridovich, 1971). In this assay, superoxide radicals are formed, which reduce NBT to blue formazan. However, if SOD is present, superoxide will be dismutated and the formation of blue formazan will be inhibited.

Purified extract (0.2 ml) and 0.1 ml 60 µM riboflavine were added to 2.7 ml phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 100 nM Na2EDTA (Del Longo et al., 1993). A positive blank was used in which the enzyme extract was replaced by 0.2 ml demineralised water. To detect the aspecific activity (caused by antioxidants other than enzymatic ones) an aliquot of extract was boiled for 30 minutes before being assayed. After thorough stirring the mixture was illuminated for 12 minutes by two fluorescent tube lights (Sylvania F36W-GRO) in an aluminium foil lined box (after Beyer & Fridovich, 1987). The temperature in the box increased with not more than 2 °C. Before and after illumination the absorption by blue formazan was measured at 560 nm (A560). In a pilot experiment it was proven that the increase in A560 was linear for at least 12 minutes. The aspecific activity was subtracted from the total activity to yield the specific SOD activity. One unit of SOD activity was arbitrarily defined as the amount of SOD that inhibits the increase of A560 with 50% compared to the blank (Beauchamp & Fridovich, 1971). In this way, we could express the SOD activity in arbitrary units per gram FW.

**Acetaldehyde production**

Plants of *R. palustris* that were grown hydroponically for 45-55 days (see above) were mounted in a two-compartment cuvette. Roots and shoot were enclosed in separate 0.5 l compartments and the root/shoot transition was sealed with plasticine. During the experiments the shoot-compartment was illuminated continuously (25-40 µmol m$^{-2}$ s$^{-1}$ PPFD, 25 °C). At the start of the experiment both compartments were flushed with pure N$_2$ gas. After 45-50 h of anoxia, the gas composition of the inlet flow was changed to 21% O$_2$ in both compartments. The flow rate was 1 l h$^{-1}$ in the shoot compartment and 5 l h$^{-1}$ in the root compartment. The acetaldehyde concentration in the gas leaving the cuvette was
measured with a laser-driven photo-acoustic trace gas detection equipment. Further details about this technique and the exact methods used are described in Te Lintel Hekkert et al. (1997).

Statistical analyses

The results of each replicate survival experiment were fitted per species according to the Weibull function. In this way, the LT50 could be determined (according to equation 1). The significant differences in LT50 between species were calculated by one-way ANOVA for the anoxia treatment and one-way ANOVA on ranks for the paraquat treatment (n=4). The survival percentages were tested by two-way ANOVA (species x time) after “arcsin square root (percentage/100)” transformation. The mean relative growth was calculated for the four replicate experiments. These data were used to statistically compare the results by two-way ANOVA (species x time) after rank transformation.

The effects of anoxia on in vitro SOD activity were analysed by two-way ANOVA (species x time). All calculations were performed with the SigmaStat statistical programme and according to Sokal & Rohlf (1995). All differences mentioned in this paper concern significant differences (p<0.05).

Results

Survival of roots and growth rate after anoxia and paraquat treatment

After 5, 6 and 7 hours of anoxia followed by reaeration, the root survival of *R. palustris* was significantly higher than that of *R. maritimus* (Fig. 1a), while the root survival of *R. thyrsiflorus* was intermediate and did not differ significantly from these two species. The same order of species was also reflected in the LT50 values derived from these curves. However, the growth rates showed a different picture since this parameter indicated that *R. thyrsiflorus* was much less resistant to anoxia and post-anoxia than *R. maritimus*, while again *R. palustris* displayed the highest resistance (Fig. 1b).

*R. palustris* showed the highest root survival of treatment with paraquat, as was the case in the anoxia treatment (Fig. 2a). *R. thyrsiflorus* performed worst in reaction to paraquat while *R. maritimus* showed root survival rates that were in between, but differed significantly from, that of the other two species; the LT50
values confirmed this order. Again, as was found in the anoxia treatment, the
growth rates led to a different order in resistance than the surviving numbers.
Judging from the growth rates, *R. thyrsiflorus* was the least sensitive species,
followed by *R. palustris* and *R. maritimus* being most sensitive to paraquat
treatment.

**Figure 1:**
a: Survival of roots of 22 days old seedlings of Rumex species after anoxia and a subsequent post-anoxia period. Survival curves are fitted according to the Weibull distribution. LT50 values are $6.46 \pm 0.18^a$, $5.36 \pm 0.24^b$ and $6.01 \pm 0.26^{ab}$ for *R. palustris*, *R. maritimus* and *R. thyrsiflorus* respectively.

b: Growth rate of 24 days old seedlings of Rumex species during an 8-day reaeration period following anoxia. Control growth rates are: $193 \pm 6$, $232 \pm 4$ and $153 \pm 2$ mg g$^{-1}$FWd$^{-1}$ for *R. palustris*, *R. maritimus* and *R. thyrsiflorus* respectively.

Different letters indicate significant differences among species for each time point (means ±SE, n=4).
Figure 2: a: Survival of roots of 22 days old seedlings of *Rumex* species after treatment of the roots with $10^4$ M paraquat. Survival curves are fitted according to the Weibull distribution. LT$_{50}$ values are $5.44 \pm 0.08^a$, $4.33 \pm 0.20^b$ and $3.78 \pm 0.17^b$ for *R. palustris*, *R. maritimus* and *R. thyrsiflorus* respectively.

b: Growth rate of 24 days old seedlings of *Rumex* species during a 8 day reaeration period following treatment of the roots with $10^5$ M paraquat. Control growth rates are: $121 \pm 14$, $166 \pm 11$ and $131 \pm 5$ mg g$^{-1}$ FW d$^{-1}$ for *R. palustris*, *R. maritimus* and *R. thyrsiflorus* respectively.

Different letters indicate significant differences among species for each time point (means $\pm$ SE, n=4).
SOD activity

Leaves of seedlings of *R. palustris* and *R. thyrsiflorus* that were treated anoxically for 0 or 3 hours did not differ in their *in vitro* SOD activity (Fig. 3). However, after anoxia periods longer than 3 hours of anoxia followed by reaeration the SOD activity in *R. thyrsiflorus* decreased, while it did not change significantly in *R. palustris*. This led to significantly lower *in vitro* SOD activities in leaves of *R. thyrsiflorus* than in leaves of *R. palustris* after 5 and 8 hours of anoxia followed by reaeration.

![Figure 3: In vitro SOD activity of 24 days old seedlings of Rumex species after a 2 day reaeration period following anoxia. Different letters indicate significant differences among species per time point and among time points per species (means ± SE, n=3).](image)

Acetaldehyde production

Both in shoots and roots of *R. palustris* a clear increase in acetaldehyde production occurred following reaeration after prolonged anoxia (Fig. 4). The time course in acetaldehyde production in the two plant parts was very similar. In shoots and roots the production increased strongly within 25 minutes after reaeration to reach a stable level after 45 to 60 minutes. The production of acetaldehyde in the roots started to decrease slowly 1.5 hours after introduction of air into the cuvette. A small peak in acetaldehyde production by the shoots was noted only 2.5 minutes after reaeration. The absolute level of acetaldehyde
production differed markedly between the two plant parts, being 39 and 8.2 nl g$^{-1}$FW h$^{-1}$ for shoots and roots respectively. Roots turned from white to grey and leaves wilted within several hours after oxygen was introduced into the cuvettes.

