Growth on low nitrogen and sufide toxicity in wet, calcareous dune slacks

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Introduction

Basiphilous pioneer vegetation types are very rare in the Netherlands. They mainly occur in wet dune slacks and often contain many Red List species. Many nature conservation programs therefore aim at preserving these early states of succession (Lammerts & Grootjans, 1997). Adema (Adema *et al.*, in prep.) suggests that alternative stable states may exist as a result of the occurrence of a positive feedback mechanism. They found two different states of succession in 'De Buiten Muy' on the Dutch Wadden Island of Texel which have been existing side by side for several decades (Adema *et al.*, in prep.).

Adema developed a mathematical model describing biotic and abiotic conditions of the dune slack system, which potentially limit growth of plants. Several assumptions were made in the model. Three of these assumptions will be discussed here:

-Pioneer species win competition for nitrogen at low concentrations;

-Later species win competition for nitrogen at high (-er) concentrations;

-Species of later stages are sensitive to sulfide.

Nitrogen

Plant growth in most primary dune slacks is limited by nitrogen (Gerlach *et al.*, 1994; Lammerts & Grootjans, 1997). At low nitrogen concentrations the growth of high productive species (species of later successional stages) is limited more than the growth of less productive species (Tilman, 1986; Van der Werf *et al.*, 1993). As a result a critical nitrogen concentration can exist. The pioneer species are more productive than and can compete better with the later species if the nitrogen concentration is below this critical concentration.

In the present study we focus on the question whether pioneer species in dune slacks can produce more biomass at low nitrogen concentrations compared to later successional species.

Sulfide

Sulfide is in some cases even more toxic to plants than it is to animals (De Kok *et al.*, 1998; Mudd, 1979). It can reach plants pedospheric and atmospheric, but in this project only the effects of hydrogen sulfide gas from the air are taken in account. Although hydrogen sulfide gas is usually only present in very low concentrations, it can be very harmful to plants. Typical consequences of hydrogen sulfide are growth reduction and - more severely – wilting of leaves (Mudd, 1979; De Kok *et al.*, 1989).

Atmospheric sulfide enters the plant through the stomata, after which it is metabolized into cysteine and it is used to assemble lipids (e.g. Hell, 1997). Although the exact mechanism of its toxicity is largely unknown still, it is obvious that hydrogen sulfide affects growth by attacking the shoot meristems. In grass-like monocot plant species the leaf shaft often surrounds the meristems of younger leaves, thus protecting the meristem from harmful influences (Stulen *et al.*, in press).

Littorella uniflora is able to adapt its morphology in case of flooding. The plants develop new leaves without stomata, but with aerenchym tissue in order to retain CO_2 for assimilation. If the water table drops, the underwater leaves die off and new leaves are formed again (Weeda *et al.*, 1988). This may not only be a good protection from flooding, but against atmospheric hydrogen sulfide as well.

In an experiment the same four selected species as in the nitrogen experiment were exposed to atmospheric sulfide (H_2S) for several weeks. This experiment was carried out to find out whether the used species from later stages of succession are sensitive to atmospheric sulfide.

For both experiments, the nitrogen experiment and the sulfide experiment, plants of four species were used: *Littorella uniflora* & *Carex nigra* and *Schoenus nigricans* & *Calamagrostis epigejos*. In natural conditions *C. nigra* will be the successor of *L. uniflora* and *C. epigejos* will be the successor of *S. nigricans*. The first two species grow in much wetter (regularly flooded) conditions than the latter.

Materials and methods

Used plants

The plants used in the experiments were grown in the greenhouse. The plants had been growing in the greenhouse for several years; the plants of *C. nigra* originated from the Buiten Muy on the Dutch Wadden Island of Texel; the plants of the other species come from the Dutch Wadden Island of Schiermonnikoog.

Before the start of each experiment the leaves of *C. epigejos* were cut in half to prevent excessive evaporating[1].

Methods used in the nitrogen experiment

Plants of the four species were hydroponically grown on Hoagland-Snyder nutrient solution (as described by Hewitt, 1966), with sand (approximately 1 gram per 30 liter) added as silicium source for *S. nigricans* (Ernst et al., 1995), in four replicates. By diluting the standard Hoagland solution several times four different nitrogen regimes (0.265, 0.200, 0.130 and 0.065 mM) were created in sixteen 30-liter containers[2]. The containers were put in the greenhouse (24/20°C day/night, 70% r.h. and approximately 300µmol m^{-2} s^{-1} photon flux). Air was permanently blown through the solutions to prevent algal growth. The nutrient solutions were refreshed weekly. Twelve plants of each species were grown in each container. All dead plants were replaced two weeks after the start of the experiment.

The dry weight (DW) and lengths of shoots and roots of twelve plants of each species were determined as initial state at the beginning of the experiment. After 60-63 days the experiment was ended and these variables were measured of all plants to calculate the relative growth rates (RGR) and the shoot/root ratios. Sigmaplot for Windows 5.0 was used to determine the cross points of the RGR curves of the species. Shoot/root ratios were determined to show in what part of the plants most of the growth takes place and the ratios were tested for differences between treatments and equality of repeats with One-Way ANOVA with Spss 9.0 for Windows ($\alpha = 0.05$; Zar, 1999).

