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Effects of Arbuscular Mycorrhiza and Phosphorus Supply on the Growth of Perennial Ryegrass

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ABSTRACT

Aim: The basic aim of this thesis was to disentangle phosphorus status-dependent and -independent effects of arbuscular mycorrhizal fungi (AMF, *Glomus hoi*) on the components of plant growth: morphology and assimilation rates, in perennial ryegrass (*Lolium perenne* L.).

Materials & Methods: In a first experiment, I assessed phosphorus response functions of leaf and plant morphological components in undisturbed plants with similar size. To this end, nonmycorrhizal and mycorrhizal plants were grown in controlled conditions at four different soluble phosphorus supplies ranging from 0 mM to 0.5 mM. In a second experiment, AMF effects on carbon economy were investigated by comparing nonmycorrhizal and mycorrhizal plants of similar size, morphology and phosphorus content. $^{13}CO_2/^{12}CO_2$ steady-state labelling was used to trace all photosynthate assimilated during one photoperiod, and a respiratory $^{13}CO_2/^{12}CO_2$ exchange system to assess dark respiration rates, and the contribution of recently fixed carbon to shoot and root respiration.

Results & Discussion: Relative growth rate of mycorrhizal plants was significantly higher as a result of improved phosphorus nutrition. The presence of AMF stimulated relative phosphorus uptake rate, decreased leaf mass per area (LMA) and increased shoot mass ratio at low phosphorus supply. Lower LMA was caused by both decreased tissue density and thickness. Variation in tissue density was almost entirely due to variations in soluble carbon, while that in thickness involved structural changes. Carbon economy analyses revealed that AMF enhanced relative respiration rate of the root-soil system by 16%, inducing an extra C flow amounting to 3% of daily gross photosynthesis. Total C drain for growth and respiration of the AMF was estimated at ≤ 8% of daily gross photosynthesis. This was associated with a greater amount of new C allocated below-ground and respired in mycorrhizal plants. AMF colonization affected the sources supplying below-ground respiration, revealing a greater importance of plant C stores in supplying respiration and/or the participation of storage pools within fungal tissues.

Conclusions: The relationships between relative phosphorus uptake rate, leaf and plant morphology were identical in mycorrhizal and nonmycorrhizal plants. Beneficial effects of mycorrhizal symbiosis were mainly mediated by adjustments in leaf morphology, which were largely dependent on AMF effects upon phosphorus capture. When ontological and nutritional effects are accounted for, AMF increased below-ground costs, which were not compensated by increased photosynthesis rates.

ZUSAMMENFASSUNG

Zielsetzung: Ziel der vorliegenden Arbeit war es, die Phosphorstatus-abhängigen und -unabhängigen Wirkungen der Mykorrhizierung auf die Komponenten des Wachstums von Deutsch Weidelgras (*Lolium perenne* L.) zu trennen. Es wurden Wirkungen auf die Morphologie und den Kohlenstoffhaushalt untersucht.

Material und Methoden: Die Untersuchungen wurden mit der arbuskuläre Mykorrhiza *Glomus hoi* und der Deutsch Weidelgras Sorte Condesa durchgeführt. Das erste Experiment befasste sich mit der Wirkung der Phosphorernährung und Mykorrhizierung auf morphologische Eigenschaften, insbesondere der Blätter. Da morphologische Eigenschaften auch ontogenetischen Veränderungen unterworfen sind, wurden nur Pflanzen ähnlicher Größe verglichen, um Verfahrenseffekte von ontogenetischen Effekten trennen zu können. Nicht-mykorrhizierte und mykorrhizierte Pflanzen wurden unter kontrollierten Bedingungen in Klimakammern bei vier verschiedenen Versorgungsniveaus von löslichem Phosphor in der Nährlösung (Bereich 0 bis 0.5 mM) angezogen. Im zweiten Experiment wurde der Kohlenstoffhaushalt an mykorrhizierten und nicht-mykorrhizierten Pflanzen mit gleichem Größe und gleichen Phosphorgehalt untersucht. Mithilfe von 'steady-state' $^{13}CO_2/^{12}CO_2$ Markierung und Gaswechselmessungen wurde die Photosyntheseleistung und die Allokation des aktuell assimilierten Kohlenstoffs, einschließlich seines Verbrauch in Spross- und Wurzelrespiration, quantifiziert.

Ergebnisse und Diskussion: Die Mykorrhizierung förderte vor allem bei geringem Phosphorangebot die Phosphoraufnahme, wodurch das Wachstums beschleunigt wurde. Die Mykorrhiza bewirkte eine Erhöhung der spezifischen Blattfläche und des Spross/Wurzel-Verhältnisses. Die höhere spezifische Blattfläche beruhte auf einer geringeren Dichte und Dicke der Blätter. Die Variation in der Blattdichte wurde fast ausschließlich durch Variation in den Gehalten wasserlöslicher Kohlenhydrate hervorgerufen, während die Veränderungen der Blattdicke durch strukturelle Veränderungen verursacht wurden. Eine verbesserte Phosphorernährung hatte bei nicht-mykorrhizierten Pflanzen dieselben Effekte. Bei Pflanzen vergleichbarer Größe und ähnlichen Phosphorstatus führte die Mykorrhizierung zu einer Stimulation der unterirdischen Respiration (Wurzel plus Boden) von 16%, entsprechend 3% der gesamten täglichen Assimilationsleistung. Der gesamte Kohlestoffkonsum für Wachstum und Respiration der Mykorrhiza wurde auf ≤8% der gesamten Assimilationsleistung der Pflanze geschätzt. Mykorrhizierung förderte die Allokation von aktuell assimiliertem Kohlenstoff in die Wurzel, sowie dessen Verbrauch in der Respiration. Gleichwohl war der Beitrag von Reservepools der Pflanzen bzw. der Mykorrhiza zur unterirdischen Respiration in mykorhizierten Pflanzen größer als in nicht-mykorrhizierten. Trotz des erhöhten Assimilatbedarfs bewirkte Mykorrhizierung keine Erhöhung der photosynthetischen Phosphor- und Stickstoffnutzungseffizienz.

Schlussfolgerungen: Die Beziehungen zwischen Phosphoraufnahme, Wachstum und Morphologie wurden durch die Mykorrhiza nicht beeinflusst. Die positiven Wirkungen der Mykorrhizierung beruhten primär auf Veränderungen der Blattmorphologie, welche hauptsächlich auf die Stimulation der Phosphoraufnahme durch die Mykorrhiza zurückzuführen war. Bei gleicher Größe und gleichem Phosphorernährungsstatus zeigten mykorrhizierte Pflanzen erhöhte unterirdische Kosten, welche nicht durch höhere Photosyntheseleistung kompensiert wurden.

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ABBREVIATIONS

Symbol	*Description*	*Units*
AMF	Arbuscular mycorrhizal fungi	
C	Carbon	
P	Phosphorus	
N	Nitrogen	
WSC	Water soluble carbohydrates	
δ	The deviation of the ^{13}C to ^{12}C ratio in CO_2 from that of the international standard, VPDB	
<Variable>		
RPUR	Relative phosporus uptake rate	mg P g^{-1} P d^{-1}
RGR	Relative growth rate	mg C g^{-1} C d^{-1}
LAR	Leaf area ratio (plant leaf area / plant C mass)	g C g^{-1} C
LMA	Leaf mass per area (lamina C mass / leaf area)	g C cm^{-2}
LMR	Leaf mass ratio (lamina C mass / plant C mass)	g C g^{-1} C
SMR	Shoot mass ratio (shoot C mass / plant C mass)	g C g^{-1} C
DEN	Lamina tissue density (lamina C mass / lamina fresh weight)	g C g^{-1} f. wt
<Variable>		
R	Respiration rate	mg C $plant^{-1}$ d^{-1}
P_G	Gross photosynthesis rate	mg C $plant^{-1}$ d^{-1}
RRR	Relative respiration rate	mg C g^{-1} C $time^{-1}$
RPR	Relative gross photosynthesis rate	mg C g^{-1} C d^{-1}
$f_{B\,new}$	Fraction of labelled C in biomass	%
$f_{R\,new}$	Fraction of labelled C in the respired CO_2	%
PPUE	Photosynthetic phosphorus-use efficiency (P_G / shoot P)	g C g^{-1} P d^{-1}
PNUE	Photosynthetic nitrogen-use efficiency (P_G / shoot N)	g C g^{-1} N d^{-1}

CHAPTER I. GENERAL INTRODUCTION

Soil phosphorus availability has been recognized as an important factor limiting production in grassland ecosystems. Symbiosis with arbuscular mycorrhizal fungi (AMF) can increase the nutritional status of grassland plants, especially with respect to phosphorus (Koide, 1991; Marschner, 1995; Smith & Read, 1997). In this way, one may expect beneficial effects of AMF on growth, and on resistance to stress and disturbance. But, since the AMF draws carbon from the host for its maintenance and function, the overall effect of AMF on the growth of the plant is dependent on the cost-benefit relationship of the symbiosis (Koide, 1991; Eissenstat *et al.*, 1993; Tinker *et al.*, 1994; Smith & Smith, 1996; Johnson *et al*., 1997; Jones & Smith, 2004).

Grassland plants are relevant objects for the study of cost-benefit relationships of mycorrhizal symbiosis since (*i*) they are generally strongly colonized, (*ii*) soil phosphorus availability is often limiting, and (*iii*) the mycorrhizal network is not frequently disrupted as in annual crops. However, little is known about the interactive effects of AMF colonization and phosphorus nutrition on carbon economy and leaf area expansion in perennial grasses. This thesis reports the effects of AMF and phosphorus supply on morphological traits, biomass allocation patterns and carbon economy of perennial ryegrass (*Lolium perenne* L.) plants under undisturbed growth conditions.

Concepts and relevance of mycorrhizal associations

Mycorrhizae are symbiotic associations that form between fungi and roots of the majority of plant species. Mycorrhizal associations vary widely in form and function. Two basic types of mycorrhizae are distinguished: (*i*) one in which the root surface is sheathed in a fungal mantle (SHEATHING: monotropoid, arbutoid and ectomycorrhizae), and (*ii*) the other where a mantle is lacking but in which hyphae proliferate internally into the plant root (ENDO: orchid, ericoid and arbuscular) (*sensu* van der Heijden & Sanders, 2002). Some ectomycorrhizal fungi (EM) are facultative symbionts, which can mineralise organic carbon from the soil. This type of mycorrhiza is of vital importance to many forest trees in temperate regions. In contrast, arbuscular mycorrhizal fungi (*Zigomycetes*, order *Glomales*) are obligate symbionts, which can only obtain carbon from the host plants, and form associations with the majority of the world's plants.

The structure of an arbuscular mycorrhiza has three principal components: (*i*) the root itself, (*ii*) the fungal structures within and between the cell of the roots, and (*iii*) the extraradical mycelium network in the soil. Inside the root, the fungus initially grows between cortical cells, but soon penetrates host cell walls forming highly branched structures called arbuscules (Smith & Read, 1997). It is known that arbuscules are the site of phosphorus transfer from the fungus to the plant. The site of carbon exchange is likely the interface between the intercellular hyphae and the root cells. It is assumed that carbohydrates, predominantly sucrose, are lost from the host cell by passive or conditioned efflux, hydrolysed in the apoplastic interface by invertase and actively absorbed as hexose (preferentially glucose) by the fungus (Smith & Read, 1997). This is rapidly incorporate into trehalose, glycerol and small amounts of mannitol. These, together with large amounts of lipids constitute the main carbohydrates and storage compounds of the fungal structure. AMF is widely thought to consume 4 to 20% of the total photosynthates (Douds *et al*., 1988; Jakobsen & Rosendahl, 1990). Thus, the mycorrhiza could be a quantitatively important component of plant carbon metabolism.

Symbiosis with AMF facilitates nutrient uptake (mainly P, but also NH_4, H_2O, Zn, Cu and other micronutrients) and increases plant tolerance to various kinds of stress (Smith & Read, 1997) in return for photosynthate supply from the host plant to the fungi. The rate of phosphorus uptake from the soil solution is largely determined by the amount of absorptive surface area, and the beneficial effect of AMF colonization is mainly due to the ability of fungal hyphae to acquire phosphorus beyond the limits of the rhizosphere depletion zone (Koide, 1991). Importantly, it was recently demonstrated that AMF colonization could result in partial or complete inactivation of the phosphorus uptake by the roots, and fungal hyphae provide then the dominated pathway for plant phosphorus supply (Smith *et al.*, 2004). This means that the functional significance of AMF is still relevant even if plant growth or nutritional status seems not to be improved, as fungal hyphae still play an active role in the nutrient uptake of the plant (Pearson & Jakobsen, 1993; Smith *et al*., 2004).

Interactive effects of phosphorus and AMF on plant growth

The relative growth rate (RGR) of a plant is equal to the product of its leaf area ratio (LAR: leaf area per unit of plant mass) and net assimilation rate (NAR) (Hunt, 1978; Lambers et al., 1989). Thus, plant

growth is dependent on a morphological component and on a 'photochemical' one *i.e.* net photosynthesis rate.

The response of leaf area to phosphorus is an important factor determining the productivity of grassland plants since leaf area determines the amount of intercepted radiation and consequently the amount of dry matter accumulation. Although phosphorus deficiency in plants is generally associated with reducing photosynthetic capacity, shoot growth and leaf area expansion are known to be more sensitive to P-supply (Freeden *et al.*, 1989). In fact, a recent meta-analysis of the published literature concluded that plant morphological components were major determinants of growth responses to nutrient deficiencies (Poorter & Nagel, 2000). It is known that both phosphorus addition and AMF could promote plant growth by increasing leaf area per unit of plant biomass (Freeden & Terry, 1988; Baas & Lambers; 1988), and it was found that AMF colonization could increase growth of plants by altering their biomass allocation patterns (Freeden & Terry, 1988; Bass *et al.*, 1989; Lovelock *et al.*, 1996). But are AMF and phosphorus supply effects on morphological determinants quantitatively equal? The first part of this thesis (CHAPTER II) is an attempt to elucidate this question.

In some scenarios, the proportion of carbon drawn by the fungus was large enough to slow down plant growth (Johnson *et al.*, 1997). But generally, the carbon cost of the fungus is overcompensated by the beneficial terms of the symbiosis (Smith & Read, 1987). Interestingly, in white clover (Wright *et al*. 1998a) and in the grass *Andropogon gerardii* (Miller *et al*., 2002), at similar phosphorus and nitrogen status in nonmycorrhizal and mycorrhizal plants, AMF colonization enhanced net photosynthesis rates. In those studies, it was suggested that the stimulation of photosynthesis was due to the additional sink activity of the fungus, which counterbalanced part of the symbiotic costs. However, this effect seems not to be general, as many reports have not shown changes in photosynthesis rates induced by AMF not mediated by changes in nutritional status (Snellgrove *et al*., 1982; Freeden & Terry, 1988; Douds *et al*., 1988; Bass *et al*., 1989; Pearson & Jakobsen, 1993; Black *et al*., 2000; Gavito *et al*., 2000; and others). Therefore, an interesting point to test is whether AMF effects on photosynthesis and consequently on plant carbon balance are entirely mediated by changes in nutritional status, or whether it can be influenced by nutrient status-independent AMF effects. We explore this issue in the second part of this thesis (CHAPTER III).

Aims

The basic aim of this thesis was to disentangle phosphorus status-dependent and -independent effects of AMF on the components of plant growth in perennial ryegrass. The specific aim of the first experiment (CHAPTER II) was (*i*) to study responses of plant growth, leaf and plant morphology of nonmycorrhizal and mycorrhizal plants as a function of relative phosphorus uptake rate. The specific aims of the second experiment (CHAPTER III) were (*ii*) to quantify the extra C drain of AMF, (*iii*) to analyse the influence of AMF on the amount of new C allocated below-ground and quantify its importance in supplying respiration and (*iv*) to test the existence of enhancements on carbon assimilation rate by AMF not mediated by improvement of nutritional status.

Outline of this thesis

The thesis was divided in two experimental parts: (*i*) "Phosphorus nutrition-mediated effects of arbuscular mycorrhiza on leaf morphology and carbon allocation in perennial ryegrass", and (*ii*) "Effects of arbuscular mycorrhiza on carbon economy in perennial ryegrass: quantification by $^{13}CO_2/^{12}CO_2$ steady-state labelling and gas exchange".

In a first experiment (CHAPTER II), nonmycorrhizal and mycorrhizal plants were grown in controlled conditions at four different soluble phosphorus supplies ranging from 0 mM to 0.5 mM. Mycorrhizal colonization, plant and leaf morphological components, leaf carbon fractions and phosphorus concentration were determined for each plant. Data were used to assess phosphorus response function of leaf and plant morphological components in undisturbed plants with similar size.

In a second experiment (CHAPTER III), AMF effects on carbon economy were quantified by $^{13}CO_2/^{12}CO_2$ steady-state labelling techniques in nonmycorrhizal and mycorrhizal undisturbed plants with similar size, morphology and low phosphorus content. An open-system, gas exchange and $^{13}CO_2/^{12}CO_2$ labelling facility was used to label all photosynthate assimilated during one photoperiod, and a respiratory $^{13}CO_2/^{12}CO_2$ exchange system to assess the contribution of recently fixed carbon to shoot and root respiration.

In both experiments, effects of phosphorus supply and AMF inoculation on leaf elongation rate and its components were additionally evaluated (APPENDICES). In the final section (CHAPTER IV), principal results are further discussed in the context of current knowledge about cost-benefit relationships of mycorrhizal symbiosis.

CHAPTER II. PHOSPHORUS NUTRITION-MEDIATED EFFECTS OF ARBUSCULAR MYCORRHIZA ON LEAF MORPHOLOGY AND CARBON ALLOCATION IN PERENNIAL RYEGRASS [1]

ABSTRACT

- The aim of this work was to disentangle phosphorus status-dependent and -independent effects of arbuscular mycorrhizal fungus (AMF) on leaf morphology and carbon allocation in perennial ryegrass (*Lolium perenne* L.).

- To this end, we assessed the phosphorus response function of morphological components in mycorrhizal and nonmycorrhizal plants of similar size.

- AMF (*Glomus hoi*) stimulated relative phosphorus uptake rate, decreased leaf mass per area (LMA) and increased shoot mass ratio at low phosphorus supply. Lower LMA was caused by both decreased tissue density and thickness. Variation in tissue density was almost entirely due to variations in soluble carbon, while that in thickness involved structural changes.

- All effects of AMF were undistinguishable from those mediated by increases in relative phosphorus uptake rate through higher phosphorus supply rates. Thus, the relationships between relative phosphorus uptake rate, leaf morphology and carbon allocation were identical in mycorrhizal and nonmycorrhizal plants. Hence, no evidence was found for AMF effects not mediated by changes in plant phosphorus status.

[1] Grimoldi A.A., Kavanová M., Lattanzi F.A. & Schnyder H. (2005) *New Phytologist* **168**, 435–444.

INTRODUCTION

Soil P availability has been recognized as a key factor limiting crop growth and grassland production on many soils. Symbiosis with arbuscular mycorrhizal fungus (AMF) is known to increase the nutritional status of grassland plants, especially with respect to P (Smith & Read, 1997). The production of hyphae seems to incur a smaller C cost per unit absorbing area than that of roots, and it allows the exploration of soil volumes that are not accessed by roots (Koide, 1991). However, as obligate symbionts, the AMF relies on the plant host for the supply of C assimilates required for its growth, maintenance and functioning. Since the AMF draws C from the host, it has been proposed that the overall effect of AMF on the growth of the host is dependent on the cost-benefit relationship of the symbiosis (Smith & Smith, 1996; Johnson *et al*., 1997). However, the mechanistic understanding of the terms of exchange of this symbiosis is far from complete (Fitter, 2005). A still unsolved aspect is whether AMF influence on growth is entirely mediated by changes in plant P status, or whether there are P status-independent AMF effects. It is known that both P addition and AMF colonization could promote plant growth by increasing the leaf area per unit of plant biomass (leaf area ratio, LAR) (Baas & Lambers, 1988), which contributes to an increase in the C assimilation on a whole plant basis (Lambers & Poorter, 1992). But are AMF and P supply effects on growth and its morphological determinants quantitatively equal? This chapter is an attempt to elucidate this question.

Efficient resource uptake and optimal biomass allocation are important determinants of competitive ability and stress tolerance. Plants have a great capacity to control the expansion of their organs, and optimise light interception by increasing the proportion of leaf area and shoot biomass if soil nutrient availability is improved (Lambers & Poorter, 1992). In fact, a recent meta-analysis of the published literature concluded that plant morphological components were major determinants of growth responses to nutrient deficiencies (Poorter & Nagel, 2000). Differences in LAR can be due to variation in the leaf mass per area (LMA) and in the fraction of plant mass present in leaves (leaf mass ratio, LMR). There is a close association between plant growth rate and the components of LMA (Lambers & Poorter, 1992), but still little is known about the effects of nutrient availability on leaf morphology (Meziane & Shipley, 1999). LMA often shows a considerable plasticity in response to environmental factors, and this can be attributed either to the density or to the thickness of the leaf

tissue (Lambers & Poorter, 1992; Witkoswski & Lamont, 1991). P deficiency in plants is generally associated with accumulation of non-structural compounds, as more photosynthates are produced than can be consumed in growth (Rao *et al*., 1989). Therefore, it is possible that the often found increases in LMA in response to P deficiency do not reflect structural changes in leaf architecture but result merely from passive accumulation of water soluble compounds (de Groot *et al*., 2001; Miller *et al*., 2002). Clearly, a functional interpretation of changes in LMA must distinguish between effects related to changes in the soluble fraction, and those associated with a change of the proportion of structural C per unit of leaf area (Witkoswski & Lamont, 1991; Miller *et al.*, 2002).

The basic aim of this study was to disentangle P status-dependent and -independent effects of AMF on leaf morphology and C allocation in perennial ryegrass (*Lolium perenne* L.). To this end, responses of plant growth, leaf morphology and C allocation of mycorrhizal and nonmycorrhizal perennial ryegrass plants of similar size were compared as a function of relative P uptake rate. Awareness of size-dependent effects is a prerequisite for plant growth studies, as many morphological ratios change in conjunction with plant size (Coleman *et al*., 1994). In order to assess unbiased treatment effects, it is therefore essential to take into account ontogenic drifts by, for example, comparing plants of common size (*e.g.* Eissenstat *et al.*, 1993; Wright *et al*., 1998a; de Groot *et al.*, 2001). Specifically, we addressed the following questions: (*i*) is there a consistent quantitative relationship between P availability, plant growth, leaf morphology and C allocation in perennial ryegrass? (*ii*) If so, is this relationship modified by P status-independent effects due to the presence of AMF colonization? Further, (*iii*) which are the specific morphological components explaining plant response to changes in P availability? To our knowledge, this is the first comparative study of AMF effects on the P response of leaf morphology and biomass allocation in a grass.

MATERIALS AND METHODS

Plant material, AMF inoculation and growth conditions

Seeds of perennial ryegrass (*Lolium perenne* L. cv. Condesa) were washed for 20 min in NaOCl (6% active chlorine) for surface sterilization and sown into tubes (diameter 5 cm, height 35 cm) filled with

quartz sand (0.3-0.8 mm). Previously, all tubes had been fertilized with fine powdered Hyperphos (0.564 g kg^{-1} sand, 30.5% P_20_5) as a P source of low plant availability. In half of the tubes, plants were inoculated with the AMF *Glomus hoi* BEG 104 (provided by Dr. A. Heinemeyer, University of York, UK), that were propagated in glasshouse conditions for three months on *Plantago lanceolata* L. plants as a host. The inoculum, consisting of roots and soil material (15 ml per tube), was mixed thoroughly with the quartz sand used as substrate. Tubes with and without AMF inoculum were placed in separate plastic boxes (56 × 76 × 37 cm) to prevent contamination of the control plants with AMF.

Plants were grown in a growth chamber (VKZPH 005-120-S, Heraeus Vötsch Gmbh, Balingen-Frommern, Germany) with relative humidity kept between 65 and 75%, temperature at 20°C during 16-h light period and 15°C during the dark, and 425 µmol m^{-2} s^{-1} of photosynthetic photon flux density at plant level. Plants were watered by automatic irrigation systems supplying 25 ml of the nutrient solution to every plant four times a day. During the first five weeks, plants were irrigated with modified half-strength P-free Hoagland's solution (2.5 mM KNO_3, 2.5 mM Ca $(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM NaCl, 0.125 mM Fe-EDTA, 23 µM H_3BO_3, 4.5 µM $MnSO_4$, 0.38 µM $ZnSO_4$, 0.16 µM $CuSO_4$, 0.05 µM Na_2MoO_4). Thereafter and till the end of the experiment, four soluble P treatments were applied: nil (0 mM), low (0.02 mM), intermediate (0.1 mM) and high (0.5 mM) P supply, delivered as KH_2PO_4 in the nutrient solution described above. Potassium supply was maintained constant between treatments by reducing proportionally the KCl supply. Once a week, tubes were flushed with 125 ml distilled water, to prevent salt accumulation.