Figure 4: Acetaldehyde production by shoots (a) and roots (b) of *R. palustris* during the shift from anoxic to normoxic conditions.
Discussion

Resistance to anoxia and oxidative stress

Both survival and growth measurements (Fig. 1) show that *R. palustris* is most resistant to anoxia and followed by a reaeration period. The survival data indicate that *R. maritimus* is least resistant, in contrast, growth measurements show that *R. thyrsiflorus* is more affected. The survival data are based on a viability stain of the roots only, while the growth measurements concern the performance of the whole plant. During flooding in the field, the performance of the whole plant is what determines its probability of survival. Therefore, it is likely that the growth measurements gave a better assessment of the overall resistance of the whole plant. This is further supported by the fact that plants of which the roots tips had died, still were able to grow (compare Figs 1a and b). Accordingly, the viability stain underestimated survival of the entire plant. Therefore, these results indicate that *R. palustris* is most resistant to anoxia and the following post-anoxic stress, followed by *R. maritimus*, while *R. thyrsiflorus* is least resistant. These results agree with those found in a previous study (Nabben *et al.*, 1999; chapter 2).

Although it was clear that the species differed in resistance, it was not clear whether anoxia stress or post-anoxia stress was most important. Therefore, we tested the resistance of the three species to oxidative stress caused by application of paraquat. The oxidative stress caused by paraquat application clearly decreased survival and growth rate of all three *Rumex* species (Fig. 2). However, again the two parameters showed a different order of the species with respect to their resistance to the stress. A high dose of $10^{-4}$ M paraquat applied to the roots resulted in a higher survival rate of roots of *R. palustris* compared to the other two species (Fig. 2), with *R. maritimus* having a slightly higher resistance than *R. thyrsiflorus*. In contrast, the growth rate of *R. thyrsiflorus* was significantly higher than that of *R. palustris* after treatment with $10^{-5}$ M paraquat for more than two hours and *R. maritimus* was affected most. Bipyridyl herbicides like paraquat are taken up rapidly by roots and are transported to the leaves (Purba *et al.*, 1995), but paraquat can be compartmentalised in plants reducing its reactivity (Matters & Scandalios, 1986). It is possible that the paraquat applied in the lower concentration of $10^{-5}$ M is more effectively sequestered in *R. thyrsiflorus* than in the other species, leading to a relatively low oxidative stress in the leaves of this species. However, when paraquat was applied to the leaves, again *R. thyrsiflorus* was least affected (results not shown). The results of paraquat application to leaves, combined with the growth measurements after paraquat application, indicate that *R. thyrsiflorus* is most resistant to oxidative stress *per se* when plants are not treated anoxically prior to the oxidative stress.
Chapter 5

Pavelic et al. (2000) found that post-anoxic lipid peroxidation in potato cells was negligible as long as membrane lipids were intact. The amounts of detoxifying enzymes appeared to be high enough to prevent build-up of ROS and peroxidation only occurred after hydrolysis of the lipids. Lipids were only hydrolysed below a certain threshold in the ATP synthesis rate (Rawyler et al., 1999) independently of the presence or absence of oxygen. This would mean that lipid peroxidation upon reaeration only occurs if the energy generation of plants during oxygen deficiency is too low to maintain sufficiently high ATP levels. It also implies that lipid peroxidation is a consequence and not a cause of post-anoxic injury. This hypothesis can explain why flooding tolerant plants are more resistant to post-anoxic reaeration than more sensitive plants, as was the case in the current study. This accords very well with a study in which we showed that the ATP status of *R. thyrsiflorus* was lower than that of *R. maritimus*, while *R. palustris* had the highest energy status during flooding (chapter 3). The different orders in resistance of species to anoxia and paraquat treatment also indicate that the resistance to oxidative stress per se can be altered during an anoxic period, as was found before (Monk et al., 1987b; VanToai & Bolles, 1991). It is clear that in this study the anoxic period was very important in determining resistance to anoxia and post-anoxic aeration, possibly by altering the resistance to oxidative stress.

**Defence mechanisms against post-anoxic injury**

The *in vitro* SOD activity of untreated leaves (0 h of anoxia) of *R. palustris* and *R. thyrsiflorus* was equal (Fig. 3). Anoxic incubation for two hours followed by reaeration did not increase the *in vitro* SOD activity in leaves of either species (Fig. 3). However, an anoxic period of five or eight hours followed by reaeration did not change *in vitro* SOD activity of *R. palustris*, while it decreased in *R. thyrsiflorus*. This strengthens the assumption that *R. palustris* is more resistant to anoxia followed by post-anoxic stress than *R. thyrsiflorus* and agrees well with other studies that show that flooding resistant species also have a high resistance to post-anoxic oxidative stress (Monk et al., 1987b; Biemelt et al., 1996; Blokhina et al., 1999).

Purification of SOD with a Sephadex column reduced the antioxidative activity by 10-20% (results not shown). The aspecific antioxidative activity after purification varied between 60 and 200% of SOD activity but on average was equal to SOD activity (results not shown). This means that besides SOD, also non-enzymatic antioxidants contributed to scavenging of superoxide. Apart from well-known antioxidants such as ascorbate, glutathione and α-tocopherol, also
other compounds can function as antioxidants. Recently it became evident that also many polyphenols are highly functional as antioxidants (Rice-Evans et al., 1997). In *Rumex* species several polyphenolic substances occur abundantly such as: quercetin, kaempferol, catechin, ferulic acid and p-coumaric acid (Hegnauer, 1969) all of which have a higher antioxidant capacity (on a molar basis) than vitamin C (ascorbate) or vitamin E (a-tocopherol; Rice-Evans et al., 1997). In addition, in *Conyza canadensis* polyphenols and polyphenol oxidases displayed SOD-like activity, even in the presence of PVPP (Turcsányi et al., 1994). Therefore, these substances probably also played a considerable role in resistance to post-anoxic injury in *Rumex* species. This could be expected since efficient destruction of ROS requires the action of several components of the antioxidant system acting in synchrony (Del Longo et al., 1993; Noctor & Foyer, 1998).

**Acetaldehyde production during reaeration**

Within half an hour after reaeration the acetaldehyde production started to increase in both shoots and roots of *R. palustris* plants and after less than one hour a stable production level was reached (Fig. 4). The acetaldehyde originated most probably from oxidation of ethanol. Upon reaeration the oxidation of ethanol to acetaldehyde can be catalysed either by alcohol dehydrogenase (ADH; Kreuzwieser et al., 1999) or by catalase using hydrogen peroxide (Monk et al., 1987a; Zuckermann et al., 1997).

Almost immediately after the switch to normoxic conditions, a temporal, small increase in acetaldehyde production was detected (Fig. 4a), while in the roots no small peak was observed (Fig. 4b). It is very well possible that the roots also showed such a temporal increase, which was not recorded because of the larger time interval with which samples were taken: 7.5 minutes (roots) instead of every 20 seconds (shoots). Up to now, we have no explanation for the temporal peak in acetaldehyde production by the shoot.