Methods used in the fumigation experiment

For *L. uniflora* two sets of plants were used in the hydrogen-sulfide fumigation experiment, because it was suspected that plants that were submerged before fumigation were more susceptible to H_2S than plants that were not. These two types will be referred to as `*L. uniflora* aerated´ and `*L. uniflora* flooded´.

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[1] The first time of fumigating all plants of C. epigejos died of drought after being planted in the pots.

[2] Dilutions: 1/56.6 for 0.265 mM, 1/75 for 0.200 mM, 1/115.4 for 0.130 mM and 1/230.8 for 0.065 mM, for exact composition of the solutions is referred to Appendix I.

For the hydrogen fumigation experiment all plants were planted in pots several days before the start of fumigation (ranging from two weeks for *C. epigejos* to three days for the *L. uniflora* aerated). The pots were put in containers with water to keep the sand wet and thus preventing drying out of the plants. The containers were in the greenhouse of the Laboratory of Plant Physiology.

Plants of the four species were planted in dune sand (from the North-Sea beach of Texel) with organic matter (sand:organic-matter ratio 4:1) and a slow-release fertilizer (approximately 0.3 gram 13+13+13 NPK, `Osmocote´, Scotts Heerlen) added as nutrient supply to ascertain a maximum influence of hydrogen-sulfide. The plants were put in three fumigation cabinets (cabinets as described by Maas et al. (1985)) at the beginning of the experiment, all with a constant temperature (20 +/- 1ºC) and 12 hours per day a photon flux of approximately 300 (+/- 30) µmol m^{-2} s^{-1}. Three different hydrogen sulfide concentrations were applied: 0, 200 and 400 ppb (each +/- 10 ppb), in different cabinets. These conditions were checked weekly.

Before the experiment the fresh and dry weights and lengths of shoots and roots of 12 plants per species were measured as initial state. After four weeks (28 days) the dry weights and lengths of roots and shoots of all fumigated plants were measured (n=6). The experiment was carried out in duplo because of the small numbers of plants used each time.

All results were tested for differences between treatments and equality of repeats with One-Way ANOVA with Spss 9.0 for Windows ($\alpha = 0.05$; Zar, 1999).

Results

Nitrogen

The graphs drawn from the calculated Relative Growth Rates based on the dry weights and the lengths of the plants are presented in figures 1 and 2 respectively. The curves represent expected RGR for the species in the 0-265µM nitrogen range, based on

