C: N: P stoichiometry and specific growth rate of clover colonized by arbuscular mycorrhizal fungi

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Abstract Ecological stoichiometry has been widely applied in aquatic ecosystems, but has limited implications in terrestrial ecosystems. The pot experiments with *Trifolium repens* L. were conducted to demonstrate the relations between C: N: P, biological components and growth rate of clover colonized by arbuscular mycorrhizal (AM) fungi. The results showed that for mycorrhizal clover, N, P concentrations increased with increasing growth rate, in

support of the Growth Rate Hypothesis (GRH). Mycorrhizal clover had higher P and RNA concentrations than non-mycorrhizal clover, indicating that the increase in P concentration would invest more RNA to meet the synthesis of protein. Results also indicated that the increase in N concentration with rapid growth rate may be attributed to the increase in the concentration of protein N. Underlying mechanisms driving the association of C: N: P with growth rate for symbiotic partners should help elucidate the allocation of major nutrients to cellular organs and trophic dynamics in terrestrial ecosystems.

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Introduction

Ecological stoichiometry is the study of the mass balance of multiple chemical elements in living systems (Reiners 1986; Hessen and Lyche 1991; Sterner 1995; Elser et al. 1996, 2000a), and recent advances have raised new questions about the implications of ecological stoichiometry for nutrient cycling and the functioning of organisms and communities of organisms in aquatic as well as terrestrial ecosystems (Elser et al. 1996; Elser and Urabe 1999; Daufresne and Loreau 2001; Sterner and Elser 2002; Vrede et al. 2004). In particular, the dynamics of carbon (C), nitrogen (N)

and phosphorus (P) have been considered to be important in this context because these elements are both structurally and functionally essential in all organisms (Sternner 1995; Elser et al. 1996; Vrede et al. 2004). Ecological stoichiometry also suggests mass-balance constraints on processes of interaction in ecosystems, such as coexistence, competition, stability (Sternner and Elser 2002) and on the evolution of organisms (Elser et al. 2006).

Recent studies have suggested a mechanistic linkage between tissue elemental stoichiometry and the growth rate of the organism (Elser et al. 2000a; Kyle et al. 2003). The Growth Rate Hypothesis (GRH) proposes that higher growth rates are coupled not only to lower C: N and C: P ratios, but also to lower N: P ratio, and the variation in P concentration in organisms is driven by differences in allocation to P-rich ribosomal RNA (rRNA) needed to meet the protein synthesis demands of increased growth rates (Hessen and Lyche 1991; Elser et al. 1996, 2000a; Vrede et al. 2004; Hessen et al. 2007). This GRH potentially connects cellular allocation to organism function and ultimately to ecological dynamics. Thus, any factor affecting growth, or physiological adjustment leading to changes in growth rate could result in differences in C: N: P of biomass and cellular allocation (Hessen et al. 2007). The GRH has been supported by a variety of evidence in different taxa. For example, significant associations between growth rate, organism C: N: P and biochemical composition have been shown for zooplankton in interspecific or interclonal comparisons (Main et al. 1997; Vrede et al. 1998; Elser et al. 2000b; Gorokhova et al. 2002; Weider et al. 2005).

The symbiosis between the majority of higher plants and arbuscular mycorrhizal (AM) fungi is one of the most ancient and abundant mutualisms in terrestrial ecosystems (Read 1991; Redecker et al. 2000). As biotrophs, mycorrhizal fungal hyphae explore the soil volume for mineral nutrients (Bolan 1991; Marschner 1998) and provide these nutrients (especially P) to plants in return for carbon (C) (Chen et al. 2004, 2007; Smith and Read 1997), so the formation of AM can impact plant C allocation. Therefore AM fungal influences on C: N: P stoichiometry in plants will affect trophic dynamics and nutrient cycling. The mechanisms of plant-fungus physiology have been well documented (Allen et al. 2003), however, few studies have attempted to

examine the connection between biomass C: N: P and growth rate and rRNA allocation in plants colonized by AM fungi.

