Effects of nitrogen supply on the anatomy and chemical composition of leaves of four grass species belonging to the genus Poa, as determined by image-processing analysis and pyrolysis–mass spectrometry

J. J. C. M. Van Arendonk, 1 G. J. Niemann, 1 J. J. Boon 2 & H. Lambers 1

1 Department of Plant Ecology and Evolutionary Biology, University of Utrecht, P.O. Box 800-84, 3508 TB Utrecht, and 2 FOM Institute for Atomic and Molecular Physics, Kruislaan 407, 1098 SJ Amsterdam, The Netherlands

ABSTRACT

Previous experiments have shown that the anatomy and chemical composition of leaves of inherently fast- and slow-growing grass species, grown at non-limiting nitrogen supply, differ systematically. The present experiment was carried out to investigate whether these differences persist when the plants are grown at an intermediate or a very low nitrogen supply. To this end, the inherently fast-growing Poa annua L. and Poa trivialis L., and the inherently slow-growing Poa compressa L. and Poa pratensis (L.) Schreb. were grown hydroponically at three levels of nitrate supply: at optimum (RGRmax) and at relative addition rates of 100 and 50 mmol N (mol N)−1 d−1 (RAR100 and RAR50), respectively.

As expected, at the lowest N supply, the potentially fast-growing species grew at the same rate as the inherently slow-growing ones. Similarly, the differences in leaf area ratio (LAR, leaf area:total dry mass), specific leaf area (SLA, leaf area:leaf dry mass) and leaf mass ratio (LMR, leaf dry mass:total dry mass) disappeared. Under optimal conditions, the fast-growing species differed from the slow-growing ones in that they had a higher N concentration. There were no significant differences in C concentration. With decreasing N supply, the total N concentration decreased and the differences between the species disappeared. The total C concentration increased for the fast-growing species and decreased for the slow-growing ones, i.e. the small, but insignificant, difference in C concentration between the species at RGRmax increased with decreasing N supply.

The chemical composition of the leaves at low N supply, analysed in more detail by pyrolysis–mass spectrometry, showed an increase in the relative amounts of guaiacyl lignin, cellulose and hemicellulose, whereas those of syringyl lignin and protein decreased.

The anatomy and morphology of the leaves of the four grass species differing in RGRmax were analysed by image-processing analysis. The proportion of the total volume occupied by mesophyll plus intercellular spaces and epidermis did not correlate with the amount of leaf mass per unit leaf area (specific leaf mass, SLM) at different N supply. The higher SLM at low N supply was caused partly by a high proportion of non-veinal sclerenchymatic cells per cross-section and partly by the smaller volume of epidermal cells.

We conclude that the decrease in relative growth rate (and increase in SLM) at decreasing N supply is partly due to chemical and anatomical changes. The differences between the fast- and slow-growing grass species at an optimum nutrient supply diminished when plants were growing at a limiting nitrogen supply.

Key-words: epidermal cells; Poa species; pyrolysis–mass spectrometry; relative growth rate; sclerenchymatic cells; specific leaf mass.

Abbreviations: CI, chemical ionization; DA, discriminant analysis; DF, discriminant function; EI, electron impact ionization; LAR, leaf area ratio [m² (kg plant)−1]; MVA, multivariate analysis; m/z, mass/charge, atomic mass unit; NAR, net assimilation rate (g plant m⁻² d⁻¹); PC(A), principal component (analysis); PyGCMS, pyrolysis–gas chromatography–mass spectrometry; PyMS, pyrolysis–mass spectrometry; RAR, relative addition rate [mg plant (g plant)⁻¹ d⁻¹]; RMR, root mass ratio [g root (g plant)−1]; RGR, relative growth rate [mg plant (g plant)⁻¹ d⁻¹]; SLA, specific leaf area [m² (kg leaves)⁻¹]; SLM, specific leaf mass (kg leaves m⁻²); SMR, stem mass ratio [g stem (g plant)⁻¹].

INTRODUCTION

Plants vary widely in the growth rate that they achieve under conditions where they have free access to nutrients (RGRmax), and this variation is closely correlated with their ecological distribution. That is, fast-growing species tend to occur in productive environments, whereas slow-growing species are found in nutrient-poor or otherwise unfavourable habitats (Grime & Hunt 1975; Chapin 1980;
Lambers & Poorter 1992; Atkin, Botman & Lambers 1996). In a comparison of fast- and slow-growing grass species, the variation in \( R_{GR_{max}} \) is largely accounted for by variation in specific leaf area (SLA, leaf area:leaf dry mass; Poorter & Remkes 1990; Garnier 1992; Van der Werf, Poorter & Lambers 1994). The low SLA of slow-growing species is associated with more non-veinal sclerenchymatous cells, relatively small epidermal cells (Van Arendonk & Poorter 1994), a low leaf water content and a high leaf mass density (Garnier & Laurent 1994; Ryser & Lambers 1995). Chemically, low SLA is associated with a comparatively high ratio of cell wall components to cytoplasmic compounds (Niemann et al. 1992; Van Arendonk & Poorter 1994), which might be the cause of a higher leaf mass density.

When plants are grown at a limiting supply of nitrogen, their relative growth rate (RGR) decreases, largely due to a decrease in leaf area ratio (LAR, leaf area:plant dry mass; Van der Werf, Schieving & Lambers 1993b). The decrease in LAR is associated with a lower leaf mass ratio (LMR, leaf dry mass:plant dry mass), and sometimes (Hirose, Freijsen & Lambers 1988), but not invariably (Van der Werf et al. 1993b), also a decrease in SLA.