Sampling procedure

Five plants per treatment combination were sampled at the end of the dark period 68, 74 and 83 days after sowing. Roots were freed from soil substrate by washing with tap water. A sample of the fresh root material was weighed and used for detection of AMF colonization. Shoots were separated into mature tillers (at least one fully developed leaf) and daughter tillers, and counted. Mature tillers were dissected into four compartments: (*i*) expanding leaf, (*ii*) youngest fully expanded leaf (defined as the youngest one having its ligule exposed), (*iii*) all other mature leaves and (*iv*) dead material. Each leaf was then dissected into lamina and sheath. The area of the laminas was measured with a Li-3100 leaf

area meter (Li-Cor Inc., Lincoln, NE, USA). Samples were then fresh-weighed, frozen in liquid nitrogen, freeze-dried, dry-weighed, ground and stored at -25°C.

AMF colonization

Mycorrhizal colonization of the roots was determined by the histological detection of mycorrhizal structures after root staining. A sample of the fresh root material was cleared in KOH (10% w/v) for 10 min at 105°C, acidified in HCl (1% v/v) for 5 min, and then stained with Trypan Blue (0.05% w/v; Sigma-Aldrich, Steinheim, Germany) in acid glycerol for 10 min at 105°C. Stained roots were briefly submerged in distilled water, arranged lengthwise on microscope slides, and then mounted in glycerol-gelatine. The percentage of root length with AMF colonization was determined in the mounted roots by evaluating 100 random intersections for each plant, using a line graticule in the eyepiece of a compound microscope at 125 × magnification (Giovannetti & Mosse, 1980).

Chemical analyses

One representative shoot sample for each plant was made by pooling proportional fractions of dry biomass of each organ. The concentration of C was then determined in roots, shoot and additionally separate laminas samples on 0.7 mg aliquots of dry ground material using an elemental analyser (NA1110, Carlo Erba Instruments, Milan, Italy). P concentration was determined on samples of roots and shoot (25 mg), which were ashed in a muffle furnace (4h at 500°C). The resulting ash was digested in HNO_3/HCl and P quantities were measured by phosphovanado-molybdate colorimetry (Hanson, 1950). Reference material of ground grass leaves was included with every ten samples to check digestion and analytical procedures. All data are presented as P and C masses.

For analyses of different C fractions, 20 mg of dry mass of the youngest fully expanded leaf were extracted with 2 ml of distilled water for 10 min at 93°C, and 45 min at room temperature (Schnyder & de Visser, 1999). After centrifugation (10 000g for 15 min), the supernatant was used for analyses of water soluble fractions, while the residual pellet was used for starch determination. The content of water soluble N and of water soluble C in 100 µl of the supernatant was analysed with the

elemental analyser, using sulphanilamide (Merck, Darmstadt, Germany) as a standard. Water soluble amino-C content was estimated as soluble N content times 2.6 (Schnyder & de Visser, 1999). Water soluble carbohydrates were analysed on an aliquot of the extract. For this, samples were hydrolysed in 0.1 M H_2SO_4 for 20 min at 93°C, and reducing power of the hydrolysed carbohydrates was detected photometrically at 425 nm after reduction of a potassium ferricyanide solution (Schnyder & de Visser, 1999). Fructose and glucose (Merck, Darmstadt, Germany) were used as standards. Finally, the residual pellets were hydrolysed in a mixture of 5 ml dimethylsulfoxide (DMSO) and 1.25 ml HCl (8M) for 30 min at 60°C. Starch was determined colorimetrically after neutralization with 1.25 ml NaOH (8M) and equilibration with citric buffer (0.112 M; pH = 4) by an enzymatic test-combination (Cat. Nr. 207748, Boehringer, Mannheim, Germany). The content of C in water soluble carbohydrates and starch were estimated as total hexose units times 0.4. Structural C fraction was then estimated as: $C_{Structural} = C_{Total} - (C_{Soluble} + C_{Starch})$.

Calculations

Relative growth rate (RGR, mg C g^{-1} C d^{-1}) and relative phosphorus uptake rate (RPUR, mg P g^{-1} P d^{-1}) during the sampling period (68 to 83 days after sowing) were calculated as the slope of the least squares regression lines of the $\log_e$-transformed values of C and P masses against time, respectively. LAR (cm^2 g^{-1} C) was determined as total plant leaf area divided by plant mass. LAR is associated with the amount of lamina mass invested to construct a unit of leaf area (LMA) and with the fraction of plant mass allocated to the leaf laminas (LMR). LMA (g C cm^{-2}) was estimated for different C fractions (total, structural and non-structural), and calculated as lamina C mass divided by leaf area. In addition, LMA was factorised into lamina tissue density (g C g^{-1} f. wt) and thickness (mm). Lamina tissue density was estimated as the ratio of lamina mass to leaf fresh weight, and tissue thickness as the ratio of leaf fresh weight to leaf area under the assumption that leaf volume is approximately equal to leaf fresh weight (Garnier & Laurent, 1994). LMR (g C g^{-1} C) was calculated as total lamina mass divided by plant mass, and factorised into shoot mass ratio (g C g^{-1} C) and lamina to shoot ratio (g C g^{-1} C). Shoot P to C ratio and all morphological components (plant and leaf) were analysed on plants of similar size (0.5-0.9 g C $plant^{-1}$) selected from the three harvests instead of a chronological analyses

in order to distinguish treatment effects from ontogenetic drifts (Coleman *et al.*, 1994). All harvested plants that fitted within this range were used. Leaf morphology and C fractions analyses were performed on laminas of the youngest fully expanded leaf from mature tillers of the size-selected plants.

Statistical analyses

All data were previously checked for normality and homogeneity of variances. Root mycorrhizal colonization was analysed by one-way ANOVA and a posteriori mean comparisons were performed with Tukey test (Steel & Torrie, 1988). Differences in RGR and RPUR within P treatments were analysed by two-way ANOVA with time and mycorrhizal inoculation as independent variables (skipping the intermediate harvest), and $\log_e$-transformed values of C or P mass as dependent variables. Significant time × treatment interaction denotes significant difference in RGR or RPUR, respectively (Poorter & Lewis, 1986). The relationship between RGR and RPUR was examined by simple correlation analysis, and F-test for differences between mycorrhizal and nonmycorrhizal plants was performed (Steel & Torrie, 1988). All other variables were analysed by two-way ANOVA, with P and AMF inoculation as main factors, on plants with similar size, and contrasts between specific treatments were performed by a priori LSD mean tests (Steel & Torrie, 1988). Variables that involve percentages were arcsine square-root transformed before analysis (Steel & Torrie, 1988). Quadratic-plateau functions and all statistical analyses were performed by the statistical package SAS version 8.2 (SAS Inc., Cary, NC, USA). Results are shown as mean and standard error of the means. Plant material and growth conditions.

RESULTS

Phosphorus uptake, AMF colonization and plant growth

Both P supply and AMF inoculation affected the P status of the plants, expressed as shoot P to C ratios (w/w; Fig. II.1a). Planned-comparison tests revealed that all P treatments differed significantly (df_e = 41; $P < 0.05$) in this parameter. Also P to C ratio was higher ($P = 0.008$) in mycorrhizal plants under low P supply (0.02 mM), but was not affected by mycorrhiza in the other P treatments (P > 0.05; Fig. II.1a). P treatment had a significant effect (df_e = 16; $P < 0.001$) on the percentage of AMF colonization (Fig. II.1b). None of the control plants observed were contaminated with AMF.

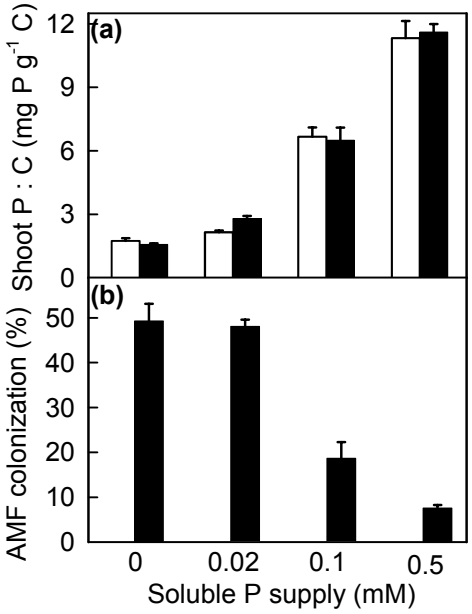

Figure II.1. Shoot P to C ratio (a) and arbuscular mycorrhizal fungus (AMF) colonization (b) in perennial ryegrass plants grown for twelve weeks with different soluble P supply. Open bars, nonmycorrhizal plants; closed bars, mycorrhizal plants. Soluble P treatments: nil (0 mM), low (0.02 mM), intermediate (0.1 mM) and high (0.5 mM). Values are means ± SE (n = 4-8).

RGR and RPUR were positive and approximately constant during the sampling period (Fig. II.2). Overall, RGR was linearly related to RPUR, and the relationship was not different between AMF treatments ($P > 0.05$), not far from the 1:1 line in both treatments (Fig. II.3). This implies that the P to C ratio of accumulated biomass was rather similar to that of the standing biomass, although somewhat lower in intermediate and high P supply. In consequence, plant P to C ratios remained relatively stable over the harvesting period. Hence, plants within each treatment can be considered to be close to a nutritional steady-state.

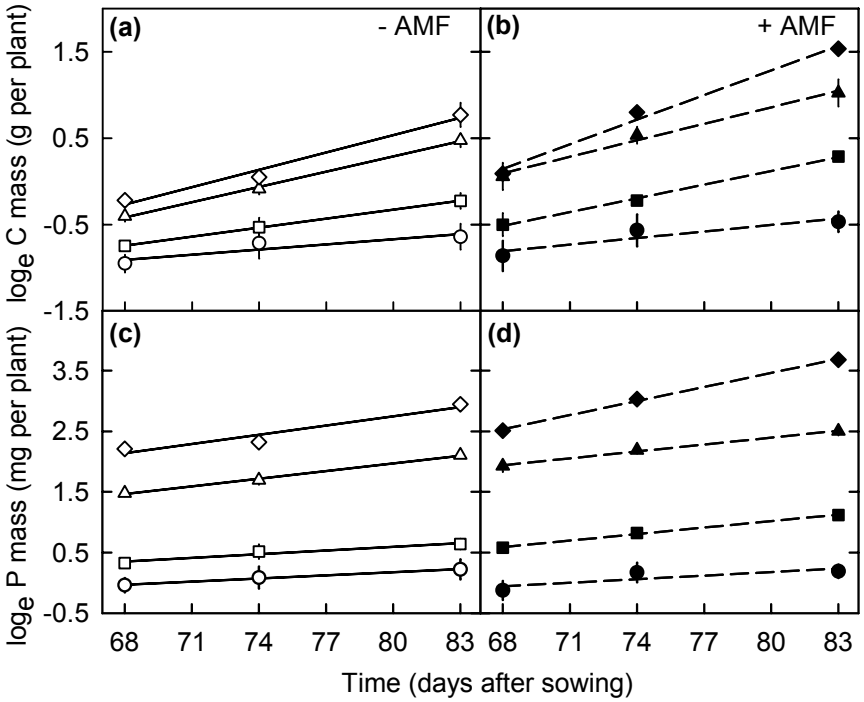

Figure II.2. C (a,b) and P (c,d) masses as affected by arbuscular mycorrhizal fungus (AMF) in perennial ryegrass plants grown for twelve weeks with different soluble P supply rates. Open symbols and full lines, nonmycorrhizal plants (-AMF); closed symbols and dashed lines, mycorrhizal plants (+AMF). Soluble P treatments: nil (0 mM; ○●), low (0.02 mM; □■), intermediate (0.1 mM; Δ▲) and high (0.5 mM; ◇♦). The slopes of the regression lines represent the relative growth rate (RGR) and the relative phosphorus uptake rate (RPUR) respectively. Slopes of the regressions differed from zero ($P < 0.01$), except for both 0 mM soluble P treatments. Values are means ± SE ($n = 5$).

In nonmycorrhizal plants, RGR increased 2.3-fold between the lowest and highest P supply rates (Fig. II.3). Two-way ANOVA analyses within P treatments indicated that RGR of mycorrhizal plants was significantly higher than nonmycorrhizal plants only when RPUR was improved (Table II.1), which occurred both under low (0.02 mM) and high (0.5 mM) P supply rates (by +40% and +52%, respectively; Fig. II.3). Similarly, at intermediate P supply (0.1 mM), C and P masses of the mycorrhizal plants were significantly higher than nonmycorrhizal ones (Fig. II.2; Table II.1), although differences in RGR and RPUR were not apparent. Similarly, RGR and RPUR of mycorrhizal plants in the 0 mM soluble P treatment tended to be higher than those of nonmycorrhizal plants (+23% and +9% respectively). However, the resolution of the data was not good enough to detect these effects as statistically significant (Fig. II.3).

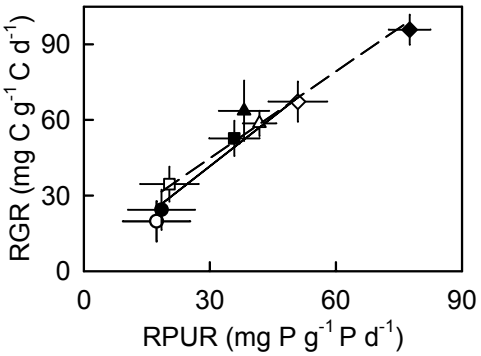

Figure II.3. Relative growth rate (RGR) to relative phosphorus uptake rate (RPUR) in perennial ryegrass plants grown for twelve weeks with different soluble P supply rates. Open symbols and full line, nonmycorrhizal plants; closed symbols and dashed line, mycorrhizal plants. Soluble P treatments: nil (0 mM; ○●), low (0.02 mM; □■), intermediate (0.1 mM; Δ▲) and high (0.5 mM; ◇♦). Correlation coefficients were 0.95 (P = 0.022) for the nonmycorrhizal and 0.94 (P = 0.030) for the mycorrhizal plants. Line intercepts were not different from zero (P > 0.4). Point values are slopes ± SE of the regression lines corresponding to three harvests.

Table II.1. Results of two-way ANOVA for the effects of arbuscular mycorrhizal fungus (AMF) and time on C and P plant masses. Associated mean squares (MS) and number of degrees of freedom (df) for each source of variation are given. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns $P > 0.05$) within soluble P treatments. Significant AMF × time interaction denotes differences in relative growth rate (RGR) and relative phosphorus uptake rate (RPUR) within soluble P treatments.

		Plant C mass – MS values				Plant P mass – MS values			
S. of Var.	df	0 mM	0.02 mM	0.1 mM	0.5 mM	0 mM	0.02 mM	0.1 mM	0.5 mM
AMF	1	0.072 ns	0.491 ***	1.074 ***	1.063 ***	0.015 ns	2.583 ***	1.411 ***	0.987 ***
Time	1	0.525 *	1.141 ***	3.579 ***	5.459 ***	0.349 ns	3.379 ***	2.828 ***	3.337 ***
AMF × T	1	0.007 ns	0.149 *	0.008 ns	0.190 *	0.002 ns	0.568 *	0.011 ns	0.170 **
Error	16	0.080	0.021	0.057	0.026	0.079	0.096	0.036	0.017

Effects of phosphorus nutrition on leaf morphology and C allocation

Leaf and plant morphology were closely related to RPUR. Increasing RPUR significantly increased LAR up to a plateau at intermediate P supply (Fig. II.4; $df_e = 41$; $P = 0.034$).

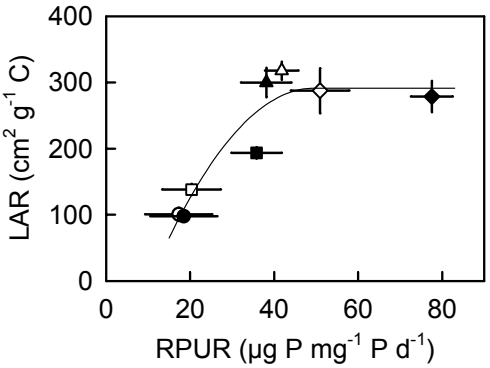

Figure II.4. Leaf area ratio (LAR) to relative phosphorus uptake rate (RPUR) in perennial ryegrass plants grown for twelve weeks with different soluble P supply rates. Open symbols, nonmycorrhizal plants; closed symbols, mycorrhizal plants. Soluble P treatments: nil (0 mM; ○●), low (0.02 mM; □■), intermediate (0.1 mM; Δ▲) and high (0.5 mM; ◇♦). Quadratic-plateau functions are shown for illustrative purposes. Values of LAR are means ± SE for plants of similar size (n = 4-8).

This plateau coincided with the attainment of minimum values of LMA (Fig. II.5a) and maximum values of LMR (Fig. II.6a). LMA decreased by 200% with increasing RPUR and was the main factor affecting LAR. This response was associated with changes in both density and thickness of the lamina tissues (Fig. II.5b, c; Table II.2).

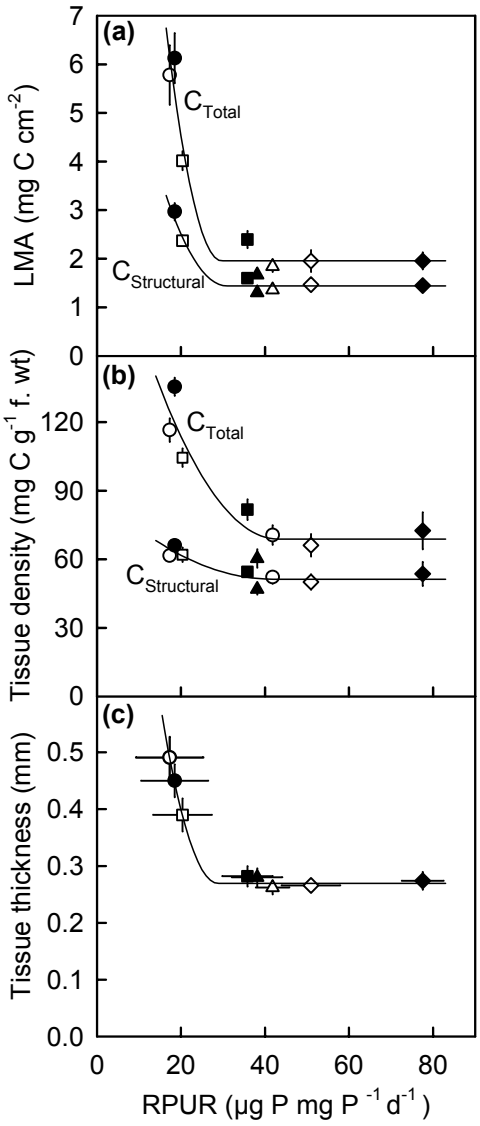

Figure II.5. Leaf mass per area (LMA) (a) and its components: lamina tissue density (b) and thickness (c), in relation to relative phosphorus uptake rate (RPUR) in perennial ryegrass plants grown for twelve weeks with different soluble P supply rates. Open symbols, nonmycorrhizal plants; closed symbols, mycorrhizal plants. Soluble P treatments: nil (0 mM; ○●), low (0.02 mM; □■), intermediate (0.1 mM; Δ▲) and high (0.5 mM; ◇♦). Structural C fraction was estimated as: $C_{Structural} = C_{Total} - (C_{Soluble} + C_{Starch})$. For further details see Table 3. Quadratic-plateau functions are shown for illustrative purposes. Values of *y*-axes are means ± SE for similar size plants (n = 4-8).

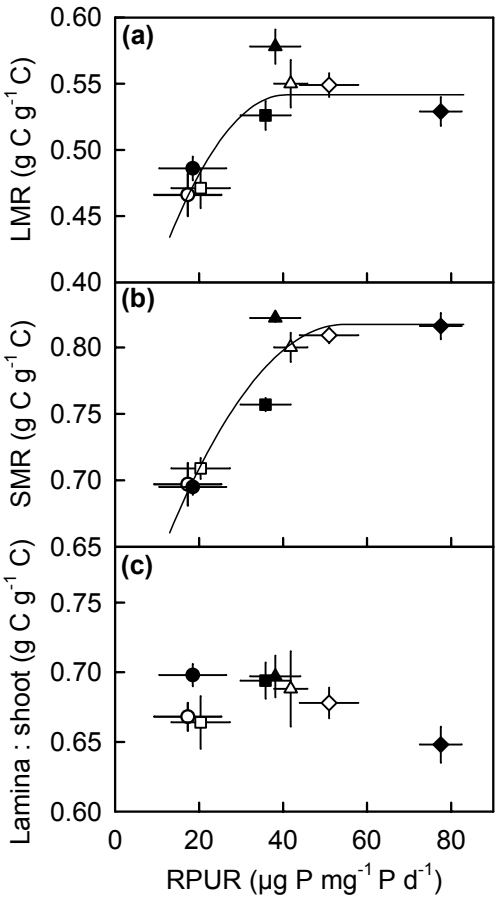

Figure II.6. Leaf mass ratio (LMR) (a) and its components: shoot mass ratio (SMR) (b) and lamina to shoot ratio (c), in relation to relative phosphorus uptake rate (RPUR) in perennial ryegrass plants grown for twelve weeks with different soluble P supply rates. Open symbols, nonmycorrhizal plants; closed symbols, mycorrhizal plants. Soluble P treatments: nil (0 mM; ○●), low (0.02 mM; □■), intermediate (0.1 mM; Δ▲) and high (0.5 mM; ◇♦). Quadratic-plateau functions are shown for illustrative purposes. Values of *y*-axes are means ± SE for plants of similar size (n = 4-8).

Table II.2. Results of two-way ANOVA for the effects of P supply rate and arbuscular mycorrhizal fungus (AMF) on different variables of similar size plants. (LAR) leaf area ratio, (LMR) leaf mass ratio, (SMR) shoot mass ratio, (LMA) leaf mass per area, (DEN) lamina tissue density. Associated mean squares (MS) and number of degrees of freedom (df) for each source of variation are given. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns $P > 0.05$).

		MS values				
S. of Var.	df	Shoot C : P	LAR	LMR	SMR	Lamina : shoot
P supply	3	9.177 ***	2.888 ***	1.016 ***	0.036 ***	0.001 ns
AMF	1	0.005 ns	0.036 ns	0.005 *	0.004 *	0.001 ns
P × AMF	3	0.052 *	0.110 *	0.003 **	0.003 *	0.002 ns
Error	41	0.017	0.035	0.001	0.001	0.001

S. of Var.	df	LMA_{Total}	$LMA_{Structural}$	DEN_{Total}	$DEN_{Structural}$	Thickness
P supply	3	2.898 ***	1.252 *	0.888 ***	0.131 ***	0.629 ***
AMF	1	0.188 *	0.137 ***	0.020 ns	0.006 ns	0.059 ns
P × AMF	3	0.238 **	0.118 **	0.106 **	0.030 ns	0.085 *
Error	35	0.039	0.024	0.022	0.015	0.023

In nonmycorrhizal plants, the concentration of soluble C decreased 3.5-fold between the lowest and highest P supply rates (Table II.3), explaining the sharp decrease in total tissue density (Fig. II.5b). Yet, a slight decrease (compared to the variation in total tissue density) in the structural tissue density was also detected at highest P supplies (Fig. II.5b; Table II.2). The decrease in soluble C at high RPUR was mainly due to decreases in WSC and soluble amino-C contents. Conversely, treatments had no effect on starch content, which was very low in all treatments (Table II.3). Lamina tissue thickness decreased with increasing RPUR, and was the main determinant of changes in structural LMA (Fig. II.5a, c). The increase in LMR between the lowest and highest value of RPUR was nearly 25% (Fig. II.6a), and was fully due to an increase in the shoot mass ratio (Fig. II.6b). RPUR had virtually no effect on the lamina to shoot ratio (Fig. II.6c; Table II.2).

A consistent feature of the above presented relationships was that they were virtually the same in mycorrhizal and nonmycorrhizal plants (Figs II.4-6). For instance, higher RPUR associated with presence of mycorrhiza at low P supply (0.02 mM) resulted in an increase of LAR and their components ($P < 0.01$) in the same way as in nonmycorrhizal plants (Figs II.4-6; Table II.3).

Table II.3. Effects of P supply and arbuscular mycorrhizal fungus (AMF) on fractions of non-structural C in leaf laminas: soluble C, water soluble carbohydrates (WSC-C), water soluble amino-C (Amino-C) and starch-C, in perennial ryegrass plants grown for twelve weeks with different P supply rates. Values are means ± SE for plants of similar size (n = 4-8). Associated mean squares (MS) and number of degrees of freedom (df) for each source of variation are given. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns $P > 0.05$).