The aim of this study was to assess the influence of AM fungi on C: N: P stoichiometry, plant growth rate and biochemical composition, and the interaction between them, and test if the GRH patterns are suitable for mycorrhizal and non-mycorrhizal plants. Our hypothesis was that AM fungal colonization can enhance P concentration in plants, thus AM fungal colonization will increase the allocation to ribosomal RNA, change C: N: P stoichiometry and plant growth rate.

Materials and methods

Mycorrhizal inoculum

The AM fungus *Glomus mosseae* BGCXJ03A was kindly provided by Beijing Academy of Agriculture and Forestry Sciences. The inoculum was a mixture of spores, mycelium, sandy soil and plant root fragments containing approximately 742 spores per 20 ml solution.

Host plants

Seeds of white clover (*Trifolium repens* L.) were surface sterilized in a 10% (v/v) solution of hydrogen peroxide for 10 min and washed by deionized water. They were then germinated on moist filter paper for about 24 h until the radicles appeared. They were selected for uniformity before sowing.

Soil properties

The soil was collected from a crop field in the hilly region of the Chinese Loess Plateau in Dingxi City, Gansu province, China. This area has a semiarid climate, and the rainfall in this area is very low and variable, therefore, available water and soil nutrients are important limiting factors for agriculture and the development of vegetation (Jiang et al. 1981; Hessel and van Asch 2003). The soil is a silt loam with the following properties (on a dry mass basis): pH (soil: water ratio 1: 2.5) 8.67, organic matter 1.04%, 0.5 mol l⁻¹ NaHCO₃-extractable P 12.25 mg kg⁻¹. The soils were passed through a 2-mm sieve, and

sterilized by γ - radiation (20 Kgy, 20 MeV electron beam).

Experimental procedure

Experiment one

White clover plants were either inoculated with 30 g of AM fungus *G. mosseae* inoculum (+M) or an equivalent amount of sterilized inoculum as controls (–M). White plastic pots (180×160 mm) were used, each pot contains 1.0 kg of soil. Firstly, 600 g sterilized soil was placed in the pot, then mycorrhizal inoculum, and finally the remainder of the sterilized soil. Germinated seeds were sown in each pot and 7 days after seedling emergence these were thinned to 12 seedlings per pot. The experiment was conducted in the glasshouse, and the plants grew from 7 April 2006 to 16 June 2006 under natural light conditions with no supplementary illumination. During the period of plant growth, the day temperature ranged from 20 to 30°C and the night temperature from 10 to 20°C, deionized water was used to maintain 75% field capacity by regular weighing.

Plants were first harvested after a month to establish baseline biomass (M_0), then a group of plants were harvested at ten day intervals. Thus, there were 10 treatments in total with 4 replicates, giving a total of 40 pots in a randomized block design. No treatments received fertilizers.

At harvest, shoots and roots were harvested separately. Samples were carefully washed in tap water and then deionized water. Weighed sub-samples of fresh roots were collected for determination of AM fungal colonization. The dry weights of shoots, and roots were determined after drying at 70°C for 48 h, then shoots were analyzed for C, N, P concentrations (see below). Total biomass weights obtained from the dried samples were then used to calculate specific growth rates for each interval as:

$$\mu(\text{day}^{-1}) = \ln(m_T/m_0)/T,$$

Where m_T is plant biomass at a given time T , m_0 is the baseline biomass at the start of the interval, and T is the given time T (days) (Sternier and Elser 2002).

Experiment two

White clover plants were grown in the same conditions, however, each treatment included eight

replicates. Weighed sub-samples of fresh shoots at the final sampling time were snap-frozen in liquid nitrogen and stored at –80°C for analysis of RNA. The dry weights of shoots, and roots were determined after drying at 70°C for 48 h, then shoots were analyzed for protein N concentration.