The differences in maximum acetaldehyde production between shoots and roots can have several causes. It could mean that more ethanol accumulated in the shoots during anoxia. However, measurements of *in vitro* enzyme activity indicate that the production of ethanol in illuminated shoots of *R. palustris* will be much lower than the production in the roots (chapter 3). Therefore, it is more likely that the higher acetaldehyde release of the shoots was due to transport of ethanol from the roots to the shoots by the transpiration stream (Kreuzwieser et al., 2000). Another cause of the difference in acetaldehyde release can be a higher resistance to outward diffusion of acetaldehyde in roots. It is also possible that the efficacy of ethanol oxidation was higher in the shoots, for instance because of a higher
catalase activity by generation of more hydrogen peroxide in shoots compared to roots.
The wilting of the leaves and the discoloration of the roots upon reaeration indicate that *R. palustris* suffered from post-anoxic injury. Since acetaldehyde is phytotoxic (Op den Camp & Kuhlemeier, 1997), it is very likely that in this experiment acetaldehyde played an important role the post-anoxic injury observed in *R. palustris*, like it does in other species (Monk *et al.*, 1987a; Perata & Alpi, 1991). However, it is likely that under flooded conditions ethanol diffuses out of the tissues more easily than in a gaseous environment (Vartapetian & Jackson, 1997) and does not lead to toxic concentrations of acetaldehyde upon reaeration. Therefore, the production of acetaldehyde in this experiment might be an artefact, caused by the conditions needed for application of the technique, and this situation will not be encountered in flooded plants.
Chapter 6

General Discussion
Flooding leading to oxygen deprivation is a major stress for plants growing in river floodplains (Blom, 1999). Oxygen deprivation results in arrest of aerobic respiration, leading quickly to an energy deficit in plants. If floods are deep and plants are not able to reach the water surface and restore contact with the atmosphere, they depend for survival on metabolic adaptations and underwater photosynthesis (leading to oxygen production) (Vartapetian & Jackson, 1997). Metabolic adaptations that enable plants to tolerate oxygen deprivation consist of 1) maintenance of glycolysis for anaerobic generation of ATP, 2) fermentation processes regenerating NAD⁺, thereby preventing that it becomes limiting for glycolysis and 3) an adequate supply of carbohydrates to fuel glycolysis (Drew, 1997).

Observations show that, apart from oxygen deprivation, renewed exposure to normal oxygen concentrations after a prolonged period of oxygen deficiency can lead to damage as well. This so-called post-anoxic injury is caused by formation of reactive oxygen species and oxidation of metabolites that have accumulated during oxygen deprivation (Armstrong et al., 1994; Crawford & Brändle, 1996).

In order to survive in frequently flooded environments, plants have to be adapted to this stress as well.

Because plants differ in flooding resistance, frequent floods lead to a zonation of species (Blom, 1999; Casanova & Brock, 2000). In this thesis, three Rumex species from habitats at different elevations in river floodplains and with different modes of adaptation to flooding were studied. *R. palustris* and *R. maritimus* inhabit low-elevated, frequently flooded mud flats near rivers (Hejny, 1960; Tüxen, 1979). These two species are able to avoid floods by their short life cycle or they can alter their morphology when flooded in order to facilitate uptake and diffusion of oxygen from the atmosphere above the floodwater. Nevertheless, the two species also differ from each other. *R. maritimus* is an annual and appears to invest more energy in submergence-induced shoot elongation and reproductive effort, while *R. palustris* is longer-lived and relies more on tolerance of oxygen deficiency by anaerobic metabolism (Van der Sman, 1992). In contrast, *R. thyrsiflorus*, the third species that was studied, grows in flooding-prone environments on high-elevated, seldom flooded sites like river dunes and dikes. This species is a perennial and has fewer adaptations to alleviate oxygen deficiency stress (Blom, 1999).

In this thesis, the flooding resistance of the three Rumex species was tested by recording their survival upon complete submergence. Additionally, their metabolic adaptations to oxygen deprivation were assessed and also the influences of underwater photosynthesis and post-anoxic injury on survival of floods were
determined. It was hypothesised that *R. thyrsiflorus*, the species that is least exposed to flooding in its natural habitat, would have the lowest resistance to flooding and would display less metabolic adaptations to oxygen deprivation than the other two species. Since *R. palustris* is supposed to rely more on metabolic adaptations due to its longer life cycle than *R. maritimus*, the former species is expected to have a higher resistance to complete submergence.

**Summary of the main results and synthesis**

*Flood characterisation and distribution of Rumex species.*

In *Rumex* species, germination of seeds and emergence of new shoots in spring takes place when the soil is uncovered for the first time after the winter floods. For floodplains of the river Waal this occurred in most years in April or May (chapter 2). The first spring or summer floods occurred in May to July, which means that very young plants may become flooded, but also much older plants, especially if these germinated in one of the previous years. In most years, one to three floods lasting between 1 and 85 days occurred in the *R. palustris/R. maritimus* zone. The period for plant growth (the interval between floods) also varied largely from 5 to 180 days. The height of the floods ranged from 5 to 200 cm leading to waterlogging (soil flooding) only or, more frequently, to deep submergence that in some cases could not be overcome by shoot elongation. Accordingly, *R. palustris* and *R. maritimus* are occasionally exposed to deep, long-lasting floods. The only way to survive these is by tolerance of oxygen deficiency at the cellular level or by oxygen production through underwater photosynthesis (Vartapetian & Jackson, 1997). In contrast, the zone inhabited by *R. thyrsiflorus* was flooded only in a few years. In those years, the flooding frequency was low (never more than twice) and of short duration (less than 17 days; chapter 2). The flooding stress imposed on *R. thyrsiflorus* plants is therefore much lower than that on the other two species. Mature plants of all three species showed relatively high survival rates, even if they were submerged in complete darkness and underwater photosynthesis was prevented (chapter 2). In addition, all three species were able to degrade the stored carbohydrates and to metabolise the resulting hexoses during submergence (chapter 4). This is a property mainly found in flooding resistant species (Perata *et al.*, 1992; 1996). Moreover, tap root slices of the three species retained accumulated solutes during anoxia and resumed ion uptake upon reaeration (chapter 3), indicating as well that the tissues were quite resistant to oxygen deprivation (Zhang & Greenway, 1994). Although all species were rather resistant
to oxygen deprivation, they clearly differed in flooding resistance, as indicated by the survival and biomass development during complete submergence (chapter 2). As was expected, *R. palustris* was most resistant, *R. thyrsiflorus* showed the lowest survival and *R. maritimus* performed intermediately.