As a continuation of our earlier work with leaves of plants grown under conditions of free access to nutrients (Van Arendonk & Poorter 1994), we compared fast- and slow-growing grass species of one genus (Poa), grown at both an optimum and a limiting nitrogen supply. To facilitate comparison of fast- and slow-growing species at optimum and limiting nitrogen supply, we grew our plants under steady-state conditions, adding nitrate in an exponential way (Van der Werf et al. 1993b). Here we report on the effect of a limiting nitrogen supply on various growth parameters: net assimilation rate (NAR, the rate of increase in plant dry mass per unit leaf area), leaf area ratio (LAR), specific leaf area (SLA, leaf area:leaf dry mass), or its inverse, the specific leaf mass (SLM, leaf dry mass:leaf area), and allocation of biomass.

Since the growth experiments show that SLA decreases with decreasing nitrogen supply, the anatomical (image-processing analysis) and chemical (pyrolysis–mass spectrometry) bases of the decrease in SLA were subsequently analysed. These techniques are rapid and require little pre-treatment of the material to be investigated. Furthermore, analytical pyrolysis is much more informative than ‘wet’ chemical analysis, because it is a multicomponent analytical technique.

### MATERIALS AND METHODS

#### Growth of plants

Four grass species common in Western Europe, Poa annua L., Poa pratensis (L.) Schreb, Poa trivialis L. and Poa compressa L., were grown from seeds commercially obtained from Kieft B.V. (Blokker, the Netherlands).

Table 1 lists these species [nomenclature according to Hubbard (1968) and Van der Meijden et al. (1987)], together with their main habitats.

After germination, seedlings were planted in trays filled with sand and supplied with half-strength modified Hoagland solution: 795 mmol m\(^{-3}\) KNO\(_3\), 603 mmol m\(^{-3}\) Ca(NO\(_3\))\(_2\), 270 mmol m\(^{-3}\) MgSO\(_4\), 190 mmol m\(^{-3}\) KH\(_2\)PO\(_4\), 41 mmol m\(^{-3}\) Fe-EDTA, 20 mmol m\(^{-3}\) H\(_3\)BO\(_3\), 2 mmol m\(^{-3}\) MnSO\(_4\), 0.85 mmol m\(^{-3}\) ZnSO\(_4\), 0.25 mmol m\(^{-3}\) Na\(_2\)MoO\(_4\) and 0.15 mmol m\(^{-3}\) CuSO\(_4\) (Poorter & Remkes 1990). The seedlings were placed in a growth room with the following conditions: a day length of 14 h; photosynthetic photon flux of 315 ± 30 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), provided by fluorescent tubes (Philips TL-33-RS, 215 W, Eindhoven, the Netherlands) and incandescent bulbs (Philips, 40 W, Eindhoven, the Netherlands) in a ratio of 4:1; temperature 20 ± 1 °C, relative humidity 70%. When the root length of the seedlings was >50 mm, the seedlings were transferred to 33 dm\(^3\) containers with aerated nutrient solution. During the first 3 d, the nutrient solution was half strength, and thereafter full strength. The pH of the solution was regularly adjusted to 5.8 using H\(_2\)SO\(_4\). To prevent nutrient depletion the solution was renewed once a week.

To minimize mutual shading, the number of plants on each container varied, depending on the size of the plants. Plants were rotated in the growth room twice a week.

The growth experiment started when the plants had a fresh mass of >100 mg (day 0). To determine the maximum relative growth rate (RGR\(_{max}\)), eight plants were harvested on days 3, 7, 10 and 14, and 16 plants on days 0 and 17. The RGR was calculated, according to Poorter (1989a), for the time interval when the plants had attained a total dry mass of 30–100 mg.

To obtain plants with a constant RGR whose growth is nitrogen-limited, we used the technique of the relative addition rate (RAR) as described by Ingestad (1981), with the exception that we only limited the nitrate supply, while the other conditions for growth were as described above. After being allowed to reach a new steady state, plants were harvested at regular intervals, freeze-dried and used

### Table 1. Values of the relative growth rate (RGR: mg g\(^{-1}\) d\(^{-1}\)) and the specific leaf area (SLA: m\(^2\) kg\(^{-1}\)) for the four grass species. Plants were grown in nutrient solution with a nitrate concentration of 2 mol m\(^{-3}\). Life form and habitat are as described in Hubbard (1968)

<table>
<thead>
<tr>
<th>Species</th>
<th>Habitat</th>
<th>RGR</th>
<th>SLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poa compressa L.</td>
<td>poor thin grassland, dry banks, waste ground, perennial</td>
<td>194</td>
<td>36.6</td>
</tr>
<tr>
<td>Poa pratensis (L.) Schreb.</td>
<td>well-drained sandy, gravelly, loamy soils, perennial</td>
<td>185</td>
<td>40.0</td>
</tr>
<tr>
<td>Poa trivialis L.</td>
<td>common in meadows and pastures of lowlands, perennial</td>
<td>231</td>
<td>44.2</td>
</tr>
<tr>
<td>Poa annua L.</td>
<td>disturbed and trampled places, annual</td>
<td>248</td>
<td>55.3</td>
</tr>
</tbody>
</table>
for chemical analyses. The RGR under N-limited conditions was also calculated according to Poorter (1989a).

Under steady-state conditions, the relative nitrate addition rate (RAR; mmol N added per mol plant N per day) equals the RGR. Relative addition rates of 50 and 100 mmol N mol⁻¹ plant N d⁻¹ were applied to each of the four Poa species.

Chemical analyses

For the chemical analyses the plants were harvested when they had reached a dry mass of ≈450 mg.

The total C and N concentrations of the freeze-dried leaf samples were determined with a CHN analyser (Carlo Erba, model 1106, Milano, Italy).