Treatments		Leaf lamina content (mg C g^{-1} f. wt)			
P supply	AMF	Soluble C	WSC-C	Amino-C	Starch-C
0 mM	-	54.7 ± 4.7	23.4 ± 2.6	16.6 ± 2.3	0.3 ± 0.1
	+	68.7 ± 4.1	35.5 ± 2.3	12.7 ± 1.6	0.8 ± 0.1
0.02 mM	-	42.0 ± 3.5	17.8 ± 1.9	16.3 ± 2.7	0.5 ± 0.2
	+	27.1 ± 3.1	11.8 ± 1.2	11.8 ± 1.1	0.2 ± 0.1
0.1 mM	-	18.1 ± 2.3	7.2 ± 0.9	10.1 ± 0.6	0.4 ± 0.1
	+	13.1 ± 1.9	5.1 ± 1.2	8.7 ± 0.7	0.5 ± 0.2
0.5 mM	-	15.6 ± 2.4	6.4 ± 1.6	9.8 ± 1.1	0.4 ± 0.2
	+	18.4 ± 2.8	8.4 ± 1.5	10.8 ± 1.6	0.5 ± 0.3

Source of var.	df	MS values			
P supply	3	4.177 ***	5.717 ***	0.404 *	0.083 ns
AMF	1	0.101 ns	0.000 ns	0.226 ns	0.051 ns
P × AMF	3	0.352 *	0.573 *	0.065 ns	0.320 ns
Error	35	0.083	0.139	0.094	0.158

DISCUSSION

AMF affected plant growth, leaf morphology and carbon allocation solely through enhancement of phosphorus uptake

All effects of mycorrhizal symbiosis on plant growth, leaf morphology and C allocation resulted from the effect of AMF upon P capture. Thus, we observed no P status-independent effects of AMF on perennial ryegrass plants. RGR of mycorrhizal plants was significantly higher as a result of improved RPUR. Interestingly, this occurred at both low and high P supply rate. Perennial grasses are generally considered less dependent of mycorrhiza than legumes and other grassland species (Schweiger *et al*., 1995; Hartnett & Wilson, 2002), because they usually possess a highly branched root architecture with very long root hairs, which seems to render benefits from increases of P uptake rate by mycorrhizal symbiosis less likely (Jakobsen *et al*., 2005). However, there is evidence of a high degree of diversity in the functional effect of mycorrhiza in relation to environmental conditions and the identity of both partners involved in the relationship (Munkvold *et al*., 2004; Smith *et al*., 2004; Jakobsen *et al*., 2005). Enhancement of growth by AMF when P availability is high, although a counterintuitive result, has already been reported in perennial ryegrass growing in association with *Glomus* species (*e.g.* Powell & Daniel, 1978; Hall *et al*., 1984). In our experiment, AMF colonization of the roots was lower at high P supply. However, the presence of mycorrhiza continued playing a positive role on P uptake and plant growth. Thus, this suggests that the degree of AMF colonization was a poor indicator of AMF activity and its effect on the host plant (Son & Smith, 1988; Jongen *et al.*, 1996; Smith *et al*., 2004). More importantly, this indicates that no parasitism-like effect was apparent over the applied range of P supply rates. We can not exclude that higher P supplies might lead to mycorrhizal induced growth depressions, but this threshold was not achieved in our experimental conditions. Parasitic mycorrhizal associations may occur when environmental factors cause net costs to exceed net benefits; a effect that is most commonly reported at low light intensity or when defoliation exacerbates C stress (Smith & Smith, 1996; Johnson *et al*., 1997).

Decreases in LMA and WSC concentration, and increases in shoot mass ratio have often been reported in mycorrhizal plants (*e.g.* Buwalda & Goh, 1982; Son & Smith, 1988; Freeden & Terry, 1988; Jongen *et al.*, 1996; Wright *et al.*, 1998a; Müller *et al.*, 1999). Our results are in agreement with these observations. These responses have been commonly attributed to C stress and growth depression due to the presence of AMF in the roots. Interestingly, our results indicate that such responses were in fact indirect effects mediated by the improvement of the P nutrition of the plants. AMF is widely thought to consume 4-20% of the total photosynthates (Douds *et al.*, 1988; Jakobsen & Rosendahl, 1990). Although the present study did not attempt at constructing a C balance of the symbiosis, it does nonetheless indicate that for a given P capture level (*i.e.* RPUR), a determined C gain was made (RGR) irrespective of the presence of AMF. Therefore, any additional C cost of the AMF would seem to be counterbalanced, *e.g.* by reductions in metabolic costs associated with P uptake and utilization and/or enhancements of C assimilation rates (Douds *et al.*, 1988; Wright *et al.*, 1998a; Black *et al.*, 2000), so that an overall neutral effect on whole plant C accumulation was observed. How these effects are physiologically controlled and whether the cost-benefit relationship of the mycorrhizal symbiosis could be affected by severe C stress (*e.g.* imposed by defoliation through herbivory or mowing) merits further experimental investigation.

Effects of phosphorus nutrition status -as a function of either phosphorus supply or AMF- on leaf morphology and carbon allocation

The improvement of P nutrition, either by the addition of P fertilizer or by AMF inoculation, affected both components of LAR: LMA and LMR. Remarkably, increasing RPUR significantly affected LAR and its components up to a plateau, and from this point, leaf characters and C allocation patterns seemed to attain morphogenetic limits. Then, further changes of RGR are no more related to morphological adjustments, and might reflect increases in net assimilation rate. There is increasing evidence that C assimilation is influenced by the demand of the sinks (Farrar, 1992; Paul & Foyer, 2001; Pieters *et al.*, 2001). If this is the case, the improvement of P capture could have altered the

number of active meristems, and thus increase the sink strength (Pieters *et al*., 2001). In our study, the increase in LMR was fully due to an increase in shoot mass ratio, representing a common response associated with increasing P supply rates (*e.g.* Baas & Lambers, 1988; Wright *et al.*, 1998a; de Groot *et al*., 2001), as lamina to shoot ratio is a quite conserved quantity in grass species. However, LMA was decreased to a much greater extent than the positive change in LMR. Therefore, our results strongly suggest that the adjustment of leaf morphology (LMA) rather than plant C allocation (LMR) was the main factor explaining differences in LAR as affected by P supply in perennial ryegrass.

This study revealed strong effects of P nutrition status on both components of LMA: tissue density and thickness. LMA was 2-fold lower under high P supply in comparison to P deficiency conditions. High LMA is often associated with P deficiency due to high concentrations of non-structural compounds (Rao *et al*., 1989; Ryser *et al*., 1997; de Groot *et al.*, 2001). Variation in leaf thickness has been reported in response to changes in light intensity and nutrient availability (Witkowski & Lamont, 1991; Meziane & Shipley, 1999), but its functional determination as related to changes in environmental conditions is still unclear. In our experimental conditions, both LMA components varied in the same direction with increasing P, thus resulting in leaves with lower tissue density and thickness. However, there was a major difference in the response of these two variables. On one hand, more than 90% of the change in tissue density was due to variations in the content of water soluble C compounds. Thus, apparently, P deficiency affected more profoundly C use than C assimilation (as in Radin & Eidenbock, 1986; Rao *et al*., 1989), leading to the reported increase in lamina density by accumulation of WSC (likely in form of fructans). In agreement, we found that P deficiency markedly reduced relative cell expansion rates (Kavanová *et al*., 2006a). Additionally, the slight increment in the structural density could be related to a decreased cell size (Radin & Eidenbock, 1986), which might increase the proportion of cell walls per unit of cell volume.

On the other hand, changes in tissue thickness are inherently associated with responses in structural tissues. Therefore, while the increase in tissue density in response to P deficiency was a consequence of a passive accumulation of soluble C, changes in thickness must have been entirely

associated with active responses of the growing tissue. In the present study, thickness was measured as the amount of water per unit leaf area (i.e. tissue thickness as defined here). The increase in water per unit area observed under P deficiency could be associated with a decrease in the amount of mesophyll intercellular spaces. Restricted cell expansion under low P availability might thus produce a more compact tissue, with more water per unit area. Decreases in LMA, either by having thinner leaves or leaves with lower tissue density, enhance light interception and photosynthetic capacity per unit of leaf mass (Lambers & Poorter, 1992; Reich *et al*., 1998; Poorter & Evans, 1998). Leaf morphological changes affect nutrient distribution per unit of leaf area, which in turn affects the productivity of nutrients allocated into leaves (Poorter & Evans, 1998). Ryser *et al.* (1997) reported a close relationship between productivity of leaf P and the ability to distribute it over a large leaf area. Interestingly, our results indicate that AMF colonization would affect such relationships by the improvement of the P status of the plants.

CONCLUSIONS

All effects of mycorrhizal symbiosis on plant growth observed in this study were strictly correlated with effects of AMF upon phosphorus capture. Furthermore, the relationships between RPUR and all analysed leaf morphological characters, and between RPUR and plant carbon allocation, were very similar in mycorrhizal and nonmycorrhizal plants. Hence, no evidence for phosphorus status-independent effects of AMF was found on undisturbed perennial ryegrass plants.

Adjustment in leaf morphology (LMA) rather than plant carbon allocation (LMR) was the main observed response. Higher LMA in phosphorus deficient plants was caused by both increased tissue density and thickness. However, variation in tissue density was almost entirely due to passive variations in soluble carbon, while that in thickness involved structural changes.

CHAPTER III. EFFECTS OF ARBUSCULAR MYCORRHIZA ON CARBON ECONOMY IN PERENNIAL RYEGRASS: QUANTIFICATION BY $^{13}CO_2/^{12}CO_2$ STEADY-STATE LABELLING AND GAS EXCHANGE [2]

ABSTRACT

- Effects of the arbuscular mycorrhizal fungus (AMF) *Glomus hoi* on carbon economy of perennial ryegrass (*Lolium perenne* L.) were investigated by comparing nonmycorrhizal and mycorrhizal plants of the same size, morphology and phosphorus status.

- Plants were grown for 10 wk in the presence of CO_2 sources with different C isotope composition ($\delta^{13}C$ –1‰ or –44‰). Relative respiration and gross photosynthesis rates, and below-ground allocation of C assimilated during one light-period ('new C'), as well as its contribution to respiration, were quantified by the concerted use of $^{13}CO_2/^{12}CO_2$ steady-state labelling and $^{13}CO_2/^{12}CO_2$ gas exchange techniques.

- AMF (*G. hoi*) enhanced relative respiration rate of the root+soil system by 16%, inducing an extra C flow amounting to 3% of daily gross photosynthesis. Total C flow into AMF growth and respiration was estimated at less than 8% of daily gross photosynthesis. This was associated with a greater amount of new C allocated below-ground and respired in mycorrhizal plants. AMF colonization affected the sources supplying below-ground respiration, indicating a greater importance of plant C stores in supplying respiration and/or the participation of storage pools within fungal tissues.

- When ontogenetic and nutritional effects were accounted for, AMF increased below-ground C costs, which were not compensated by increased photosynthesis rates. Therefore, the instantaneous relative growth rate was lower in mycorrhizal plants.

[2] Grimoldi A.A., Kavanová M., Lattanzi F.A., Schäufele R. & Schnyder H. (2006) *New Phytologist* (in press).

INTRODUCTION

As obligate symbionts, arbuscular mycorrhizal fungi (AMF) entirely depend on the supply of C substrates from the host plant (Ho & Trappe, 1973), and therefore are an integral part of the C economy of colonized plants. For instance, AMF typically enhance below-ground demand for photoassimilates (Snellgrove *et al.*, 1982; Harris *et al.*, 1985; Douds *et al*., 1988; Jakobsen & Rosendahl, 1990; Eissenstat *et al*., 1993). In some scenarios, the proportion of C flow to the AMF was large enough to slow down plant growth (Johnson *et al*., 1997). More generally, the C cost of the fungus seems overcompensated by the beneficial terms of the symbiosis, which mainly consists in the improvement of nutrient uptake (principally P, but also NH_4, Zn, Cu and other micronutrients) and water status (Koide, 1991; Marschner, 1995; Smith & Read, 1997), increases in plant tolerance to various kinds of stress (Smith & Read, 1997) and protection against root pathogenic fungi (Newsham *et al.*, 1995). In the grass species *Lolium perenne*, we have recently shown that AMF effects on plant morphology (Grimoldi *et al.*, 2005) and leaf growth (Kavanová *et al.*, 2006a) were largely mediated by the improvement of the phosphorus nutrition status. Some authors reported that AMF could cause an enhancement of photosynthesis rates not mediated by nutritional effects (Brown & Bethlenfalvay, 1988; Wright *et al*., 1998a; Miller *et al.*, 2002), which was attributed to the additional sink activity of the fungus. But this effect, which counterbalanced part of the symbiotic costs, seems not to be general (*cf* Snellgrove e*t al.*, 1982; Freeden & Terry, 1988; Douds *et al*., 1988; Bass *et al.*, 1989; Pearson & Jakobsen, 1993; Black *et al*., 2000; and references therein).

The C flow to the AMF is equivalent to anything between 4 and 20% of daily gross photosynthesis (Smith & Read, 1997). This variability in C demand of the AMF seems to be related to the particular identity of the partners involved in the symbiosis (Pearson & Jakobsen, 1993; Lerat *et al.*, 2003; Heinemeyer & Fitter, 2004; Munkvold *et al*., 2004; Smith *et al*., 2004; Jakobsen *et al*., 2005). But the actual cause(s) behind it are still unclear, as the mechanistic understanding of how

plants regulate carbon partitioning to the fungal partner is far from complete (Fitter, 2005). In part, this is due to methodological problems in assessing daily gross photosynthesis, and the share and sources of C substrates taken by the AMF. Further, it is of no minor importance that experimental designs are used that disentangle the effects of AMF from those of ontogenetic and nutritional status (Staddon *et al.*, 1999; Heinemeyer & Fitter, 2004; Grimoldi *et al*., 2005).

The present study investigates the effects of the AMF *Glomus hoi* on the carbon economy of perennial ryegrass (*Lolium perenne* L.). The specific aims were: (*i*) to estimate the C flow to the AMF, (*ii*) to analyse the influence of the AMF on the amount of recently assimilated C (termed 'new C') allocated below-ground and *(iii)* to quantify the importance of new C in supplying respiration. To this end, the carbon balance components of nonmycorrhizal and mycorrhizal plants of the same size, morphology and phosphorus status were assessed by the concerted use of $^{13}CO_2/^{12}CO_2$ steady-state labelling and $^{13}CO_2/^{12}CO_2$ gas exchange techniques so as to (*i*) label all photosynthate assimilated during one light period, and (*ii*) follow the contribution of new (labelled) and old C (unlabelled) to above- and below-ground respiration over the next dark period. To the best of our knowledge, this is the first whole plant C balance comparing AMF effects in a grass species. We also provide the first quantitative analysis of allocation and respiratory use of new C as affected by mycorrhizal symbiosis.

MATERIALS AND METHODS

Plant material, AMF inoculation and growth conditions

Seeds of perennial ryegrass (*Lolium perenne* L. cv. Condesa) were surface sterilized for 20 min in NaOCl (6% active chlorine) and sown into pots (diameter 5 cm, depth 35 cm). Pots were filled with quartz sand (0.3−0.8 mm) and fertilized with fine powdered Hyperphos (63 mg P per pot). Half of the pots were inoculated with the AMF *Glomus hoi* BEG 104 (provided by Dr. A. Heinemeyer, University of York, UK). For each pot of the AMF treatment, 15 mL of the inoculum, consisting of a mixture of

dead fine roots of mycorrhizal *Plantago lanceolata* and fine sand, was mixed thoroughly with the quartz sand (Grimoldi *et al.*, 2005). Pots with and without AMF inoculum were placed in separate plastic containers, to prevent colonization of the nonmycorrhizal plants with AMF.

Plants were grown for 10 wk in two controlled environment chambers (E15, Conviron, Winnipeg, Canada) with 80% relative humidity, 20/15°C (day/night) and 525 μmol m^{-2} s^{-1} photosynthetic photon flux density at plant height for 16 h d^{-1}. Two containers (−AMF and +AMF) were placed in each chamber. Plants were watered by an automatic irrigation system supplying, to each plant, four times a day, 25 ml of a modified half-strength low-P Hoagland's solution (0.02 mM KH_2PO_4, 2.5 mM KNO_3, 2.5 mM Ca $(NO_3)_2$, 1 mM $MgSO_4$, 0.98 mM KCl, 0.5 mM NaCl, 0.125 mM Fe-EDTA, 23 μM H_3BO_3, 4.5 μM $MnSO_4$, 0.38 μM $ZnSO_4$, 0.16 μM $CuSO_4$, 0.05 μM Na_2MoO_4). Weekly, pots were flushed with distilled water, to prevent salt accumulation.

Sampling protocol

All variables were analysed in plants of similar size (1.1–1.5 g C $plant^{-1}$) selected from a large group of plants in order to isolate AMF effects from ontogenetic drift and phosphorus status. Immediately after harvest, roots were freed from the soil substrate by washing with tap water. The washing caused the loss of most of the extraradical mycelium of the AMF. A sample of the fresh root material was weighed and used for detection of AMF colonization. Shoots were separated into individual tillers, which were counted and dissected into lamina and sheath. The area of the laminas was measured with a LI-3100 leaf area meter (Li-Cor Inc., Lincoln, NE, USA). Leaf area ratio (LAR; cm^2 g^{-1} C) was determined as total leaf area divided by plant C mass, and specific leaf area (SLA; cm^2 g^{-1} C) as plant leaf area divided by lamina C mass. Samples were frozen in liquid nitrogen, freeze-dried, weighed, ground and stored at −25°C.

Chemical and isotopic analyses, and AMF colonization

The concentrations of C and N and $^{13}CO_2/^{12}CO_2$ isotope ratios were determined on aliquots of 0.7 mg dry ground material using an elemental analyser (NA1110, Carlo Erba Instruments, Milan, Italy) interfaced to a continuous-flow isotope-ratio mass spectrometer (IRMS, Delta Plus, Finnigan MAT, Bremen, Germany). All C-isotope data are expressed in the conventional form: $\delta^{13}C$ sample = ($^{13}C/^{12}C$ in sample / $^{13}C/^{12}C$ in the VPDB standard – 1) × 1000. Phosphorus concentration was determined on 25 mg dry weight aliquots, ashed in a muffle furnace (4 h at 500°C), and then digested in HNO_3/HCl. Phosphorus was quantified by phosphovanado-molybdate colorimetry (Hanson, 1950). Water soluble carbohydrates were analysed as in Schnyder & de Visser (1999). The content of C in water soluble carbohydrates was estimated as total hexose units times 0.4.

Mycorrhizal colonization of roots was determined by histological detection of mycorrhizal structures after root staining as in Grimoldi *et al.* (2005). Briefly, a sample of fresh material was cleared in KOH (10% w/v) for 10 min at 105°C, acidified in HCl (1% v/v) for 5 min, and then stained with Trypan Blue (0.05% w/v; Sigma-Aldrich, Steinheim, Germany) in acid glycerol for 10 min at 105°C. The percentage of root length with AMF colonization was determined in glycerol-gelatine-mounted roots by evaluating 100 random intersections for each plant, using the gridline interception method (Giovannetti & Mosse, 1980).

$^{13}CO_2/^{12}CO_2$ labelling procedure

The $^{13}CO_2/^{12}CO_2$ labelling system described by Schnyder *et al.* (2003) was used. During the whole experiment, one growth chamber received CO_2 from a mineral source ($\delta^{13}C$ –1‰), and the other CO_2 from an industrial source ($\delta^{13}C$ –44‰; both CO_2 sources from Linde AG, Höllriegelskreuth, Germany). The system allowed an independent control of CO_2 concentration (360 µl L^{-1}), and isotopic

composition ($\delta^{13}C$ +3‰ or –40‰ at the outlet of the growth chambers). As an example, the isotopic compositions ($\delta^{13}C$; ‰) of shoot biomass of nonmycorrhizal plants continuously grown in the same chamber (end members) were –22.2 ± 0.3‰ and –64.6 ± 0.2‰ respectively. Plant tissue signatures were more negative and thus contained relatively less ^{13}C in their C than the atmospheric CO_2, due to ^{13}C discrimination (Farquhar *et al.,* 1989). After 10 wk growth, carbon assimilation was quantified by labelling the plants with the different CO_2 isotopic sources. For this, plants were swapped between chambers (mineral → industrial CO_2, and vice versa) shortly before the lights went on and labelled during one entire light period (16 h) as described by Lattanzi *et al.* (2005). Labelling for one light period caused a shift of the $\delta^{13}C$ of total plant biomass of nonmycorrhizal plants of approx. 4‰, which was equivalent to *ca.* 10% new C in total biomass. The δ-shift brought about by labelling was virtually the same in the different chambers and approximately ten times larger than the variability among plants.

Respiratory $^{13}CO_2/^{12}CO_2$ exchange system and measurement

The respiration measurement system has been described in detail by Lötscher *et al.* (2004) and Klumpp *et al*. (2005). The system allowed a simultaneous measurement of dark respiration rate and isotopic composition of respired CO_2, of shoot and root+soil compartments, of four individual plants. Respiration measurements were performed at dark period temperature (15°C). Respiration rates of unlabelled and labelled plants were measured during the dark period following the labelling light-period, allowing quantification of the contribution of 'new' (labelled) and 'old C' (unlabelled) to the total respiration rate (see below). Pots were removed from chambers at the beginning of the dark period, flushed with 0.5 l distilled water and rinsed with CO_2-free nutrient solution, removing all CO_2 from the root+soil compartment. System tests demonstrated that up to 1 h was required to purge the system from all extraneous CO_2 (Klumpp *et al*., 2005). Dark respiration rates were measured every 38

min for 5 to 6 h, after which plants were harvested. This procedure was performed during four subsequent days, and every day one unlabelled and one labelled individual plant from each treatment (–AMF and +AMF) were included. Thus, labelled target plants (–AMF and +AMF) were measured in pairs, and a daily whole carbon balance was constructed for each individual plant (see below).

Gas exchange parameters and carbon balance

C isotope data ($\delta^{13}C$) were used to calculate the fraction of new C incorporated into biomass and respired during a complete diurnal cycle. The fraction of new C in biomass ($f_{B\ new}$) was calculated as in Schnyder & de Visser (1999): $f_{B\ new} = (\delta_P - \delta_{PO}) / (\delta_{PL} - \delta_{PO})$, where δ_P is the δ of a given sample from a labelled plant, δ_{PL} and δ_{PO} are the δ of the same tissue collected from plants continuously grown in the labelling chamber (to which the plant was transferred for labelling during one light period) and in the chamber of origin (in which the plant was grown before the transfer). In the same way, the fraction of new C in the respired CO_2 ($f_{R\ new}$) was calculated as: $f_{R\ new} = (\delta_R - \delta_{RO}) / (\delta_{RL} - \delta_{RO})$, where δ_R is the δ of the respired CO_2 of a plant after one labelling light period, and δ_{RL} and δ_{RO} are the δ of the respired CO_2 of plants continuously grown in the labelling chamber and the chamber of origin, respectively.

^{13}C discrimination ($\Delta^{13}C$) provides information on the relative importance of stomatal conductance and photosynthetic capacity in limiting photosynthesis (Farquhar *et al*., 1989). Non-labelled plants from each chamber were used to determine $\Delta^{13}C$ as: $\Delta^{13}C$ (‰) = $[(\delta_{CO2} - \delta_{Biomass}) / (1000 + \delta_{Biomass})] \times 1000$ (Farquhar *et al*., 1989).

Respiration rate for the whole day (16 h light, 8 h dark) was calculated as: R (mg C d^{-1} $plant^{-1}$) = 16 R_{light} + 8 R_{dark}, where R_{light} and R_{dark} are the hourly respiration rates (shoot and root+soil) during the light and dark period, respectively. Respiration of root+soil (including the AMF) in the light period ($R_{light\ R}$) was estimated from R_{dark} of root+soil as: $R_{light\ R} = R_{dark\ R} \times Q_{10}^{(TL - TD)/10}$, where T_L and T_D are

the temperatures of the light and dark period, respectively. Shoot respiration in light ($R_{light\ S}$) was calculated accordingly, but values were corrected due to the inhibition of dark respiration by light observed at leaf (Krömer, 1995 and references therein) and canopy level (Schnyder *et al*., 2003). In the present experimental conditions, light caused a ~30% inhibition of shoot respiration in perennial ryegrass (Piel, Schäufele & Schnyder, unpublished). Thus, $R_{light\ S} = 0.7 \times R_{dark\ S} \times Q_{10}^{(TL - TD)/10}$. A Q_{10} of 2 for both shoot and root+soil respiration was used to account for short-term effects of the warmer conditions in the light than in the dark period (Amthor, 1989; Lötscher *et al.*, 2004; Lötscher & Gayler, 2005).

Relative respiration rates (RRR; mg C g^{-1} C d^{-1}) indicate respiration rate per unit C mass of shoot or root (including intraradical mycelium). The respiration rate of new (*i.e.* labelled) C during the dark period was calculated as: $R_{dark\ new}$ (mg C h^{-1} $plant^{-1}$) = $f_{R\ new} \times R_{dark}$. For the light period, the evolution of $f_{R\ new}$ was assumed to be linear, starting from zero at the beginning of the labelling period and reaching the value found during the subsequent dark period by the end of the light period (Lötscher & Gayler, 2005). Thus, $R_{light\ new} = 0.5 \times f_{R\ new} \times R_{light}$.

Gross photosynthesis rate (P_G; mg C d^{-1} $plant^{-1}$) was calculated for each plant as the mass of labelled C in the plant at the end of the dark period (C_{new}) *plus* labelled C respired during the whole day: $P_G = C_{new} + R_{light\ new} + R_{dark\ new}$. Relative gross photosynthesis rate (RPR; mg C g^{-1} C d^{-1}) is P_G per unit of total plant C mass (excluding extraradical mycelium). The instantaneous relative growth rate (RGR_i; mg C g^{-1} C d^{-1}) was calculated as: $RGR_i = (P_G - R_{light} - R_{dark})$ / plant C mass. Photosynthetic nutrient-use efficiencies for phosphorus (PPUE; g C g^{-1} P d^{-1}) and nitrogen (PNUE; g C g^{-1} N d^{-1}) were calculated as P_G divided by shoot nutrient mass.

