CSIRO PUBLISHING

Crop & Pasture Science, 2016, **67**, 629–640
http://dx.doi.org/10.1071/CP15257

Competition and growth of a grass–legume mixture fertilised with nitrogen and phosphorus: effect on nutrient acquisition, root morphology and symbiosis with soil microorganisms

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Abstract. Achieving a fast initial growth is crucial for legumes because grasses grow more rapidly and compete much better with forbs. In a pot experiment with a nutrient-deficient soil, we added nitrogen (N), phosphorus (P) and N + P to pure and mixed stands of *Lotus tenuis* and *Festuca arundinacea* and investigated the effects of on plant growth, nutrient uptake and symbiotic associations with arbuscular mycorrhizae and rhizobia. Plant yield, N and P acquisition, mycorrhizal colonisation, rhizobial nodulation and root length were measured and root diameter and root surface area were calculated after two harvests. Species responded differently to specific nutrients when grown pure or mixed. Comparing pure with mixed stands in soils fertilised with P and N + P, *L. tenuis* showed decreased shoot and particularly root biomass, whereas *F. arundinacea* showed increases in both biomasses. This suggests that the competitiveness of the grass with the legume increased upon P and N + P addition. In mixed stands, *F. arundinacea* produced 51–64% of the total shoot biomass and 69–74% of the total root biomass with P and N + P, respectively. Root length and root surface area were greater and the roots thinner in *F. arundinacea* than in *L. tenuis*. Addition of P and N + P increased rhizobial nodulation in legume roots but decreased mycorrhizal colonisation in both plants. Supply of N does not necessarily favour grasses, whereas P supply favours legumes. Optimisation of P nutrition might help to maximise N inputs into grasslands by symbiotic N-fixation and decrease inputs of inorganic N by fertilisation.

Additional keywords: nutrient interactions, nutrient-limited environment, microorganisms, root morphology.

Received 4 August 2015, accepted 24 January 2016, published online 28 June 2016

Introduction

Land use and/or environmental conditions in temperate grasslands have led to the dominance of perennial and annual grasses associated with a decline or absence of native legumes (Muir *et al*. 2011). This lack of legumes plants in turn affects species diversity and herbage quality in those grasslands (Ledgard and Steele 1992). Generally, because grasses have fibrous root systems that give them an advantage when competing for shallow moisture and soil nutrients, they tend to establish more easily and recover from grazing more quickly than legumes (Muir *et al*. 2011). Furthermore, increasing the access to available nitrogen (N) tends to favour the dominance of grasses over legume plants (Aydin and Uzun 2005; Høgh-Jensen and Schjoerring 2010). However, legumes may have an advantage over grasses in N-deficient soils because legumes are able to fix atmospheric N_2 in ecosystems that are poor in N, such as native grasslands (Temperton et al. 2007). Legumes may have an additional advantage over grasses in such systems when available phosphorus (P) is relatively sufficient for plant growth; for example, an increase in P supply has been reported to enhance the competitive ability of white clover (*Trifolium repens*) growing in association with ryegrass (*Lolium rigidum*) (Høgh-Jensen and Schjoerring 2010).

The temperate grasslands of the Buenos Aires Province (The Pampas), a most productive area for beef and dairy cattle farms, comprise 30–40% lowlands affected by salinity, alkalinity and low availability of nutrients for plant growth, especially P, along with either an excess or deficit of water in the soil (Escudero and Mendoza 2005; García *et al*. 2008). These aspects, in addition to the lack of legumes, strongly affect the quality and quantity of forage production of these grasslands (Cahuépé 2004).

Lotus tenuis Waldst. & Kit. ex Willd. (narrow-leaf trefoil) is a widespread legume growing in winter–spring–summer that occurs naturally in the lowlands of the Argentine Pampas. This legume strongly responds to the addition of P in nutrient-deficient soils (Mendoza and Pagani 1997), can tolerate conditions of waterlogging or drought conditions for more

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than 1 month (Mendoza *et al*. 2005; García *et al*. 2008), and is dependent on arbuscular mycorrhizae to grow in P-deficient soils (Mendoza 2001). The presence of *L. tenuis* in mixed communities has been shown to be crucial for improving the quality of natural grasslands for beef production (Cahuépé 2004). *Festuca arundinacea* Schreb, (tall fescue) is a fast-growing perennial, autumn–winter–spring grass that produces high-quality pasture (Frame 1990). This grass tolerates moderate moisture excess and salinity in soil, has shown some resistance to drought, and can grow adequately with *L. tenuis* in mixed stands (Bañuelos and Beuselinck 2003).

Mixing grasses with legumes is an effective and economical management option to improve the quality and/or quantity of forage production in grasslands from regions where both temperate grasses and temperate legumes are adapted (Høgh-Jensen and Schjoerring 2010; Nyfeler *et al*. 2011). Enhanced productivity in mixtures compared with monospecific grasslands is mainly due to two major processes: complementarity and facilitation (Fridley 2001). In the case of a grass–legume mixture, a sufficient level of P is needed for adequate N_2 fixation, and differences between root architecture of the associated species for exploring the soil may facilitate and/or complement the uptake of N and/or P of one or both of the plant species (Hinsinger *et al*. 2011). Promoting the presence of native or naturalised perennial legumes, as in the example of *L. tenuis*, would result in greater plant diversity and wider adaptation to diverse climate and soil conditions (Bennett *et al*. 2011).

In grassland soils deficient in nutrients for plant growth, fertilisation with N and P, seeding legumes and mixing legumes with grasses have all been used to improve forage yield (Muir *et al*. 2011). In general, enhancing N supply may favour the growth of the grass over the legume, whereas enhancing P supply may promote the legume component (Jouany *et al*. 2011). Nevertheless, increasing nutrient supply in nutrient-deficient soils may vary the competitive pressure exerted by the two co-cultivated plants, such that the plants are incapable of responding to variations in the environmental conditions. The level of nutrient deficiency, the association of plants with soil microorganisms, the amount of nutrient addition and the ability of the roots to access nutrients in soil are some of the factors that may alter the competitive pressure between co-cultivated plants when nutrients are added to deficient soils.