Pyrolysis–mass spectrometry (PyMS) was performed on a JEOL SX 102 A double-focusing spectrometer (B/E, Tokyo, Japan) equipped with a platinum-rhodium filament in-source pyrolysis probe (Pt/Ph wire of 0·1 mm diameter, 10% Rh). Aliquots of 1–2·5 mm³ of a suspension of 0·5 mg dry homogenized leaf material per cm² of demineralized water were loaded on the filament tip and dried in vacuo before the PyMS analysis. Three leaf samples from each plant were analysed to enable multivariate analysis of the data.

The pyrolysis conditions after insertion of the probe into the ion source were as follows: the source temperature was 180 °C (10⁻⁴ Pa), and the heating rate of the wire was 16 °C s⁻¹ up to a final temperature of 800 °C. The equilibration time before pyrolysis was 10 s for standardization of the procedure.

The mass spectrometric conditions were as follows: the pyrolysis and evaporation products were electron-ionized (EI; ion voltage 16 eV, in order to minimize fragmentation during the ionization), accelerated to 3 kV, mass-analysed over a range of 20–750 Da with a scan cycle of 1 s, and post-accelerated to 10 kV. Chemical ionization was performed at an ammonia pressure of 20 Pa (NH₃-Cl; ion voltage 250 eV, ammonia as the reaction gas to maximize the formation of pseudo-molecular ions) and ions were accelerated to 3 kV, mass-analysed over a range of 60–1000 Da, and post-accelerated to 5 kV. A complete description of pyrolysis–mass spectrometry methods is given in Boon (1989, 1992), Van der Hage, Mulder & Boon (1993) and Van Arendonk, Niemann & Boon (1997); we restrict ourselves therefore to a short introduction.

Pyrolysis–mass spectrometry

PyMS has increasingly been used in the analysis of macro-molecular systems such as plant and animal cells (Boon 1989, 1992; Lapierre 1993). This is not surprising as the method has several advantages over a number of chemical techniques normally applied in plant analysis. Analytical pyrolysis is rapid, sensitive and more informative because it is a multicomponent analytical technique which requires little pre-treatment of the material to be investigated. Analytical pyrolysis involves first polymer breakdown into lower molecular weight components and secondly the characterization of these dissociation products. Common techniques are pyrolysis–mass spectrometry (PyMS) and pyrolysis–gas chromatography–mass spectrometry (PyGCMS), whereby the pyrolysis device is directly coupled on line with the analytical device. For the ionization, which is necessary for mass determination in PyMS methods, mainly soft ionization (low voltage EI, chemical ionization and field ionization) is used to avoid further fragmentation of the molecular ions obtained by the pyrolysis step.

Pyrolysis-mass spectra of plant material contain the summed masses of ionized fragments and molecules originating from desorption of lower molecular weight components and from pyrolysis of the different biopolymers. Single masses may represent several structurally different fragments which also may differ in origin. Phenol (EI, m/2 ṽ 94), for instance, may be derived either from lignin or from protein; EI m/z 180 represents at least six different fragments, all identified by GC-MS, from several (fractions) of plant species (Pouwels et al. 1987; Van Smeerdijk & Boon 1987; Van Boon 1989; Hempfling & Schulten 1990; Niemann, Baayen & Boon 1990b; Van der Hage et al. 1993), most of which, however, are derived from guaiacyl lignin, and thus m/z 180 can still be used as a marker for this polymer.

Biomacromolecular systems such as bacterial cells and plant cells have been the subject of many Py(GC)MS studies in which the fragments pyrolytically released have been identified and classified. These studies have shown that, despite the presence of nominally isobaric ions, many fragments are, as such or in combination, still specific for certain polymers or molecules.

PyMS fragments were for a long time mainly been used as qualitative and semiquantitative markers. Recently, however, quantitative determinations based on relative intensities of ions in PyMS (Niemann et al. 1991, 1995; Sorge, Schnitzer & Schulten 1993; Agblevor, Evans & Johnson 1994; Van Arendonk et al. 1997), or peak areas in PyGCMS (Kleen, Lindblad & Backa 1993; Mellon et al. 1994; Kleen & Gellerstedt 1995), were shown to correlate well with determinations using conventional wet chemical methods. Quantification can be rather difficult when large variations in sodium and/or potassium concentration are present (Van der Kaaden et al. 1983; Boon 1992; Kleen & Gellerstedt 1995).

Direct interpretation of results is usually difficult and often also requires multivariate chemometric procedures (Boon et al. 1984; Kleen et al. 1993; Valcarce et al. 1993) such as used in the present work.

Leaf anatomy

Leaf sections of P. annua, P. pratensis, P. trivialis and P. compressa were taken from the middle part of the youngest fully grown leaves, which had been stored in FPA-fixative (formalin, propionic acid, ethanol and demineralized water in a ratio of 1:0·1:0·12:6·5:4 (v/v/v/v/v)).

The leaf sections were embedded in historesin (LKB Bromma, Sweden), and 5 mmol m⁻³ slices were cut by microtome and coloured with safranine, which stains
phenolics red, and astro-blue, which stains cellulose blue. We assume that the distortion and collapse of the epidermal cells of the four grass species were equal for all species. Light microscopy and image analyses by an image-processing system (Interactiv Bild Analyse System, I.B.A.S.-2000, Germany) were used to quantify the anatomical differences in the leaf cross-sections. The total area of each cross-section was determined, as well as the area occupied by epidermis, veins (including the sclerenchyma around them), non-veinal sclerenchyma and intercellular spaces. The area of the mesophyll was then determined by subtraction of the areas from the total area. The width of the sections was measured through the middle of all veins. All measurements were made in triplicate.