**The central role of tap roots in survival of floods**

In general, leaves and lateral roots are more sensitive to oxygen deprivation than tubers or rhizomes (Armstrong *et al.*, 1994). Likewise, tap roots were the longest surviving organ during flooding of the *Rumex* species tested here (chapter 2). This was probably due to higher fermentation rates, resulting in a higher energy status of tap roots compared to other plant parts (chapter 3). Juvenile plants (5-6 weeks old) of all species were less resistant to submergence than mature plants (12-16 weeks old; chapter 2). This can be explained by the presence of high amounts of carbohydrates in tap roots of mature plants (chapter 4), while this organ was not yet present in juveniles. Moreover, upon submergence a large part of the non-structural carbohydrates in juvenile *Rumex* plants was consumed within one day (unpublished results AJ Visser). In contrast, the dry weight and the carbohydrate content of the tap roots decreased only slowly during submergence (chapters 2 and 4). This can be due to either a low metabolic rate (see below) or allocation of carbohydrates (which form a large portion of the dry weight) from other plant parts. In contrast, the dry weight (chapters 2 and 4) and the carbohydrate content (unpublished results AJ Visser) of shoots of *Rumex* species decrease considerably during flooding and therefore it is plausible that some carbohydrates are allocated from shoots to tap roots.

In tap roots of *R. palustris* and *R. thyrsiflorus* starch formed the major carbohydrate store and was preferentially used, while *R. maritimus* stored and consumed equal amounts of starch and fructans (chapter 4). However, the main form of carbohydrate storage or use (starch or fructans) was not correlated with flooding resistance. Moreover, in none of the species the carbohydrates in tap roots were exhausted by the end of the experiment (chapter 4), which means that not carbohydrate depletion, but some other factor caused the death of mature plants during flooding. This absence of a correlation between carbohydrate quantity (reviewed by Ricard *et al.*, 1994) and quality was found before in many other plants like *Iris* species (Hanhijärvi & Fagerstedt, 1995).

Apart from fuelling fermentation, fructans seemed to have no specific function in *Rumex* species during submergence stress, like they do in drought and cold stress (Pollock & Cairns, 1991; Hendry, 1993). However, carbohydrates still present at the end of the submergence period can also be important for survival after
submergence as was found for rice and other species (Setter et al., 1987b; 1997). Especially the easily degradable fructans might be very efficient in this respect (Albrecht et al., 1994; Morvan-Bertrand et al., 1999).

In addition to being the longest surviving organ and the main location of carbohydrates, tap roots are also the location of new root and shoot primordia (Armstrong et al., 1994). Therefore, tap roots of Rumex species are the organ from which regeneration can occur after the floods have receded. Consequently, survival of this organ and regrowth of shoots and lateral roots from it are crucial for survival of the entire plant (cf. Crawford, 1996).

**Underwater photosynthesis**

*Rumex* plants that were submerged in the light showed a higher survival (chapter 2), a slower decrease in shoot biomass (chapters 2 and 4) and slower depletion of carbohydrates in tap roots (chapter 4) than plants submerged in the dark. Additionally, the carbohydrate content in leaves of submerged *R. palustris* and *R. acetosa* decreased mainly during the night (unpublished results AJ Visser). These beneficial effects of light on submerged plants are commonly found in many species (e.g. Setter et al., 1987a; Laan & Blom, 1990; He et al., 1999) and can be attributed to underwater photosynthesis. Underwater photosynthesis leads to an increased carbohydrate (Setter et al., 1989) and oxygen (Setter et al., 1987a; Rijnders et al., 2000) availability, resulting in provision of readily respirable carbohydrates or conservation of carbohydrates by more efficient aerobic respiration. Photosynthesis can indeed alleviate oxygen deprivation stress, as was indicated by low ethanol production in ice-encased cereals incubated in the light compared to plants incubated in the dark (Andrews, 1988) and by low activities of fermentation enzymes in illuminated *Rumex* shoots during submergence (chapter 3). The extent to which underwater photosynthesis actually contributes to survival of flooding under field conditions depends on the availability of CO₂ and light (Setter et al., 1987b; 1989) and the capacity for underwater photosynthesis (Vervuren et al., 1999). Both CO₂ concentrations (He et al., 1999) and light levels (unpublished results PJA Vervuren) can be rather low in flooded forelands of the river Waal, which may hamper underwater photosynthesis. Under such conditions, completely submerged plants depend to a large extent on metabolic adaptations to tolerate oxygen deficiency.
Contribution of metabolic adaptations to flooding resistance

The in vitro activity of enzymes involved in anaerobic regeneration of NAD$^+$ by ethanolic fermentation (PDC and ADH), lactate fermentation (LDH) and nitrate reduction (NR) were very low in submerged leaves of the three Rumex species (chapter 3). This indicates that the oxygen status in the submerged leaves was sufficiently high to enable aerobic respiration. In contrast, the in vitro activity of fermentative enzymes clearly increased in the belowground parts during submergence. Judging from in vitro enzyme activities and in vivo fermentation products, lactate fermentation and nitrate reduction appeared to be of minor importance in NAD$^+$ regeneration. Instead, ethanol was the main fermentation product in all three Rumex species. This is consistent with the observation that ethanolic fermentation generally is the most important pathway for regenerating NAD$^+$ in oxygen-deprived plants (reviewed by Perata & Alpi, 1993; Ricard et al., 1994). Because the species with the lowest fermentation rates (i.e. R. thyrsiflorus) also showed the fastest decrease in energy status (chapter 3) and displayed the lowest resistance to flooding, ethanolic fermentation rates correlated positively with flooding resistance.

Some plants show a high glycolytic (and fermentative) rate upon oxygen deprivation (Pasteur effect), leading to increased energy generation by glycolysis. Wetland plant rhizomes often exhibit these high reaction rates only at the beginning of a flooding period. These plant organs thus economise reserves and produce less toxic compounds than with high fermentation rates (Albrecht & Wiedenroth, 1994b; Armstrong et al., 1994). No indications for a Pasteur effect in Rumex species were found (chapter 4), which agrees with previous results in other Rumex species (Laan, 1990). On the other hand, the content of carbohydrates in tap roots mainly decreased during the first week of submergence, with a much slower decrease thereafter (chapter 4). In addition, the in vitro and in vivo fermentation rate decreased during the first week of oxygen deprivation (chapter 3). High rates of fermentation in the first days of flooding can supply energy for survival. Moreover, the energy can also be used for developing amelioration tactics like enhanced shoot elongation and aerenchyma formation, which are observed in R. palustris and R. maritimus (Van der Sman et al., 1993b; Laan et al., 1990). Nonetheless, when floods last longer it is more advantageous to slow down the fermentation rate so that carbohydrates can be saved for prolonged resistance. All three Rumex species appeared to employ this strategy and down-regulated their energy generating metabolism during oxygen deprivation.

Rhizomes of some wetland plants with large carbohydrate reserves can enter a quasi-dormant state that permits survival of temporal anoxia (Brändle, 1991; Vartapetian & Jackson, 1997). For instance, the maintenance energy required in
aged beetroot tissues under anoxia is 10 to 25 times lower than in air (Zhang and Greenway, 1994). In addition, a low ATP/ADP ratio during oxygen deficiency may prevent overconsumption of ATP and carbohydrates by inhibiting glucose and fructose degrading enzymes (Renz & Stitt, 1993). It is conceivable that also in *Rumex* species energy requirements are lowered during oxygen deficiency, thereby requiring only slow rates of fermentation and, consequently, saving carbohydrates. An indication that this actually occurred was the slow rate of energy dependent trans-membrane transport of solutes in tap root discs of the three species during anoxia (chapter 3). On the other hand, some energy was allocated to the maintenance of membrane integrity as was shown by the limited loss of accumulated solutes during anoxia and the resumption of solute transport after reaeration.