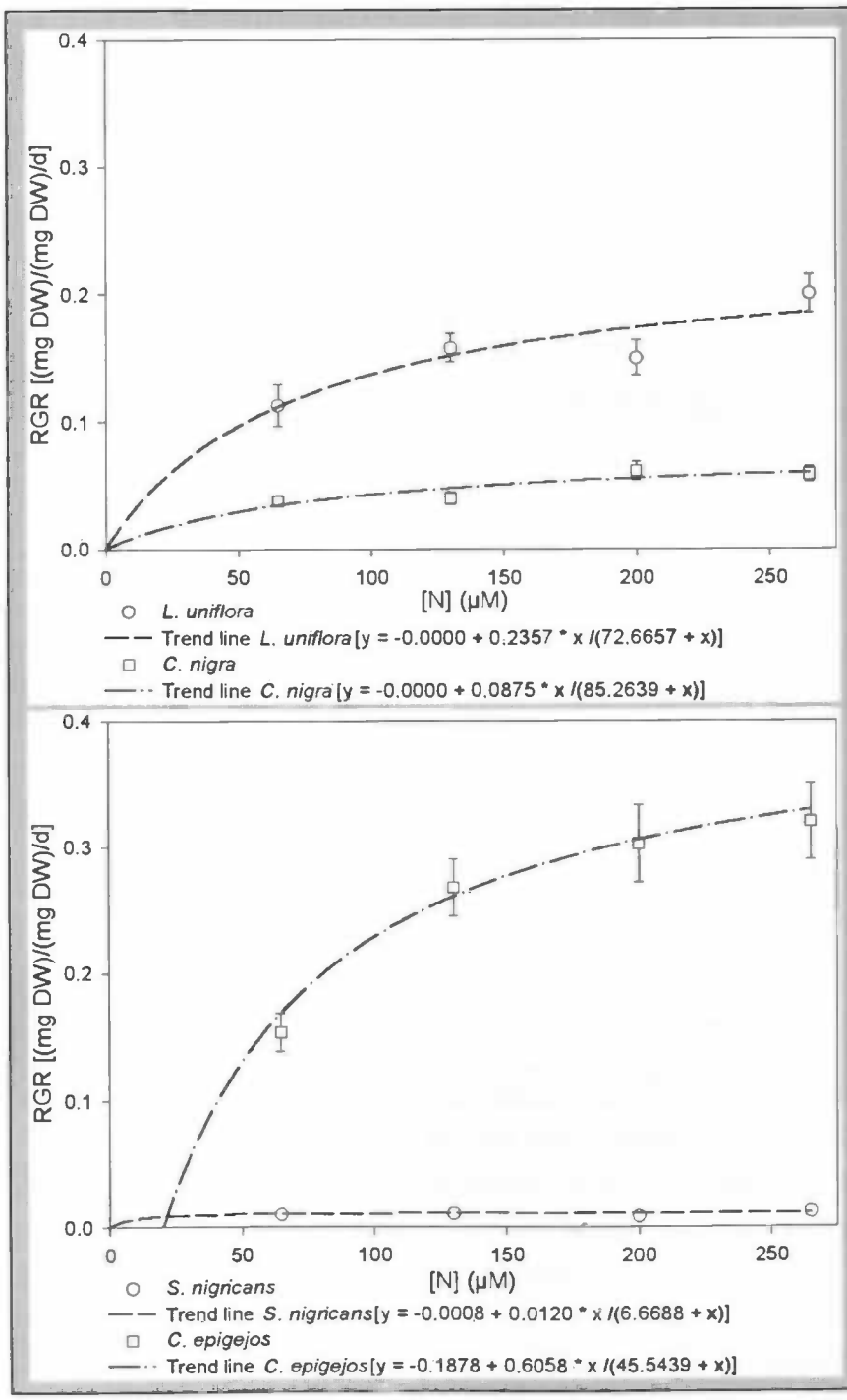

Figure 1. *Relative Growth Rates (RGR) ±S.E. of the four species, grouped in couples. RGR and S.E. are calculated from dry-weight (DW) data and expressed as increase of dry weight per milligram initial dry weight per day in milligrams. The data used to calculate the curves and the nitrogen concentrations at which the curves cross are presented in table 1.*

Michaelis-Menten equations derived from the data. With these equations the cross points of the curves in the two couples are calculated, for both the length and the dry-weight based curves (see also Table 1.).