AM fungal colonization

Sub-samples of fresh roots were cleared and stained according to Phillips and Hayman (1970). Percentage root colonization was determined by the grid-line intersect method (Giovannetti and Mosse 1980).

Macronutrient analysis

Oven-dried sub-samples of shoots were milled and digested with a mixture of hydrogen peroxide and sulphuric acid for nitrogen and phosphorus analysis. Total N concentrations were determined using Kjeldahl analysis (Jones 1991). P was determined colorimetrically by the vanadomolybdate method (Jackson 1958). C concentration was determined after digestion with potassium dichromate solution and sulphuric acid, and then analyzed by the titration method of Lu (1999).

RNA extraction and estimation/quantification of concentration

RNA was extracted by the modified guanidinium isothiocyanate (GITC) method (Chomczynski and Sacchi 1987). Mortars, pestles and spatulas used to manipulate and homogenize the tissue were treated with alcohol. Plant tissue was ground with a mortar and pestle under liquid nitrogen, the fine powder was transferred to a 1.5 ml centrifuge tube with a spatula and mixed with pre-cooled GITC extraction solution consisting of 4 M GITC, 25 mM sodium citrate, 0.5% (*m/v*) Sodium Lauroyl Sarcosine, and 0.1 M β -mercaptoethanol. Then 2 M Sodium Acetate (pH=4.0) was added and vortexed fully. Subsequently, PCI reagent (phenol: chloroform: isoamyl alcohol = 125:24:1) was added, vortexed and incubated on ice for 15 min. The mixture was then centrifuged at 12,000 *g* for 30 min at 4°C to facilitate phase separation. The resulting supernatant was transferred to a new tube, where RNA was precipitated by the addition of isopropyl alcohol at –20°C for 1 h. RNA was isolated

by centrifugation at 12,000 *g* for 20 min at 4°C. The resulting pellet was washed twice with 70% ethanol, then the pellet was dried and dissolved in GITC extraction solution, RNA was precipitated by the addition of isopropyl alcohol at –20°C for 30 min–1 h again, RNA was isolated by centrifugation at 12,000 *g* for 20 min at 4°C. The resulting pellet was washed with 70% ethanol and dried, then the pellet was dissolved in autoclaved diethyl pyrocarbonate (DEPC) water for further analysis.

The extracted RNA (5 µl) was checked by 1.0% agarose gel electrophoresis in TAE buffer. The gels were stained with ethidium bromide and determined by electrophoresis.

Concentration and purity of total RNA were assessed in a NanoDrop® -ND 1000 Spectrophotometer (NanoDrop Technologies, New Zealand), using 2 µl aliquot of the total RNA solutions. RNA purity was estimated from the A_{260}/A_{280} absorbance ratio, which provides an estimation of contamination mainly from proteins and phenol. Generally speaking, the A_{260}/A_{280} ratio is from 1.8 to 2.2. Putative DNA contamination was not directly assessed; however, a very low DNA contamination cannot be excluded (Portillo et al. 2006). An internal or external standard was not used for the estimation of the recoveries in the protocol of RNA extraction, so the concentration of RNA was not very accurate and rather semi-quantitative. RNA concentration was treated as “supportive data” in the text.

Protein N

The milled sub-samples were mixed with 5% trichloroacetic acid and heated for 15 min at 90°C, the solution was centrifuged at 4,000 rpm for 15 min, and the precipitate was washed with 5% trichloroacetic acid twice, then with distilled water on filter paper. The filter paper with the precipitate was dried at 50°C and digested with a mixture of hydrogen peroxide and sulphuric acid for protein N analysis (Li 2000).

Data analysis

The relationships between growth rate and total biomass, root/shoot (R/S) ratio, shoot nutrient concentrations, protein N concentration were analyzed using the linear regression of SPSS 11.5.