Estimation of C flow to extraradical mycelium

Although the respiration rate of extraradical mycelium has been measured successfully (Heinemeyer *et al.*, 2006), direct measurements of *whole*-AMF respiration are (so far) not possible. However, there is good evidence that the surplus of RRR of mycorrhizal relative to nonmycorrhizal root systems is attributable to (maintenance and growth) respiration of the AMF (Baas *et al.*, 1989; Peng *et al.*, 1993; Nielsen *et al.*, 1998). Thus, the contribution of the AMF to the relative respiration rate of the root (RRR_{AMF}) can be estimated as: $RRR_{AMF} = RRR_{NMR} - RRR_{MR}$, where NMR and MR refer to nonmycorrhizal and mycorrhizal root systems, respectively. But, respiration rate is also closely related to tissue nitrogen content (Amthor, 1989), which differed slightly, but significantly, between root systems of mycorrhizal and nonmycorrhizal plants. We estimated RRR_{AMF} from RRR_{NMR} and RRR_{MR} of root systems normalized for nitrogen content, thus: $RRR_{AMF} = RRR_{MR} - (RRR_{NMR} \times [N_{MR}/N_{NMR}])$, where N refers to nitrogen concentration (mg N g^{-1} C) in the root system.

An estimate of the maximum total daily C flow to the AMF (F_{AMF}, mg C d^{-1}) was derived from the respiration rate of the AMF (R_{AMF}, where $R_{AMF} = RRR_{AMF} \times C_R$, and C_R is the total C in the root system) and a carbon use efficiency (CUE) of the AMF of 0.6. CUE_{AMF} is defined as production of fungal biomass (ΔC_{AMF}, mg C $plant^{-1}$) per unit of total C flow to the AMF. Thus, $CUE_{AMF} = \Delta C_{AMF} / F_{AMF}$; with $F_{AMF} = \Delta C_{AMF} + R_{AMF}$. Substitution and rearranging of the above equations yields $F_{AMF} = R_{AMF} / (1 - CUE_{AMF})$, showing that C flow to AMF can be obtained from respiration rate and knowledge of CUE. CUE (a synonymous term is 'apparent growth efficiency') is not a constant, but depends on maintenance respiration, relative growth rate and the efficiency of biosynthesis (Penning de Vries, 1975; Amthor, 1989). A maximum CUE of 0.6 (g biomass C per g substrate C utilized) has been found for many aerobic heterotrophs growing on a variety of substrates (Payne, 1970). Where growth is nil and all imported C is used in (maintenance) respiration, the $CUE_{AMF} = 0$.

The fraction of new C in the C flow to the AMF was assumed to be equal to that in respiratory CO_2 of the nonmycorrhizal plants. Thus, $F_{AMF\ new} = f_{R\ new} \times F_{AMF}$.

Statistical analyses

All data were examined for normality. Nutritional status and morphological variables were compared by *t*-test. Physiological measurements were performed during four subsequent days, and every day one unlabelled and one labelled individual plant from each treatment were included. Thus, all physiological parameters were analysed by *t*-test for paired samples, since all data of the daily whole carbon balance were derived from nonmycorrhizal and mycorrhizal paired-plants corresponding to the same day of measurements. Variables that involved percentages were arcsine square root transformed before analysis. Statistical analyses were performed using the statistical package STATISTICA version 6.0 (Stat Soft Inc., Tulsa, OK, USA).

RESULTS

AMF colonization, plant size and morphological traits

Plants inoculated with *G. hoi* averaged 32 ± 3% root length colonized. Noninoculated plants remained nonmycorrhizal. All plants were carefully selected to have a similar size. Thus, there was no difference in plant biomass ($df_e = 14$; $P = 0.71$) and total number of tillers per plant ($df_e = 14$; $P = 0.20$) between nonmycorrhizal and mycorrhizal plants (Table III.1). Also, there was no significant difference in plant morphological traits: leaf area ratio, root to shoot ratio and specific leaf area (Table III.1; $df_e = 14$; $P > 0.46$ for all variables).

Table III.1. Morphological traits of nonmycorrhizal (–AMF) and mycorrhizal (+AMF) perennial ryegrass (*Lolium perenne*) plants grown for 10 weeks at low soluble phosphorus supply. Differences were not significant ($P > 0.05$). Values are means ± SE (n = 8).

Variable	Treatments	
	–AMF	+AMF
Tillers per plant	14.7 ± 1.1	12.8 ± 0.8
Plant biomass (g C)	1.32 ± 0.07	1.36 ± 0.09
Leaf area ratio ($cm^2\ g^{-1}$ C)	161 ± 7	154 ± 5
Root : shoot ratio (g C g^{-1} C)	0.32 ± 0.02	0.34 ± 0.01
Specific leaf area ($cm^2\ g^{-1}$ C)	300 ± 15	293 ± 11

Respiration and carbon balance

Relative respiration rates (RRR) of the root+soil system were 16% greater in mycorrhizal than in nonmycorrhizal plants (Fig. III.1; $df_e = 6$; $P < 0.001$ for all measurements). No differences were found in RRR of the shoot compartment (Fig. III.1; $df_e = 6$; $P > 0.23$).

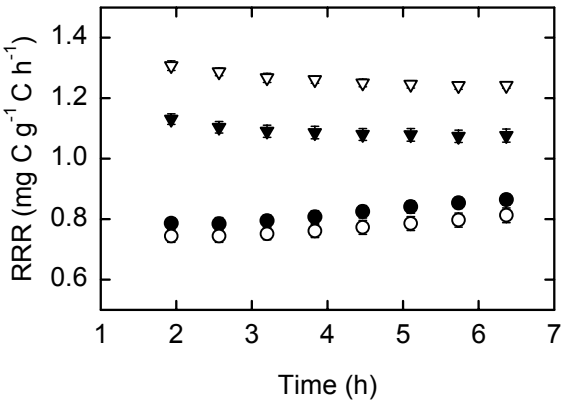

Figure III.1. Relative respiration rate (RRR) during one dark period (8 h, 15°C) in the shoot (circles) and the root+soil compartment (triangles) of perennial ryegrass (*Lolium perenne*). Closed symbols, nonmycorrhizal plants; open symbols, mycorrhizal plants. Values are means ± SE (n = 4).

Relative gross photosynthesis rate (RPR) tended to be higher in nonmycorrhizal compared with mycorrhizal plants (Fig. III.2; $df_e = 6$; $P = 0.09$). This was, in a small part, due to the loss of new C in the extraradical mycelium that was not recovered when harvesting the plants (see Materials & methods). The amount of new C flow to extraradical mycelium tissues was estimated to be 1.3 ± 0.1 mg C g^{-1} C d^{-1}. Accounting for this amount reduced the difference in RPR between mycorrhizal and nonmycorrhizal plants to 18% (Fig. III.2), which was not significant ($df_e = 6$; $P > 0.16$).

Instantaneous relative growth rate (RGR_i; mg C g^{-1} C d^{-1}) was lower in mycorrhizal than in nonmycorrhizal plants (Fig. III.2; $df_e = 6$; $P < 0.05$), due to the reduced relative gross photosynthesis rate (–18%), higher root respiration (+3%), and C flow to growth of extraradical mycelium tissues (estimated up to approx. 5% of plant carbon) in mycorrhizal plants. For this calculation, the amount of C (new and old) flow to extraradical mycelium tissues was estimated to be 4.1 ± 0.4 mg C g^{-1} C d^{-1} (Fig. III.2).

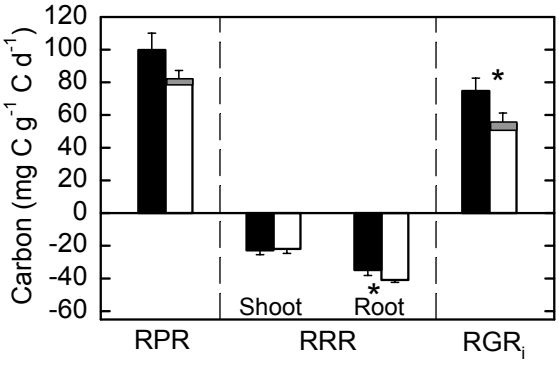

Figure III.2. Daily C balance of perennial ryegrass (*Lolium perenne*). Plants were harvested at the end of the dark period following the 16-h-light labelling period. Respiration rates were measured during the dark period (8 h, 15°C), and estimated for the preceding light period (16 h, 20°C). Each value of relative gross photosynthesis (RPR), relative respiration (RRR) and instantaneous relative growth (RGR_i) rates and estimated C cost of the production of AMF biomass correspond to the same individual plant. Closed bars, nonmycorrhizal plants; open bars, mycorrhizal plants; grey bar, estimation of the C cost of the production of AMF biomass. Values are means ± SE of labelled plants (n = 4). Significant differences: *, $P < 0.05$.

Allocation of new carbon

Most of the new C in both treatments was allocated to shoots (Fig. III.3), and the proportion tended to be higher in nonmycorrhizal compared with mycorrhizal plants (Fig. III.3; $df_e = 6$; $P < 0.08$). But, no difference ($df_e = 6$; $P = 0.54$) was found in the amount of new C allocated to shoot respiration (Fig. III.3). The amount of new C allocated below-ground was 15% higher ($df_e = 6$; $P < 0.05$) in mycorrhizal than nonmycorrhizal plants (Fig. III.3). This was due to an increased amount of new C respired below-ground, and, likely, flow of new C to growth of extraradical mycelium. The amount of new C retained in mycorrhizal roots was slightly, but not significantly, higher compared with nonmycorrhizal plants (Fig. III.3, $df_e = 6$; $P = 0.21$).

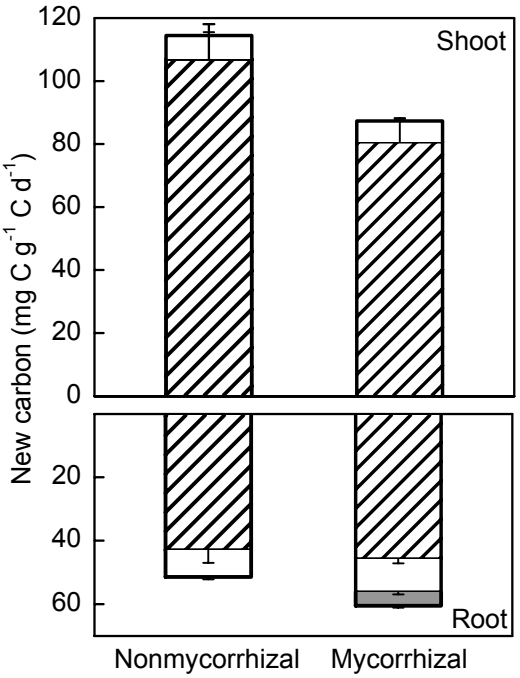

Figure III.3. Allocation of new C in perennial ryegrass (*Lolium perenne*). Shoot and root allocation are expressed per unit of organ C mass. Biomass and respiration data and estimated C cost of the production of AMF biomass correspond to the same individual plant. Plants were harvested at the end of the dark period following the 16-h-light labelling period. Respiration rates were measured during the dark period (8 h, 15°C), and estimated for the preceding light period (16 h, 20°C). Hatched bars, new C in end-of-day biomass; open bars, daily new C respired; grey bar, estimation of the C cost of the production of AMF biomass. Values are means ± SE of labelled plants (n = 4).

Fraction of labelled carbon in respired CO_2

The fraction of new C in respired CO_2 in the shoot and root+soil compartment was constant during the dark period in both AMF treatments (Fig. III.4). The presence of AMF did not affect this parameter in the respiration of the shoot compartment (Fig. III.4; $P = 0.56$). In contrast, the fraction of new C in respiration was lower in mycorrhizal roots compared with nonmycorrhizal ones (Fig. III.4; $P < 0.05$).

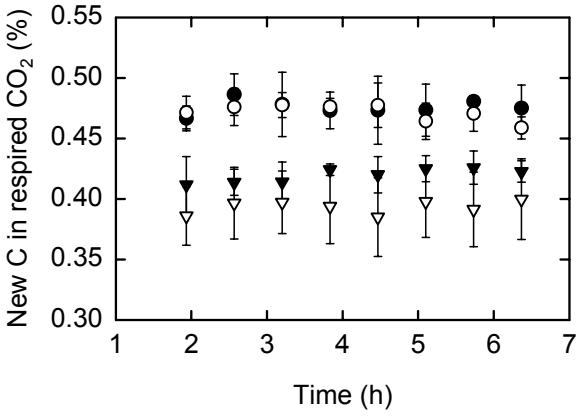

Figure III.4. Fraction of new C in respired CO_2 of one dark period (8 h, 15°C) in shoot (circles) and root+soil (triangles) of perennial ryegrass (*Lolium perenne*). Closed symbols, nonmycorrhizal plants; open symbols, mycorrhizal plants. Values are means ± SE of labelled plants (n = 4).

Plant nutrition and photosynthetic nutrient-use efficiency

The presence of AMF had no statistically detectable effect ($df_e = 14$; $P > 0.05$) on plant phosphorus content, expressed as shoot and root P : C ratios (w/w; Table III.2). Nitrogen concentrations in the shoot and root were 14% and 12% respectively higher in nonmycorrhizal compared with mycorrhizal plants (w/w; Table III.2; $df_e = 14$; $P < 0.05$). The N : P ratio of the total biomass was 14% higher in nonmycorrhizal compared with mycorrhizal plants. Concentrations expressed per unit structural C (*i.e.* when C in water soluble carbohydrates was subtracted from total C), showed these same relationships (data not shown). Mycorrhizal plants had a higher concentration of water soluble carbohydrates (mg C g^{-1} C) in leaf sheaths (–AMF: 204 ± 8 vs. +AMF: 265 ± 11; $df_e = 14$; $P < 0.001$). But there were no differences in water soluble carbohydrate concentration in roots (–AMF: 91 ± 9 vs. +AMF: 102 ± 10;

df_e = 14; P = 0.45). Photosynthetic nutrient-use efficiencies for phosphorus (PPUE; g C g^{-1} shoot P d^{-1}) and nitrogen (PNUE; g C g^{-1} shoot N d^{-1}) were slightly (+7 to +12%) higher in nonmycorrhizal plants, but again, these differences were not statistically significant (Table III.2; df_e = 6; P > 0.35 for both cases). ^{13}C discrimination ($\Delta^{13}C$; ‰), as expressed in total plant biomass, was identical in the two treatments (–AMF: 25.5 ± 0.1 ‰ vs. +AMF: 25.5 ± 0.2 ‰; df_e = 14; P = 0.99).

Table III.2. Nutritional status and photosynthetic nutrient use efficiency of nonmycorrhizal (–AMF) and mycorrhizal (+AMF) perennial ryegrass (*Lolium perenne*). Nutrient analyses were performed on eight plants per treatment. Photosynthetic nutrient-use efficiencies for phosphorus (PPUE) and nitrogen (PNUE) were calculated for each labelled plant as gross photosynthesis rate (P_G) per unit of shoot nutrient (n = 4). All values are means ± SE. Significant differences: *, P < 0.05.

	Treatments	
Variable	–AMF	+AMF
Shoot P (mg P g^{-1} C)	2.12 ± 0.16	2.01 ± 0.10
Root P (mg P g^{-1} C)	3.08 ± 0.37	3.38 ± 0.22
Shoot N (mg N g^{-1} C)	66.3 ± 2.2	58.3 ± 2.0 *
Root N (mg N g^{-1} C)	58.2 ± 1.7	52.2 ± 0.9 *
PPUE (g C g^{-1} shoot P d^{-1})	46.2 ± 6.2	43.2 ± 2.3
PNUE (g C g^{-1} shoot N d^{-1})	1.73 ± 0.17	1.55 ± 0.12

DISCUSSION

The demand for C substrates of mycorrhizal roots

Gas exchange measurements revealed an increased consumption of assimilates in below-ground respiration in mycorrhizal plants. When compared with nonmycorrhizal plants of the same size and phosphorus status, the presence of the AMF *G. hoi* enhanced relative respiration rate of the root+soil system of perennial ryegrass by 16% (Fig. III.1). This is not surprising, since 'extraradical mycelium + colonized roots' often have enhanced relative respiration rates compared with nonmycorrhizal roots (*e.g.* Baas *et al.*, 1989; Rygiewicz & Andersen, 1994), which has usually been attributed to the respiratory activity of the fungus. Root respiration rate reflects diverse processes, such as nutrient uptake, tissue biosynthesis, and maintenance of structures. But none of these seemed a plausible cause of the difference observed in the present study. The nitrogen status of mycorrhizal plants was lower than nonmycorrhizal plants (Table III.2), indicating that C costs associated with protein turnover and N uptake and assimilation were smaller than in nonmycorrhizal plants. Further, the amount of new C allocated below-ground and retained in root biomass was not affected by the presence of AMF (Fig. III.3), indicating that root growth rates were similar. These relationships all support the notion that the enhanced consumption of photoassimilates in below-ground respiration of mycorrhizal plants was related to the maintenance and growth respiration of the fungal partner.

In the present study, C flow to AMF accounted for 3% (estimated assuming a $CUE_{AMF} = 0$) to 8% (estimated using a maximum CUE of 0.60 of the AMF) of daily gross photosynthesis. Recently, Heinemeyer *et al.* (2006) reported an estimation of hyphal biomass production of *Glomus mosseae*, together with the first direct measurement of respiration rate of AMF external mycelium. From these values, it is possible to estimate a CUE of *ca.* 0.28 at low temperature and *ca.* 0.51 at higher temperature. Additionally, Peng *et al.* (1993) presented construction costs of citrus roots colonized by the AMF *Glomus intraradices*. From these values, assuming that 20% of the root system biomass correspond to intraradical hyphae (as suggested by the authors), it is possible to estimate a CUE of AMF intraradical hyphae of *ca.* 0.41 (see Fig. 5, Peng *et al.*, 1993). For our analysis, a CUE of 0.3 and

0.45 would yield a C flow to the AMF equivalent to 4.8% and 6% of daily gross photosynthesis, respectively. To the best of our knowledge, this is the first estimation of C flow to a mycorrhizal partner in a grass species, and it is at the low end of the range of published data. From our results, while the AMF (*G. hoi*) did affect the plant C balance, it represented a relatively small C cost for perennial ryegrass plants. This is because, first, the contribution of the AMF to the relative respiration rate of the root system was low compared to other species (16%), and second, below-ground respiration was a relatively minor component of the plant C balance, possibly due to the low fraction of root biomass (25%), and the optimal water and nutrient (other than phosphorus) status. In undisturbed plants of perennial ryegrass, the additional C drain of the AMF *G. hoi* seems to be easily over-ridden by the beneficial nutritional effects provided by the mycorrhizal symbiosis (Black *et al.,* 2000). In this species, the C-sink demand of AMF might be more significant in scenarios of C stress and higher root to shoot ratios as was suggested following events of intense defoliation (Johnson *et al.*, 1997). Whether this situation turns to the opposite when defoliation drastically decreases assimilation rate and increases the relevance of below-ground respiration, is currently under investigation.

The C flow from the host to the AMF has been reported to vary from 4 to 20% of total photoassimilates (Smith & Read, 1997). This wide range is in good agreement with the high variability reported in the increase in relative respiration rates induced by AMF colonization: white clover with AMF field-inoculum (+18%; Wright *et al.*, 1998b), cucumber with *G. caledonium* (+32%; Pearson & Jakobsen, 1993), citrus (+37%; Peng *et al*., 1993) and common bean with *G. intraradices* (+70%; Nielsen *et al.*, 1998), *Plantago major* with *G. fasciculatum* (+80%; Baas *et al.*, 1989). The actual causes behind the large variability in observed C demand of AMF are still unclear. On one hand, evidence is accumulating of a high functional diversity in the effect of AMF in relation to the identity of both partners (Pearson & Jakobsen, 1993; Lerat *et al.*, 2003; Heinemeyer & Fitter, 2004; Munkvold *et al*., 2004; Smith *et al*., 2004; Jakobsen *et al*., 2005), suggesting particular associations can be more (or less) C-costly for the plant. In this sense, perennial grasses are generally less colonized by AMF and considered less dependent of mycorrhiza than legumes and other grassland

species (Schweiger *et al*., 1995; Hartnett & Wilson, 2002), because they usually possess a highly branched root architecture with very long root hairs, which seems to render benefits from increases of P uptake rate by mycorrhizal symbiosis less likely (Jakobsen *et al*., 2005). In principle, our results agree with the general view that AMF are of relatively small importance for grass species (Hartnett & Wilson, 2002). Moreover, carbon flow to the AMF, a sink for C substrates, may be influenced by the relative activity, hierarchy and developmental stage of the different sinks within the plant (Farrar & Jones, 2000) and by other root symbionts (Brown & Bethlenfalvay, 1988). In this context, the (scarce) available estimations of turnover rate (Staddon *et al.*, 2003) suggest that the short half-life of fungal tissues could be a major factor controlling C drain. In an experiment in which plants were exposed to 'fossil' (^{14}C-dead)-CO_2, those authors found a half-life of hyphae that was less than four days, suggesting that the relative rate of fungal biomass production must be large to maintain the AMF. Why the turnover is so fast is unknown, and is perhaps the key for understanding the variability in C demand of the different plant-fungus combinations (Staddon & Fitter, 1998). Further experiments should assess this issue in controlled and natural conditions.

Consequences for plant growth

The overall effect of AMF on the growth of the host is believed to depend on the balance between the benefits of increased nutrient uptake and the C cost of the fungus (Koide, 1991; Eissenstat *et al.*, 1993; Johnson *et al*., 1997). It has been reported that AMF colonization could promote C assimilation rate even in the absence of an effect on plant nutritional status (Brown & Bethlenfalvay, 1988; Wright *et al.*, 1998a; Staddon *et al.*, 1999; Miller *et al.*, 2002). This was suggested to occur *via* the easing of a (hypothetical) sink limitation of photosynthesis. In this study, when nonmycorrhizal and mycorrhizal perennial ryegrass plants of similar size, morphology and phosphorus status were compared, AMF colonization did not enhance the rate of gross photosynthesis. This is in agreement with Black *et al.* (2000) who showed that increases in photosynthesis rates in cucumber plants were strictly limited to situations where the AMF improved plant phosphorus status.

The lower instantaneous relative growth rate of mycorrhizal plants seemed to be only partly due to C flow to the AMF. Approximately half of the difference in relative growth rate was the consequence of an 18% lower gross photosynthesis rate in mycorrhizal plants. This could not be ascribed to a specific effect of AMF on stomatal conductance or photosynthetic capacity: ^{13}C discrimination was high and virtually identical in the two treatments, suggesting that the relationship between stomatal conductance and photosynthetic capacity was very similar in the two treatments, and that photosynthesis was mainly limited by photosynthetic capacity in both (Farquhar *et al.*, 1989). The difference was neither due to light capture, because plants had the same leaf area (Table III.1), nor to differences in phosphorus concentrations, which were very similar in the two treatments. However, shoot nitrogen concentration was 14% lower in mycorrhizal plants, which compares favourably with the lower gross photosynthesis rate. The observation of a decreased N uptake was unexpected (Table III.2), as full nitrate-N supply was applied to both treatments, but it might be related to the fact that AMF seem unable to deliver nitrate-N to the host plant (Tanaka & Yano, 2005). Such AMF effects on nitrogen economy are not unequivocal, particularly in perennial grasses (Miller *et al.*, 2002; Reynolds *et al.*, 2005), and certainly require further study. In any case, no evidence for beneficial AMF effects on gross photosynthesis rate per unit nutrient (phosphorus or nitrogen) was apparent (Table III.2), confirming former results (Grimoldi *et al*., 2005; Kavanová *et al*., 2006a) indicating that AMF effects on perennial ryegrass are largely dependent on changes in nutritional status.

Allocation of new carbon and sources supplying below-ground respiration

The total amount of new C allocated below-ground was increased by the presence of AMF in otherwise very similar perennial ryegrass plants (Fig. III.3). This result, indicating AMF as a competitive sink, is even more remarkable in the light of the somewhat lower gross photosynthesis rate of mycorrhizal plants. None of this extra amount of new C allocated below-ground was in fact retained in root biomass. This result agrees with observations of Wright *et al*. (1998b). AMF increased partitioning of new C to respiration within the root system, as was previously suggested for

mycorrhizal barley by interpretation of enzyme analyses (Müller *et al.*, 1999). These results highlight the relevance of AMF as a C sink, indicating that AMF activity affected the distribution of newly available C within the plant.

Recent studies found that AMF provides a rapid pathway of C flux from plants back to the atmosphere (Johnson *et al.*, 2002; Staddon *et al.*, 2003; Heinemeyer *et al.*, 2006), which led Johnson *et al.* (2002) to suggest that AMF primarily use current plant photoassimilates. Our work adds some new information to this point. In spite of the higher allocation of new C to below-ground and to respiration, the fraction of new C in respired CO_2 was actually lower in mycorrhizal roots (Fig. III.4). Thus indeed, there was a differential use of new and old C sources in respiration, but it was in the opposite direction of that suggested by Johnson *et al.* (2002). Even though the total flux of new C respired below-ground was higher in mycorrhizal plants (due to the much higher amount of total respiration), the lower proportion of new C in respired CO_2 indicates either a greater importance of plant C stores in supplying the respiration process, and/or the existence (and involvement in respiration) of storage pools within AMF tissues. (note that this finding is not directly related to the turnover rate of the whole fungus biomass, as it only refers to the importance of storage pools in supplying respiration). Evidence for kinetically different C pools supplying respiration has been reported previously in plants (Farrar & Jones, 2000; Schnyder *et al.*, 2003). The higher contribution of a longer-term pool in mycorrhizal roots may result from the use of lipids (a major component of fungus metabolism) as a respiratory substrate.