**Statistical and multivariate analysis**

The correlations between the various parameters and the RGR and SLM were tested with a linear regression analysis. Terms were considered significant at the 5% level

Principal-component (PC) and discriminant analyses were performed on the PyMS data files, using a modified ARTHUR package, adapted to PyMS data (Boon et al. 1984). In this method, spectra are considered to be points in a multidimensional space with the mass numbers as coordinate axes. The relative distribution of mass intensities in each spectrum determines the position in multidimensional space. Similar spectra cluster together. From the file of selected spectra an overall average spectrum (zero point) was calculated which served as a reference point for the individual spectra. Mathematically, the differences between the individual spectra are determined by comparison with the zero point spectrum. These data are factor-analysed to produce sets of correlated mass peaks (factors), which can be represented by reconstructed mass spectra of principal components. When multiple analyses are available, discriminant analyses can be performed. The covariant mass peaks are linearly combined to new independent variables (discriminant functions), which are represented graphically by reconstructed mass spectra. Dissimilarity is quantitatively expressed in discriminant function scores. For multivariate analyses, average PyMS spectra were used, which were summarized over the total pyrolysis time. In general, multiple analyses were carried out, which allowed the performance of discriminant analysis. The geometric distance between the sample coordinates for each leaf analysis is a measure of the analytical reproducibility. When only one mass spectrum was available, data were analysed by principal-component analysis, instead of discriminant analysis.

For all other statistical analyses, the SAS statistical package was used (SAS Institute Inc., Cary).

**RESULTS**

**Growth with free access to nitrate**

The grass species differed significantly in their maximum relative growth rate (RGR) when grown with free access to nutrients, ranging from 184 mg g$^{-1}$ d$^{-1}$ for the slowest-growing *P. pratensis* to 248 mg g$^{-1}$ d$^{-1}$ for the fastest-growing *P. annua* (Fig. 1a). The RGR can be factorized into a ‘morphological’ component, the leaf area ratio (LAR, m$^2$ kg$^{-1}$), and a ‘physiological’ component, the net assimilation rate (NAR, g m$^{-2}$ d$^{-1}$): RGR = LAR * NAR (West, Briggs & Kidd 1920). Despite differences in NAR between the four species, RGR was not correlated with NAR, but it was significantly correlated with LAR (Figs 1b & c). The LAR can be factorized into a ‘morphological’ component (SLA, m$^2$ kg$^{-1}$) and a leaf ‘allocation’ component (LMR, g g$^{-1}$): LAR = SLA * LMR (West et al. 1920). The higher LAR of the faster growing grasses was entirely due to their higher SLA, whereas LMR tended to be somewhat higher for the slower growing species (Figs 2a & b). No correlation was found between RGR and root mass ratio (RMR, root dry mass:plant dry mass) or stem mass

![Figure 1](image-url)
Effects of N supply on Poa leaves

The measured RGR values at relative nitrate addition rates (RAR) of 100 and 50 mmol mol⁻¹ d⁻¹ corresponded very well with the expected ones (Fig. 1a). The decrease in RGR was largely accounted for by the decrease in LAR, whereas NAR changed rather erratically, without correlation with RGR (Figs 1b & c). Comparing treatments, both SLA and LMR decreased with decreasing RAR. Comparing the SLA within a treatment, the differences between the fast- and slow-growing species more or less persisted, until RAR₅₀ was achieved (Figs 2a & b). RMR increased significantly with decreasing RAR, whereas there was no clear trend for SMR (Figs 3a & b).

Anatomy

The differences in SLA, or rather its inverse, the specific leaf mass (SLM, leaf mass: leaf area), amongst species and treatments inspired us to investigate further the anatomical structure of the different leaves. Examples of cross-sections are given in Fig. 4, for the fast-growing P. pratensis at the maximum growth rate (RGR₉₀), the intermediate growth rate (RAR₁₀₀) and the lowest growth rate (RAR₅₀).

Figure 5 shows the proportion of the area of the leaf cross-section occupied by various cell types. Comparing the four species at the three rates of N supply, there was no clear correlation between SLM and the relative areas occupied by mesophyll cells plus intercellular spaces (Fig. 5a) [we pooled mesophyll cells and intercellular spaces, because it was virtually impossible to separate them with the I.B.A.S.-technique, and since Van Arendonk & Poorter (1994) found no significant correlation of these separate parameters with the SLM], veins (Fig. 5b) or epidermal cells (Fig. 5d). However, the total area occupied by non-veinal sclerenchymatic cells did show a positive correlation with the SLM (Fig. 5c; $P < 0.001$, $r^2 = 0.56$). This positive correlation was due to variation in the number of sclerenchymatic cells per unit area (Fig. 6a; $P < 0.001$, $r^2 = 0.61$). The average size of the sclerenchymatic cells did not vary systematically (Fig. 6c). This contrasts with the data on the epidermics, which show that the number of epidermal cells per section correlated positively with SLM (Fig. 6b; $P < 0.001$, $r^2 = 0.56$), whereas the average area of a single epidermal cell showed a negative correlation (Fig. 6d; $P < 0.01$, $r^2 = 0.52$). The size of the mesophyll cells decreased but the number stayed the same at decreasing SLM (data not shown).

Morphology

The thickness (Fig. 7a; $P < 0.05$, $r^2 = 0.38$) and width (Fig. 7b; $P < 0.05$, $r^2 = 0.38$) of the leaves showed a negative correlation with the SLM, in agreement with data from the literature (Poorter et al. 1995; Ryser & Lambers 1995).

Chemical composition (analysed by elemental analyser and pyrolysis–mass spectrometry)

The observed differences in SLA and leaf anatomy between species and treatments led to a further analysis of

Figure 4. Cross-sections of leaves of *Poa pratensis* grown (a) at an optimum N supply (RGR$_{\text{max}}$, RGR = 185 mg g$^{-1}$ d$^{-1}$) and (b) at intermediate N supply (RAR$_{100}$, RGR = 98 mg g$^{-1}$ d$^{-1}$) and at the lowest N supply (RAR$_{50}$, RGR = 51 mg g$^{-1}$ d$^{-1}$). The slices are 5 mmol m$^{-3}$ thick, and the stains are safranine and atro-blue.