It has been suggested that metabolic adjustments are useful only for short-term adaptation to oxygen deficiency (Laan, 1990). However, when down-regulation of energy generation and consumption occur in addition to fermentation processes, some plants can survive for months on solely metabolic adaptations (Crawford & Brändle, 1996). Additionally, also the *Rumex* species tested in this study survived for weeks when they were submerged in the dark (chapter 2). Therefore, metabolic adaptations can contribute considerably to both short- and longer-term resistance to flooding in these *Rumex* species.

*The impact of post-anoxic injury*

Since ethanol is the main fermentation product in these *Rumex* species, it was hypothesised that its oxidation to acetaldehyde upon reaeration can comprise a substantial part of post-anoxic injury. Acetaldehyde was indeed formed by roots and shoots of *R. palustris* within half an hour after reaeration after anoxia in a gaseous environment. In addition, post-anoxic injury occurred as shown by the discoloration of roots and wilting of leaves (chapter 5). To obtain a high time resolution, highly sensitive photo acoustic detection of acetaldehyde was applied. This method of detection requires that the plants are incubated in a gaseous atmosphere, which will have impeded diffusion of ethanol out of the tissues, presumably leading to accumulation of ethanol. In contrast, during submergence ethanol can easily diffuse out of the tissues and into the water surrounding the plants (Vartapetian & Jackson, 1997). Therefore, it remains questionable if acetaldehyde formation by oxidation of ethanol also contributes to post-anoxic injury in flooded plants. Nevertheless, some plants that looked healthy immediately after submergence died in the subsequent reaeration period. Chlorotic and necrotic spots occurred on
the leaves and some leaves disintegrated quickly (chapter 2), which are typical symptoms of post-anoxic injury (Armstrong et al., 1994). Especially plants with a low flooding resistance (juvenile plants and plants of R. thyrsiflorus) died after the floodwater had receded (chapter 2) or after experimental anoxia followed by reaeration (chapter 5). By comparing these data with results from an experiment with paraquat application (a herbicide leading to oxidative stress), it was deduced that resistance to post-anoxic injury was mainly determined in the ability to generate energy during anoxia and only to a small extent by their resistance to oxidative stress (chapter 5). The activity of superoxide dismutase (an enzyme involved in detoxification of oxygen radicals) was maintained in a flooding resistant species, while it decreased in a less resistant species during anoxia (chapter 5). Apparently, resistance to oxidative stress was altered by oxygen deprivation as was found before with other species (Monk et al., 1987b; VanToai & Bolles, 1991). It is likely that the resistance to post-anoxic stress is related to the generation of energy during the preceding period of oxygen deficiency (Rawyler et al., 1999). In addition to radical scavenging enzymes, non-enzymatic antioxidants appeared to be important in detoxifying superoxide in these Rumex species (chapter 5). It is hypothesised that polyphenols that occur abundantly in Rumex species (Hegnauer, 1969) are part of this antioxidative capacity (as in many other species, Rice-Evans et al., 1997).

From the results presented in this thesis it is clear that post-anoxic injury can indeed form a serious threat to submerged plants, as was noted before in rice (Ushimaru et al., 1992) and maize (Yan et al., 1996). Hence, in order to survive in frequently flooded areas, plants not only need to be able to withstand the oxygen deficiency, but also the period of normal oxygen concentrations following the floods.

Differences among species in tolerance to oxygen deprivation

The lower resistance to flooding of R. thyrsiflorus was probably caused by a slow fermentative rate. This was indicated by the low in vitro enzyme activities of PDC and ADH during flooding, resulting in lower ethanol production rates during anoxia. This appeared to lead to a relatively low total amount of adenylates and AEC (chapter 3). Additionally, R. thyrsiflorus also seems to be more susceptible to post-anoxic injury than the other species (chapters 2 and 5), which might be related as well to a lower generation of energy in the oxygen deficient phase (see above). Nevertheless, mature plants of R. thyrsiflorus were able to survive complete submergence in the dark for up to five weeks (chapter 2), enabling them to survive the flooding period (up to two weeks) they are confronted with in their
natural habitat (chapter 2). This means that even a relatively slow fermentation rate can generate sufficient energy to keep the tissues viable for some time. As was indicated in a previous study (Van der Sman et al., 1993a), R. palustris was more resistant to complete submergence than R. maritimus (based on survival and biomass; chapter 2). R. maritimus died if floods lasted too long and underwater photosynthesis was impeded (chapter 2; Laan & Blom, 1990), while R. palustris survived floods lasting eight weeks, even without underwater photosynthesis. In contrast to R. thyrsiflorus, both other species showed a clear induction of fermentative enzymes and higher ethanol production rates. This led to a higher AEC and total adenylate content in R. palustris, but not in R. maritimus, as compared to R. thyrsiflorus (chapter 3). In other words, R. palustris and R. maritimus do not clearly differ in fermentative capacity, but they do differ in flooding resistance. Accordingly, fermentation rate was not the only factor determining tolerance to flooding-induced oxygen deficiency in these species.

Submergence-induced shoot elongation can lead to a lower allocation of carbohydrates or energy to other processes, resulting in lower flooding tolerance (Laan & Blom, 1990; Setter & Laureles, 1996). Van der Sman (1992) hypothesised that R. maritimus invests more energy in shoot elongation, sexual reproductive effort and early reproduction, while R. palustris relies more on tolerance strategies. Possibly, R. maritimus is not able to tolerate stress for a long time because of the allocation to other survival tactics (Carter & Grace, 1990).

The large intervals between two floods (chapter 2) are long enough to enable flowering of R. maritimus (Van der Sman et al., 1993b), thereby surviving the following flood as a dormant seed and avoiding oxygen deprivation. The higher resistance of R. palustris to complete submergence in the dark found in the present study further supports the hypothesis as posed by Van der Sman (1992).

In addition to aspects related to survival of the oxygen deficient period, R. maritimus is also less resistant to post-anoxic injury than R. palustris. This is shown by the lower root survival and slower growth rate of R. maritimus after anoxia followed by reaeration (chapter 4). R. maritimus also appeared to be more sensitive to oxidative stress caused by paraquat application. This lower resistance to post-anoxic injury can also contribute to a lower resistance to flooding of R. maritimus compared to R. palustris.