CONCLUSIONS

AMF (*Glomus hoi*) colonization of otherwise very similar perennial ryegrass plants enhanced relative respiration rate of the root+soil system by 16%, and the C flow to the AMF represented 3% to a maximum of 8% of daily gross photosynthesis. This was closely associated with a greater amount of new C allocated below-ground and respired in mycorrhizal plants. AMF colonization affected the sources supplying below-ground respiration, indicating a greater importance of plant C stores in supplying respiration and/or the participation of storage pools within fungal tissues.

CHAPTER IV. SUMMARIZING DISCUSSION

The basic aim of this thesis was to disentangle phosphorus status-dependent and -independent effects of AMF on the components of plant growth: morphology and carbon economy components, in undisturbed perennial ryegrass plants. In a first phase (CHAPTER II), the study involved the assessment of phosphorus response functions of leaf morphological components and biomass allocation patterns. In a second phase (CHAPTER III), AMF effects on carbon economy were quantified by $^{13}CO_2/^{12}CO_2$ steady-state labelling techniques in plants with similar morphology and phosphorus content. Additionally, effects of phosphorus supply and AMF inoculation on leaf elongation rate and its components were evaluated (APPENDICES). In this section, results are summarized and discussed in the context of current knowledge about cost-benefit relationships of mycorrhizal symbiosis.

Cost-benefit relationships and specificity on mycorrhizal associations

The functional role of mycorrhizal symbiosis is frequently considered in relation to the current demand for phosphorus by plants and current soil availability (Koide, 1991), analysing nutritional benefits of the AMF versus increased carbon cost for the plant along a mutualism-parasitism continuum (Eissenstat *et al.*, 1993; Tinker *et al.*, 1994; Smith & Smith, 1996; Johnson *et al.*, 1997; Jones & Smith, 2004). Consequently, to understand the terms of the plant-fungus association it is necessary to assess the relationships between resource gain (benefits) by the plant and resource investment (costs) into the fungal partner. Basically, the terms of the relationship are the benefits arising from an enhanced ability to acquire phosphorus beyond the limits of the rhizosphere depletion zone by fungal hyphae versus the additional carbon cost for growth and maintenance of the fungal tissues.

Generally, the carbon cost of the fungus is overcompensated by the beneficial terms of the symbiosis (Smith & Read, 1997). The literature is replete with reports of increased growth of single plants associated with AMF colonization in pot and field experiments (Smith & Read, 1997; van der Heijden & Sanders, 2002). The cost for the production of a unit length of mycorrhizal hyphae are

normally considered much less than for a unit length of root (Koide, 1991; Smith & Read, 1997). However, evidence for parasitic relationships were also obtained (Buwalda & Goh, 1982; Eissenstat *et al.*, 1993; Peng *et al.*, 1993; Graham & Eissenstat, 1998). In situations of sufficient phosphorus supply and/or carbon stress, the costs for construction and maintaining mycorrhizal roots could exceed those of non-mycorrhizal ones (Koide, 1991; Peng *et al.*, 1993; Eissenstat *et al.*, 1993). Defoliation, low light and low temperature conditions, which can result in limitation of photosynthesis rates, are scenarios that could cause reduced growth of mycorrhizal relative to nonmycorrhizal plants (Smith & Smith, 1996; Johnson et al., 1997). In these scenarios, the AMF may function as a parasite.

It is known that arbuscules are the site of phosphorus transfer from fungus to plant, but the location of carbon exchange is still under discussion (Fitter *et al.*, 2000; Jakobsen, 2002; Fitter, 2005). Histochemical evidence and the fact that AMF colonization occurs effectively even without producing arbuscules suggest that the intercellular hyphae might be at least in part the location where C is lost from plant cells and absorbed by fungal tissues (Smith & Smith, 1996; Fitter *et al.*, 2000). Then, if carbon and phosphorus transfers are spatially dislocated, there is no basis to assume that they are fully metabolically coupled (Smith & Smith, 1996; Fitter *et al*., 2000; Jakobsen, 2002). This feature provides a possible explanation for the differences in cost-benefit relationships and the high functional diversity of AMF effects in relation to the identity of both partners (Smith & Smith, 1996; Fitter 2000). In this context, the importance of fungus-plant specificity and physiological variability in AMF interactions has been recognized only recently, and functional diversity in AMF has been currently analysed in much detail (*e.g*. van der Heijden *et al.*, 1998; Helgason *et al.*, 2002; Klironomos, 2003; Lerat *et al.*, 2003; Munkvold *et al*., 2004; Smith *et al*., 2004; Jakobsen *et al*., 2005).

Integration of results: phosphorus supply and AMF effects on the growth of perennial ryegrass

In the first experiment, relative growth rate of mycorrhizal perennial ryegrass plants was significantly higher as a result of improved phosphorus nutrition (Fig. II.2). The presence of mycorrhiza played a positive role on phosphorus uptake and plant growth both at low and high phosphorus supply rate (Fig. II.3). Accordingly, leaf elongation rate was linearly related to phosphorus status of the leaf growth zone (Kavanová *et al.*, 2006a; Appendix 1). Phosphorus status affected leaf growth of perennial

ryegrass directly through effects on cell division and relative tissue expansion rates (Kavanová *et al.*, 2006b; Appendix 2). When size effects were taken into account, no phosphorus independent effects of mycorrhiza on plant growth (Chapter II) and leaf elongation rate components (Kavanová *et al.*, 2006a and b; Appendices 1 and 2) were observed. Remarkably, perennial grasses are generally considered less dependent of mycorrhiza than legumes and other herbaceous dicot species of grassland (Schweiger *et al.*, 1995; Hartnett & Wilson, 2002). But, there is a general expectation that wild plant species would be less responsive to mycorrhizal symbiosis for phosphorus uptake than fast growing cultivated ones (Koide, 1991). Nutrient deficit, as defined by Koide (1991), is the combination of nutrient supply by the soil and nutrient demand by the target plant. On one hand, the demand for phosphorus by cultivated plants with high growth rates, as is the case of perennial ryegrass, normally is so high that it surpasses the uptake of phosphorus by the roots. Hence, the general result of this kind of association is expected to be a positive effect of AMF colonization (Koide, 1991; Smith & Read, 1997).

On the other hand, as was explained above, there is increasing evidence of a high degree of diversity in the functional effect of AMF in relation to the identity of both partners involved in the relationship (Munkvold *et al*., 2004; Smith *et al.*, 2004; Jakobsen *et al*., 2005). Concerning phosphorus exchange, it was observed that arbuscules in grass species were generally larger than in the non-grasses species, with consequent effects on the increase in volume of the plant cytoplasm and the surface area for phosphorus exchange between both symbionts (Smith & Read, 1997). In relation to the C cost of the fungi, the second experiment revealed that additional respiration was not a major expensive cost for undisturbed perennial ryegrass plants (Figs. III.1-2). In all mycorrhizal symbiosis the cost in terms of C assimilated is real in a physical sense, but the question to answer is whether paying the cost carries expensive penalties in terms of slower plant growth (Tinker *et al.*, 1994). It seems that this question has to be analysed for each type of plant-fungus association and environmental conditions. In undisturbed plants of perennial ryegrass, the additional C drain of AMF seems to be relatively easy to over-ride by beneficial nutritional effects provided by the mycorrhizal symbiosis (Black *et al.*, 2000). In this species, the C-sink demand of AMF might be more significant

in scenarios of C stress and higher root to shoot ratios as was suggested following events of intense defoliation (Johnson *et al.*, 1997). This last hypothesis needs further experimental work.

The amount of photosynthate available to the plant is dependent on the light intercepting area and the photosynthetic rate. Thus, relative growth rate (RGR) can be separated into leaf area ratio (LAR: leaf area per unit of plant mass) and assimilation rate (Hunt, 1978; Lambers *et al*., 1989). The first of which is composed of the leaf mass per area (LMA) and the fraction of plant mass present in leaves (LMR; leaf mass ratio). Any response of plants to stressful conditions normally involves an internal adjustment of resource allocation between and within the different organs. On that note, a difference between plant species in the ability to adjust plant morphology in response to nutrient supply provides further potential mechanisms underlying differences in responsiveness to mycorrhizal symbiosis (Johnson *et al.*, 1997; Jakobsen *et al.*, 2002). It was stated that due to the improvement of phosphorus nutrition, either by the addition of phosphorus to the soil or by the presence of arbuscular mycorrhizal symbiosis, it would be optimal for a plant to allocate a greater proportion of its resources to leaf area production (Baas & Lambers, 1988; Freeden & Terry, 1988). In our experiment, beneficial effects of mycorrhizal symbiosis were mainly mediated by adjustments in leaf morphology, which were largely dependent on AMF effects upon phosphorus capture (Figs. II.4-6). Accordingly, compilations of the available data concluded that there is a close association between leaf morphology and plant potential growth rate, and leaf mass per area (LMA) can thus be considered as a major factor determining differences in relative growth rates (Lambers & Poorter, 1992; Poorter & Nagel, 2002).

When morphological and nutritional effects are accounted for, AMF increased below-ground costs (Fig. III.1), which were not a major cost for undisturbed perennial ryegrass plants (Fig. III.2). However, this additional cost was not compensated by increases in photosynthesis rates, and therefore inevitably led to some degree of growth depression (Fig. III.2). Moreover, mycorrhizal colonization was closely associated with a greater amount of new C allocated below-ground and respired in mycorrhizal plants (Fig. III.3), indicating that mycorrhiza was an integral part of the C economy of perennial ryegrass plants. Despite the enhanced consumption of photoassimilates for below-ground respiration, photosynthetic nutrient-use efficiencies for phosphorus and nitrogen remained unaffected (Table III.2). Some authors reported that AMF might cause an enhancement of photosynthesis rates

not mediated by nutritional effects (Brown & Bethlenfalvay, 1988; Wright *et al*., 1998a; Miller *et al.*, 2002). In those studies, it was suggested that the stimulation of photosynthesis was due to the additional sink activity of the fungus, which counterbalanced part of the symbiotic costs. In the second experiment of this thesis, when nonmycorrhizal and mycorrhizal perennial ryegrass plants of similar size, morphology and phosphorus status were compared, AMF colonization did not enhance the rate of gross photosynthesis. This is in agreement with Black *et al.* (2000) who demonstrated that increases in photosynthesis rates in cucumber plants were strictly limited to situations where the AMF improved plant phosphorus status. Accordingly, many other reports have not shown evidence of changes in C assimilation rates induced by AMF (Snellgrove *et al.*, 1982; Freeden & Terry, 1987; Douds *et al.*, 1988; Bass *et al.*, 1989; Pearson & Jakobsen, 1993; Black *et al.*, 2000; Gavito *et al*., 2000; and references therein).

Conclusion and outlook

Relative growth and leaf elongation rates of mycorrhizal plants was significantly higher as a result of improved phosphorus nutrition. The relationships between relative phosphorus uptake rate, leaf and plant morphology were identical in mycorrhizal and nonmycorrhizal plants. Beneficial effects of mycorrhizal symbiosis were mainly mediated by adjustments in leaf morphology, which were largely dependent on AMF effects upon phosphorus capture. When ontological and nutritional effects are accounted for, AMF increased below-ground costs, which were not compensated by increased photosynthesis rates.

Ryegrass species are the most important grasses of temperate regions. They are intensively used for grazing and mowing in humid temperate grassland ecosystems in both hemispheres. Future research with plants suffering drastic increases in root to shoot ratios (*e.g*. caused by defoliation through grazing) and contrasting nutrient scenarios are needed to gain more insight into the trade-off between increased uptake of limited nutrients and C cost of AMF in perennial grasses. How the observed effects are controlled and whether the recruitment and persistence of the host is influenced by AMF colonization under field conditions also deserves further investigation.

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APPENDICES

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In the framework of this project and the experimental set-up of my thesis, I have contributed to the analyses of different aspects of the regulation of leaf growth in perennial ryegrass as affected by phosphorus supply and arbuscular mycorrhizal colonization. These analyses are part of the Doctoral studies of Monika Kavanová and the principal findings are described in the following articles:

Appendix 1:

Kavanová M, Grimoldi AA, **Lattanzi FA, Schnyder H. 2006a.** Phosphorus nutrition and mycorrhiza effects on grass leaf growth. P status- and size-mediated effects on growth zone kinematics. *Plant Cell & Environment* **29**: 511–520.

Appendix 2:

Kavanová M, Lattanzi FA, Grimoldi AA, Schnyder H. 2006b. Phosphorus deficiency decreases cell division and elongation in grass leaves. *Plant Physiology* **141**: 766–775.

Appendix 1

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Phosphorus nutrition and mycorrhiza effects on grass leaf growth. P status- and size-mediated effects on growth zone kinematics

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ABSTRACT

This study tested whether leaf elongation rate (*LER*, mm h^{-1}) and its components – average relative elemental growth rate ($REGR_{avg}$, mm mm^{-1} h^{-1}) and leaf growth zone length (L_{LGZ}, mm) – are related to phosphorus (P) concentration in the growth zone (P_{LGZ}, mg P g^{-1} tissue water) of *Lolium perenne* L. cv. Condesa and whether such relationships are modified by the arbuscular mycorrhizal fungus (AMF) *Glomus hoi*. Mycorrhizal and non-mycorrhizal plants were grown at a range of P supply rates and analysed at either the same plant age or the same tiller size (defined by the length of the sheath of the youngest fully expanded leaf). Both improved P supply (up to 95%) and AMF (up to 21%) strongly increased *LER*. In tillers of even-aged plants, this was due to increased $REGR_{avg}$ and L_{LGZ}. In even-sized tillers, it was exclusively due to increased $REGR_{avg}$. $REGR_{avg}$ was strictly related to P_{LGZ} ($r^2 = 0.95$) and independent of tiller size. Conversely, L_{LGZ} strictly depended on tiller size ($r^2 = 0.88$) and not on P_{LGZ}. Hence, P status affected leaf growth directly only through effects on relative tissue expansion rates. Symbiosis with AMF did not modify these relationships. Thus, no evidence for P status-independent effects of AMF on *LER* was found.

Key-words: arbuscular mycorrhizal fungi; *Glomus hoi*; leaf elongation rate; leaf growth zone; *Lolium perenne* L.; relative elemental growth rate

Abbreviations: AMF, arbuscular mycorrhizal fungus/fungi; *LER*, leaf elongation rate; L_{LGZ}, length of the leaf growth zone; P_{LGZ}, concentration of P in leaf growth zone; $REGR_{avg}$, average relative elemental growth rate; WSC, total water soluble carbohydrates.

INTRODUCTION

Phosphorus (P) is an essential macronutrient required for plant growth and development, but plants have to cope with limiting soil P availability in many terrestrial ecosystems (Schachtman, Reid & Ayling 1998). Low P availability activates a series of responses that maximize P acquisition or are directed to maintain the internal P homeostasis of the plant (Raghothama 1999; Ticconi & Abel 2004). An early response to P deficiency is the reduction of leaf growth rate, which is usually more pronounced than the reduction of root growth rate (Plaxton & Carswell 1999).

A widespread adaptive evolutionary response to low soil P availability is symbiosis with AMF, whose beneficial effects are usually manifested as improved P uptake and increased plant growth rate (Smith & Read 1997). As yet, the physiological bases of the AMF effect on plant growth are not fully understood. A still unsolved question is whether AMF affect the leaf growth directly by improving the P status of leaf meristems and growing leaves, or if other (additional or alternative) factors are involved.

Grasses offer a convenient system for studying the effects of P nutrition and AMF on leaf growth processes. This is because the complexity of two-dimensional leaf area expansion rate of dicotyledons is reduced to predominantly one-dimensional elongation. In grasses, leaf growth is confined to a short region which is located at its base, close to the point of attachment of the leaf to the stem base (Davidson & Milthorpe 1966; Kemp 1980a). This region was named 'leaf growth zone' and includes a short meristematic region that continuously produces cells that enter into the subsequent zone of cell expansion (Fig. 1).

The effects of diverse factors on leaf growth, including nutrient deficiency, can be studied using kinematic analysis (Silk & Erickson 1979; Silk 1984). In the spatial (Eulerian) approach of kinematic analysis, longitudinal profiles of relative elemental growth rate provide a measure of the magnitude of expansion and of its spatial location. *LER* (mm h^{-1}) is the definite integral of relative elemental growth rates (*REGR*, mm mm^{-1} h^{-1}) over the L_{LGZ} (mm) or, more simply, the product of $REGR_{avg}$ and L_{LGZ}. Assuero, Mollier & Pellerin (2004) found that in maize, the only monocot in which the effects of P deficiency on components of *LER* have been studied so far, the lower *LER* of P-deficient plants was mainly related to a shorter L_{LGZ} while $REGR_{avg}$ was only marginally decreased in P-deficient plants.

Nutrient deficiencies result in decreased grass tiller size, since *LER* and final leaf length are generally correlated (Fournier *et al.* 2005). Such a treatment-related change in

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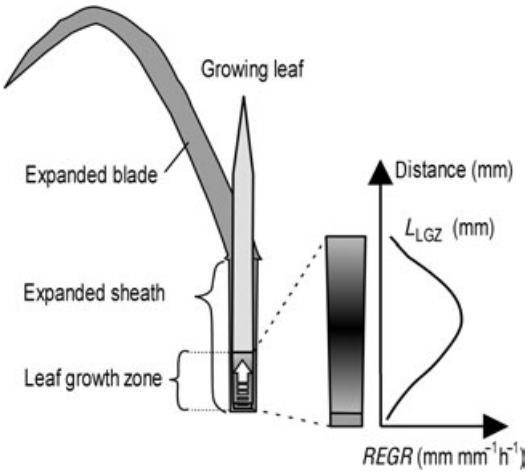

Figure 1. Schematic representation of the leaf growth zone in a grass tiller. Growth is limited to the basal part of the growing leaf (leaf growth zone) surrounded by the enclosing sheaths of expanded leaves. Cells are produced by an intercalary meristem and then enter the zone of cell expansion. Relative elemental growth rate (*REGR*, mm mm^{-1} h^{-1}) is a measure of the magnitude of tissue expansion per unit existing tissue. The location where *REGR* decreases to zero marks the end of leaf growth zone (L_{LGZ}, mm). Leaf elongation rate (*LER*, mm h^{-1}) is the definite integral of *REGR* from the beginning of elongation zone to L_{LGZ}, or more simply, the product of L_{LGZ} and the average *REGR* ($REGR_{avg}$).

tiller size might subsequently affect plant growth independently of the nutrient status (Niklas 1994). It is presently unknown whether the decrease in *LER* under P deficiency is related exclusively to a decreased plant P status or it is also partly related to effects mediated by the reduced tiller size. Interestingly, an allometric relationship between the length of the sheath enclosing the growing leaf and one of the *LER* components, L_{LGZ}, has been observed in a study of successive leaves in wheat (Kemp 1980b) and in eight grasses compared at different sheath lengths (Arredondo & Schnyder 2003). Furthermore, Casey *et al.* (1999) showed that artificial shortening of sheaths reduced *LER* by decreasing L_{LGZ}. Usually, studies of the effects of environmental or nutritional factors on leaf growth have not distinguished direct from size-dependent treatment effects. However, such a distinction is essential for understanding the mechanisms controlling leaf growth.

The aims of this study were: (1) to assess the effects of P supply on *LER* and its components, $REGR_{avg}$ and L_{LGZ}; (2) to verify if these parameters are related to growth zone P status; and (3) to investigate if these relationships are modified by AMF. *LER* and its components were determined in perennial ryegrass (*Lolium perenne* L. cv. Condesa) grown over a wide range of P supply, with and without AMF (*Glomus hoi*). In order to account for both P status- and tiller size-mediated effects, comparisons were made between the responses of even-aged plants with different tiller sizes and plants with even-sized tillers.

MATERIALS AND METHODS

Plant culture, AMF inoculation and growth conditions

Surface-sterilized seeds of perennial ryegrass (*L. perenne*) were sown in plastic pots (diameter 5 cm, height 35 cm) containing a mixture of quartz sand supplemented with fine powdered Hyperphos (63 mg P per pot), providing a source of P with low availability for all plants. Each pot contained one plant. Half of the pots were inoculated with AM fungus *G. hoi* (15 mL inoculum per pot). The inoculum consisted of a mixture of sand and roots originating from a single-spore pot culture of *G. hoi* BEG104 propagated on *Plantago lanceolata* L. Pots with and without inoculation were placed in separate containers ($76 \times 56 \times 37$ cm) in growth chambers. Two independent experiments were conducted.

Experiment 1: comparison at even age

The aim of this experiment was to analyse the response of *LER* and its components to P supply and AMF in even-aged tillers. Thus, plants were grown on different levels of soluble P supply for the same time period: 61–63 d after sowing (DAS). Growth room (VKZPH 005-120-S, Heraeus Vötsch, Balingen, Germany) conditions were 20/15 °C (day/night), 70% relative air humidity and 425 μmol m^{-2} s^{-1} photosynthetically active photon flux density (PPFD) at plant height for 16 h day^{-1}. In order to promote AMF colonization, all plants were initially (first 34 DAS) irrigated four times a day with 25 mL of modified P-free half-strength Hoagland's solution [2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM NaCl, 0.125 mM Fe-EDTA, 23 μM H_3BO_3, 4.5 μM $MnSO_4$, 0.38 μM $ZnSO_4$, 0.16 μM $CuSO_4$ and 0.05 μM Na_2MoO_4]. Thereafter and until the end of the experiment, four concentrations of soluble P (0, 0.02, 0.1 and 0.5 mM) in the form of KH_2PO_4 were supplied to both mycorrhizal and non-mycorrhizal plants.

Experiment 2: comparison at even size

The aim of this experiment was to analyse the response of *LER* and its components to P supply and AMF of plants with even-sized tillers, that is, similar sheath length. To this end, seeds for low-P plants were germinated 14 d in advance of seeds for high-P plants. Measurements were performed at 60–61 DAS in low-P plants and at 46–47 DAS in high-P plants when plants in the different treatments had mature tillers of similar size (sheath length of the youngest expanded leaf: 100 ± 11 mm). The plants were grown in four growth chambers (E15, Conviron, Winnipeg, Canada), with 20/15 °C (day/night), 70% relative air humidity and 525 μmol m^{-2} s^{-1} PPFD at plant height for 16 h day^{-1}. All plants were first irrigated for 21 DAS with the nutrient solution as described above, except that 0.02 mM KH_2PO_4 was included. Thereafter, two levels of soluble P supply were applied in both the mycorrhizal and non-mycorrhizal plants: 0.02 mM (low P) and 1 mM (high P).

AMF colonization

Root colonization by AMF was determined at 68 DAS in experiment 1 and at 63 DAS (low-P treatments) and 49 DAS (high-P treatments) in experiment 2. Colonization was estimated on roots stained with Trypan Blue by using the gridline intersect method (McGonigle *et al*. 1990). The root length colonized (%) is represented by the percentage of total intercepts where hyphae were present.

LER

Representative mature tillers (i.e. tillers having at least two fully expanded leaves) were selected for measurement of *LER* and leaf growth zone properties. Leaf elongation rate (*LER*, mm h^{-1}) was measured on the most rapidly growing leaf on a tiller. The measured leaf was the youngest visible leaf during the phase of its maximal expansion when *LER* was near constant (Schnyder *et al*. 1990). *LER* was determined as the rate of change of the distance between the tip of the elongating blade and the ligule of the youngest fully expanded leaf, which was measured daily with a ruler. The length of the sheath of the youngest expanded leaf was recorded at every measurement. In experiment 1, *LER* was measured on four plants per treatment at 61, 62 and 63 DAS. In experiment 2, five plants per treatment were measured at 60 and 61 DAS in the low-P treatments, and at 46 and 47 DAS in the high-P treatments. No difference in *LER* was observed between sampling dates ($P > 0.1$) or growth chambers ($P > 0.1$). Therefore, data from different sampling times and chambers were pooled.

Components of *LER*: L_{LGZ} and *REGR*

LER components were estimated by determining the growth spatial distribution within the leaf growth zone of mature tillers immediately after *LER* measurements on the same measured leaves using a pin-pricking method (Schnyder, Nelson & Coutts 1987). Briefly, 2 h after the start of the light period, a series of holes 3 mm apart along the basal 40–60 mm of a tiller was made with a fine needle. The plants were returned to the growth chamber for 4–6 h. Thereafter, distances between the holes both along the base of the growing leaf and along the non-growing surrounding sheath were measured with 0.1 mm accuracy. Leaves with the ligule located farther than 2 mm from the point of attachment were discarded, which assured that only the blade elongation was assessed.

Segmental elongation rate (SER_i, mm h^{-1}) was then calculated as:

$$SER_i = \frac{L_{i,t1} - L_{i,t0}}{\Delta t} \times \frac{LER_{control}}{LER_{pierced}}, \quad (1)$$

where $L_{i,\,t1}$ is the length of a segment delimited by two neighbouring holes in the growing blade (measured Δt, h, after pinning) and $L_{i,\,t0}$ is the length of the corresponding segment measured in the surrounding non-growing leaf sheath. SER_i was corrected by the ratio between the *LER* of a non-pierced leaf ($LER_{control}$, measured on the same leaf before pinning) and the *LER* of the pierced leaf ($LER_{pierced}$, determined as the sum of all SER_i along the leaf). This was done to account for the effects of pinning on *LER*. It has been repeatedly shown that growth reductions caused by pinning do not modify the relative distribution of growth rates and L_{LGZ} (Schnyder *et al*. 1987, 1990; Hu & Schmidhalter 2000), validating its use for the assessment of the spatial distribution of growth rates (e.g. Ben-Haj-Salah & Tardieu 1995; Fricke & Peters 2002; Assuero *et al*. 2004).