The aim of the present experiment was to study the effects of addition of N, P and N + P to *L. tenuis* and *F. arundinacea* cultivated in monoculture or mixture on plant growth, N and P uptake, arbuscular mycorrhizal symbiotic associations, and rhizobial nodulation in the legume plant when grown in a typical N- and P-deficient soil. We were especially interested to examine the response of each plant species to the supply of a specific nutrient (N and/or P) when co-cultivated, because the achievement of a fast initial development is crucial for leguminous plants to compete with forbs and grasses over time.

We tested the following hypotheses. First, under N- and P-limiting conditions, *F. arundinacea* grows better when mixed with *L. tenuis* than in monoculture. This is because the legume fixes atmospheric-N_2, which increases the availability of soil inorganic N to the grass, and because the grass increases the proportion of roots colonised by arbuscular mycorrhizae when associated with a mycotrophic plant such as *L. tenuis*. Second, increasing nutrient supply varies the competitive pressure exerted by the co-cultivated plants. We wanted to test, in N- and P-deficient systems, whether improving N supply may favour the growth of the grass and enriching P may favour the growth of the legume. Third, the competitive pressure exerted between *F. arundinacea* and *L. tenuis* in mixed stands is associated with the ability of the root system to acquire nutrients from the soil because the roots of *F. arundinacea* are longer and thinner and have greater surface area than the roots of *L. tenuis*. These root properties therefore give the grass a competitive advantage over the legume plant.

Materials and methods

Soil characteristics and experimental setup

Soil samples were collected from the top 0.15 m layer in early autumn from a natural grassland in Chascomús (35°37 S, 58°50 W), Province of Buenos Aires, Argentina. The sampled site was a lowland affected by flooding in wet periods (autumn–winter–spring). It is currently grazed by beef cattle, which are sustained mainly by natural pastures dominated by perennial and seasonal grasses. A lack of legumes decreases the forage quality of these pastures. The soil of the sampled site is a Typical Natraquoll (Mendoza 2001), limited in NO_3^- and P for plant growth, and represents typical characteristics of the area in terms of soil properties. The soil was air-dried and sieved through 2-mm mesh screen. The main soil properties of the top 0.15 m (upper A1–2 horizon) were: pH 5.9, electrical conductivity (EC) 1.5 dS m^{-1}, total organic carbon 2.0%, total N 0.26%, organic matter 3.9%, available P (Bray I) 3.1 µg g^{-1}, NO_3^- 10.5 µg g^{-1}, exchangeable sodium percentage (ESP) 7.1%, clay content 33%, loam 51%, sand 16%, and field water capacity 36% (w/w). The upper horizon was followed by B2t of soil pH 8.0, EC 3.0 dS m^{-1} and ESP 23%.

Closed-bottom pots (capacity 2 L) were filled with 1.7 L of soil, with fertiliser treatments as follows: 100 µg N g^{-1} as $(NH_2)_2CO$; 100 µg P g^{-1} as H_2KPO_4; N and P together at the indicated doses; unfertilised control pots. Based on previous experiments, the doses of fertilisers applied to the soil used were sufficient to achieve maximum yield (Mendoza and Pagani 1997). The fertilisation treatment and the control pots were sown with three different plant stands: *F. arundinacea* and *L. tenuis* each in pure culture, and *F. arundinacea* plus *L. tenuis* in mixed culture.

Pre-germinated seeds of *F. arundinacea* cv. Arizona and *L. tenuis* cv. Esmeralda were sown on the soil surface and covered with a 0.2-cm layer of sand to reduce water evaporation. The pots were then placed on mobile tables and transferred to a greenhouse with mean day temperature 30 ± 4°C, mean night temperature 19 ± 3°C, mean relative humidity 65 ± 15%, photoperiod length 10–12 h, and midday photon flux density 900–1300 µmol m^{-2} s^{-1} (sunny days) and 400–700 µmol m^{-2} s^{-1} (with cloud cover) during the experimental period.

The pure stands of each species had six plants per pot and the mixed stands three plants of each species per pot. The resulting 12 treatments were replicated five times. The soil in the pots was maintained near 80% of field capacity by watering daily and bringing to a constant weight. The pots were completely

randomised and the mobile tables rotated daily to minimise potential gradient effects of the greenhouse environment. After two harvests (at 55 and 90 days after sowing), the shoots and roots of the 12 experimental groups were harvested and the shoot and root tissues from each species separated.

Plant yield and analytical determinations in tissue

At the first harvest 55 days after sowing, the shoot biomass was clipped 2.5 cm above the soil surface. After a further regrowth for 35 days, shoot biomass was clipped once again, but at soil level. The total root biomass of each pot was harvested and separated from the soil, and by plant species in mixed stand, in tap water. The roots were then washed three times with distilled water and weighed after drying with absorbent paper to eliminate excess water. The biomass of the clipped shoots was oven-dried at 70°C for 48 h and weighed for subsequent determination of plant yield and tissue N and P content. Biomass of fresh roots was divided into two portions, one oven-dried to make the same determinations as for shoots, and the other kept fresh to measure total root length, mycorrhizal colonisation (MC), and the number of rhizobial nodules in the roots of *L. tenuis*. The biomass of dried shoot and root was digested in sulfuric acid and analysed separately to determine N concentration by the Kjeldahl method (Jackson 1964) and P concentration by a modification of the Murphy and Riley method (John 1970).