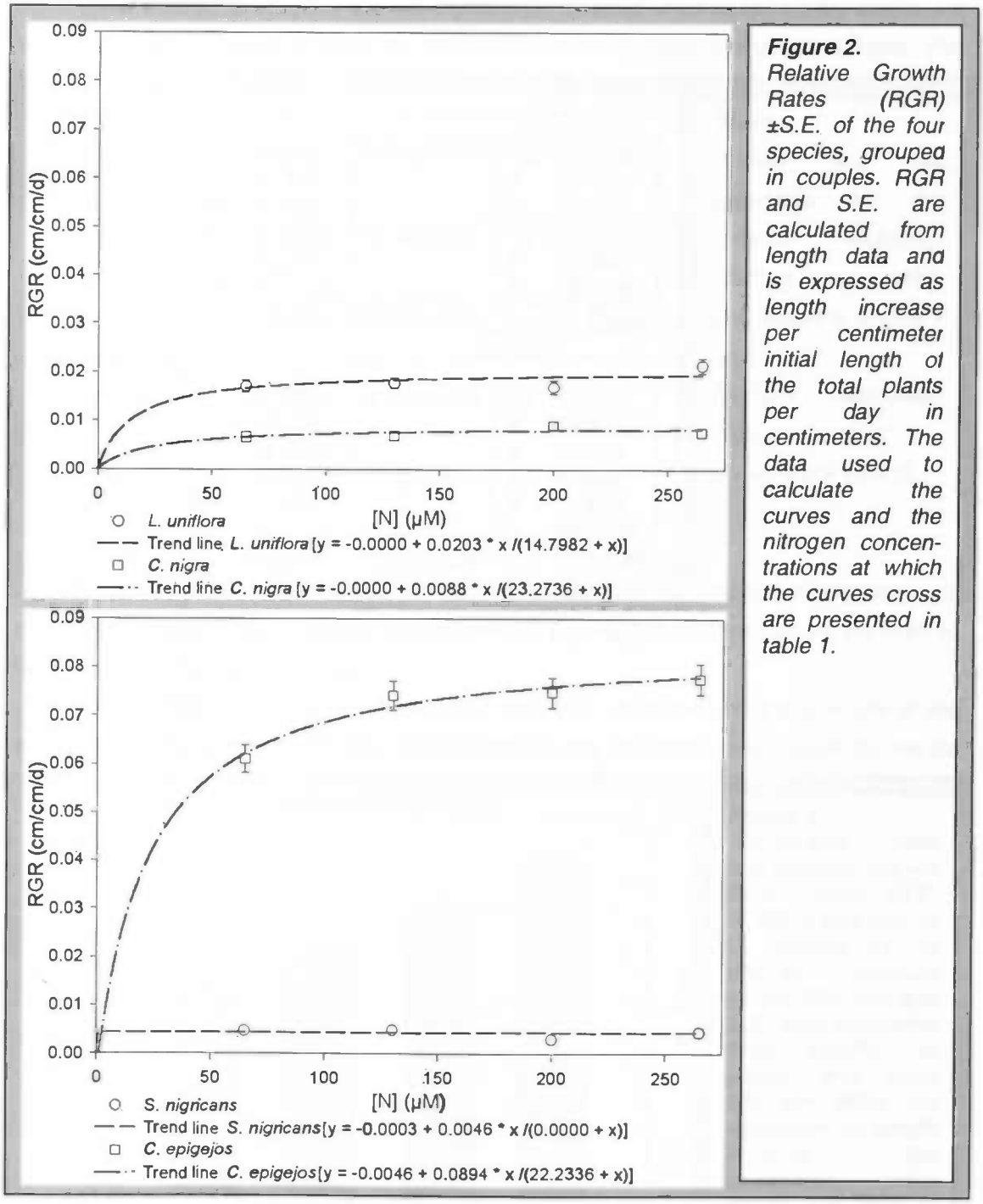

Figure 2. *Relative Growth Rates (RGR) ±S.E. of the four species, grouped in couples. RGR and S.E. are calculated from length data and is expressed as length increase per centimeter initial length of the total plants per day in centimeters. The data used to calculate the curves and the nitrogen concentrations at which the curves cross are presented in table 1.*

The figures show that *L. uniflora* has a higher RGR than *C. nigra* at all applied nitrogen concentrations. *S. nigricans* apparently hardly grows at all and *C. epigejos* has a high RGR; higher than *S. nigricans* already at very low nitrogen concentrations (1.34 or 6.16µM, depending on the data set considered).

The shoot/root ratios of *S. nigricans* and *C. nigra* did not differ significantly for the different nitrogen treatments. *S nigricans* has most of its biomass above ground (S/R: 2.61) and *C. nigra* has approximately as much underground as aboveground (S/R: 0.95). The ratios were neither significantly different when based on lengths.

The ratio for *C. epigejos* was not significantly different when based on dry weight, but there was a significant increase of the ratio based on the lengths of shoots and roots. The

RGR (DW)					
L. uniflora	Y0	-0.0000	*S. nigricans*	Y0	-0.0008
	A	0.2357		A	0.0120
	B	72.6657		B	6.6688
C. nigra	Y0	-0.0000	*C. epigejos*	Y0	-0.1878
	A	0.0875		A	0.6058
	B	85.2639		B	45.5439
Cross point		0.00µM	Cross point		21.81µM

RGR (length)					
L. uniflora	Y0	-0.0000	*S. nigricans*	Y0	-0.0003
	A	0.0203		A	0.0046
	B	14.7982		B	0.0000
C. nigra	Y0	-0.0000	*C. epigejos*	Y0	-0.0046
	A	0.0088		A	0.0894
	B	23.2736		B	22.2336
Cross point		0.00µM	Cross point		13.10µM

Table 1.
Shown are the constants for the different Michaelis-Menten curves which are plotted in figures 1 and 2.
General Michaelis-Menten equation:
$y = y0 + a*x/(b + x)$.

difference is visible in figure 3 as an upward trend in the RGR of the shoot where the RGR of the root does not show such trend.

The length based ratios of shoots and roots in *L. uniflora* differed only in one of the repeats. The dry-weight based ratio differed between the treatments and though the results

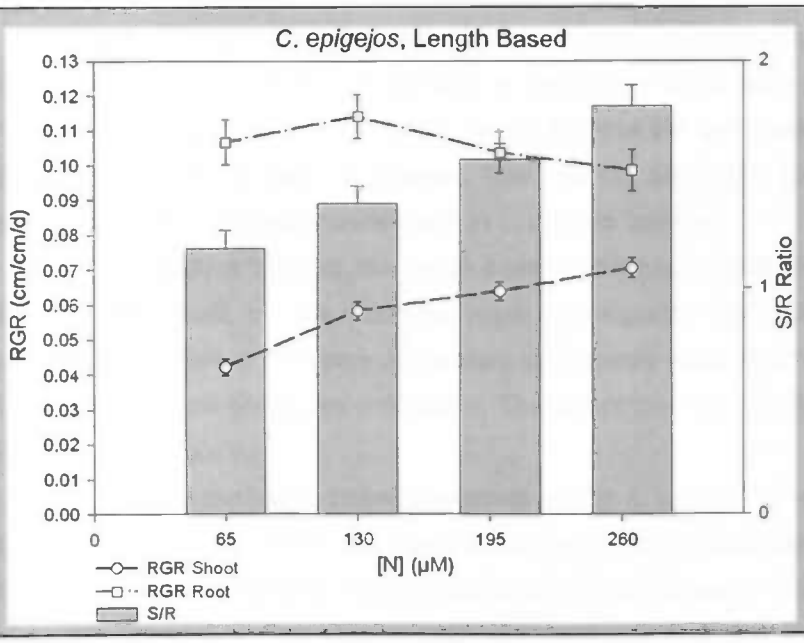

Figure 3.
Shoot/Root ratio and Relative Growth Rates (RGR) ±S.E. of shoot and root of C. epigejos *at the different nitrogen levels. The ratio and S.E. were calculated from lengths of shoots and roots and the RGR are expressed as length increase per centimeter initial length per day in centimeters.*

from the different repeats differed significantly, there was a clear coincidence between increasing nitrogen concentration and the ratio in all repeats. In figure 4 are the averaged dry-weight ratios of *L. uniflora* plotted together with RGR of shoot and root. It can be concluded that the increase of the shoot/root ratio coincides with the increase of RGR of the shoot with increasing nitrogen concentrations.