The velocity of displacement (V_i, mm h^{-1}) of a given segment i was calculated as the sum of elongation rates of all segments located more basally. The Richards function was fitted to each V_i profile (all fittings $r^2 \geq 0.99$; TableCurve 2D v.5.01, Systat, Richmond, CA, USA):

$$V_i = a \frac{1}{(1 + \exp^{(b-cx)})^{\frac{1}{d}}}, \quad (2)$$

where x is the distance from the leaf base, a is the asymptotic maximal V_i, and b, c and d are constants.

Relative elemental growth rate (*REGR*, mm mm^{-1} h^{-1}) was estimated as the first derivative of the fitted Richards function at the midpoint of each 3-mm-long segment:

$$REGR = \frac{a\,c \exp^{\left(c\frac{x}{d}+b\right)} (\exp^{(cx)} + \exp^{b})^{-\left(\frac{d+1}{d}\right)}}{d}. \quad (3)$$

L_{LGZ} (mm) was defined as the distance from the leaf base to the midpoint of the last segment where SER_i was positive. The average relative elemental growth rate ($REGR_{avg}$) was then determined as:

$$REGR_{avg} = \frac{LER_{control}}{L_{LGZ}}. \quad (4)$$

L_{LGZ} was alternatively calculated as the position where 95% of the a value (the asymptotic V_i predicted by the fitted Richards function) was reached:

$$L_{LGZ} = \frac{-\ln(20^d - 19^d)}{c} + \frac{d \ln 19}{c} + \frac{b}{c}. \quad (5)$$

The different estimations of L_{LGZ} gave near-identical results because of the high values of r^2 fittings of the Richards function (data not shown).

Sampling and chemical analyses

In experiment 1, five plants were sampled at the end of the dark period at 68, 74 and 83 DAS. A piece of tissue 1.7 times the length of the L_{LGZ} was dissected out from the base of elongating leaves of mature tillers similar to those used in *LER* measurements. In experiment 2, 12 plants were sampled at the end of the light period at 63 DAS in low-P and 49 DAS in high-P treatments, and leaf growth zones (L_{LGZ} determined by the pin pricking) were dissected out. The rest of the shoot tissue was pooled. In all cases, fresh weight was recorded and the samples were immediately frozen in liquid N_2, freeze-dried for 72 h at –80 °C, weighed, ground and stored at –25 °C before analyses.

P concentration was determined on 10–20 mg of pooled samples by a modified phosphovanado-molybdate colorimetric method following acid digestion (Hanson 1950).

Concentrations of C and N were determined on 0.7 mg samples with an elemental analyser (NA1500, Carlo Erba Instruments, Milan, Italy). In experiment 1, WSC were extracted from 2–10 mg of the sample and quantified as in Schnyder & de Visser (1999). In experiment 2, 20 mg of the sample were extracted as in Morvan-Bertrand *et al.* (2001), and WSC were quantified using a refractometer following separation by high performance liquid chromatography (HPLC) on a cation exchange column (Sugar-PAK, Millipore Waters, Milford, MA,USA). All concentrations are expressed on a tissue water basis (mg g^{-1} tissue water).

Experimental design and statistical analysis

Both experiments were complete two-way factorials. The first experiment consisted of two levels of AMF treatment and four levels of P supply arranged in a completely randomized design. The second experiment consisted of two levels of AMF treatment and two levels of P supply arranged in a randomized complete block (growth chambers) design. Analysis of variance (ANOVA) revealed no effect of growth chamber on *LER* ($P > 0.1$). In both experiments, the effects of P supply and AMF on the nutritional status and parameters of the kinematic analysis were then tested by two-way ANOVA, with the main factors P supply and AMF treatment. The relationships between P_{LGZ} and *LER* and its components were tested by linear regression analyses of treatment averages, and slopes and intercepts for AMF treatments were compared with *F*-test (Statistica 6.0, Statsoft, Tulsa, OK, USA). Results are shown as means ± 1 SE.

RESULTS

Plant growth at different levels of P supply and AMF treatment

When sampled at the same age (experiment 1), plants grown at low P supply and/or in the absence of AMF had substantially lower plant biomass (Grimoldi *et al.* 2005) and had mature tillers with shorter sheaths than plants grown at high P and/or in the presence of AMF (Fig. 2a). In contrast, and as aimed for, tiller sheath length did not differ among treatments when plants were sampled at different dates in experiment 2 (60 and 46 DAS) (Fig. 2b). Thus, the responses of *LER* and its components to P supply and AMF were analysed in plants where any possible tiller size-mediated effects could (experiment 1) or could not (experiment 2) interfere with P status-mediated effects.

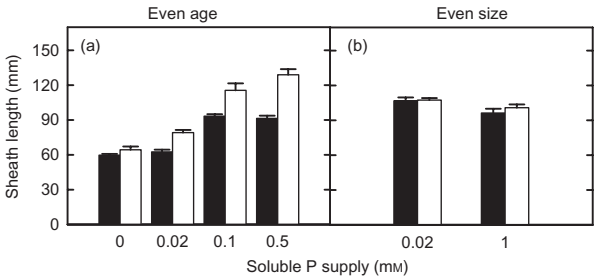

Figure 2. Sheath length of the youngest expanded leaf enclosing the growing leaf. Perennial ryegrass was grown under different levels of soluble P supply for (a) 61 DAS (even age) or (b) 60 DAS (0.02 mM P, even size) and 46 DAS (1 mM P, even size) in the absence (■) or presence (□) of AMF. Bars indicate ±1 SE ($n = 5$–12).

Spatial distribution of growth along the leaf base

The profile of velocity of displacement along the leaf growth zones had the expected form in all treatments. Velocity increased with distance from the leaf base until the end of the leaf growth zone, where it became constant and equal to *LER*. P deficiency consistently reduced the maximal displacement velocity, i.e. *LER*, by up to 60% in mature tillers of even-aged plants and by 45% in even-sized mature tillers ($P < 0.05$; Fig. 3a–c).

On the other hand, P deficiency had contrasting effects on L_{LGZ} in the two experiments. It shortened L_{LGZ} by up to 46% when mature tillers of even-aged plants were compared ($P < 0.05$). However, neither P supply nor AMF affected L_{LGZ} when even-sized tillers were compared ($P > 0.05$; Fig. 3c), even though they clearly differed in P_{LGZ}. Likewise, mycorrhizal plants had higher *LER* and longer

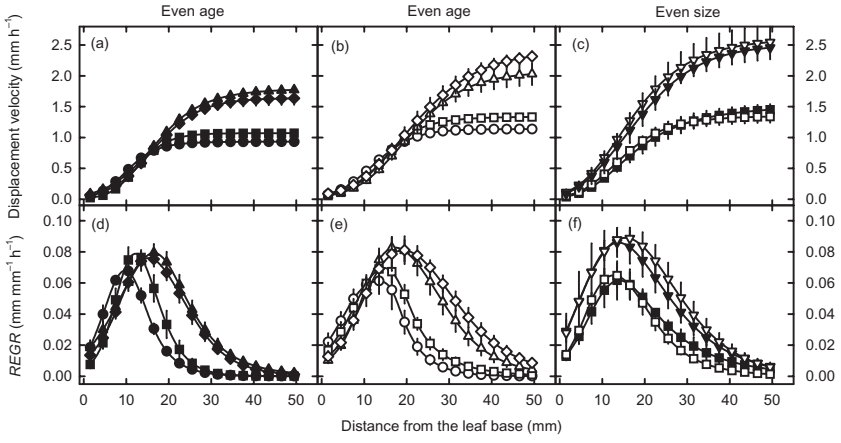

Figure 3. Spatial distributions of (a–c) displacement velocity and (d–f) relative elemental growth rate (*REGR*) along the leaf growth zone of the most rapidly growing leaf of perennial ryegrass tillers. Plants were grown with 0 mM (●○), 0.02 mM (■□), 0.1 mM (▲△), 0.5 mM (◆◇) or 1 mM (▼▽) soluble P supply in the absence (●■▲◆▼) or presence (○□△◇▽) of AMF. Bars indicate ±1 SE ($n = 5$–12).

Table 1. Nutrient status of perennial ryegrass plants. The effect of soluble P supply and presence (AMF+) or absence (AMF–) of the arbuscular mycorrhizal fungus *Glomus hoi* on the concentrations of P, N and C in the form of WSC, and total C in the leaf growth zone biomass (P_{LGZ}, N_{LGZ}, $C_{WSC\text{-}LGZ}$, C_{LGZ}, respectively). Values in brackets are SE ($n = 2$–10)

Experiment	AMF treatment	P supply (mM)	P_{LGZ}	N_{LGZ}	$C_{WSC\text{-}LGZ}$	C_{LGZ}
			mg g^{-1} tissue water			
Even age	AMF–	0	0.42 (0.02)	4.77 (0.22)	23.4 (0.7)	73.0 (4.3)
		0.02	0.67 (0.04)	6.23 (0.29)	24.9 (2.3)	59.6 (2.8)
		0.1	0.90 (0.05)	6.85 (0.18)	18.9 (1.7)	57.3 (2.3)
		0.5	0.89 (0.08)	6.35 (0.40)	17.0 (1.9)	50.4 (4.4)
	AMF+	0	0.47 (0.08)	5.07 (0.07)	22.0 (1.7)	63.5 (3.6)
		0.02	0.66 (0.05)	5.53 (0.26)	21.9 (2.3)	56.4 (4.2)
		0.1	0.83 (0.07)	5.97 (0.17)	20.7 (2.4)	55.8 (3.3)
		0.5	1.10 (0.05)	6.06 (0.37)	15.1 (1.2)	50.4 (3.1)
Even size	AMF–	0.02	0.71 (0.03)	6.97 (0.21)	46.0 (2.8)	71.8 (1.9)
		1.00	1.21 (0.11)	8.63 (0.24)	20.8 (4.3)	55.0 (1.5)
	AMF+	0.02	0.77 (0.10)	7.25 (0.28)	56.2 (2.6)	76.9 (3.1)
		1.00	1.47 (0.14)	8.82 (0.30)	23.8 (1.8)	59.1 (2.1)

L_{LGZ} than non-mycorrhizal plants in tillers of even-aged plants ($P < 0.05$). Such an effect was not found when even-sized tillers were compared ($P > 0.05$).

The spatial distribution of *REGR* within the growth zone had a common bell shape in all treatments, with a maximum near the centre of the growth zone (Fig. 3d–f). P deficiency lowered maximum *REGR*, an effect particularly evident in even-sized tillers.

The effect of P supply and AMF on P, N and WSC concentrations

P_{LGZ} increased strongly with increasing P supply in both experiments ($P < 0.01$; Table 1). AMF increased P_{LGZ} at the highest P supply level in the experiment comparing even-aged plants ($P < 0.05$; Table 1), although AMF colonization was low compared with the other treatments (Table 2). In the other treatments, AMF had no significant effect on P_{LGZ} ($P > 0.05$, Table 1).

Table 2. Mycorrhizal colonization of perennial ryegrass roots. The effect of soluble P supply on the percentage of root length colonized by the AMF *Glomus hoi* was determined in even-aged plants at 68 DAS, and in even-sized plants at 63 DAS (0.02 mM P) and 49 DAS (1 mM P) on Trypan Blue stained roots by the gridline intersect method. Mycorrhizal colonization of the even-aged plants was reported before (Grimoldi *et al.* 2005). Non-mycorrhizal plants had null root colonization in both experiments. Values in brackets are SE ($n = 4$–9)

Experiment	P supply (mM)	Root length colonized (%)
Even age	0	49.3 (3.8)
	0.02	48.1 (1.7)
	0.1	15.2 (1.6)
	0.5	8.3 (0.3)
Even size	0.02	33.7 (3.5)
	1	2.4 (0.4)

Other components of leaf growth zone biomass were also affected by P supply. In both experiments, increasing P supply led to 20–33% higher nitrogen (N) and 27–58% lower WSC concentrations in the leaf growth zone (Table 1). Because increasing P supply had a greater effect on P concentrations (by 70–134%), growth zone N:P and WSC:P ratios (w w^{-1}) were highest at the lowest P supply. AMF colonization increased N and decreased WSC concentrations in the growth zone only when P_{LGZ} was also improved.

Relationship between growth zone P status and *LER* and its components

In both even-aged and even-sized tillers, *LER* was linearly related to P_{LGZ} ($P < 0.05$; Fig. 4a & b, Table 3). The slope and intercept of the regression tended to be higher in even-aged mycorrhizal plants, but this effect was not statistically significant ($P > 0.1$; Table 3).

Regression analyses also demonstrated a positive linear relationship between $REGR_{avg}$ and P_{LGZ} in both even-aged and even-sized tillers ($P < 0.05$; Fig. 4c & d, Table 3). The slope of this relationship was somewhat higher in even-sized tillers, possibly because of a slightly different definition of the growth zone tissue sampled for P analysis (see Materials and methods).

The relationship between L_{LGZ} and P_{LGZ} was more complex. When treatments differed in tiller sheath length (even-aged plants), L_{LGZ} was related linearly to P_{LGZ} ($P < 0.05$; Fig. 4e, Table 3). Furthermore, AMF colonization increased L_{LGZ} over the whole range of P_{LGZ} in even-aged plants ($P > 0.1$; Table 3). Notably, in this experiment, AMF also increased the tiller size over the whole range of P supplies (Fig. 2a). Conversely, none of these effects were observed when size-mediated effects were avoided: P_{LGZ} and L_{LGZ} were unrelated, and AMF had no effect on L_{LGZ} when tillers of the same size were compared ($P > 0.1$; Fig. 4f, Table 3), even though their P_{LGZ} were very different.

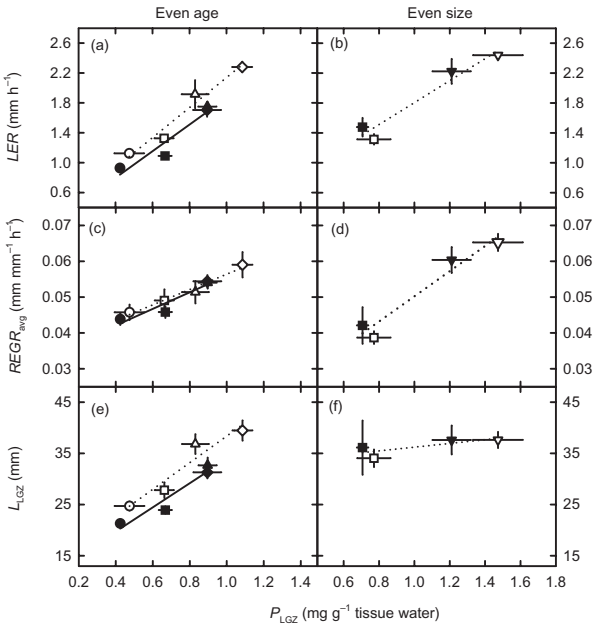

Figure 4. (a & b) Leaf elongation rate (*LER*), (c & d) average relative elemental growth rate ($REGR_{avg}$) and (e & f) length of the leaf growth zone (L_{LGZ}) of the most rapidly growing leaf of perennial ryegrass tillers in relation to leaf growth zone P concentration (P_{LGZ}). Plants were grown with 0 mM (●○), 0.02 mM (■□), 0.1 mM (▲△), 0.5 mM (◆◇) or 1 mM (▼▽) soluble P supply in the absence (●■▲◆▼) or presence (○□△◇▽) of AMF. Bars indicate ±1 SE (n = 3–4 for P_{LGZ} and 5–12 for growth parameters). Lines are linear regressions (see Table 3) for non-mycorrhizal (full) and mycorrhizal (dashed line) plants in the experiment with even-aged tillers and for both AMF treatments in the experiment with even-sized tillers.

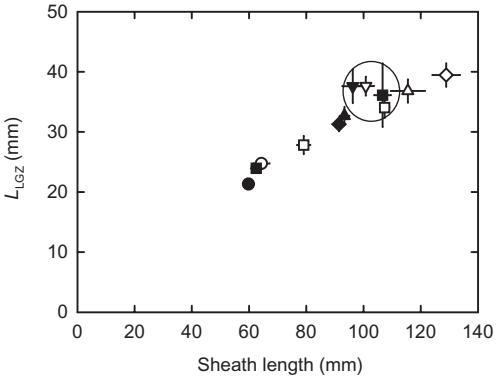

Figure 5. Relationship between the sheath length of the youngest expanded leaf enclosing the growing leaf and the leaf growth zone length (L_{LGZ}) in perennial ryegrass. Plants grown with 0 mM (●○), 0.02 mM (■□), 0.1 mM (▲△) or 0.5 mM (◆◇) soluble P supply in the experiment with even-aged plants. Plants grown with 0.02 mM (■□) or 1 mM (▼▽) soluble P supply in the experiment with even-sized tillers (encircled data points). Plants were grown in the absence (●■▲◆▼) or presence (○□△◇▽) of AMF. Bars indicate ±1 SE (n = 5–12).

Accounting for sheath length effects on *LER* and its components

The data already presented revealed a strict allometric relationship between L_{LGZ} and the sheath length of the youngest expanded leaf through which the growing leaves emerged (Fig. 5). This relationship was not modified by AMF and did not differ between the two experiments.

The correlation between L_{LGZ} and sheath length in Fig. 5 includes data from different P supply treatments. We verified that the same relationship also existed in data subsets with uniform P status. Figure 6 shows one such subset including even-aged tillers with 0.83–0.90 mg P g^{-1} tissue water in the leaf growth zone. In these tillers, *LER* was positively correlated with the sheath length of the youngest expanded leaf ($r^2 = 0.51$, $P < 0.05$; Fig. 6a). The correlation was entirely due to the relationship between L_{LGZ} and sheath length ($r^2 = 0.63$, $P < 0.05$; Fig. 6c) because $REGR_{avg}$ was independent of sheath length ($r^2 = 0.00$, $P > 0.1$; Fig. 6b). These results were confirmed by regression analyses within each treatment in both experiments, and $REGR_{avg}$ never correlated with sheath length (data not shown).

Table 3. Linear regression analysis of the relationship between leaf growth variables and P status of the growth zone in non-mycorrhizal (AMF–) and mycorrhizal (AMF+) plants. Parameters of the linear regression of leaf elongation rate (*LER*) and its two components, average relative elemental growth rate ($REGR_{avg}$) and leaf growth zone length (L_{LGZ}) against P_{LGZ} are presented. In the experiment with even-sized tillers, AMF– and AMF+ data were combined. Values in brackets are SE (n = 4) for parameters different from zero ($P < 0.05$)

Experiment	Variable	AMF treatment	Slope	Intercept	r^2
Even age	*LER*	AMF–	1.78 (0.39)	0.08 (n.s.)	0.91
		AMF+	2.01 (0.29)	0.13 (n.s.)	0.94
	$REGR_{avg}$	AMF–	0.022 (0.005)	0.033 (0.004)	0.91
		AMF+	0.023 (0.003)	0.035 (0.002)	0.97
	L_{LGZ}	AMF–	23.6 (4.8)	10.3 (n.s.)	0.93
		AMF+	26.0 (5.6)	12.3 (n.s.)	0.92
Even size	*LER*	AMF–/AMF+	1.47 (0.25)	0.33 (n.s.)	0.95
	$REGR_{avg}$	AMF–/AMF+	0.035 (0.006)	0.015 (n.s.)	0.95
	L_{LGZ}	AMF–/AMF+	3.65 (n.s.)	32.6 (2.2)	0.62

n.s., not significant at $P = 0.05$.

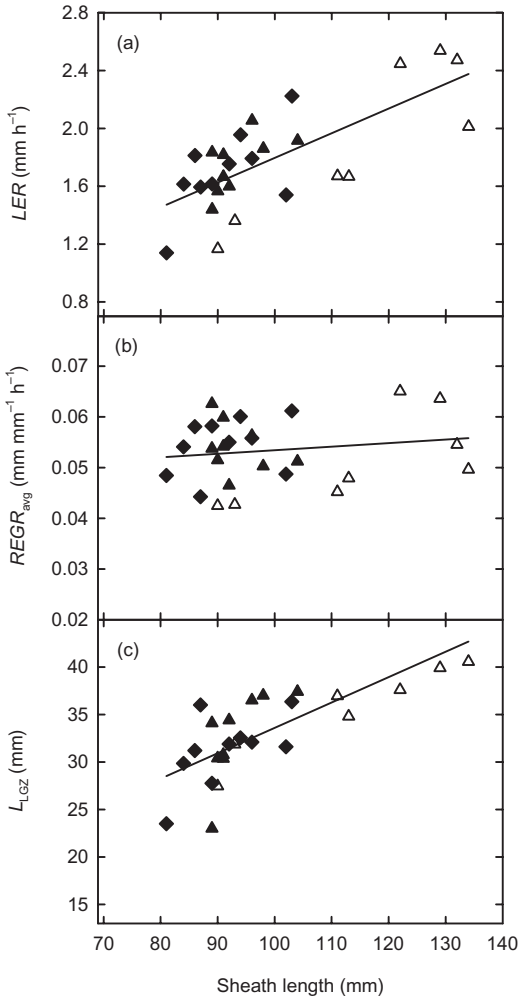

Figure 6. Relationships between the sheath length of the youngest expanded leaf enclosing the growing leaf and (a) leaf elongation rate (*LER*) (b) average relative elemental growth rate ($REGR_{avg}$) and (c) leaf growth zone length (L_{LGZ}) in the experiment with even-aged tillers. Data correspond to a subset of plants with similar growth zone P concentration (0.83–0.90 mg g^{-1} tissue water), with symbols as for Fig. 3. Linear regression equations are: y(*LER*) = 0.017x + 0.088, $r^2 = 0.52$ $P < 0.001$; y($REGR_{avg}$) = 0.000x + 0.046*, $r^2 = 0.03$ $P = 0.41$; y(L_{LGZ}) = 0.27x + 6.84, $r^2 = 0.65$ $P < 0.001$. Intercepts different from zero are marked with an asterisk ($P < 0.05$).

DISCUSSION

AMF and P supply control leaf growth via the same P status- and size-dependent mechanisms

This is the first quantitative assessment of the relationship between the P status of leaf growth zones and the components of *LER*. The results revealed that AMF and P supply enhanced leaf growth via identical mechanisms. These included both P status-dependent and tiller size-mediated effects on *LER* (Fig. 7). Remarkably, $REGR_{avg}$ was strictly related to the P status of the leaf growth zone (i.e. P_{LGZ}) and was independent of tiller size and AMF. Conversely, L_{LGZ} was a function of the sheath length of the youngest expanded leaf through which the growing leaf emerged (i.e. tiller size) and was independent of P status and AMF. Accordingly, in plants of similar tiller size, P supply and AMF affected *LER* only via the effect of P status on $REGR_{avg}$. Conversely, in plants of the same age, differences in tiller size contributed to treatment differences in *LER* via the effect of tiller size on L_{LGZ}. These results illustrate the need for a distinction between P status-dependent and size-mediated effects on *LER*.

Our results for the even-aged plants agree with those of the only other study on effects of P supply on leaf growth kinematics in grasses reported by Assuero *et al.* (2004). In their study with even-aged (but uneven-sized) plants, P deficiency caused a 63% reduction in *LER*, which resulted from a 56% shorter L_{LGZ}, and a 7% reduction in $REGR_{avg}$. Their work also demonstrated a higher cell production rate in the P sufficient tillers. The present data suggest that the responses observed by Assuero *et al.* (2004) resulted mainly from the size-mediated changes of *LER* and only marginally from the direct effect of growth zone P status on *LER*. Thus, it raises the question whether cell production rate is also related to tiller size. Clearly, the cellular mechanisms underlying the effects of P supply and AMF on leaf growth merit further study.

Our data also directly demonstrate that P deficiency did not limit leaf growth through a reduced C availability. In fact, the growth zones of P deficient plants had a higher WSC concentration than the growth zones of P sufficient plants, thus corroborating previous findings that P deficiency has a stronger effect on C utilization than on assimilation (Rao & Terry 1989; Rodríguez, Andrade & Goudriaan 2000). AMF have been reported to consume up to 20% of C fixed by the plant (Jakobsen & Rosendahl 1990). However, this cost was not reflected in WSC concentrations in the growth zone, suggesting that the presence of AMF did not negatively affect the amount of C available for leaf growth.

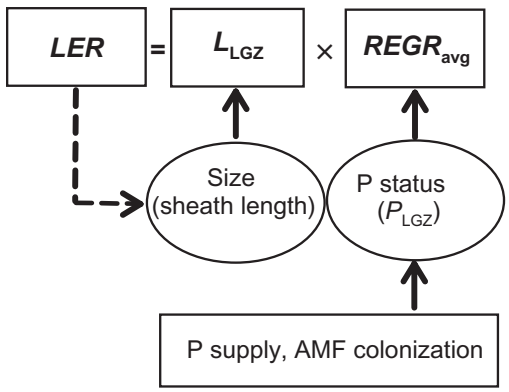

Figure 7. Graphical summary of the effects of P supply and mycorrhizal colonization on *LER* and its components. P supply and AMF treatment affect the leaf growth zone P status, which then directly affects *LER* through a change in $REGR_{avg}$. In turn, changes in the sheath length of the youngest expanded leaf (related to either the direct effect of P status on *LER* or to any other P-independent influence) will affect *LER* through an effect on L_{LGZ}.