Arbuscular mycorrhizal colonisation and root nodulation

Mycorrhizal root colonisation of both species was measured after clearing them in 10% KOH for 10 min at 90°C, and staining in 0.05% lactic acid–glycerol trypan blue (Phillips and Hayman 1970). In addition, the roots of *F. arundinacea* were cleared in 3% H_2O_2 for 15 min at room temperature. In total, 25 root segments per plant sample were examined under a microscope at 200× magnification. Root length was determined with the line-intercept method (Giovannetti and Mosse 1980). The fraction of root length colonised (MC index), and root length containing arbuscules (AC index), vesicles and hyphae only, were determined following McGonigle *et al*. (1990). The number of entry points was measured every 3 mm along root fragments at 200× magnification (Amijee *et al*. 1989) and expressed as the number of entry points per mm colonised root. Rhizobial nodules in the roots of *L. tenuis* were counted under a binocular stereomicroscope (7.5×).

Root measurements

In addition to the total root length (RL) and the total root weight (RW) of each plant species, the root diameter (RD) and the root surface area (RSA) were estimated through the following equations (Yang *et al*. 2004):

$$\mathrm{RD} = \sqrt{\mathrm{RW(g)} \times 1(\mathrm{cm^3/g})/\pi \times \mathrm{RL\ (cm)}} \quad (1)$$

$$\mathrm{RSA} = \mathrm{RD\ (cm)} \times \pi \times \mathrm{RL\ (cm)} \quad (2)$$

The value of 1 cm^3 used in Eqn 1 is an approach assuming that the roots of both species occupying a volume of 1 cm^3 weigh 1 g. This is not strictly correct because the value may differ between plant species.

Soil analyses

At the end of the experiment, soil samples of ~200 g from the centre of each pot were taken and analysed for pH, EC and available P (Bray and Kurtz 1945). Additionally, total organic carbon, total N, organic matter, NO_3^- and NH_4^+ were analysed (Marban 2005).

Statistical analyses

Datasets were tested for normality and variance heteroscedasticity by standard methods. Non-normal data were appropriately transformed for comparing treatment means. Two-way analysis of variance (ANOVA) was used to test the equality of treatment means, and separation among means was performed by the Tukey test. The main-plot treatment was the plant stands and the sub-plot treatment the fertilisation. Statgraphics 5.0 plus software (Statpoint Technologies, Warrenton, VA, USA) was used for statistical analyses.

Results

Plant yield

Accumulated shoot and root dry yields were affected by plant stand and type of fertilisation, and there was a significant interaction between the two variables (Fig. 1). The shoot yield of the second harvest was much higher than that of the first harvest (Fig. 1*a*). The accumulated dry yield per plant of *F. arundinacea* pure or mixed with *L. tenuis* did not respond to N supply, whereas the dry yield of *L. tenuis* pure or mixed with *F. arundinacea* increased with respect to the control treatment (Fig. 1*b*). Fertilisation with P alone or N + P increased the dry yield in all plant stands in both pure and mixed cultures. *Lotus tenuis* pure with P alone produced higher shoot yield than *F. arundinacea* pure or mixed and *L. tenuis* mixed, whereas *F. arundinacea* pure or mixed and fertilised with N + P showed increased shoot yield and produced statistically the same yield as *L. tenuis* pure (Fig. 1*b*). With N + P supply, *L. tenuis* accumulated higher shoot yield per plant when grown pure than when grown mixed with *F. arundinacea*, whereas *F. arundinacea* had an opposite response, accumulating higher yield when grown mixed with *L. tenuis* than when grown in pure culture (Fig. 1*b*). The root dry weight of both plants showed a different response from that of shoots with respect to N and P fertilisation. In mixed stands, the growth of the roots of *F. arundinacea* increased with P and even more with N + P fertilisation in contrast to the *L. tenuis* component, in which the root yield remained the same with either P or N + P fertilisation (Fig. 1*c*). The relative differences in growth between the shoots and the roots of the three plant stands upon P and N + P fertilisation are reflected in the shoot : root ratio, whereby *L. tenuis* pure or mixed showed a higher ratio than *F. arundinacea* either alone or mixed (Fig. 1*d*).

Nitrogen and P in plant tissue

Nitrogen content in shoot and root tissues was affected by plant stand, fertilisation and the plant stand × fertilisation interaction (Fig. 2*a*, *b*). In the unfertilised control, the two plant species pure or mixed contained statistically the same amount of N in their shoots (5.9 ± 0.5 mg N $plant^{-1}$), whereas the *L. tenuis* roots in pure culture differed significantly from *F. arundinacea* roots in