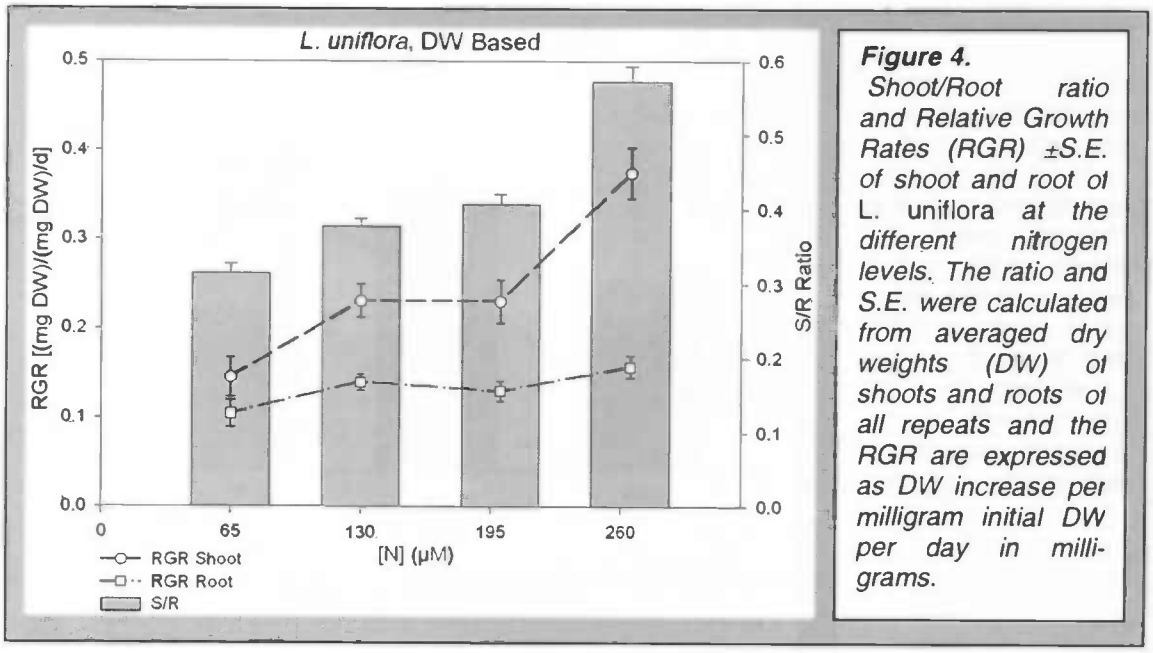

Figure 4. *Shoot/Root ratio and Relative Growth Rates (RGR) ±S.E. of shoot and root of* L. uniflora *at the different nitrogen levels. The ratio and S.E. were calculated from averaged dry weights (DW) of shoots and roots of all repeats and the RGR are expressed as DW increase per milligram initial DW per day in milligrams.*

Sulfide

S. nigricans and *C. epigejos* did not show any significant response to the applied hydrogen sulfide concentration. The dry-weight based shoot/root ratio of *C. nigra* differed significantly after the second experiment, but no up- or downward trend could be recognized. None of the other variables differed significantly in *C. nigra*.

In *L. uniflora* 'aerated' the length based shoot/root ratio differed significantly after the first experiment. The ratio was smaller in the plants when 400ppb hydrogen sulfide was applied. In the same plants the length based RGR of the shoot was also significantly, while the RGR of the roots was not affected. This was not seen after the second experiment. No other significant differences were found in *L. uniflora* 'aerated'.

In *L. uniflora* 'flooded' the length based shoot/root ratio also differed significantly after the first experiment, but the ratio was larger in the plants if 400ppb had been present. The length based RGR of the roots decreased significantly when hydrogen sulfide was applied, but the RGR of the shoot was unaffected. The differences in *L. uniflora* 'aerated' and 'flooded' are shown in figure 5.

Although the length based shoot/root ratio in *L. uniflora* 'aerated' showed a downward trend in the first experiment, the trend in the second experiment was upward, as is shown in figure 6. *L. uniflora* 'flooded' showed the same contrast between the trends in shoot/root ratio in both experiments (not shown), although only the first repeat there was a significant decrease of RGR.