The uptake and subsequent metabolism of N are also known to be reduced under P deficiency (Rufty *et al*. 1993). In the present study, N concentration in leaf growth zone biomass decreased with P deficiency, but the N:P ratio of the growth zone biomass was highest in P-deficient plants. This typical response of P-limited plants (but not of N-limited plants) (Agren 2004) indicates that the reduction of $REGR_{avg}$ under P deficiency was related exclusively to the low P status of the growth zone.

Tissue expansion rate is directly related to P status of the growth zone

This study demonstrated a direct and strong relationship between the P_{LGZ} and $REGR_{avg}$. Because *REGR* is a relative rate, it does not depend on the number of cells or cell length, and for a given position is equal to the relative rate of cell expansion (Schnyder *et al*. 1990; Ivanov, Dobrochaev & Baskin 2002). The actual mechanism by which expansion rate is affected by P status is not known. However, relative cell expansion rate is a function of cell wall extensibility and turgor pressure in excess of the yield threshold of the cell wall (Van Volkenburgh 1999). Arguably, P deficiency can affect both parameters because it leads to low leaf ATP concentrations (Jacob & Lawlor 1993) and causes a variety of transcriptional and hormonal changes (Franco-Zorrilla *et al*. 2004). It also remains to be elucidated whether the growth reduction is mediated by unavailability of P as a substrate or by P as a signal (Ticconi & Abel 2004). Certainly, observed lower P_{LGZ} under P deficiency may result from decreased availability of P in xylem/phloem and may directly limit the synthesis of P-containing cell components, although this can be partially compensated, e.g. by galactolipids and sulfolipids partly replacing phospholipids in membranes (Dörmann & Benning 2002). Yet, decreased P_{LGZ} may result from lower P deposition due to cytokinin-mediated decreases in cell division/expansion as well (Werner *et al*. 2003).

What controls the length of the leaf growth zone?

The mechanism how the length of the leaf growth zone is actually regulated is so far unknown. Possible determinants include both positional and temporal controls of tissue expansion. We evaluated the possibility that cells require a fixed time interval to complete expansion. However, time-position trajectories (cf. Gandar & Hall 1988) generated from the present data suggested that such a 'time-control' mechanism was unlikely. This is because P deficiency led to significantly longer residence times of tissue in the growth zone when size effects were taken into account (data not shown). The present – and also previous (Kemp 1980b; Casey *et al*. 1999; Arredondo & Schnyder 2003) – studies showed that L_{LGZ} is proportional to the sheath length of the youngest expanded leaf, suggesting a positional rather than temporal control. It is known that termination of cell expansion is associated with increased apoplastic peroxidase activity (Bacon, Thompson & Davies 1997; de Souza & MacAdam 1998). In turn, peroxidase activity in maize coleoptiles responded to changes in light quality in a phytochrome-mediated response (Kim, Shinkle & Roux 1989). Thus, coordination between L_{LGZ} and enclosing sheath mediated by morphogenic effects of light quality seems a reasonable hypothesis (cf. Skinner & Simmons 1993; Gautier & Varlet-Grancher 1996), albeit direct experimental evidence is still missing.

A review of kinematic studies evaluating the control of leaf elongation under different abiotic stresses indicates that when experimental designs produced no substantial difference between sheath length of control and treated plants, changes in *LER* were chiefly due to changes in $REGR_{avg}$ and not L_{LGZ} (e.g. light-dark cycles: Schnyder & Nelson 1988; salinity: Fricke & Peters 2002; N deficiency: Fricke, McDonald & Mattson-Djos 1997; general nutrient deficiency: Snir & Neumann 1997; source limitation: Fricke 2002; temperature: Ben-Haj-Salah & Tardieu 1995; ABA accumulation: Dodd & Davies 1996). Conversely, in studies where treatments altered sheath length substantially, both L_{LGZ} and $REGR_{avg}$ contributed to differences in *LER* (e.g. irradiance: Schnyder & Nelson 1989; salinity: Bernstein, Läuchli & Silk 1993; N deficiency: Gastal & Nelson 1994, Tóth *et al*. 2002; P deficiency: Assuero *et al*. 2004). Therefore, it seems these abiotic stresses initially affected *LER* solely by reducing *REGR*. Eventually, however, the treatment-related changes in tiller sheath length brought about an additional (indirect) effect: a change of L_{LGZ}.

In conclusion, this study showed that the effects of P supply and AMF on $REGR_{avg}$, and thus on *LER*, were closely and linearly related to their effects on P_{LGZ}. The other component of *LER*, L_{LGZ}, was strictly related to the sheath length of the youngest expanded leaf independently of P supply or AMF treatment. Thus, tissue expansion rate was directly associated with P status, but the position at which expansion stopped was unrelated. AMF and P supply affected leaf growth through identical mechanisms.

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Appendix 2

Phosphorus Deficiency Decreases Cell Division and Elongation in Grass Leaves[1]

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Leaf growth in monocotyledons results from the flux of newly born cells out of the division zone and into the adjacent elongation-only zone, where cells reach their final length. We used a kinematic method to analyze the effect of phosphorus nutrition status on cell division and elongation parameters in the epidermis of *Lolium perenne*. Phosphorus deficiency reduced the leaf elongation rate by 39% due to decreases in the cell production rate (−19%) and final cell length (−20%). The former was solely due to a lower average cell division rate (0.028 versus 0.046 cell cell^{-1} h^{-1}) and, thus, a lengthened average cell cycle duration (25 versus 15 h). The number of division cycles of the initial cell progeny (five to six) and, as a result, the number of meristematic cells (32–64) and division zone length were independent of phosphorus status. Accordingly, low-phosphorus cells maintained meristematic activity longer. Lack of effect of phosphorus deficiency on meristematic cell length implies that a lower division rate was matched to a lower elongation rate. Phosphorus deficiency did not affect the elongation-only zone length, thus leading to longer cell elongation duration (99 versus 75 h). However, the substantially reduced postmitotic average relative elongation rate (0.045 versus 0.064 mm mm^{-1} h^{-1}) resulted in shorter mature cells. In summary, phosphorus deficiency did not affect the general controls of cell morphogenesis, but, by slowing down the rates of cell division and expansion, it slowed down its pace.

Although essential for plant growth and development, inorganic phosphorus is one of the least available nutrients in soils of many terrestrial ecosystems (Vance et al., 2003). Plants are profoundly affected by phosphorus deficiency because phosphorus is an indispensable constituent of nucleic acids and membrane phospholipids. Moreover, phosphorus plays a pivotal role in energy transfer, as a regulator of enzyme activity, and in signal transduction. Thus, not surprisingly, low phosphorus availability activates a series of morphological and physiological responses that maximize phosphorus acquisition (Raghothama, 1999) and are directed to maintain internal phosphorus homeostasis (Ticconi and Abel, 2004). Leaf growth depression under phosphorus deficiency is well documented (Radin and Eidenbock, 1984; Chiera et al., 2002; Assuero et al., 2004; Kavanová et al., 2006). Ultimately, this growth reduction must be due to an alteration of cell division or cell elongation parameters.

We have chosen a grass leaf system to investigate the cellular bases of growth reduction under phosphorus deficiency. In grasses, growth is confined to a short tissue segment located at the base of the developing leaf enclosed by older sheaths (Kemp, 1980). Here, meristematic cells proliferate, undergoing a number of cell cycles before entering a phase of elongation-only growth. This creates a clearly defined spatial pattern of cell development along the longitudinal axis, giving place to a basal division zone, where meristematic cells elongate and divide, and an elongation-only zone, where cells undergo postmitotic elongation. Together, the two zones form the leaf growth zone (Fig. 1). Kinematic analysis provides the appropriate analytical tools to translate back the spatial patterns into the time history of an individual cell, making it possible to derive, from the spatial profiles of cell length and displacement velocity, rates and durations of cell division and elongation (Green, 1976; Silk and Erickson, 1979; Silk, 1992). The leaf elongation rate (mm h^{-1}), the flux of leaf tissue out of the growth zone, can then be analyzed in terms of the cell production rate (cell h^{-1}) and final cell length (mm cell^{-1}; Volenec and Nelson, 1981). In turn, the cell production rate is determined by the number of cells in the division zone and their division rate (cell cell^{-1} h^{-1}), whereas the final cell length is determined by the length of cells leaving the meristem (mm cell^{-1}), and their relative elongation rate (mm mm^{-1} h^{-1}) and elongation duration (h; Fig. 1).

The contribution of the different cellular parameters to leaf growth reduction under nutrient stress is not well understood. Few studies addressed the effects of phosphorus deficiency and gave different results. In cotton (*Gossypium hirsutum*), Radin and Eidenbock (1984) concluded that reduced cell expansion underlay reduced leaf size, whereas, in soybean (*Glycine max*), Chiera et al. (2002) concluded that reduced cell division was the major cause. Although this divergence may be related to a different species response, it may also arise from the fact that neither of the studies

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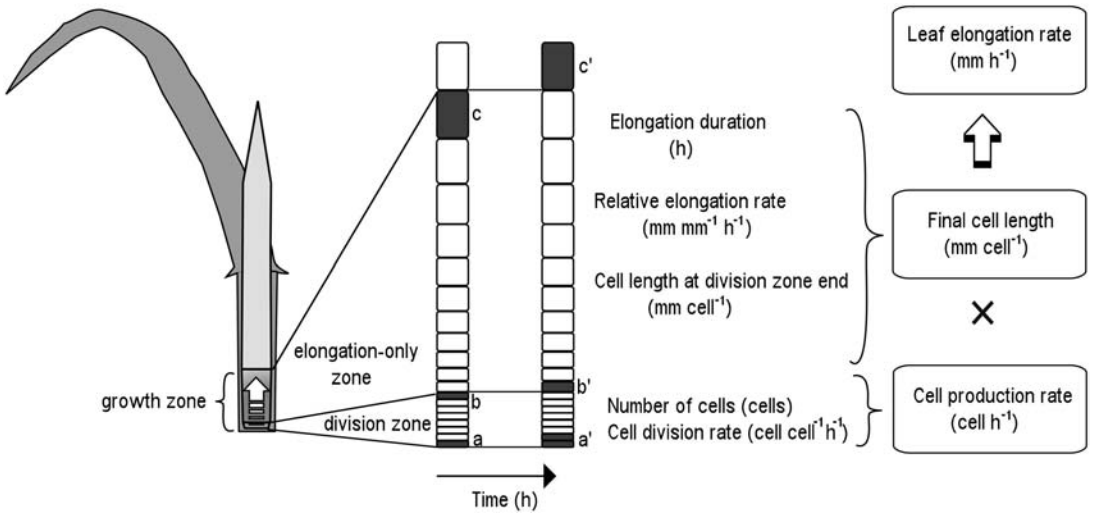

Figure 1. Growth zone of a grass leaf. Growth is limited to the basal part of the growing leaf, the growth zone, which is enclosed by the sheaths of expanded leaves. Meristematic cells in the division zone elongate and divide simultaneously (a→a′). The progeny of the initial cell at the base of the meristem goes through a certain number of division cycles, thus determining the number of cells per meristematic file. Upon entering the elongation-only zone (b→b′), cells elongate without further divisions until they reach their final length at the distal end of the growth zone (c→c′).

directly measured these parameters. Instead, the role of cell expansion was inferred from smaller leaf cells and the role of cell division was inferred from reduced cell number. In maize (*Zea mays*), the first monocot studied, Assuero et al. (2004) attributed the reduction in leaf growth to a decreased cell production rate. In the only other kinematic study of phosphorus effects on leaf growth in the grass *Lolium perenne*, low relative elongation rates along the elongation-only zone caused a severe reduction of leaf growth (Kavanová et al., 2006).

This study provides a comprehensive analysis of the cellular responses underlying reduction of the leaf elongation rate in *L. perenne* leaves growing under phosphorus deficiency. Using a kinematic approach, we evaluated which parameters determining the number of produced cells and their final length responded to changes in phosphorus status and which did not. This included (1) number of meristematic cells as controlled by a (constant) number of division cycles of the initial cell progeny; (2) duration of cell elongation as determined by a spatially controlled elongation-only zone length; (3) rate of cell division as determined by the growth rate of meristematic cells and a (constant) mitotic cell length; and (4) rate of mitotic and post-mitotic elongation.

RESULTS

Leaf Elongation Rate

L. perenne plants grew at low (0.02 mM) or high (1 mM) phosphorus supply. Growth at low phosphorus supply caused a 42% reduction in the phosphorus concentration in the leaf growth zone ($P < 0.001$; Table I) and a 39% reduction in the leaf elongation rate ($P < 0.001$; Fig. 2). In both treatments, leaves selected for measurement elongated at a steady rate over time (Fig. 2).

The treatment effect on the leaf elongation rate was entirely due to the different phosphorus nutrition status because selected tillers of low- and high-phosphorus plants did not differ in size or developmental variables:

Table I. *Phosphorus status, tiller size, and developmental stage of leaves selected for analysis of leaf growth and underlying cellular dynamics*

L. perenne plants were grown for 47 d at high (1 mM) and 61 d at low (0.02 mM) phosphorus supply. Data are averages of six plants (±SE), along with the significance of the difference between phosphorus treatments based on a *t* test. ***, $P \leq 0.001$; NS, not significant, $P > 0.05$.

Parameter	High Phosphorus	Low Phosphorus	Significance
Phosphorus in the growth zone (mg g^{-1} fresh weight)	1.07 ± 0.01	0.61 ± 0.01	***
Number of green leaves per tiller	4.8 ± 0.19	4.7 ± 0.24	NS
Sheath length of the youngest expanded leaf (mm)	90 ± 5	101 ± 5	NS
Blade length of the youngest expanded leaf (mm)	305 ± 14	342 ± 20	NS
Blade length of the growing leaf (mm)	206 ± 13	190 ± 24	NS
Blade length expanded: blade length growing leaf	0.68 ± 0.05	0.57 ± 0.08	NS

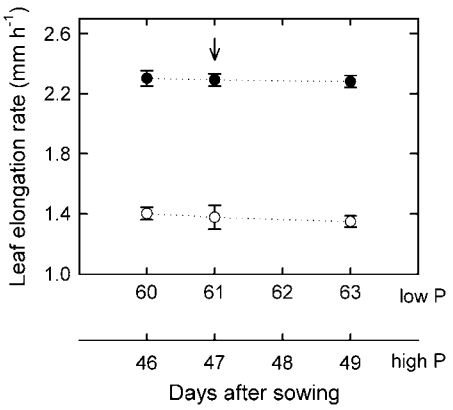

Figure 2. Effect of phosphorus supply on the leaf elongation rate. *L. perenne* plants were grown at high (1 mM, ●) and low (0.02 mM, ○) phosphorus supply. The arrow indicates the time when the kinematic analysis was performed. Data are means of five to six plants on each date (±SE).

Leaf blades and sheaths had similar lengths, and the tillers held a similar number of leaves (Table I). Further, growing leaves were in the same developmental stage, indicated by the ratio of the growing blade length to the blade length of the youngest expanded leaf (Table I). This ensured that effects of phosphorus status on growth were not confused with effects of size and development (Kavanová et al., 2006).

Cell Proliferation

The reduction of leaf growth under low phosphorus originated partly from decreased cell proliferation in the division zone. Phosphorus deficiency reduced the cell production rate (i.e. the cell flux out of the division zone estimated from the leaf elongation rate and final cell length [Eq. 2]) by 19% ($P < 0.01$; Table II).

This difference arose entirely from a different average cell division rate, that is, the number of cells produced per cell present in the division zone per unit time. Meristematic cells divided at a 39% lower rate in low-phosphorus plants ($P < 0.01$; Table II). As a result, the average cell cycle duration (Eq. 8) was 10 h longer in low-phosphorus plants (Table II).

Phosphorus deficiency did not affect the average number of cells in a meristematic cell file ($P > 0.1$; Table II). Cell division was confined to the basal 0.9 ± 0.1 mm in low-phosphorus plants and to 0.6 ± 0.1 mm in high-phosphorus plants, but this difference was not statistically significant ($P = 0.06$). It is important to note that we did not derive the length of the division zone and the number of meristematic cells from cell deposition rates. Instead, we counted all cells present in individual meristematic cell files from the leaf base to the position of the last recently formed perpendicular cell wall. A closer examination of these data revealed that the number of cells per meristematic cell file was a weighted average of two major groups of files: files with approximately 32 cells and files with approximately 64 cells. Low- and high-phosphorus plants had a similar frequency distribution of these two groups (data not shown).

The number of division cycles necessary to displace a transversal cell wall from the basal to the distal boundary of the division zone (i.e. the average number of division cycles of the progeny of a cell formed by the division of the initial cell at the base of the meristem) can be derived from the number of cells in the division zone (Eq. 10). In both phosphorus treatments, the number of division cycles was, on average, five to six ($P > 0.1$; Table II). Frequency distribution of the number of division cycles in different meristematic cell files revealed distinct peaks around four, five, and six, indicating that variability exists between cell files within one division zone (Fig. 3). Whereas in high-phosphorus plants cell files were equally distributed around five and six division cycles, low-phosphorus plants tended to have a frequency distribution shifted toward six division cycles.

As a consequence of a similar number of division cycles but longer average cell cycle duration, the average residence time of a cell in the division zone tended

Table II. *Effect of phosphorus deficiency on kinematic parameters*

L. perenne plants were grown for 47 d at high (1 mM) and 61 d at low (0.02 mM) phosphorus supply. Data are averages for epidermal cells of six plants (±SE), along with the significance of the difference between phosphorus treatments based on a *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; NS, not significant, $P > 0.05$.

Parameter	High Phosphorus	Low Phosphorus	Significance
Leaf elongation rate (mm h^{-1})	2.30 ± 0.05	1.41 ± 0.05	***
Cell production rate (cell h^{-1})	1.91 ± 0.10	1.52 ± 0.10	**
Average cell division rate (cell $cell^{-1}$ h^{-1})	0.046 ± 0.008	0.028 ± 0.005	**
Average cell cycle duration (h)	15.1 ± 3.1	24.9 ± 2.0	***
Number of cells per meristematic cell file	41 ± 7	55 ± 9	NS
Number of division cycles	5.4 ± 2.7	5.8 ± 2.3	NS
Final cell length (mm)	1.208 ± 0.057	0.929 ± 0.052	**
Cell length in the meristem (μm)	19.3 ± 3.2	18.8 ± 2.4	NS
Postmitotic relative elongation rate (mm mm^{-1} h^{-1})	0.064 ± 0.005	0.045 ± 0.005	*
Elongation duration (h)	75 ± 6	99 ± 9	*

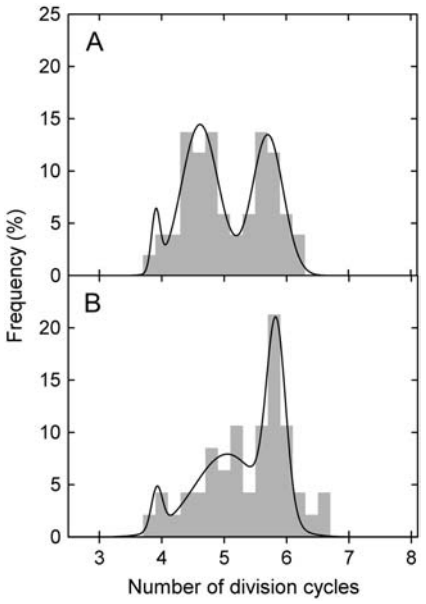

Figure 3. Frequency distribution of cell files with different numbers of division cycles. *L. perenne* plants were grown for 47 d at high (1 mM; A) and 61 d at low (0.02 mM; B) phosphorus supply. For the analysis, data for eight to 10 cell files of each of the six plants per treatment were combined. In every file, the number of division cycles of the progeny of the cell formed by the division of the initial cell at the base of the meristem was calculated as $\log_2$ (number of cells in the meristem; Eq. 10). Triple Gaussian normal distribution curves best fitted the frequency distributions ($r^2 = 0.95$ for high-phosphorus plants; $r^2 = 0.91$ for low-phosphorus plants), with peaks located at 3.9 ± 3.46, 4.6 ± 0.03, and 5.7 ± 0.03 divisions per cell (high phosphorus), and 3.9 ± 0.38, 5.1 ± 0.15, and 5.8 ± 0.02 divisions per cell (low phosphorus).

to be longer under phosphorus deficiency (144 ± 55 h versus 81 ± 37 h in high-phosphorus plants). Thus, cells in low-phosphorus plants tended to maintain meristematic activity for a longer period of time.

Relative Elongation Rate of Meristematic Cells

Cell length was typically constant within each individual meristematic cell file up to the position where division stopped (Fig. 4, inset). When averaged over each treatment, average cell lengths were stable along the first half of the division zone and phosphorus deficiency did not affect this pattern (Fig. 4), but average cell length increased in the second half of the division zone by 23% in low- and 17% in high-phosphorus plants. This increase was due to the fact that in some meristematic cell files the number of division cycles of the initial cell progeny was five (meristem length approximately 32 cells), whereas in others it was six (meristem length approximately 64 cells). Hence, in the second half of the meristem, dividing cells (maintaining their average length unaltered) coexisted with nondividing cells (that were increasing in length). This is also appreciated by comparing the stability of minimal cell lengths against the increase in maximal cell lengths.

The stability of cell length along the division zone provides important information on the balance between relative rates of cell division and elongation (for discussion, see Green, 1976). It implies that the relative rates of meristematic cell elongation were very close to the average cell division rates (0.028 ± 0.005 h^{-1} in low- versus 0.046 ± 0.008 h^{-1} in high-phosphorus plants). Phosphorus deficiency did not affect the size at which cells divided or, consequently, their length at birth: The 39% higher average cell division rate of high-phosphorus meristematic cells means they had a 39% higher relative elongation rate.

Final Cell Length

Whereas one-half of the leaf growth reduction under phosphorus stress was due to a reduced cell production rate, the other half originated from a decrease in final cell length. Mature epidermal cells were 20% shorter in low-phosphorus plants ($P < 0.01$; Table II; Fig. 5A). The final length of a cell depends on three factors: the length of the cell leaving the meristem (i.e. when it enters the elongation-only zone) and the relative rate and duration of the elongation-only phase. Phosphorus deficiency affected the latter two, but cell size at the position where elongation started was not different: 24.2 ± 3.8 μm in low- versus 22.5 ± 3.4 μm in high-phosphorus plants ($P > 0.1$; Fig. 4).

Spatial Analysis of Postmitotic Elongation

Cell elongation was confined to the basal 31 to 36 mm of the growing leaf in low- and high-phosphorus plants, respectively ($P > 0.1$; Fig. 5A). The number of cells in the elongation-only zone was also not affected by phosphorus deficiency (136 ± 9 in low- and 117 ± 9 in high-phosphorus plants; $P > 0.1$). This confirms our previous observation (Kavanová et al., 2006) that

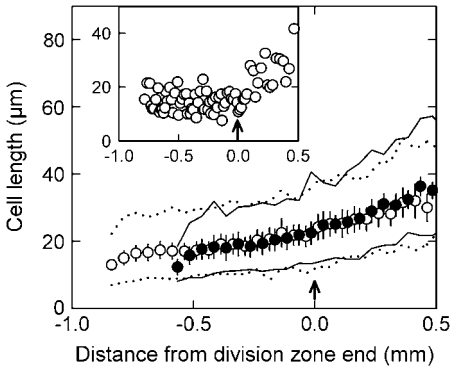

Figure 4. Effect of phosphorus supply on epidermal cell length along the basal part of the leaf growth zone. *L. perenne* plants were grown for 47 d at high (1 mM, ●) and 61 d at low (0.02 mM, ○) phosphorus supply. The length of the shortest and the longest cell over 50-μm intervals is indicated by continuous lines (1 mM phosphorus) and dashed lines (0.02 mM phosphorus). Data are means of six plants (±SE). Arrows indicate the distal end of the division zone. Inset shows the raw cell length data for an individual cell file of a low-phosphorus plant.

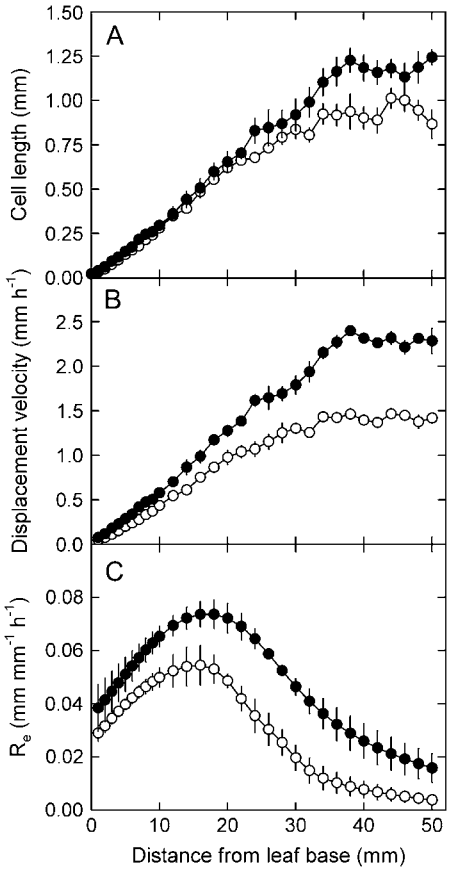

Figure 5. Effect of phosphorus supply on spatial profiles of epidermal cell length (A), displacement velocity (B), and relative elongation rate (R_e; C) along the base of the growing leaf. *L. perenne* plants were grown for 47 d at high (1 mM, ●) and 61 d at low (0.02 mM, ○) phosphorus supply. Data are means of six plants (±SE).

phosphorus deficiency has no (direct) effect on the length of the elongation-only zone.