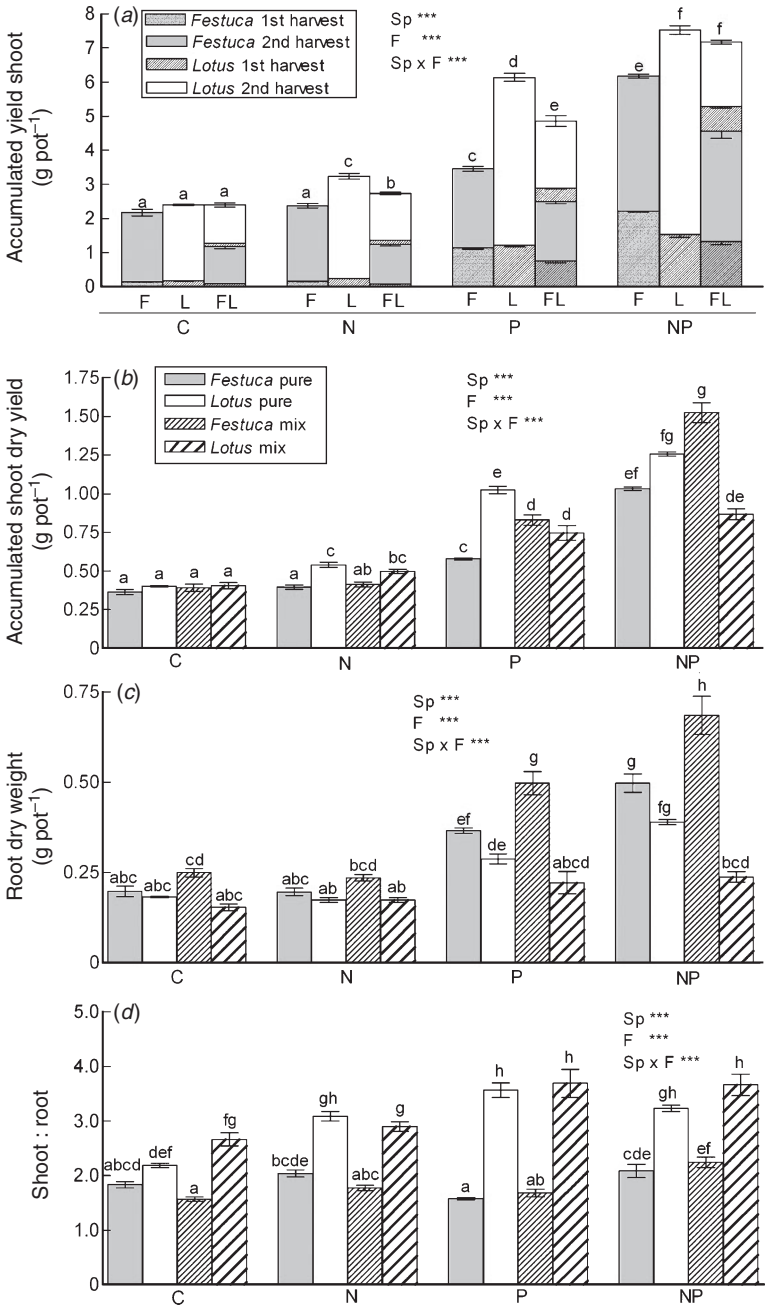

Fig. 1. Accumulated shoot dry yield of the two harvests expressed (*a*) per pot and (*b*) per plant; (*c*) root dry weight; and (*d*) shoot : root ratio of *F. arundinacea* and *L. tenuis* cultivated in pure or mixed stands in soils unfertilised (C) or fertilised with nitrogen (N), phosphorus (P) and N + P (NP). Results are presented as means ± standard errors. Parameter means with the same letter are not significantly different among treatments ($P > 0.05$) according to the Tukey test. Results of the 2-way ANOVA are indicated for each parameter (***$P < 0.001$); Sp, species stand; F, fertilisation; Sp × F, interaction.

pure culture (3.0 ± 0.2 *v.* 2.1 mg N plant^{-1}) (Fig. 2*a*, *b*). With N fertilisation, *L. tenuis* had higher N content than *F. arundinacea* in both shoot and root tissues when the species were in pure culture, but N contents were statistically the same in mixed stands. Fertilisation with P alone increased the N content in shoots and roots of *L. tenuis* in pure and mixed stands, and those levels were considerably higher than the N content in both tissues of *F. arundinacea* in pure culture. In mixed stands, *L. tenuis* had higher N content in the shoots than *F. arundinacea* but the two species had statistically equivalent amounts in roots tissues (Fig. 2*a*, *b*). In addition, the N content in shoots and roots of *F. arundinacea* increased in mixed stands