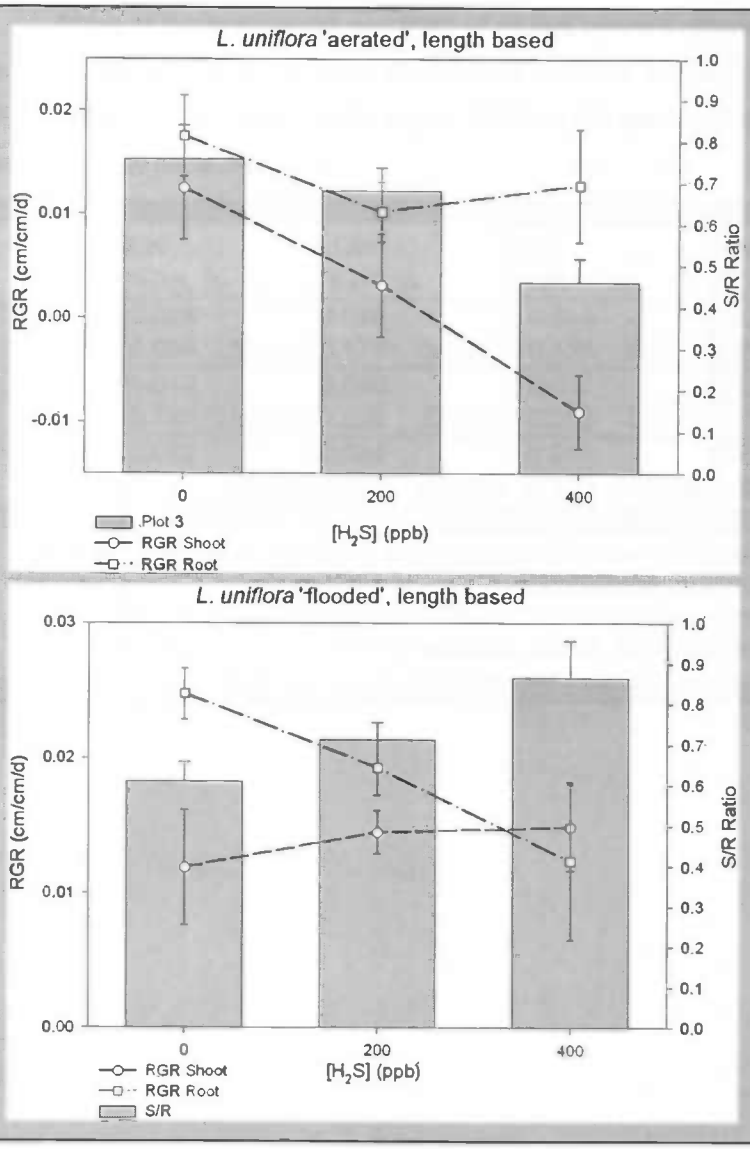

Figure 5.
Length based Shoot/root Ratios and RGR of shoots and roots (all ±S.E.) of L. uniflora *'aerated' and* L. uniflora *'flooded'. Ratios differ significantly within species. RGR of shoot in* L. uniflora *'aerated' and RGR of roots in* L. uniflora *'flooded' are significantly different if hydrogen sulfide was applied.*

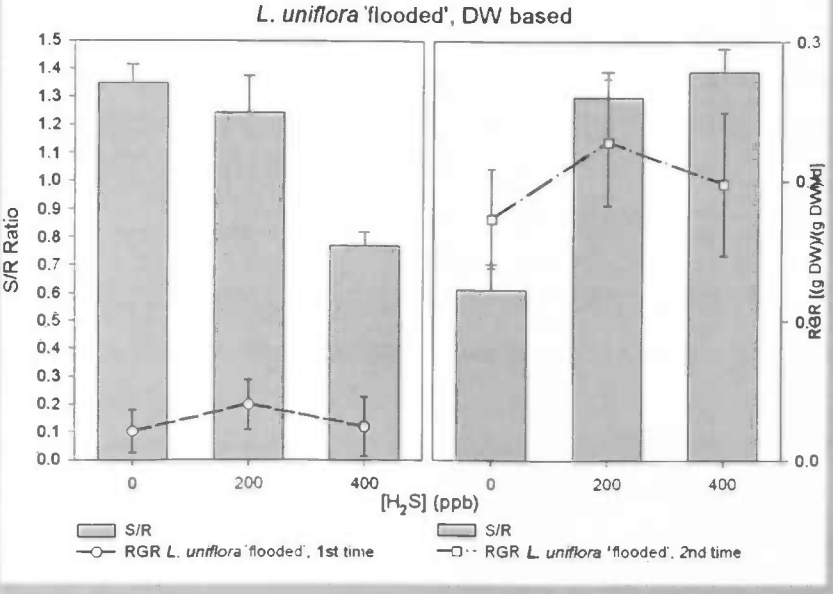

Figure 6.
Dry-weight based Shoot/Root ratios and RGR of total plants (both ±S.E.) of L. uniflora *'aerated' after the first and the second experiment.*
Differences in ratios are significant within and between experiments; RGR do not differ significantly between repeats.

Many of the considered variables of all five species differed significantly between the two experiments. This is in figure 5 shown (for L. uniflora 'flooded' only) and table 2. The most striking difference, the significantly higher RGR of the second experiments found back in all species, except for *S. nigricans*.

[H_2S]		CN RGR	%	LUW RGR	%	LUA RGR	%	SN RGR	%	CE RGR	%
0ppb	1	0.028		0.020		0.015		0.028		0.032	
	2	0.084	300.97	0.173	849.59	0.135	903.06	0.022	77.77	0.095	294.87
200ppb	1	0.012		0.040		0.017		0.021		0.024	
	2	0.185	1519.28	0.228	574.53	0.068	395.57	0.020	95.75	0.066	280.25
400ppb	1	0.032		0.024		-0.011		0.032		0.027	
	2	0.130	408.09	0.198	828.03	0.124	-1109.24	0.029	89.78	0.074	276.73

Table 2. *Differences between experiments: Dry-weight based RGR of total plants of all species at different hydrogen sulfide concentrations and the differences between the experiments expressed as a percentage of the first experiment. CN*=C. nigra, *LUW*=L. uniflora *'flooded', LUA*=L. uniflora *'aerated', SN*=S. nigricans *and CE*=C. epigejos.