Considering all aspects related to flooding resistance tested in this study, R. palustris resembles plants that are renowned for their anoxia tolerance such as Iris pseudacorus (Hanhijärvi & Fagerstedt, 1995; Crawford & Brändle, 1996) and Acorus calamus (Joly & Brändle, 1995; Weber & Brändle, 1996) in their adaptation to oxygen deficiency.
Main conclusions

1. Tap roots play an important role in resistance of *Rumex* species to submergence since they contain reserve carbohydrates, survive longest and therefore are the organs from which regeneration of the whole plant occurs after the floods have receded.
2. Underwater photosynthesis enhances resistance of *Rumex* species to submergence, probably by generation of oxygen and/or carbohydrates.
3. Ethanol fermentation is the main pathway for anaerobic NAD\(^+\) regeneration in these species, while lactate fermentation and nitrate reduction are of minor importance.
4. No Pasteur effect occurs in these species; instead metabolic rates appear to be down-regulated during prolonged oxygen deprivation, thereby saving carbohydrates and allowing plants to survive submergence for weeks by metabolic adaptations only.
5. Post-anoxic injury can form a constraint for survival of flooding in these species, and the plants most tolerant to submergence are also least negatively affected by reaeration. Resistance to oxidative stress decreases during anoxia, especially in the most flooding sensitive species. This is probably linked to a decreased availability of energy during oxygen deprivation.
6. All three species are quite resistant to oxygen deprivation and are able to degrade stored carbohydrates and metabolise the resulting sugars. Nevertheless, *R. palustris* is most resistant to complete submergence, followed by *R. maritimus*, while *R. thyrsiflorus* is most sensitive. Neither quality nor quantity of carbohydrates could explain the differences in resistance to submergence between mature plants of the species. The lower fermentation rate of *R. thyrsiflorus* probably causes its lower resistance to complete submergence, as indicated by its energy status. The difference in resistance to submergence between *R. palustris* and *R. maritimus* is probably related to allocation of energy to different adaptive strategies. *R. maritimus* seems to invest more energy and resources in avoidance and amelioration, while *R. palustris* depends most on tolerance mechanisms.
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Samenvatting (Summary in Dutch)

Overstromingen

Natuurlijke overstromingen leiden wereldwijd tot een verlaagde opbrengst van landbouwgewassen. Ook rijst, het belangrijkste voedsel voor de helft van de wereldbevolking, wordt voornamelijk verbouwd in gebieden die vaak overstroomd worden. Overstromingen kunnen derhalve leiden tot economische verliezen en voedselgebrek. Daarnaast bepalen overstromingen de verspreiding van veel wilde plantensoorten en beïnvloeden ze natuurlijke processen in de bodem. Een goed inzicht in de reactie van planten op overstroming kan daardoor enerzijds een bijdrage leveren aan de verhoging van de landbouwproductie en anderzijds leidt het tot een beter begrip van bodemprocessen en de verspreiding van wilde plantensoorten in ecosystemen die beïnvloed worden door overstroming.

Het belangrijkste probleem voor planten tijdens overstroming is de sterk geremde gasuitwisseling tussen plant en omgeving. Dit leidt tot ophoping van sommige gassen in de plant of uitputting van andere gassen. Uit literatuurgegevens blijkt dat verlaging van de zuurstofconcentratie de belangrijkste verandering in gassensamenstelling in overstroomde planten is. Net als veel andere organismen hebben ook planten zuurstof nodig voor energieproductie via respiratie. Overstromingen kunnen daardoor tot energiegebrek leiden. Toch blijken sommige terrestrische plantensoorten goed tegen overstromingen bestand te zijn, omdat ze specifieke aanpassingen aan zuurstofgebrek hebben ontwikkeld.

Aanpassingen van planten aan overstromingen

Sommige plantensoorten die in vaak overstroomde gebieden voorkomen kunnen snel groeien, bloeien en zaad zetten. Wanneer ze hun hele levenscyclus voltooien tussen twee overstromingen door, kunnen ze de overstroming overleven als zaad (dat vrij ongevoelig is voor zuurstofgebrek). Andere soorten strekken snel hun stengels en bladeren als ze onder water staan, zodat ze het wateroppervlak bereiken en zuurstof uit de lucht kunnen opnemen. Indien er daarnaast ook luchtkanalen in de stengels en een nieuw wortelstelsel met veel luchtkanalen gevormd worden, dan kan zuurstof door de hele plant diffunderen. Bovendien zijn planten, zelfs onder water, in staat zelf zuurstof te maken door middel van fotosynthese, waarbij tevens koolhydraten worden gevormd, indien er voldoende licht en CO₂ aanwezig zijn.
Als de overstroming diep is kunnen planten het wateroppervlak niet bereiken. Is het water daarnaast ook troebel, dan zal er geen onderwater fotosynthese plaatsvinden. Enkele plantensoorten kunnen deze omstandigheden toch overleven door hun metabolisme zodanig aan te passen dat zonder zuurstof energie wordt geproduceerd (metabole aanpassingen). Deze anaërobe respiratie bestaat met name uit fermentatieprocessen, waarvan ethanolfermentatie en lactaatfermentatie de belangrijkste zijn. Omdat de efficiency van deze processen veel lager is dan die van aerobe respiratie, zijn meer koolhydraten nodig als brandstof. Planten hebben dan ook grote koolhydraathoeveelheden nodig om langdurig zuurstofgebrek te overleven. Sommige soorten versnellen hun anaërobe respiratie zodat per tijdseenheid toch relatief veel energie geproduceerd wordt (Pasteur effect).

Naast zuurstofgebrek zelf, kan ook hernieuwde blootstelling aan zuurstof (als het waterpeil zakt) na een lange zuurstofarme periode schade aan planten toebrengen en een aanzienlijk aandeel vormen van de sterfte als gevolg van overstroming. Deze zogenaamde “post-anoxia schade” wordt veroorzaakt door de vorming van, zeer reactieve, zuurstofradicalen. Antioxidanten bieden bescherming tegen zuurstofradicalen door ze om te zetten in minder reactieve moleculen. Het antioxidantsysteem van planten bestaat uit natuurlijke antioxidanten (zoals ascorbaat, glutathion en α-tocopherol) en enzymatische antioxidanten (zoals catalases, peroxidases en superoxide dismutase). Een tweede oorzaak van post-anoxia schade is de omzetting van metabolieten die in de plant ophopen tijdens zuurstofgebrek, in andere, toxische stoffen, zoals de vorming van acetaldehyde uit ethanol.


Nederlandse uiterwaarden en zuringsoorten

Uiterwaarden van de Rijn en haar zijrivieren zijn vooral in de winter en het vroege voorjaar aan overstroming onderhevig. Smeltwater uit de bergen van
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Zwitserland en Zuid-Duitsland veroorzaakt deze verhoogde waterniveaus. Daarnaast komen ook pieken in de waterstand voor die samenhangen met overvloedige regenval. De pieken kunnen het gehele jaar door plaatsvinden en zijn onvoorspelbaar wat betreft frequentie, duur en hoogte. Mede omdat deze regenpieken ook in het groeiseizoen van planten kunnen optreden, hebben ze een grote invloed op de groei van plantensoorten in Nederlandse uiterwaarden en resulteren ze in een variatie in verspreidingspatroon van plantensoorten. Nauw verwante soorten uit het geslacht *Rumex* (zuring) blijken in veel vegetatiertypen van de uiterwaarden vertegenwoordigd te zijn en komen verspreid over de hele uiterwaard voor, van zelden overstroomde tot vaak overstroomde plaatsen. Dit geslacht vormt dan ook een uitstekend modelsysteem om verschillen in aanpassing aan overstroming te onderzoeken.