Results

Mycorrhizal colonization

No root colonization was detected in the non-inoculated plants, while the colonization of inoculated plants was relatively high, and sampling time had no detectable influence on root colonization. Mycorrhizal colonization was from $30\% \pm 3.09$ to $36\% \pm 0.95$. However, at final sampling time, mycorrhizal colonization was only $14\% \pm 0.09$ (data not shown). During the experiments, all plants remained free from rhizobial colonization.

Plant biomass and specific growth rate

AM fungal colonization positively correlated with the specific growth rate and biomass production of clover compared with the non-mycorrhizal clover (Figs. 1 and 2). Irrespective of mycorrhizal colonization, specific growth rate decreased with the increase in development time (Fig. 1); and specific growth rate of mycorrhizal clover decreased more strongly (Table 1). For mycorrhizal clover, growth rate was negatively related to plant biomass, while total plant biomass of non-mycorrhizal clover did not vary significantly with increasing growth rate (Fig. 2 and Table 1).

Root biomass and R/S ratio

AM fungal colonization increased root biomass compared with non-mycorrhizal clover. For mycorrhizal clover, root biomass increased with time, ranging from 0.4 g to 1.1 g, while root biomass of

Fig. 1 The association between specific growth rate (d^{-1}) and time (d) for mycorrhizal and non-mycorrhizal clover. Error bars indicate standard errors ($n=4$)

Fig. 2 The association between total plant biomass and specific growth rate (d^{-1}) for mycorrhizal and non-mycorrhizal clover. Error bars indicate standard errors ($n=4$)

non-mycorrhizal clover did not increase significantly with time (Fig. 3).

Compared with non-mycorrhizal clover, R/S ratio was lower for +M treatments at some harvests. R/S ratio of mycorrhizal clover enhanced first, declined thereafter with time, while R/S ratio of non-mycorrhizal clover did not vary with time (Fig. 4).

Macronutrient uptake

Figure 5 shows that AM fungal colonization had different effects on N and P concentrations of clover plants. Shoot N concentration was lower in mycorrhizal plants; however P concentration was higher in mycorrhizal plants than that in non-mycorrhizal plants. C concentration, which accounted for about 40% dry weight, was not different between mycorrhizal and non-mycorrhizal treatments (Fig. 5a). N-concentrations were higher when plants had a higher relative growth rate. P concentration of mycorrhizal

clover increased significantly with increasing growth rate, while there was no correlation between P concentration and growth rate of non-mycorrhizal clover (Fig. 5b, c and Table 1).

C: N: P stoichiometry and specific growth rate

C: N ratio of mycorrhizal clover was much higher than that of non-mycorrhizal clover (Fig. 6a), and C: P and N: P ratios of mycorrhizal clover were lower than those of non-mycorrhizal clover (Fig. 6b and c). Irrespective of mycorrhizal colonization, C: N ratio decreased with increased growth rate (Fig. 6a). For mycorrhizal clover, C: P ratio was related negatively to growth rate (Fig. 6b and Table 1), while N: P ratio was associated positively with growth rate (Fig. 6c and Table 1). For non-mycorrhizal clover, C: P and N: P ratios did not vary significantly with increasing growth rate.

RNA and Protein N concentrations

The RNA concentration of non-mycorrhizal clover was 40% of that of mycorrhizal clover. The ratio of A_{260}/A_{280} was about 2, irrespective of mycorrhizal colonization (Table 2).

At high growth rate, mycorrhizal clover had a higher protein N concentration than non-mycorrhizal clover (Fig. 7). In both mycorrhizal and non-mycorrhizal plants protein N concentration was positively related to specific growth rate, with a better relationship between protein N concentration and growth rate for mycorrhizal clover than non-mycorrhizal clover (Fig. 7 and Table 1).