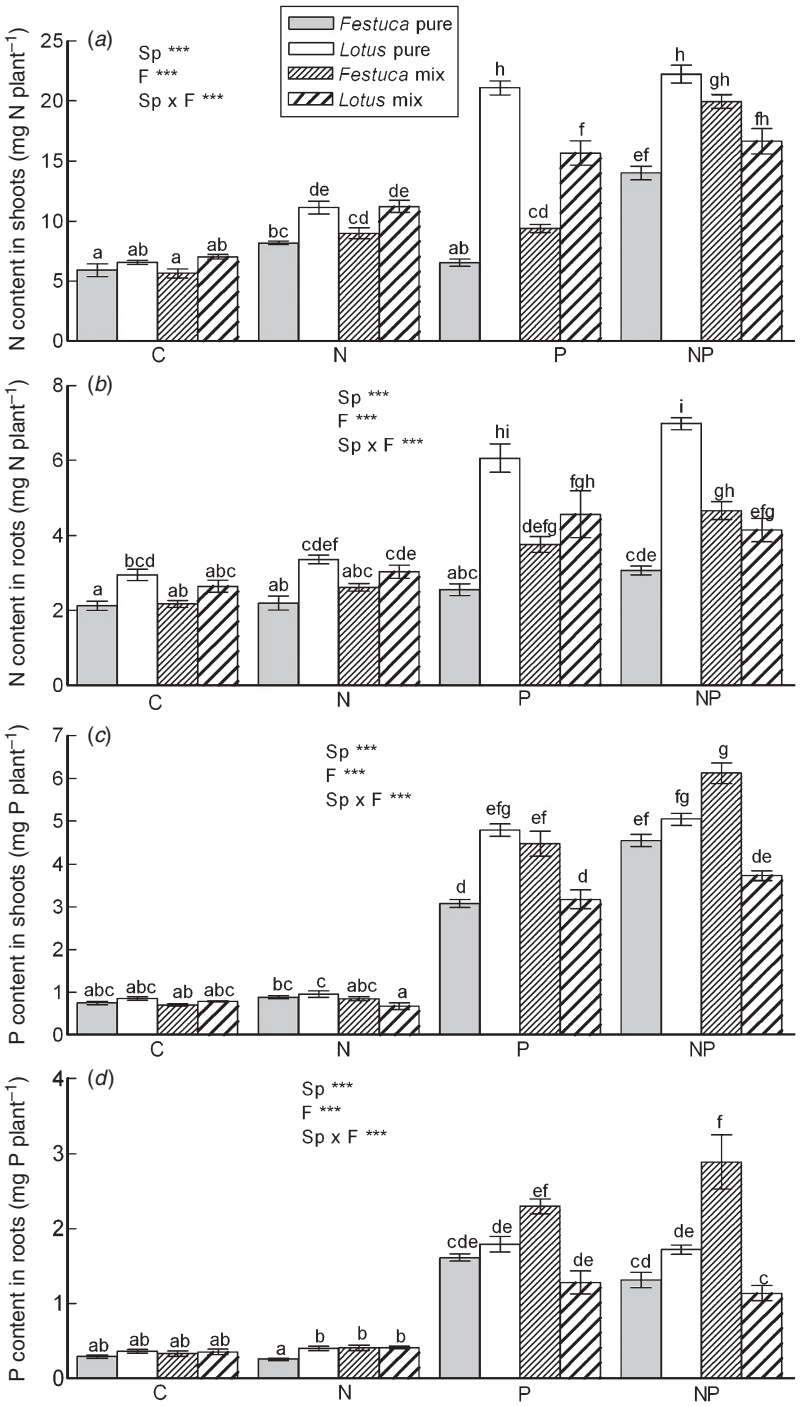

Fig. 2. Nitrogen content in (*a*) shoots and (*b*) roots, and phosphorus content in (*c*) shoots and (*d*) roots, of *F. arundinacea* and *L. tenuis* cultivated in pure or mixed stands in soils unfertilised (C) or fertilised with nitrogen (N), phosphorus (P) or N + P (NP). Results are presented as means ± standard errors. Parameter means with the same letter are not significantly different among treatments ($P > 0.05$) according to the Tukey test. Results of the 2-way ANOVA are indicated for each parameter (***$P < 0.001$); Sp, species stand; F, fertilisation; Sp × F, interaction.

relative to pure stands, whereas the N contents in *L. tenuis* were lower in mixed stands than in pure stands. With N + P added, the N contents in the shoots and roots of *L. tenuis* in both pure and mixed culture were similar to those after fertilisation with P alone, whereas the N contents of *F. arundinacea* in mixed stands were higher than those in monoculture (Fig. 2*a*, *b*).

Phosphorus content in the shoot and root tissues was affected by plant stand, fertilisation and the plant stand × fertilisation interaction (Fig. 2*c*, *d*). The pattern of P content in shoots and roots differed from that of N. The P content of the tissues of both plant species in pure or mixed stands showed no statistical differences in the control treatment. Fertilisation with N alone decreased the shoot P content of *L. tenuis* in mixed stands below that of pure stands (Fig. 2*c*), but did not affect P content in the roots (Fig. 2*d*). By contrast, in *F. arundinacea*, N fertilisation alone did not affect P content in shoot tissue of either stand (Fig. 2*c*) but increased P content in roots of mixed stands (Fig. 2*d*). Fertilisation with P alone or N + P increased the P contents in the shoots and roots of both species in both stand types relative to the respective contents in the control or N-fertilised plants. For *L. tenuis*, the P contents in the shoots and roots in mixed culture were lower than in pure culture, whereas the opposite effect occurred with *F. arundinacea*, with the P content of both tissues increased in mixed stands compared with pure cultures (Fig. 2*c*, *d*).

Root measurements

Root length, root diameter and root surface area differed with plant stand and type of fertilisation, and there was an interaction between the two variables (Fig. 3). The root length of *F. arundinacea* plants was always greater than of *L. tenuis* plants in pure or mixed stands (Fig. 3*a*). With N-fertilisation in both pure and mixed stands, the root length of the plant species did not change relative to the control plants. The addition of P and N + P increased the root length of both species, especially that of *F. arundinacea* (Fig. 3*a*). In addition, the root length of each species fertilised with P or N + P did not change between pure and mixed stands, except for *L. tenuis* in mixed stands with N + P where the root length decreased compared with pure stands (Fig. 3*a*).

The root diameter as estimated by Eqn 1 was significantly different between plant stands and fertilisation types, and the two variables interacted (Fig. 3*b*). Roots of *F. arundinacea* were thinner than of *L. tenuis* in any combination of plant stand and fertilisation treatment (Fig. 3*b*). Within each fertilisation treatment, the root diameter of *F. arundinacea* plants was statistically the same regardless of plant stand, whereas *L. tenuis* root diameter decreased in mixed stands fertilised with P and N + P compared with that in pure stands (Fig. 3*b*). The estimated root surface area per plant in contact with the soil was higher for both plants species when grown in mixed than in pure stands, either with or without fertilisation (Fig. 3*c*). Moreover, *F. arundinacea* plants in mixed stands fertilised with P and N + P had a higher root surface area than *L. tenuis* plants (Fig. 3*c*).

Arbuscular mycorrhizal colonisation and root nodulation

Roots of *L. tenuis* were more extensively colonised by arbuscular mycorrhizae than those of *F. arundinacea* after all the fertilisation treatments and were much more colonised in unfertilised and N-fertilised soils than in P- and N + P-fertilised soils (Fig. 4*a*). Fertilisation with N alone did not affect the MC index, but fertilisation with P and N + P decreased the MC index in both plant species. For both species, the MC index in pure or mixed stands did not change with N fertilisation (Fig. 4*a*). Roots of *L. tenuis* plants in pure and mixed stands fertilised with P or N + P were colonised by arbuscular mycorrhizae on ~62% of the length, whereas roots of *F. arundinacea* were colonised on ~50% of the length in pure stands and 35% in mixed stands. Responses to the three fertilisation treatments observed for the AC index were highly correlated ($r = 0.91$) with those for the MC index (Fig. 4*b*). The number of the entry points per mm in the colonised roots showed a similar response after all fertilisation treatments, with values recorded in the *L. tenuis* roots significantly higher than in *F. arundinacea* roots (Fig. 4*c*). Fertilisation with P and N + P markedly decreased the development of the entry points compared with unfertilised or N-fertilised soil for both plant species in pure and mixed stands.

Numbers of rhizobial nodules on *L. tenuis* roots were not significantly affected by N fertilisation relative to the control but nodule numbers increased significantly with P and N + P fertilisation in the roots of both pure and mixed stands (Fig. 4*d*).