Figure 5. A quantitative analysis of the leaf anatomy of four *Poa* species ($\square$ = RAR$_{50}$, $\bullet$ = RAR$_{100}$, and $\bigcirc$ = RAR$_{\text{max}}$), grown at three relative nitrogen addition rates (RAR). The proportion of the total cross-sectional area occupied by (a) mesophyll cells plus intercellular spaces ($y = -0.160x + 68.4$, $P < 0.1$, $r^2 = 0.28$), (b) veins ($y = 0.052x + 5.46$, $P < 0.1$, $r^2 = 0.21$), (c) non-veinal sclerenchymatic cells ($y = 0.102x + 0.10$, $P < 0.001$, $r^2 = 0.58$) and (d) epidermal cells ($y = 0.017x + 26.1$, ns) is shown.

the chemical composition of the different leaves. We first addressed the question of whether the correlation of RGR with plant chemistry, as observed for plants grown under near-optimum conditions, persists when plants are grown at a limiting supply of nitrogen. In addition, we investigated whether leaf chemistry becomes more similar when plants are made to grow at the same RGR, imposing the same limiting relative nitrogen addition rate.

Figure 8a does not show a significant negative correlation between the RGR max and the total nitrogen concentration in the leaves for the fast-growing *P. annua* and *P. trivialis* and the slow-growing *P. compressa* and *P. pratensis*. When the nitrogen supply was made limiting for growth, the N concentration in the leaves decreased dramatically. When the plants were grown with free access to nitrate, the carbon concentration in the leaves did not show a significant correlation with RGR (Fig. 8b). The carbon concentration decreased for the fast-growing *P. annua*, whereas it increased in the slow-growing species, when the nitrogen supply was limiting for growth (Fig. 8b). Consequently, the difference in carbon concentration between the inherently fast-growing (*P. annua* and *P. trivialis*) and the inherently slow-growing (*P. compressa* and *P. pratensis*) species increased significantly when the nitrogen supply was limiting for growth. It should be noted that the present group of four species is possibly just too small for significant statistical differentiation.

Pyrolysis–mass spectrometry yielded a vast amount of spectral data for each sample. In order to detect variations in chemical composition, principal-component analysis (PCA), followed by discriminant analysis (DA), was used.

PCA was applied both for separate data sets (per species and per treatment; not shown) and for the complete set of data. In contrast to earlier results with 11 grass species belonging to different genera (Niemann *et al.* 1992), the
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species within the genus Poa were separated by PCA and DA on the basis of some unidentified phenolic fragments. No differentiation with respect to cell wall components versus cytoplasm was apparent for plants grown with free access to nitrogen (not shown). At limiting nitrogen supply, however, polysaccharides and protein determine the sample discrimination. This can also be seen from the complete data set, for which the score plots and parts of the overall average spectrum, the discriminant functions (DFs), are shown in Fig. 10 (Py-EI-MS) and Fig. 11 (ammonia-CI-MS). There was little variance within each group of samples (Fig. 9). The discriminant analyses, followed by simple regression analysis, show that the independent chemical variation (=60% of the total variation) in the first function, DF1, is significantly correlated with RGR (EI: $P < 0.0001$, $r^2 = 0.66$; NH$_3$-CI: $P < 0.0001$, $r^2 = 0.65$) and SLM (EI: $P < 0.001$, $r^2 = 0.42$; NH$_3$-CI: $P < 0.001$, $r^2 = 0.55$).

For identification of biopolymers, intensities of certain characteristic mass peaks were used, expressed as a percentage of the total intensity of peaks within the mass range used. Characteristic PyMS mass fragments representing the major biopolymers were the same as those used before (Niemann et al. 1992, 1995; Van Arendonk et al. 1997), except for protein. For recognition of this biopolymer we used a number of fragments, which were recently shown to have a high quantitative correlation to the nitrogen concentration (Van Arendonk et al. 1997). Thus, for relative protein quantification, the EI masses $m/z$ 34, 48, 54, 70, 84, 91, 92, 100, 107, 108, 117, 130, 131, 174, 176, 186, 188, 190, 202, 209, 216, 225 and 243 and the NH$_3$-CI masses $m/z$ 70, 72, 75, 84, 86, 89, 98, 99, 101, 111, 113, 125, 127, 129, 131, 136, 139, 141, 146, 153, 155, 165, 167, 169, 183, 195, 197, 201, 211, 226, 229, 244, 262, 281 and 295 were used.

In the reconstructed mass spectrum, DF$_{1+}$ shows protein masses such as $m/z$ 34, 48, 100, 108, 117 and 186 for EI (Fig. 10c) and 111, 113, 125, 127, 129, 139, 153, 195, 201, 209, 211 and 229 for NH$_3$-CI (Fig. 11c) and (for EI only; Fig. 10c) also some higher masses of the sterols ($m/z$ 382–400 and 396–414) and lipids ($m/z$ 262). In DF$_{1-}$, lignin was only represented by a few guaiacyl lignin mass peaks.
Figure 10. (a) Discriminant function spectra DF$_1^-$, (b) the overall average spectrum, (c) discriminant function spectra DF$_1^+$ and (d) scoreplot for low-voltage EI mass spectra of leaf samples of fast- and slow-growing Poa species (▲ = RAR$_{50}$; ● = RAR$_{100}$ and ▲ = RAR$_{max}$), describing 61.0% of the total variation. The y-axis in (a), (b) and (c) gives percentages of the intensity of the highest mass peak present.