Table 1 Comparison of relationships between linear models describing the relationships between growth rate and total biomass, nutrient and protein N concentrations in non-mycorrhizal (-M) and mycorrhizal (+M) plants

		Intercept		Slope		R^2		P	
		-M	+M	-M	+M	-M	+M	-M	+M
Biomass	Growth rate	0.13	4.34	1.28	-23.50	0.20	0.71	0.09	<0.001
C (%)		40.18	40.49	32.71	26.95	0.04	0.30	0.45	<0.05
N (%)		3.84	-0.76	74.17	34.18	0.55	0.78	<0.01	<0.001
P (%)		0.07	0.17	0.90	1.68	0.10	0.64	0.25	<0.001
C: N		10.35	53.02	-122.69	-332.99	0.55	0.75	<0.01	<0.001
C: P		529.67	197.02	-627.14	-672.19	0.00	0.52	0.91	<0.01
N: P		52.90	0.33	677.20	72.94	0.06	0.67	0.36	<0.001
Protein N (%)		0.69	0.36	18.17	10.24	0.38	0.60	<0.05	<0.001

Fig. 3 The association between root biomass (g) and time (d) for mycorrhizal and non-mycorrhizal clover. Error bars indicate standard errors ($n=4$)

Discussion

Many studies have demonstrated a strong positive correlation between N and P concentrations and growth rate in bacteria and zooplankton, supporting the GRH (Main et al. 1997; Elser et al. 2000b; Karpinets et al. 2006). However, little information is available for terrestrial plants, particularly in relation to different nutrient uptake mechanisms, such as the mycorrhizal symbiosis. Our findings indicated that N and P concentrations in mycorrhizal clover significantly increased with growth rate, supporting the GRH of Sterner and Elser (2002) that plants with rapid growth rate can absorb more nutrients to achieve fast growth (Lovelock et al. 2007). Specific growth rate was negatively related to biomass C: N, while there were no trends in N: P with rapid growth rate, contrary to expectations of decreasing N: P at higher growth rate (Elser et al. 2000a). In the present study the inoculation of clover with AM fungi increased P concentration, which may result in an altered balance between N and P. According to the results of short-fertility experi-

Fig. 4 The association between R/S ratio and time (d) for mycorrhizal and non-mycorrhizal clover. Error bars indicate standard errors ($n=4$)

Fig. 5 The association between elemental composition and specific growth rate (d^{-1}) for mycorrhizal and non-mycorrhizal clover. (a) C concentration (b) N concentration (c) P concentration. Error bars indicate standard errors ($n=4$)

ments (Güsewell 2004), when N: P ratio <10 , plant growth was limited by N. N may therefore be the primary factor limiting growth rate of mycorrhizal clover. However, for non-mycorrhizal plants, clear-cut relationships between C: P, N: P and specific growth rate were not observed. Factors limiting growth such as nutrient deficiency, lack of soil microorganisms and large sample variance may obscure the correlations between growth rate and stoichiometry.

In this study we found that AM fungal colonization exerted a beneficial effect on plant growth, which was attributed to mycorrhizal colonization and the increase in P concentration. However, mycorrhizal plants had a

Fig. 6 The association between C: N: P and specific growth rate (d^{-1}) for mycorrhizal and non-mycorrhizal clover. (a) C: N (b) C: P (c) N: P. Error bars indicate standard errors ($n=4$)

lower N concentration than non-mycorrhizal plants, owing to the increase in plant biomass which dilutes the N (Figs. 3 and 5b) and changes the C: N: P. RNA makes a large contribution to organism mass, has high P content, and rRNA which is the machine of growth in the cell making up approximately 80–90% of the total RNA in most cells (Brandhorst and McConkey

Table 2 The effect of AMF colonization on RNA concentration

Treatment	RNA ($\mu\text{g g}^{-1}$)	A_{260}/A_{280}
–M	86.9 (± 6.1)	1.98
+M	226.6 (± 18.6)	2.13

Error bars indicate standard errors ($n=4$)

Fig. 7 The association between protein N and specific growth rate (d^{-1}) for mycorrhizal and non-mycorrhizal clover. Error bars indicate standard errors ($n=4$)