Soil analyses

Soil pH was affected by plant stand, fertilisation and the plant stand × fertilisation interaction (Fig. 5*a*). Soil pH before cropping was ~5.9 and decreased afterwards. For *F. arundinacea* pure and mixed stands, soil pH was lowest after N fertilisation, but in soils of *L. tenuis* pure stands, the pH was statistically the same in all treatments. Soil pH of the *F. arundinacea* pure stands was statistically the same for the control, P and N + P fertilisation treatments. In mixed stands, soil pH was ~5.7 with P fertilisation, which was significantly higher than the control (~5.4) and N-fertilised soil (~5.1); the N + P treatment was intermediate and not significantly different from the P treatment and the control. The EC of the soil decreased after cropping, from 0.45 to ~0.27 dS m^{-1} in the control treatment and with P and N + P fertilisations, but N fertilisation did not change the EC after cropping (Fig. 5*b*). As expected, the available P in soil increased with P and N + P fertilisations but it was not affected by plant stand (Fig. 5*c*). The soil NO_3^- concentration increased with N, P and N + P fertilisation, but especially with N alone in *F. arundinacea* soils under pure or mixed stands, compared with the 10.5 mg NO_3^- kg^{-1}measured before cropping (Fig. 5*d*).

Discussion

Growth, the uptake of N and P, and associations with soil microorganisms of *F. arundinacea* and *L. tenuis* depended on whether they grew in pure or mixed culture, the supply of a specific nutrient alone or in combination, and the interaction between plant stand and type of fertilisation. Grasses can grow better under nutrient-deficient conditions when mixed with legumes (Høgh-Jensen and Schjoerring 2010; Nyfeler *et al.* 2011), but they may have a disadvantage relative to legumes in N-deficient soils because legumes are able to fix atmospheric N_2 in N-poor ecosystems (Temperton et al. 2007). In the present experiment, *F. arundinacea* mixed with *L. tenuis* in unfertilised soils did not grow better than in pure culture, which disagrees with our first hypothesis. In unfertilised soils, the accumulated

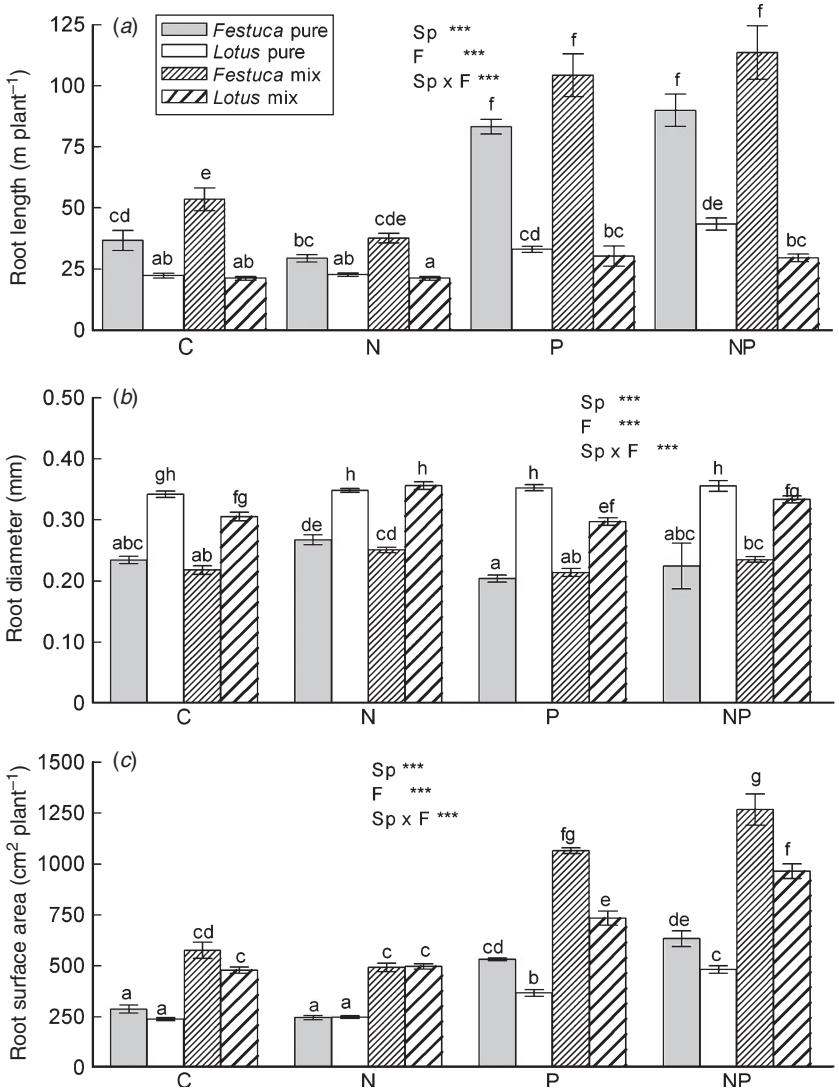

Fig. 3. (*a*) Root length, (*b*) root diameter, and (*c*) root surface area of *F. arundinacea* and *L. tenuis* cultivated in pure or mixed stands in soils unfertilised (C) or fertilised with nitrogen (N), phosphorus (P) or N + P (NP). Results are presented as means ± standard errors. Parameter means with the same letter are not significantly different among treatments ($P > 0.05$) according to the Tukey test. Results of the 2-way ANOVA are indicated for each parameter (***$P < 0.001$); Sp, species stand; F, fertilisation; Sp × F, interaction.

shoot yield, root biomass, and N and P absorbed by the two plant species were statistically the same in pure and mixed stands. Rhizobial nodulation in the roots of *L. tenuis* was restricted in the absence of P supply, which meant that the N_2 fixation needed for the legume's positive effect on grass could not occur (Bardin *et al*. 1996; Monaghan *et al*. 2007). In addition and also in disagreement with the first hypothesis, the proportion of *F. arundinacea* roots colonised by arbuscular mycorrhizae did not increase in mixed stands relative to pure stands to exert a positive effect of mycorrhizal colonisation on access of *F. arundinacea* roots to inorganic P in soil. When available P is markedly deficient in soil (as in the unfertilised soil used here, with only 3.1 mg P kg^{-1}), both the growth of the root within the soil and the development of the fungus within the roots become limited. Hence, there is no effect on the length of the roots colonised by arbuscular mycorrhizae (Bolan *et al*. 1984; Mendoza and Pagani 1997). These results are consistent with the low availability of NO_3^- and P measured in unfertilised soils at the end of the experiment. The conclusion is that *F. arundinacea* did not grow better when mixed with *L. tenuis* than in monoculture because the availabilities of N and P in the soil were insufficient for rhizobial nodulation in the roots of *L. tenuis* and/or for mycorrhizal colonisation in the roots of the grass. At the same time, both nutrients were too low before and after cropping to enable the growth of the grass and the acquisition of nutrients from the soil.