Discussion

Nitrogen

For the couple of *S. nigricans* and *C. epigejos* it is likely that a nitrogen concentration exists below which the pioneer species will grow faster than the later species. This coincides with the growth responses found in earlier research (Tilman, 1986; Lammerts & Grootjans, 1997; Veenstra, 1999). This concentration is very low however, and it was only calculated from results in this experiment because the nitrogen concentrations used were too high. The cross point in the length data may be an error due to the calculation used; maybe the cross point does not exist for length growth.

For the other couple (*C. nigra* and *L. uniflora*) a critical nitrogen concentration apparently does not exist within the range of concentrations used in this experiment and in the experiment by Veenstra (1999). Veenstra concluded that he did something wrong, but after transformation of his data into the same measures as used here (RGR) it became evident that the concentration does exist, but out of the nitrogen range in his experiment (8.6mM, see Appendix II).

From the shoot length data it is clear that *C. epigejos* invests more in the length of the shoot if there is plenty of nitrogen, which could be a strategy to take away light from competitors and to raise the organic-matter concentration in the soil to his own advantage.

L. uniflora also invests more in its shoot at maximum nitrogen concentrations. *Littorella* is capable of radial oxygen leaking in the rooting zone to protect themselves from the toxic abiotic conditions in the field and it is expected that this also enhances nitrogen loss from the soil (Adema *et al.*, in prep.). This could be a good investment in a higher photosynthesis rate.

Sulfide

C. nigra and *C. epigejos* are species from later stages of succession. Neither of these species has reduced growth rates for shoot, root or total plant when atmospheric hydrogen sulfide was applied in potentially very toxic concentrations. *S. nigricans* does not show any reduced growth if fumigated with the gas either. Possibly the anatomy of these monocots is a good protection against the harmful gas (Stulen *et al.*, in press).

Of the species investigated here only *L. uniflora* shows reduced growth if exposed to atmospheric hydrogen sulfide. In *L. uniflora* 'aerated' the length based RGR of the shoot was reduced during the first experiment. The length based RGR of the roots was reduced simultaneously in *L. uniflora* 'flooded' as result of the fumigation with hydrogen sulfide. As a consequence, the shoot/root ratios also changed.

In general there does not seem to be a real difference between the two types of *L. uniflora*; the differences were not observed in both experiments. The second time the 'flooded' type had not been flooded for about a month. Maybe that was long enough to adapt to the non-flooded conditions, but then the plants of both types should show the same response to

the sulfide. Both types did not show any response to the fumigating in the second experiment, but this is not convincing since there were large difference between the experiments and repeats in almost all species.

For the differences between the experiments several possible explanations can be given. First of all, the increase of the RGR happened while the plants used in the second experiment were accommodating in the warm, light and humid greenhouse of the Laboratory of Plant Physiology after they were planted in the pots before the experiment. Before, the plants were grown in the colder and less (not artificially) lighted greenhouse of the Laboratory of Plant Ecology. The greenhouse of the Laboratory of Plant Physiology was also warmer and more humid than the fumigation cabinets. The influence of the beginning of the growing season (April, Spring) is also a plausible explanation. For *L. uniflora* 'flooded' the difference may be caused by the adaptation to the non-flooded condition, the construction of new leaves.

To eliminate these seasonal and pre-treatment effects it might a good option to repeat the experiment when plants are near the optimum of the growing season and perhaps fumigate the plants longer than 28 days in order to observe better responses.

For both experiments the use of seedlings in stead of the full-grown plants would have been more elegant because differences would be more obvious and the results of the different species would have been easier to compare. It was not done here, because we did not know what triggers the germination process for all species and there were not enough seedlings for the experiment. Furthermore it was expected that *L. uniflora* would grow too slowly.

The measure 'length', basis for RGR, was not as useful as the measure 'dry weight' when describing growth of full-grown plants. Growth comes to expression in an increasing number of shoots or roots when at full length. This is reflected in a simultaneous increase of dry weight. Length may be a more useful measure when seedlings are used.

As assumed by Adema, pioneer species win competition for nitrogen at low nitrogen concentrations and the later successional species win competition at high (-er) concentrations, although there are large differences between species when defining low and high (-er) concentrations. However, the sensitivity of later species to sulfide needs still to be confirmed.