Relative elongation rates along the elongation-only zone obtained by differentiating displacement velocity profiles (Fig. 5B) were uniformly lower at all positions in low-phosphorus plants (Fig. 5C). Thus, phosphorus deficiency did not modify the spatial distribution of relative elongation rates, which were, on average, 30% lower in low-phosphorus plants ($P < 0.05$; Table II).

Temporal Analysis of Postmitotic Elongation

Furthermore, we carried out a temporal analysis of the elongation of an individual cell from the moment it enters the elongation-only zone. The spatial profiles of cell length, displacement velocity, and relative elongation rate were transformed into time courses using the growth trajectory function, which relates spatial position of a cell to time coordinates (Eq. 5). This analysis revealed that cells expanded for a substantially shorter period in high-phosphorus plants ($P < 0.05$; Table II) because they moved more rapidly through the elongation-only zone. Thus, the higher relative elongation rate of high-phosphorus plants was partially offset by a shorter elongation duration (Fig. 6).

DISCUSSION

Growth regulation constitutes a major field of interest in plant physiology. However, the cellular bases of growth reduction under stress conditions are not fully understood. This kinematic study showed that, under phosphorus deficiency, the reduction of leaf growth in the grass *L. perenne* arose from inhibition of cell division and elongation rates, leading to reductions in both the cell production rate and the final cell length. The lengthened average cell cycle duration in low-phosphorus plants was linked to a slower elongation rate so that meristematic cell length was not modified. Notably, other variables were unrelated to phosphorus status. In the division zone, phosphorus deficiency did not affect the number of division cycles (of the progeny of a cell formed by the division of the initial cell at the base of the meristem). In the elongation-only zone, phosphorus deficiency did not modify the

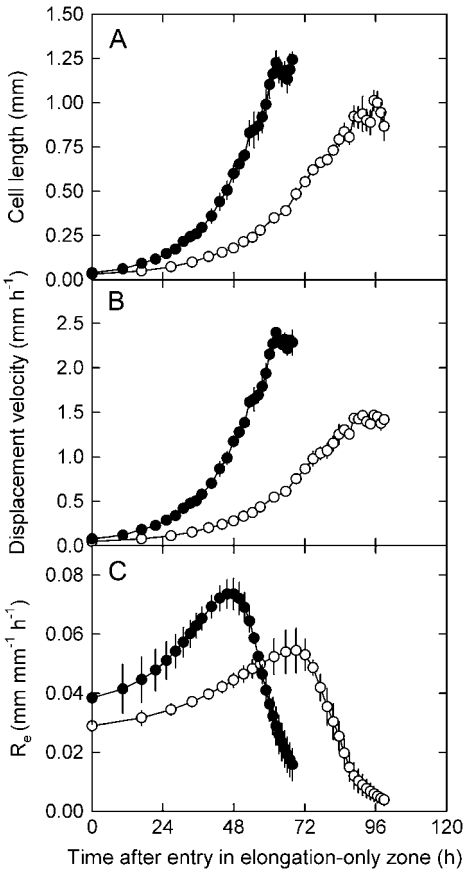

Figure 6. Effect of phosphorus supply on temporal profiles of cell length (A), displacement velocity (B), and relative elongation rate (R_e; C) of an individual epidermal cell from the time it enters the elongation-only zone. *L. perenne* plants were grown for 47 d at high (1 mM, ●) and 61 d at low (0.02 mM, ○) phosphorus supply. Data are means of six plants (±SE).

position where postmitotic elongation stopped. Hence, phosphorus deficiency did not affect the putative controls of the cell morphogenetic program, but, by slowing down the rates of cell division and elongation (and thus increasing the residence time in both zones), it slowed down the pace at which it was carried out.

Cell Proliferation Is Modulated in Response to Phosphorus Status

Coupling between cell elongation and cell division has been observed under undisturbed conditions in plant meristems, where cells double in size from birth until the next division (Cánovas et al., 1990; Korn, 2001; Ivanov et al., 2002). However, only scarce knowledge exists on the links between cell growth and cell division (Li et al., 2005) and how cell size, nutrient status, or other signals impinge upon cell cycle progression in multicellular plants (De Veylder et al., 2003).

Our study shows that phosphorus deficiency decreased the average division rate of meristematic cells. But phosphorus deficiency did not affect meristematic cell length (Fig. 4), implying that a decrease in the division rate was accompanied by an equivalent reduction in the elongation rate. Therefore, phosphorus deficiency did not affect the close coordination between cell growth and cell division in the leaf meristem (see Fig. 7, trajectory A).

What would be the result of uncoupling cell division from elongation in the meristem? Figure 7 illustrates the possible outcomes. A factor that decreases cell division rate but does not affect elongation will increase meristematic cell length (Fig. 7, trajectory B). This would also increase the initial length at which cells start expanding, and result in longer mature cells, even though their elongation rate is not affected. The same will occur when a factor increases the elongation rate but does not affect the division rate (Fig. 7, trajectory C). This analysis also illustrates the risks of inferring changes in cell division or elongation rates based only on meristematic or mature cell length.

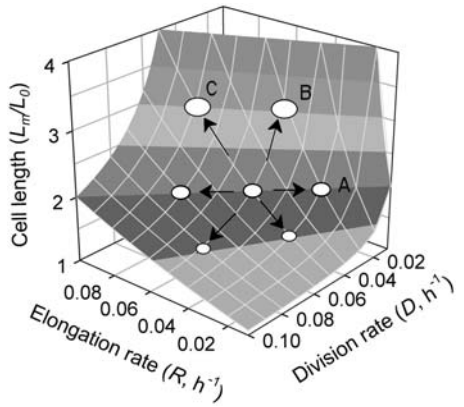

Figure 7. Coordination between rates of cell division, elongation, and cell length. A cell growing exponentially at a relative rate R (h^{-1}) and dividing at a relative rate D (h^{-1}) increases its size during one cell cycle from the initial length (L_0, mm) to its mitotic length (L_m, mm): $L_m = L_0 \times \exp(R_m \times \ln 2/D)$. Solving this equation for the relative increase in length gives: $L_m/L_0 = 2\wedge(R_m/D)$. The ratio of $L_m/L_0 = 2$ indicates that the cell progeny maintains constant size; the ratio of $L_m/L_0 > 2$ indicates that R is higher than D, resulting in longer cells; and the ratio of $L_m/L_0 < 2$ indicates that D is higher than R, resulting in shorter cells. Three cases are shown. A, Equal decrease in D and R does not affect meristematic cell length. B, Lower D with no change in R leads to longer meristematic cells. C, Faster R with no change in D results in longer meristematic cells.

The coupling of cell growth and division in proliferating cells may be achieved by alternative means: The cell division rate may affect the cell growth rate, the cell growth rate may influence the cell division rate, or both processes may respond to a common signal. In the first scenario, phosphorus deficiency would have inhibited cell cycle progression, and the reduced cell division rate would have decreased the cell elongation rate. Some authors have indeed suggested that cell division might affect cell growth (Doerner et al., 1996; Cockcroft et al., 2000). However, several studies showed that cell cycle modulators, either accelerating or slowing cell division rates, decoupled cell division from cell growth. Overexpression of the cyclin-dependent kinase inhibitors KRP1 and KRP2 (De Veylder et al., 2001) and expression of a dominant negative allele of the Arabidopsis (*Arabidopsis thaliana*) *CDKA* gene in tobacco (*Nicotiana tabacum*; Hemerly et al., 1995) resulted in fewer, but bigger, meristematic cells. Similarly, meristematic and mature cells of plants overexpressing cyclin D3 were increased in number, but were of smaller size due to accelerated progression through the G1 phase (Dewitte et al., 2003). Thus, it seems improbable that a lower cell division rate drove reductions in the elongation rate of meristematic cells under phosphorus stress.

The second scenario puts forward that a lower elongation rate of meristematic cells lengthened the average cell cycle duration. This view is supported by the fact that the probability of G1-to-S transition, a major cell size checkpoint, increased with increasing cell size (Cánovas et al., 1990). Further, Pien et al. (2001) showed that local induction of expansin expression led to the formation of normal leaf primordia, suggesting that increased cell expansion was driving cell division. In our study, a decrease in the elongation rate of meristematic cells under phosphorus deficiency would have prolonged the time needed to reach the critical length (see Fig. 7, trajectory A) and thus would have lengthened the average cell cycle duration. Consequently, phosphorus deficiency may primarily affect cell elongation and the effect on cell cycle duration may be a consequence of the reduction in growth rate. Nonetheless, a further study is needed to determine whether phosphorus deficiency extends the G1 phase specifically or all cell cycle phases.

It is not clear which signal could regulate both cell growth and division rate in the third scenario. Cytokinins are a putative candidate because phosphorus deficiency decreases their shoot levels (Horgan and Wareing, 1980), and they affect both cell cycle progression

at the G1-to-S and G2-to-M transitions (del Pozo et al., 2005) and have an effect on the expression of expansins and thereby on cell wall expansibility as well (Downes et al., 2001).

Relative Elongation Rate Is Related to Phosphorus Status

A decrease in the relative elongation rate along the elongation-only zone led to shorter mature epidermal cells in phosphorus-deficient plants and thus contributed to a decrease in the leaf elongation rate (Fig. 5). Other parameters influencing final cell length were little affected by phosphorus status (the length of cells leaving the division zone; Fig. 4) or even increased under phosphorus stress (elongation duration; Table II).

Proliferating cells grow primarily by an increase in the cytoplasmic volume, whereas cells in the postmitotic phase expand primarily through an increase in the vacuolar volume (Fagerberg, 1984). Thus, it is telling that a reduction in the relative elongation rate was of similar magnitude in both the division and elongation-only zones (30%–40%), raising the question of whether this was due to the same mechanism. The relative elongation rate depends on cell wall extensibility, tissue hydraulic conductance, and turgor pressure in excess of the yield threshold of the cell wall (Fricke, 2002). No information exists concerning phosphorus effects on these processes. Changes in turgor have been found to play only a minor role in leaf growth responses to nitrogen and carbon stress and salinity (Fricke, 2002, and refs. therein). Thus, it is more likely that phosphorus status induced either changes in cell wall properties (mediated by expansins, for example) or changes in tissue hydraulic conductivity (possibly mediated by aquaporins, which are highly expressed in dividing and elongating cells; Chaumont et al., 1998). We believe that understanding the effects of phosphorus deficiency on leaf growth will progress little until the mechanism of reduction in the relative elongation rate is understood.

Phosphorus Deficiency Does Not Affect Cell Number But Increases Residence Time in the Growth Zone

Whereas division and elongation rates varied in response to phosphorus status, the size of the division and elongation-only zones remained unaffected. Two main models of growth zone regulation have been proposed for roots and may also be valid for grass leaves. The first one proposes that a spatial gradient of growth regulators determines the developmental state of cells at any position along the growth zone (Barlow, 1984). An alternative model claims that spatial patterns result from a certain developmental program followed by each cell (González-Fernández et al., 1968).

We evaluated whether the length of the cell division zone could be determined by a temporally limited proliferation of meristematic cells. Under low phosphorus, cells were proliferative for a longer time than under high phosphorus, suggesting either that the termination of cell proliferation was not time regulated or that the temporal control changed. The spatial dimensions of the division zone might be related to the constant number of division cycles of the progeny of a cell formed by the division of the initial cell at the base of the meristem. Regardless of phosphorus status, the number of division cycles before cells entered into the elongation-only zone was four to six. There was more variation between cell files within a division zone than between plants of different phosphorus status, showing the importance of evaluating meristem parameters for individual cell files.

A review of the literature provided further support for the observed constancy. The length of the division zone in leaves of different C3 grass species has been reported to vary between 1 and 8 mm (Beemster et al., 1996; Fiorani et al., 2000; Masle, 2000; Bultynck et al., 2003). The range of number of division cycles of the progeny of the initial cell is, however, narrower. We calculated from the published data that the number of division cycles was six to eight, which suggests that this parameter may be relatively conservative.

Interestingly, this study indicated that the length of the elongation-only zone was not affected by phosphorus deficiency. As discussed previously, phosphorus deficiency decreased the flux of cells through this zone but did not affect the elongation-only zone length, thus increasing the duration of an individual cell's elongation. This contradicts the view that the termination of cell elongation is time regulated. Support for the temporal regulation has been obtained by finding the opposite; namely, that the size of the elongation-only zone is proportional to the number of cells

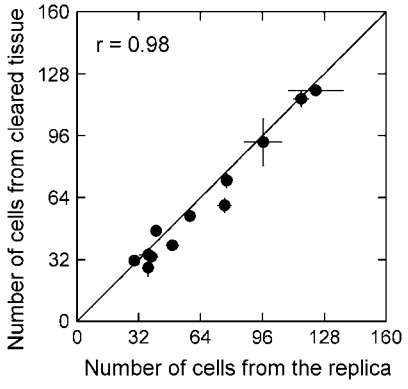

Figure 8. Comparison of the number of cells in the division zone determined from the transparent Formvar replica versus the number of cells determined from fixed and cleared leaf tissue. Each leaf was cut into two equal pieces along the central midrib. One-half was fixed and cleared following the procedure of Beemster et al. (1996). A Formvar replica was taken from the other half. The number of cells in the meristem was counted from the basal end (the ligule) to the position of the last recently formed (visually thinner) cell wall. Each data point represents the average of five to 10 cell files per leaf along with its SE. The line indicates the $y = x$ relationship.

flowing through it (i.e. cell production rate) because each cell has a temporal program of elongation to execute (Beemster and Baskin, 1998). Following this reasoning, a change in cell flux should lead to a change in elongation-only zone length. This did not happen in our study, suggesting either that termination of cell elongation was not time regulated or that this control changed. Previously, we have shown that the elongation-only zone length correlates with tiller size (Kavanová et al., 2006) and suggested that morphogenic effects of light quality could provide a mechanism for the spatial control of its length (Barlow, 1984).

In contrast to the only other kinematic study of phosphorus effects on leaf growth (Assuero et al., 2004), we found no difference in the division and elongation-only zone lengths in plants differing in their phosphorus status. This might be due to a species difference. However, the length of both zones varies during leaf development, and with changing tiller size (Durand et al., 1999; Kavanová et al., 2006). Thus, the discrepancy might arise from size differences between phosphorus treatments in the study of Assuero et al. (2004). In the primary root of Arabidopsis, phosphorus deficiency did not affect meristem length but decreased the length of the rapid elongation zone (Ma et al., 2003). Similar to leaves, regulation of the growth zone length in roots is not well understood. The comparison between phosphorus deficiency effects on leaf and root elongation suggests that root and leaf growth zones may differ in the cellular mechanisms underlying the growth response.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Surface-sterilized seeds of *Lolium perenne* L. cv Condesa were sown in pots (diameter 5 cm, height 35 cm) on a mixture of quartz sand with 63 mg phosphorus per pot in the form of finely ground Hyperphos (Deutsche HyperPhos-Gesellschaft), providing a source of phosphorus with low availability. Each pot contained one plant. Plants grew in a growth chamber (E15; Conviron), with 20°C (day)/15°C (night), 70% relative air humidity, and 525 μmol m^{-2} s^{-1} photosynthetic photon flux density at plant height for 16 h/d. Plants were irrigated for 21 d after sowing four times a day with 25 mL of modified one-half-strength Hoagland solution [0.02 mM KH_2PO_4, 2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM NaCl, 0.125 mM Fe-EDTA, 23 μM H_3BO_3, 4.5 μM $MnSO_4$, 0.38 μM $ZnSO_4$, 0.16 μM $CuSO_4$, and 0.05 μM Na_2MoO_4]. Thereafter, two levels of soluble phosphorus were applied: 0.02 mM KH_2PO_4 (low phosphorus) and 1 mM KH_2PO_4 (high phosphorus).

Leaf Elongation Rate

To avoid confounding phosphorus status with tiller size effects (see Kavanová et al., 2006), the leaf elongation rate (mm h^{-1}) and its components were analyzed in tillers with similar sheath length of the youngest fully expanded leaf (Table I). To this end, the leaf elongation rate was measured 46 to 49 d after sowing in high- and 60 to 63 d after sowing in low-phosphorus plants.

In five to six plants per treatment, representative tillers with at least three fully expanded leaves were selected at each date. The leaf elongation rate was determined on the youngest, most rapidly growing blade during the phase of maximal growth, when the leaf elongation rate was near constant. During this developmental stage, leaf growth is due exclusively to the activity of the blade growth zone, and cell division in the blade meristem and blade expansion are approximately steady (developmental stage A → B; Schnyder et al., 1990). The leaf elongation rate was calculated as the rate of change of the distance between the tip of the elongating blade and the ligule of the youngest fully expanded leaf, which was measured with a ruler every 24 h.

Sampling and Phosphorus Analysis

Twelve plants per treatment were sampled at the end of the light period 49 d after sowing in high- and 63 d after sowing in low-phosphorus plants. Leaf growth zones were dissected from leaves similar to those used for leaf elongation rate measurements. Fresh weight was recorded, samples were frozen in liquid N_2, freeze-dried, weighed, ground, and stored at −25°C. Phosphorus concentration was determined on 10- to 20-mg pooled samples as described by Kavanová et al. (2006).

Cell Length Measurement

The growing blade was carefully freed from surrounding older leaves in six plants per treatment 47 d after sowing in high- and 61 d after sowing in low-phosphorus plants. A transparent replica of the abaxial epidermis along the basal 50 mm of the growing leaf was taken as described by Schnyder et al. (1990). Briefly, a thin layer of 4% (w/w) polyvinylformaldehyde (Formvar 1595 E; Merck) in chloroform was spread along the basal part of the growing leaf. Then the film was transferred with transparent adhesive tape to a microscope slide.

Images were captured using a digital camera (Camedia C-5050Z; Olympus) fitted to an optical microscope (Olympus BX50). Leaves were excluded if the ligule was situated more than 1 mm from the leaf insertion to ensure that only the blade growth zone was contributing to the leaf elongation (Schnyder et al., 1990). Starting from the base of the growing blade (i.e. the ligule), images were taken every 1 mm (0–10 mm from the base) or 2 mm (>10 mm from the base). Images were captured at magnifications of 400× to 40× (according to increasing cell lengths), and subsequently analyzed in Sigma Scan Pro 5.0 (SPSS). The mean epidermal cell length at each distance from the base was determined by measuring the length of 20 to 80 cells in cell files located midway between files containing stomata.

In addition, a sequence of overlapping images was taken along the basal 2 mm (starting from the ligule), and composite images were created. The length and distance from the leaf base of every cell in eight to 12 cell files located midway between files containing stomata were recorded in each leaf. Mean epidermal cell length over 50-μm intervals was determined for each plant and then averaged over plants of the same treatment. Similarly, the length of the longest and shortest epidermal cell over 50-μm intervals was determined for each plant and then averaged over plants of the same treatment. In each cell file, we recorded the most distal position of a newly formed (visually thinner) perpendicular cell wall, which was used as a marker for the distal end of the cell division zone.

This is a novel method based on the same rationale as that introduced by Beemster et al. (1996) and used by Masle (2000). The latter method assessed newly formed perpendicular cell walls on cleared fixed tissue instead of replicas of the leaf surface. We validated the new method by comparison with that of Beemster et al. (1996). To this end, the base of 12 growing leaves in different stages of development of *L. perenne* cv Agenta was halved along the midrib. One-half of the leaf was treated as in Beemster et al. (1996); from the other half, a Formvar replica was taken. The position of the last newly formed perpendicular wall in epidermal cell files midway between files with stomata was measured in both sets of samples. The two methods yielded virtually identical results (Fig. 8).

Analysis of Cell Elongation

Final cell length (L_f, mm) and leaf growth zone length (L_{LGZ}, mm) were determined by fitting a Richards function (Morris and Silk, 1992) to plant cell length profiles (TableCurve 2D; SYSTAT):

$$y = e + a\frac{1}{(1+\exp^{(b-cx)})^{\frac{1}{d}}}, \quad (1)$$

where y is the cell length, x is the distance from the leaf base, $e + a$ is the asymptotic final cell length, e is the average meristematic cell length, and b, c, and d are constants. Because a reaches the maximal value only at an infinite distance, L_f was estimated as 95% of the value of a and L_{LGZ} as the position where this was reached.

Cell flux (F, cells h^{-1}), the rate at which cells are displaced past a particular position, was estimated at the distal end of the elongation-only zone from the leaf elongation rate (LER) and final cell length (L_f):

$$F = \frac{LER}{L_f}. \quad (2)$$

Under steady-state growth, when the leaf elongation rate and the cell length profiles do not change with time, cell flux is uniform beyond the division zone, and equal to the cell production rate (Silk, 1992).

In the elongation-only zone, the displacement velocity of a cell at a certain position is the result of the elongation of all cells located more basally in the growth zone. Therefore, displacement velocity increases with distance from the leaf base and finally becomes constant and equal to the leaf elongation rate. Under steady-state growth, there is strict correspondence between local cell length [$L(x)$, mm] and local displacement velocity [$v(x)$, mm h^{-1}] in the elongation-only zone (Morris and Silk, 1992; Silk, 1992):

$$v(x) = \frac{L(x)}{L_f} \times LER. \quad (3)$$

The relative elongation rate in the elongation-only zone (R_e, mm mm^{-1} h^{-1}; synonymous terms that have been used before are strain rate, relative elemental growth rate, and segmental elongation rate) was estimated by differentiating numerically the displacement velocity with respect to position. This parameter provides a measure to compare the magnitude of the elongation rate independently from the absolute cell length at a given position (Silk, 1992).

The average relative elongation rate in the elongation-only zone ($\overline{R_e}$, mm mm^{-1} h^{-1}) was calculated as:

$$\overline{R_e} = \frac{v_e - v_d}{L_e}, \quad (4)$$

where v_e and v_d are displacement velocity (mm h^{-1}) at the end of the elongation-only zone and division zone, respectively, and L_e is the elongation-only zone length (mm).

The spatial profiles of cell length, displacement velocity, and relative elongation rate were transformed in temporal profiles by calculating the trajectory function that describes the time it takes for a cell located at position x to be displaced to the end of the elongation-only zone (Silk et al., 1989):

$$t(x) = c \times N_e(x), \quad (5)$$

where c, the cellochron (h $cell^{-1}$), is the time required to displace a cell forward by one position in a cell file within the elongation-only zone and is equal to the inverse of cell flux, and $N_e(x)$ is the number of cells present between position x and the distal limit of the elongation-only zone.

The average elongation duration (i.e. the residence time of a cell in the elongation-only zone; T_e, h) was then calculated as:

$$T_e = \frac{N_e}{F}, \quad (6)$$

where N_e is the total number of cells present in the elongation-only zone and F is the cell flux.

Analysis of Cell Division

The average division rate of cells in the meristem can be determined by relating the cell production rate to the number of cells per meristematic cell file in the division zone (Ivanov and Dubrovsky, 1997). This estimation assumes all cells in the meristem are proliferative, which is supported by studies showing that the proliferative fraction is close to 1 (Ivanov and Dubrovsky, 1997; Ivanov et al., 2002, and refs. therein). Also, the constancy of the division rate along the meristem has been shown (Beemster et al., 1996) and discussed (Baskin, 2000).

The average cell division rate (D, cell $cell^{-1}$ h^{-1}) was calculated as:

$$D = \frac{F}{N_{div}}, \quad (7)$$

where F is the cell production rate and N_{div} is the number of cells in a meristematic cell file in the division zone (Green, 1976; Ivanov and Dubrovsky, 1997).

The number of cells in a meristematic cell file (N_{div}) was directly counted from the basal end of the division zone (i.e. the ligule) to the position of the last recently formed perpendicular cell wall. Meristematic cell length was determined as the average cell length between the basal and the distal end of the cell division zone.

The average cell cycle duration (T_c, h), the time from a cell's formation to the next cytokinesis that yields two daughter cells, was calculated as follows (Green, 1976; Ivanov and Dubrovsky, 1997):

$$T_c = \frac{\ln(2)}{D}. \quad (8)$$

The real residence time for an individual cell in the division zone is equal to T_c. However, it is possible to estimate the time needed for a perpendicular cell wall situated at the basal end of the division zone to reach the distal end of it. The residence time in the cell division zone (T_{div}, h) is then related to the number of division cycles necessary to form all cells in the division zone (Korn, 1993; Beemster and Baskin, 1998):

$$T_{div} = T_c \times \log_2(N_{div}). \quad (9)$$

The average number of division cycles of the progeny of a cell formed by the division of the initial cell at the base of the meristem (i.e. the number of division cycles necessary to displace a transversal cell wall from the basal to the distal boundary of the division zone) was determined as follows (González-Fernández et al., 1968):

$$N = \log_2(N_{div}). \quad (10)$$

Statistical Analysis

Differences between treatments were tested by Student's t test (Statistica 6.0; Statsoft). The error associated with parameters calculated from averages (e.g. cell production and average cell division rate) was estimated by Gaussian error propagation. Results are shown as means ± SE.

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