Figure 11. (a) Discriminant function spectra DF$_1^-$, (b) overall average spectrum, (c) discriminant function spectra DF$_1^+$ and (d) scoreplot for NH$_3$-CI mass spectra of leaf samples of fast- and slow-growing Poa species (▲ = RAR$_{50}$; ● = RAR$_{100}$ and ▲ = RAR$_{max}$), describing 58.9% of the total variation. The y-axis in (a), (b) and (c) gives percentages of the intensity of the highest mass peak present.
peaks such as \( m/z \) 137, 150 and 180 (EI, Fig. 10a) or 142, 151 and 198 (NH\(_3\)-CI, Fig. 11a) of low intensity. DF\(_1\)-mainly shows polysaccharide (hemicellulose, cellulose) mass fragments such as \( m/z \) 57, 60, 73, 85, 114, 126 and 144 (EI, Fig. 10a) and \( m/z \) 132, 134, 144, 150 and 162 (NH\(_3\)-CI, Fig. 11a). Thus, reduction in growth rate was accompanied by a comparative increase in cell wall polysaccharides and (to a lower extent) in guaiacyl lignin accompanied by a decrease in cytoplasmic components, such as proteins, cytoplasmic terpenoid fragment \( m/z \) 136, sterols and lipids (Figs 10 & 11).

Table 2 summarizes the regression coefficients for the correlation between the summed intensities of characteristic mass peaks of single polymers and the RGR and SLM. These results confirm the trend found by DF analysis. In addition, they show a correlation with syringyl lignin as well, be it the opposite of that with the other cell wall components.

**DISCUSSION**

**Growth, anatomy and morphology**

The 1·3-fold difference in the RGR\(_{\text{max}}\) between the fast-growing (\( P. \) annua and \( P. \) trivialis) and the slow-growing (\( P. \) compressa and \( P. \) pratensis) species disappeared at low nitrogen supply (Fig. 1a). N limitation also affected NAR and LAR (Figs 1b & c). The decrease in LAR at growth-limiting N supply was due partly to a decrease in specific leaf area (SLA) and partly to a shift in biomass allocation from leaves to roots, i.e. the leaf mass ratio (LMR) decreased and the root mass ratio (RMR) increased (Figs 2a,b & 3b). This is in accordance with data from the literature for other species (e.g. Van der Werf et al., 1993a; Ryser & Lambers 1995).

We conclude that the decrease in relative growth rate at limited N supply is mainly due to a decrease in LAR, rather than in NAR. The decrease in LAR in turn is caused by a decrease in both LMR, as found before by others (e.g. Hirose et al., 1988), and SLA (Van der Werf et al., 1993a). This contrasts with results of Poorter et al. (1995), who ascribed the decrease in relative growth rate at a limiting N supply to the relatively large decrease in LMR and NAR, when compared to that in SLA. The decline in LMR, as found in the present work and in other studies, must reflect an effect of N supply on cell elongation and division in the leaf meristem. Indeed, leaf cells of plants grown with a limiting nitrogen supply do tend to be smaller and the total number of cells per leaf is also reduced (Terry 1970), and leaf expansion rates are decreased at a low nitrogen supply (Gastal & Belanger 1993).

Variation in SLA, or its inverse, SLM, can be the result of differences in leaf thickness or in leaf biomass density, i.e. leaf dry mass per leaf volume (Witkowski & Lamont 1991). Our previous work showed that differences in SLM between fast- and slow-growing grasses are due to variation in leaf biomass density and in leaf anatomy (Van Arendonk & Poorter 1994), rather than leaf thickness. The present experiments also show a higher leaf mass density for the grasses at the lowest nitrogen supply with a high SLM (or low SLA, as shown in Figs 1c & 2a). A decrease in SLA (or increase in SLM) is sometimes accompanied by a decrease in leaf thickness (Thompson, Stocker &

![Table 2. Relationships between the summed intensities of the mass fragments (expressed as a percentage of mass fragments with \( m/z \) 20–450) in the four Poa species, by different N supply (RAR50, RAR100 and RARmax), with either RGR, SLM or LMR obtained by linear regression](image)

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<th></th>
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<th>SLM</th>
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<tr>
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<td>*</td>
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<td>0.66</td>
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<td>0.41</td>
<td>*</td>
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</tr>
<tr>
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<td>**</td>
<td>−0.012</td>
<td>0.56</td>
<td>**</td>
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<td>#</td>
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*Significance: ns = not significant; # 0·05 < \( P < 0·1 \); * \( P < 0·05 \); ** \( P < 0·01 \); *** \( P < 0·001 \). rc = regression coefficient, X = coefficient or slope.

The differences in leaf mass density were due to a high proportion of dense material: the non-veinal sclerenchymatic cells showed a positive correlation with the SLM (Fig. 5c), in agreement with our earlier work (Van Arendonk & Poorter 1994). On the other hand, mesophyll plus intercellular spaces and veins showed a weak correlation with SLM (Figs 5a & b). Van Arendonk & Poorter (1994) found a small decrease in percentage epidermis with increasing SLM, when plants were grown with free access to nitrate. Here we confirm the decrease in percentage epidermis for the slow-growing grass species (P. compressa and P. pratensis) grown under high nitrogen supply. However, the proportion of the leaf cross-sections occupied by the epidermis showed no correlation with the SLM when the four grass species were compared at the three rates of N supply (Fig. 5d).

The higher proportion of sclerenchymatic tissue in leaves with a higher SLM is accounted for by a larger number of cells per unit leaf area and not by a smaller cell size (Figs 6a & c). In contrast, the lack of a significant difference in the proportion of the cross-section occupied by epidermis was associated with a positive correlation of cell number and a negative correlation of cell size with SLM (Figs 6b & d).