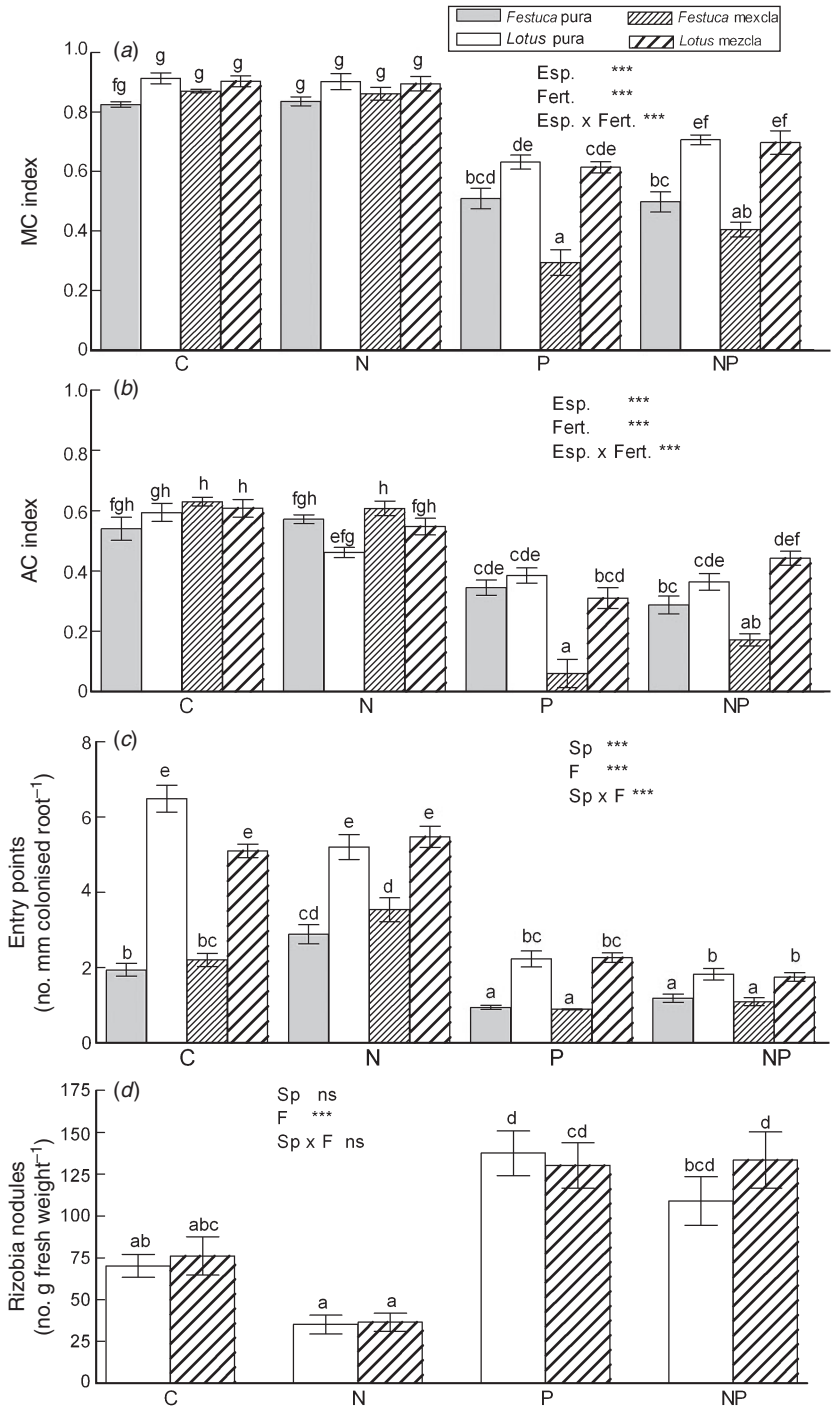

Fig. 4. (*a*) Arbuscular mycorrhizal colonisation (MC index), (*b*) arbuscular colonisation (AC index), (*c*) entry points of *F. arundinacea* and *L. tenuis*, and (*d*) rhizobia nodules in *L. tenuis* roots cultivated in pure or mixed stands in soils unfertilised (C) or fertilised with nitrogen (N), phosphorus (P) or N + P (NP). Results are presented as means ± standard errors. Parameter means with the same letter are not significantly different among treatments ($P > 0.05$) according to the Tukey test. Results of the 2-way ANOVA are indicated for each parameter (***$P < 0.001$; n.s., not significant); Sp, species stand; F, fertilisation; Sp × F, interaction.