Geoorde zuring (*R. thyrsiflorus*) komt in uiterwaarden voor in hooggelegen en daardoor zelden overstroomde habitats, zoals dijken en rivierduinen. Uit voorgaand onderzoek van de afdeling Experimentele Plantenecoloogie blijkt dat in *R. thyrsiflorus* slechts een geringe spruitstrekking optreedt tijdens overstroming. Bovendien vormt deze soort weinig luchtkanalen en nieuwe wortels. Omdat *R. thyrsiflorus* meerjarig is zullen er tijdens overstromingsperioden ook vegetatieve planten aanwezig zijn en kan deze soort de overstroming niet enkel in de vorm van zaad overleven. Deze soort heeft die aanpassingen ook niet nodig aangezien ze nauwelijks overstroomd wordt.

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*Doel van dit proefschrift*

Het doel van dit proefschrift is te onderzoeken welke metabole aanpassingen aan zuurstofgebrek en post-anoxia R. palustris, R. maritimus en R. thyrsiflorus hebben en in welke mate deze de overstromingsresistentie bepalen.

Om dit doel te bereiken zijn de overleving, fermentatiecapaciteit en de energiestatus van de drie soorten tijdens volledige onderdompeling gemeten. Als maat voor de energiestatus is tevens gemeten in hoeverre stukjes penwortel van de drie soorten tijdens zuurstofgebrek in staat zijn om actief, opgeloste stoffen te transporteren en om opgehoopte ionen vast te houden. Omdat koolhydraten nodig zijn om fermentatieprocessen ook op langere termijn te laten doorgaan, zijn ook de hoeveelheid en type koolhydraten van de drie soorten onderling vergeleken. Zuurstof- en koolhydraatproductie door onderwater fotosynthese kunnen de kans op overleving van overstroming in belangrijke mate verhogen. Daarom is het effect van onderwater fotosynthese op overleving en het koolhydraatmetabolisme tijdens volledige onderdompeling onderzocht. Tenslotte is onderzocht of post-anoxia schade inderdaad ook in deze soorten bijdraagt aan sterfte na overstroming en of de soorten in verschillende mate bestand zijn tegen deze stressfactor.

*De belangrijke rol van penwortels en koolhydraten in overstromingsresistentie*

Bij alle drie de soorten was de penwortel het orgaan dat het langst overleefde tijdens volledige onderdompeling (hoofdstuk 2). Dit werd waarschijnlijk veroorzaakt door de hogere fermentatiecapaciteit die leidde tot een hogere energiestatus in dit orgaan vergeleken met de andere plantendelen (hoofdstuk 3). Daarnaast waren de reserve koolhydraten ook gelokaliseerd in de penwortels (hoofdstuk 4). Volgroeide planten met een penwortel vertoonden daardoor een veel betere overleving bij overstroming dan juveniele planten zonder een penwortel (hoofdstuk 2).

In volwassen planten van de drie soorten werd de koolhydraatvoorraad niet uitgeput tijdens overstroming (hoofdstuk 4). Blijkbaar was de hoeveelheid koolhydraten in de penwortels voldoende voor langdurige overleving tijdens overstroming, werden er koolhydraten vanuit de spruit naar de penwortel gealloceerd en/of werd de snelheid van het algehele metabolisme verlaagd (zie later) waardoor het verbruik van koolhydraten afnam. Ook de vorm waarin de koolhydraten zijn opgeslagen (fructanen of zetmeel) kan de verschillen in overstromingsresistentie van de drie soorten niet verklaren (hoofdstuk 4). Naast het feit dat penwortels het langst overleven en de koolhydraatvoorraad van de plant
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bevatten, is het ook de locatie van groeipunten voor nieuwe bladeren en wortels. Daardoor zijn de penwortels het orgaan van waaruit de hele plant kan regenereren nadat het water is gezakt.

**Onderwater fotosynthese**

Zuringplanten die voldoende licht kregen tijdens volledige onderdompeling hadden een hogere overleving (hoofdstuk 2), een langzamere afname in biomassa (hoofdstuk 2 en 4) en in koolhydratenhoeveelheid (hoofdstuk 4) dan planten die in het donker overstroomd waren. Hoogstwaarschijnlijk zijn deze positieve effecten van licht het gevolg van een verhoogde productie van zuurstof en koolhydraten door onderwater fotosynthese. Dit kan leiden tot aanvoer van gemakkelijk respiereerbare suikers of besparing van koolhydraten door aërobe respiratie, aangezien dit proces efficiënter is wat betreft koolhydraatverbruik dan anaërobe respiratie. De activiteiten van enzymen die betrokken zijn bij fermentatie waren laag in bladeren van ondergedompelde planten die gedurende een deel van de dag licht kregen (hoofdstuk 3), hetgeen erop duidt dat er inderdaad zuurstof werd geproduceerd. Gezien de troebelheid en de lage CO₂-concentratie van het water in overstroomde uitwaarden, blijft het de vraag in hoeverre onderwater fotosynthese inderdaad voordelen biedt onder natuurlijke omstandigheden. Onder zulke omstandigheden zijn metabole aanpassingen aan zuurstofgebrek dus noodzakelijk voor de overleving van planten.

**De bijdrage van metabole aanpassingen aan overstromingsresistentie**

Net als in de meeste andere plantensoorten, bleek in alle drie de zuringsoorten ethanolfermentatie het belangrijkste proces voor anaërobe energie productie te zijn, terwijl lactaatfermentatie en nitraatreductie van gering belang waren (hoofdstuk 3). Aangezien de soort met de laagste fermentatiesnelheid (R. thyrsiflorus) ook de laagste energiestatus en de slechtste overleving had, lijkt fermentatiesnelheid in deze soorten positief gecorreleerd te zijn met overleving bij overstroming. In geen van de soorten werd een Pasteur effect waargenomen (hoofdstuk 4). De gemeten verlaging van de fermentatiesnelheid (hoofdstuk 3), vertraging van het koolhydraatgebruik en verminderde afname van biomassa (hoofdstuk 2 en 4) wijzen juist op een verlaging van het metabolisme. Een verlaging van het algehele metabolisme vermindert het koolhydraatverbruik en de productie van mogelijk toxische metaboliënen en kan daardoor overleving van langdurige overstroming bevorderen. Een voorwaarde hierbij is wel dat er
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Voldoende energie wordt geproduceerd om basale processen in stand te houden. Over het algemeen wordt aangenomen dat metabole aanpassingen alleen nuttig zijn voor kortdurende perioden van zuurstofgebrek. Als echter ook verlaging van de algehele metabole activiteit in ogenschouw wordt genomen, blijkt dat metabole processen langdurige overleving van zuurstofgebrek kunnen bewerkstelligen. Ook in de onderzochte zuringsoorten waren metabole aanpassingen voldoende voor overleving van volledige onderdompeling in het donker gedurende meer dan vijf weken (hoofdstuk 2).