The increase in SLM might be associated with changes in the chemical composition of the leaf biomass or with smaller leaf cells containing less protein and more cell wall material at limiting N supply. Therefore, the observed differences in the nitrogen and carbon concentration in the Poa leaves at different N supply were scrutinized.

Nitrogen concentration and anatomy

As expected, the nitrogen concentration in the leaves decreased with decreasing N supply, and the interspecific differences disappeared (Fig. 8a). This demonstrates the greater plasticity of the fast-growing species for this trait, in agreement with Ryser & Lambers (1995). The decrease in leaf nitrogen concentration is likely to have been partly accounted for by a decrease in the leaf nitrate and protein concentration. The stronger decrease in leaf nitrogen concentration in the fast-growing Poa annua may well have been due to the relatively large change in epidermal cell size, since vacuoles in the epidermal cells are a major storage site for inorganic nitrogen (Dietz, Hollenbach & Hellwege 1994; Fricke et al. 1994). The enormous decrease (≈50%) in the nitrogen concentration between the maximum N supply (RAR_max) and the intermediate N supply (RAR_100) might have resulted from the decline in epidermal cell size. The difference in cell size between the RAR_90 and the minimum N supply (RAR_50) was not very large (Fig. 6d). Combining the results on nitrogen concentration (Fig. 8a) with the anatomical data (Figs 6b & d), we suggest that at a high N level the fast-growing grass species show a higher storage capacity for nitrogen in their epidermis than the slow-growing ones. It should be noted that nitrate does not tend to accumulate in leaves, except when available in excess of the plant’s need for growth.

Carbon concentration and anatomy

The anatomical results (Figs 6a & d) suggest that the high SLM is mainly caused by more dense material. Earlier studies showed that species with a high RGR_max contain more cytoplasm and showed that species with a high SLM contain more chemical compounds with a high carbon concentration (Niemann et al. 1992; Van Arendonk & Poorter 1994). Our results do not show a significant difference in carbon concentration between the fast- and slow-growing species growing with free access to nitrate (RAR_90, Fig. 8b). This is in contrast with the results of Poorter & Bergkotte (1992), who found a negative correlation between RGR_max and C concentration for 24 wild species. For their eight grass species, which are in the same RGR_max range as the Poa series, however, no significant differences were found. The difference in C concentration increased when the N supply was diminished (RAR_100 and RAR_50): for the fast-growing species the concentration tended to decrease whereas it tended to increase for the slow-growing species (Fig. 8b). How can this be explained?

It is possible that the fast-growing species produce relatively more cellulose and hemicellulose, which contain relatively little carbon (46%), and relatively less proteins, which have a higher carbon concentration (53%), when the nitrogen supply decreases. In contrast, the slow-growing species possibly produce more lignin, which has a high carbon content of 69% (Penning de Vries, Brunsting & Van Laar 1974; Poorter & Bergkotte 1992). Such a contrasting response might explain the larger difference in carbon concentration between the species when plants are grown at a severely limiting N supply. It should be noted that the present series of four species is just too small for a significant statistical differentiation. To obtain a better understanding of this shift of the chemical composition in the four grass species at different N supply, we used pyrolysis–mass spectrometry (Boon 1989, 1992; Niemann et al. 1992, 1995).

Pyrolysis–mass spectrometry

Which of the chemical constituents in the leaves can explain the 40 g m \(^{-2}\) variation in SLM between the species at RGR_max and at RAR_50? Table 2 shows the quantitative, and Figs 9 and 10 the qualitative, shift of the different chemical constituents under limiting N supply.

Table 2 illustrates that the chemical shift as affected by N supply correlates with RGR (SLM and LMR), similar to earlier results for 11 grass species grown with free access to nutrients (Niemann et al. 1992). The latter is based on a simultaneous shift to cell wall polymers such as polysaccharides, guaiacyl lignin and syringyl lignin. A reduced RGR (higher SLM), however, is associated with a significant
increase in the ratio of guaiacyl/syringyl lignin. The production of guaiacyl lignin increases whereas that of syringyl lignin decreases when the N supply is limiting for growth (Table 2).

In monocotyledonous species, lignin is made up mainly of $p$-hydroxyphenyl propane, guaiacyl and syringyl units, quantitatively in that order (Higuchi 1985). The lignification could be controlled by the rate of synthesis and degradation of appropriate enzymes, the substrate specificity, and the compartmentalization of the enzymes in tissues (Higuchi 1985). More specific enzymes are involved in the production of syringyl lignin than in the production of guaiacyl lignin (Higuchi 1985). Previous studies also showed that the guaiacyl-to-syringyl ratio of lignin varies in different morphological regions. The secondary wall of the vessels in angiosperms consists mostly of guaiacyl residues and the secondary wall mostly of fibres of syringyl residues (Goring 1971; Monties 1989). The cell-extension zone of the grasses Holcus lanatus and Deschampsia flexuosa near the leaf base of a growing leaf contains only traces of lignin, in contrast with the mature zone of the same leaves (Groeneveld & Bergkotte 1996). Lignification is a common response to fungal infection or wounding (Grisebach 1981). Wound lignin (Niemann, Baayen & Boon 1990a) and lignin in young tissues (Niemann, Baayen & Boon 1990b) contain relatively more guaiacyl lignin. This indicates that the formation of lignin differs considerably for different tissues and depends on environmental conditions and the stage of leaf development. Thus, a higher guaiacyl/syringyl ratio at the lowest N supply could suggest a shift in the biosynthetic pathway of lignin. Figures 10 and 11 illustrate the qualitative correlation between the four Poa species with their different growth rates, together with the reconstructed mass spectra (DFs1). When the N supply was limiting for growth, a relative enrichment of cell wall material (cellulose, hemicellulose and lignin) was found. The same results have been obtained with samples of leaf material extracted with ethanol and enzyme-digested, which were used for other studies (Van Arendonk 1997) (data not shown).