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Appendix I

Composition of the nutrient solutions applied in the nitrogen experiment

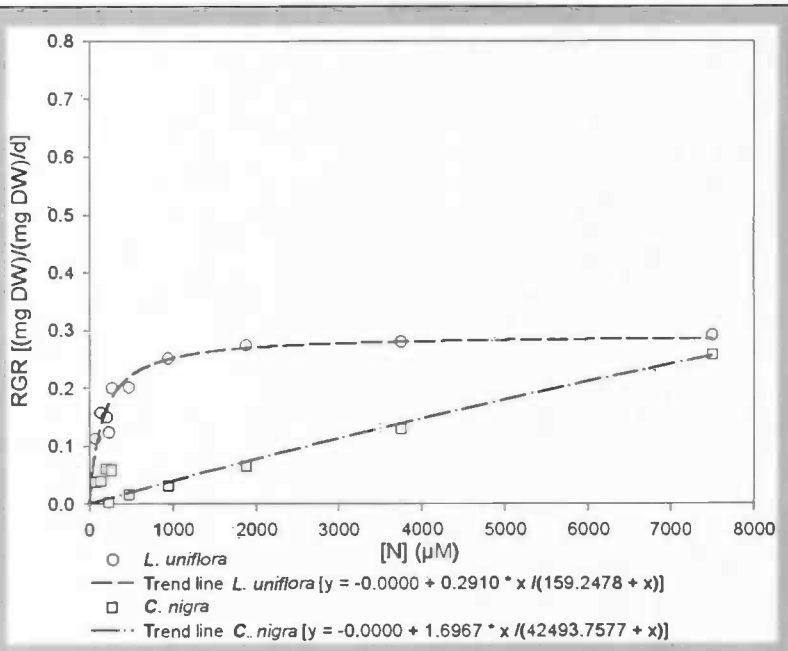
Added per 30 liter, in ml

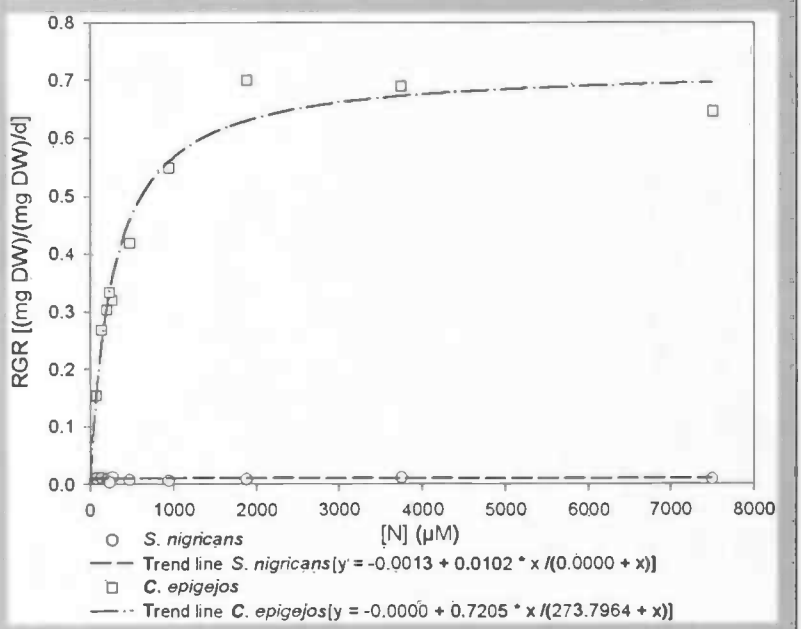
[N]	0,265mM	0,200 mM	0,130 mM	0,065 mM
KNO_3	5.3	4.0	2.6	1.3
$Ca(NO_3)_2$	5.3	4.0	2.6	1.3
$MgSO_4$	6.0	4.0	3.0	1.5
KH_2PO_4	6.0	4.0	3.0	1.5
Micro nutrients	0.6	0.4	0.3	0.2
Fe	0.06	0.04	0.03	0.02
$NaHCO_3$	125.00	125.00	125.00	125.00
SiO_2	+/- 1 g	+/- 1 g	+/- 1 g	+/- 1 g

Micro nutrients: Ba, Mn, Zn, Cu, Mo.
Fe added as 5% Ferro-rexonol.
0.72M $NaHCO_3$ added to keep pH +/- 7.
SiO_2 added as sand; S. nigricans uses it for the production of its fruits (Ernst *et al.*, 1995).

Appendix II

RGR of total plants – data of Rutger Veenstra and René Eschen





[N] (µM)	LU	CN	SN	CE
65	0.113	0.037	0.010	0.154
130	0.158	0.040	0.011	0.268
200	0.150	0.061	0.009	0.303
265	0.200	0.058	0.012	0.320
230	0.124	0.003	0.004	0.334
470	0.201	0.016	0.008	0.419
940	0.251	0.032	0.006	0.548
1880	0.274	0.065	0.009	0.701
3750	0.280	0.129	0.012	0.689
7500	0.292	0.258	0.009	0.646

Figure.
*RGR of total plants. In the table are the data from which the plots were drawn and the equations derived. LU=*L. uniflora, CN=C. nigra, *SN=*S. nigricans *and CE=*C. epigejos. *Presented data are calculated from data from both the experiment by Rutger Veenstra (Veenstra, 1999) and this project. Plots are drawn from averaged values from all repeats of each nitrogen concentration.*
Nitrogen concentration at which the curves of L. uniflora *and* C. nigra *will cross: 8604.6 µM.*