De invloed van post-anoxia schade

Ethanol is het belangrijkste fermentatieproduct in de onderzochte zuringsoorten (hoofdstuk 3). Daarom ligt het voor de hand dat de vorming van acetaldehyde door oxidatie van ethanol bij reaeratie een belangrijke vorm van post-anoxia schade kan zijn in deze soorten. In *R. palustris* planten bleek inderdaad een verhoogde afgifte acetaldehyde op te treden als de omgeving van de planten plotseling van een zuurstofloze atmosfeer in lucht veranderde (hoofdstuk 5). Tegelijkertijd traden bij blootstelling aan zuurstof ook verschijnselen van post-anoxia schade op zoals het plotseling verkleuren van de wortels van wit naar grijsbruin en het verwelken van bladeren. Aangezien dit experiment werd uitgevoerd met planten in een gasvormige omgeving, is het waarschijnlijk dat ethanol slecht uit de plant kon diffunderen en dus ophoopte in de plant. Onder natuurlijke omstandigheden wordt een plant echter door water omgeven, waardoor ethanol gemakkelijk naar de omgeving diffundeert en is ophoping in de plant minder waarschijnlijk. Het is daarom twijfelachtig of ook in overstroomde planten de oxidatie van ethanol tot acetaldehyde een belangrijke rol speelt bij het optreden van post-anoxia schade. Hoewel oxidatie van ethanol geen rol lijkt te spelen in overstroomde situaties, trad er in alle drie de soorten toch post-anoxia schade op bij het boven water halen van de planten na overstroming (hoofdstuk 2). Er vormden er zich necrotische en chlorotische plekken op de bladeren, wat duidt op oxidatieve schade. Met name juveniele planten en planten van de meest overstromingsgevoelige soort (*R. thyrsiflorus*) waren gevoelig voor reaeratie na zuurstofgebrek (hoofdstuk 2 en 5). De activiteit van superoxide dismutase, betrokken bij het onschadelijk maken van zuurstofradicalen, nam eveneens sterker af in *R. thyrsiflorus* (de meest overstromingsgevoelige soort) dan in *R. palustris* (de meest resistente soort). Door deze gegevens te vergelijken met een experiment waarin planten behandeld werden met paraquat (een herbicide dat zuurstofradicalen genereert), kon worden afgeleid dat de gevoeligheid voor zuurstofgebrek gevolgd door hernieuwde
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blootstelling aan zuurstof, vooral werd bepaald door de hoeveelheid energie die geproduceerd werd in de zuurstofarme periode (hoofdstuk 5).

Uit de resultaten bleek dat ook in zuringsoorten, oxidatieve stress en post-anoxia schade een belangrijke bijdrage kunnen leveren aan sterfte als gevolg van overstroming (hoofdstuk 2 en 5). Dit betekent dat plantensoorten die in overstromde gebieden voorkomen niet alleen aangepast moeten zijn aan zuurstofgebrek tijdens de overstroming, maar ook aan hernieuwde blootstelling aan zuurstof als het water is gezakt.

Verschillen tussen soorten in overstromingsresistentie

Volgroeide planten van alle drie de soorten waren vrij resistent tegen overstroming. Dit bleek uit de overleving (hoofdstuk 2), het vermogen om reserve koolhydraten af te breken en de gevormde suikers te metaboliseren tijdens overstroming in het donker (hoofdstuk 2 en 4) en uit de handhaving van intacte membranen tijdens zuurstofloosheid (hoofdstuk 3). Hoewel de drie soorten allemaal een vrij hoge overstromingsresistentie hadden, waren er wel verschillen in de mate van resistentie (hoofdstuk 2). De overstromingskarakteristieken van de habitats van de soorten (hoofdstuk 2) toonden aan dat *R. thyrsiflorus* inderdaad korter en minder vaak wordt overstromd dan de andere twee soorten. Zoals verwacht, was *R. thyrsiflorus* ook minder resistent tegen volledige onderdompeling, zoals bleek uit zowel de overlevingsresultaten als uit de biomassa gegevens. Deze lagere resistentie werd waarschijnlijk veroorzaakt door een lagere fermentatiecapaciteit, die tot lagere energieniveaus in de plant leidde (hoofdstuk 3). Desondanks waren volwassen planten van *R. thyrsiflorus* in staat om onderdompeling in het donker gedurende vijf weken te overleven, wat overleving van de overstromingsduur waarmeeze in hun natuurlijke omgeving geconfronteerd worden (tot 2 weken) mogelijk maakt (hoofdstuk 2). Dit betekent dat zelfs een relatief langzame fermentatiesnelheid voldoende energie kan opleveren om weefsels gedurende enige tijd in leven te houden.

*Rumex palustris* was resistentener tegen volledige onderdompeling dan *R. maritimus*, zoals bleek uit overlevings- en biomassa gegevens (hoofdstuk 2). *R. maritimus* ging dood als de overstromingen lang duurden en onderwater fotosynthese geremd was. *R. palustris*, daarentegen, overheefde zelfs acht weken volledige onderdompeling zonder onderwater fotosynthese. In tegenstelling tot *R. thyrsiflorus*, waren in beide andere soorten een duidelijke inductie van fermentatie en een hogere ethanolproductie waarneembaar (hoofdstuk 3). Dit leidde tot een hogere energiestatus in *R. palustris* in vergelijking met *R. thyrsiflorus*, maar dit
was niet het geval in *R. maritimus*. Kortom, *R. palustris* en *R. maritimus* verschilden niet duidelijk in fermentatiecapaciteit, maar verschilden wel in overstromingsresistentie. Fermentatiesnelheid is dus niet de enige factor die resistentie tegen onderdompeling in het donker bepaalt. Het is mogelijk dat *R. maritimus* meer energie investeert in strekking van de spruit en snelle voortplanting wat ten koste kan gaan van energie voor overleving als het wateroppervlak niet wordt bereikt. De perioden tussen twee overstromingen zijn voldoende lang (hoofdstuk 2) zodat *R. maritimus* zijn volledige levenscyclus kan voltooien en als zaad de overstroming kan overleven. *Rumex palustris* lijkt vooral afhankelijk te zijn van metabole aanpassingen om overstroming te overleven en lijkt in zijn aanpassingen op soorten waarvan bekend is dat ze uitermate goed aan zuurstofgebrek zijn aangepast.

**Belangrijkste conclusies**


In hun natuurlijke habitat worden *R. palustris* en *R. maritimus* vaker, langer en dieper overstroomd dan *R. thyrsiflorus*. Alle drie de soorten zijn relatief resistent tegen zuurstofgebrek, maar *R. palustris* is duidelijk het meest resistent, terwijl *R. thyrsiflorus* het meest gevoelig is. De hoeveelheid en het type reservekoolhydraten kunnen de verschillen in overstromingsgevoeligheid niet
Dankwoord

Dankwoord (Acknowledgements)

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Dankwoord

dan wel als “unpublished results” of als basis voor verdere experimenten. Een groot deel van het (soms taaie) praktische werk is door jullie uitgevoerd en de discussies met jullie hebben mij geïnspireerd tot nieuwe ideeën. Daarmee hebben jullie een grote bijdrage aan dit proefschrift geleverd. Bedankt ook voor de gezelligheid! (Vooral de periode met vier studenten tegelijkertijd zal ik nooit vergeten.)

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Dankwoord

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Naast zijn promotie onderzoek volgde hij een onderwijsprogramma voor AiO’s bij de onderzoeksschool Functionele Ecologie en de KUN. Daarnaast begeleidde hij vijf doctoraal studenten Biologie bij hun stage en assisteerde hij diverse cursussen voor biologen. Tijdens zijn promotieperiode bezocht hij diverse congressen en symposia in binnen- en buitenland. Hij gaf lezingen op onder andere plantenfysiologie congressen in Florence (Italië) en Varna ( Bulgarije).

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