Figure 10d shows a clear separation between the fast- and slow-growing grass species at a relative addition rate of 50 mg g$^{-1}$ d$^{-1}$. The fast-growing species, P. annua and P. trivialis, at RAR$_{50}$ appeared richer in polysaccharides and lignin masses, while the slow-growing species, P. pratensis and P. compressa, contained more cytoplasmic components. Similar traits were found with the discriminating analysis (DA) of the separate set of RAR$_{50}$ samples. Analyses of the summed relative intensities of the separate biopolymers at RAR$_{50}$ show significantly higher values for cellulose, guaiacyl- and syringyl-lignin for the fast-growing species in comparison to the slow-growing species. This is completely opposite to the general trend shown before for a wider range of species in the grass family at RGR$_{max}$ (Niemann et al. 1992; Van Arendonk & Poorter 1994) and for dicotyledonous species (Niemann et al. 1995). Although the present (inherent, intra-genus) difference in RGR$_{max}$ between the four species is not in accordance with this trend, we think this might be a consequence of the small number of species investigated, especially since plants of the slow-growing species grown at the intermediate limiting N supply (RAR$_{100}$) clearly show a comparatively high contribution of cell wall components in separate analyses (DA and relative intensities; data not shown). This finding of a higher cell wall contribution in fast-growing than in slow-growing species under low N supply suggests a greater adjustment (plasticity) in the fast-growing grasses under these circumstances, because the chemical composition of the slow-growing grasses was more or less the same under intermediate and high nitrogen stress.

At RAR$_{50}$, fast-growing species contained a higher concentration of (‘low-carbon’) cellulose and hemicellulose but also of (‘high-carbon’) guaiacyl- and syringyl-lignin. Differences in cell wall components obviously cannot explain the comparatively high C concentration of the slow-growing species at RAR$_{50}$ (Fig. 8b). In other words, our PyMS data do not lend support to the suggestion that the carbon concentration in leaves of slow-growing species is increased because they produce relatively more lignin, while their protein concentration diminishes.

Another explanation might be found in differences in the contribution of other components with an exceptionally high or low carbon concentration such as lipids (carbon content 78%), organic acids (38%) and/or minerals (0%). Lipids (EI: m/z 129, 228, 236, 256 and 262). CO$_2$ (EI: m/z 44, a possible marker for organic acids) and minerals [our data are restricted to those for potassium, EI: m/z 39; for 24 wild plant species the relative intensity of this fragment was found to be significantly correlated ($P < 0.01$, $r^2 0.65$) to the potassium concentration (G. J. Niemann & H. Poorter, unpublished results)] did not show significant quantitative differences between the fast- and slow-growing grasses (data not shown). It should also be noted that the plants in the present work were grown at low irradiance (315 mmol m$^{-2}$ol m$^{-2}$ s$^{-1}$). Had the plants experienced higher irradiance, differences in growth, leaf properties and chemistry might have resulted and might have increased the differences between species and N treatments.

**Ecological implications**

Interspecific variation in SLA, as observed in this growth chamber experiment for the optimum nitrogen supply, correlates with variation in the natural habitat of the Poa species. That is, P. annua and P. trivialis, which have a high SLA, occur in productive habitats, whereas P. compressa, which has a low SLA, occurs in nutrient-poor environments (Table 1). This in agreement with findings of Grime & Hunt (1979), Chapin (1980) and Poorter & Remkes (1990). However, it should be noted that the difference between the present grasses is not that big: P. pratensis appears to be associated with moderately fertile, rather than nutrient-poor, habitats, whereas the annual P. annua favours more fertile sites.

In this experiment, the potentially fast-growing species have the same RGR at the lowest N supply as the inherently slow-growing species. This suggests a greater plasticity of the fast-growing species (see e.g. Chapin 1980; Lambers & Poorter 1992). The higher amount of cell wall material in leaves of species from nutrient-limited environments points to an increase in the strength (Bell 1981; Kokubu, Kuraishi & Sakurai 1990) and reduced digestibility (Bazzaz et al. 1987; Kephart, Buxton & Hill 1990) of the leaves of species from environments where the nutrient supply is scarce. Both fast- and slow-growing grasses show a higher concentration of cell wall components under limiting nitrate supply. The question arises: why do potentially fast-growing grasses not occur in environments with growth-limiting amounts of nutrients? Aerts & Van der Peijl (1993) showed that, at the beginning of a long-term experiment, the fast-growing perennials produce more biomass than slow-growing ones, but after a few years this was reversed. This can probably be largely accounted for by a greater investment in structural components, such as sclerenchyma, in the slow-growing species, which increases their leaf longevity.

We conclude that the greater production of cell wall constituents, which inexorably increases the SLM, in slow-growing species is important for their functioning in a nutrient-limited environment.

Conclusions

Differences in growth rate between Poa species grown at an optimum N supply are associated with differences in LAR (SLA and LMR) and leaf anatomy. At a growth-limiting N supply, the inherent differences in these parameters disappear and the carbon concentration becomes significantly higher for the slow-growing species. Under these conditions, the plants produce relatively more lignin, cellulose and hemicellulose, whereas the protein concentration is reduced. For lignin also, the ratio of guaiacyl/syringyl lignin increases with decreasing N. These changes in leaf chemistry are associated with an increased leaf mass density. This higher mass density at limiting nitrogen supply is partly due to the increased number of sclerenchymatic cells and a decrease in epidermal cell size. We conclude that an inherently slow-growing and the growing season on photosynthesis of field-grown tall fescue (Festuca arundinacea Schreb.) canopies. Annals of Botany 72, 401–408.


References


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