1974; Campana and Schwartz 1981; Vrede et al. 2004). Protein synthesis in the cell is limited by the amount of rRNA. Therefore, the variation in organismal RNA concentration may be driven primarily by the change in P concentration. For mycorrhizal plants, an increase in P requirements exhibited an increased investment in P-rich RNA, thus, the increase in P concentration in mycorrhizal plants can enhance the rate of protein synthesis for a higher growth rate. This was also observed in the data showing that mycorrhizal plants had higher protein N than non-mycorrhizal plants at rapid growth rates. N is an essential component of all enzymes and P is necessary for rRNA synthesis (Elser et al. 2003), consequently changes in N and P concentrations will affect nutrient allocation to cellular components, life history strategy and physiological function.

This study analyzed root biomass and R/S ratio as measures of the C-cost of AM establishment and maintenance. Root biomass in non-mycorrhizal clover was too small to meet all analyses needs and additional analysis of C, N and P concentrations could not be undertaken on the clover model. To date, studies of ecological stoichiometry have mainly focused on leaves or shoots of plants, and there have been more data collected for leaves or shoots than roots. However, further understanding of the influence of different nutrient acquisition mechanisms, such as AM symbiosis, will benefit from more detailed analysis of total plant C budgets. Future work should consider a range of model plant species for a wider range of analysis useful for understanding ecological stoichiometry.

The results showed that AM fungal colonization increased root biomass, and R/S ratio of mycorrhizal clover was lower than that of non-mycorrhizal clover at

several harvest times, indicating that AM fungal colonization could enhance investment in shoot biomass, increasing plant photosynthesis and providing AM fungi with more carbohydrate. Root biomass in mycorrhizal clover increased strongly with time, and R/S ratio was lower at the final harvest in mycorrhizal than non-mycorrhizal clover. This may be due to investment in root biomass in order to require more nutrients at the beginning of plant growth, with a switch to investment in shoot biomass after seedling establishment.

AM fungal colonization changed the C: N: P stoichiometry of clover, indicating a possible pathway of influence of the symbiosis in terrestrial ecosystems. The effects of stoichiometry on evolutionary and ecological dynamics are driven by the biochemical requirements of the cellular machinery required for contrasting life strategies (Vrede et al. 2004) and may affect biogeochemical cycling (such as C and N) in terrestrial ecosystem. In this study, AM fungal colonization altered plant quality, potentially affecting nutrient mineralization, and resulting in a correlation between C: N: P ratios and nutrient release of plant residues. Lower C: P ratios in mycorrhizal clover can enhance P availability in soil, eventually impacting nutrient cycling in terrestrial ecosystems (Chen et al. unpublished data). This may have additional implications for plant-herbivore dynamics by linking herbivore population dynamics with nutrient cycling. The change in plant stoichiometry could affect the characteristics of herbivore nutrition (Schade et al. 2003). The experiments of Schade et al. (2003) showed that high C: P ratio of plants can lead to a decline in body %P and abundance of herbivores. Feeding of herbivores on low C: P and N: P foods may lead to herbivore species shifts from low P demand, N-limited to high P demand, P-limited (Vrede et al. 2004). Based on the GRH, herbivore communities may become dominated by species with high growth rates. The association between plants colonized by AM fungi and herbivore dynamics deserves further investigation. The stoichiometric constraints on organism growth may have important consequences at large scales if they influence structure and function in food webs and alter trophic dynamics in terrestrial ecosystems.

Here we tested the GRH for mycorrhizal clover, and present data suggesting that AM fungal colonization changed C: N: P stoichiometry, and the changes in C:

N: P stoichiometry were associated with specific growth rate and plant cellular components. Investigating the consequences of stoichiometric shifts in mycorrhizal or non-mycorrhizal plants may help us understand underlying mechanisms driving the association between stoichiometry and growth rate, and how these changes in C: N: P stoichiometry will influence the outcomes of the interactions and nutrient cycling in terrestrial ecosystems.

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