Improvement of N supply in nutrient-deficient grasslands composed of legumes and grasses favours the growth of the grasses (Laidlaw and Withers 1998; Loiseau *et al*. 2001). The present research, however, has demonstrated that *F. arundinacea* in pure or mixed cultures with *L. tenuis* did not growth better after N fertilisation of the soil. The N content of the shoot

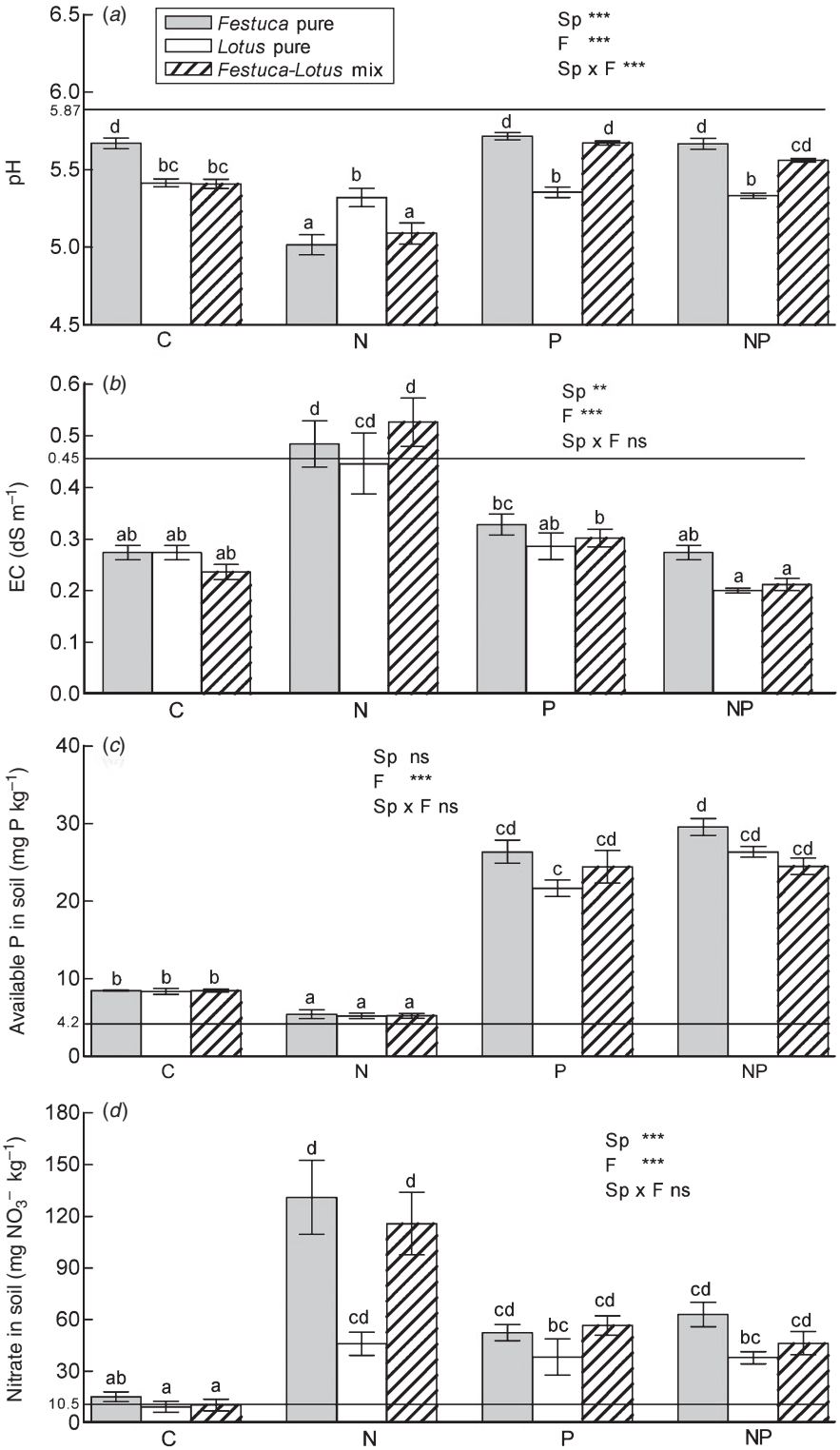

Fig. 5. Measurements of (*a*) pH, (*b*) electrical conductivity (EC), (*c*) available phosphorus and (*d*) nitrate at the end of the experiment in soils of *F. arundinacea* and *L. tenuis* cultivated in pure or mixed stands unfertilised (C) or fertilised with nitrogen (N), phosphorus (P) or N +P (NP). Horizontal lines indicate the value of the variable before cropping. Results are presented as means ± standard errors. Parameter means with the same letter are not significantly different among treatments ($P > 0.05$) according to the Tukey test. Results of the 2-way ANOVA are indicated for each parameter (**$P < 0.01$; ***$P < 0.001$); Sp, species stand; F, fertilisation; Sp × F, interaction.

tissue of *F. arundinacea* increased with N supply but the shoot yield did not change with respect to the unfertilised treatment. These results suggest that, even when NO_3^- availability in soil is increased by N application, utilisation of the N absorbed by *F. arundinacea*, which is necessary for increasing shoot growth, remains restricted. The absence of an expected response by *F. arundinacea* to N supply is ascribed to the low availability of P, which, in turn, prevented plant growth. These results are in disagreement with second hypothesis, because the increase in N supply did not favour the growth of the grass component in mixed stands. We conclude that improving N supply in grassland soils deficient in N will not necessarily favour the growth of the grasses in pastures mixed with legumes if the availability of P is insufficient to promote utilisation of the absorbed N within the plant for growth. Nevertheless, a small but significant response to N supply occurred with *L. tenuis*; the legume exhibited better growth and absorbed more N from the soil in either pure or mixed culture than observed in unfertilised soil. The increase in shoot biomass in *L. tenuis* was mainly associated with greater N availability through fertilisation, given that rhizobial nodulation was depressed by N supply. A greater increase in plant growth due to N fertilisation than N_2 fixation has been reported for other legume crops (Lowther and Kerr 2011).

Improvement of P supply in nutrient-deficient grasslands composed of legumes and grasses favours the growth of the legumes (Høgh-Jensen and Schjoerring 2010). The present study shows that both *L. tenuis* and *F. arundinacea* respond strongly to increasing P supply in both pure and mixed stands. Nevertheless, the response of both species to P fertilisation in pure culture was different from the response when grown in association. Compared with pure stands, the grass component increased shoot growth but the legume component decreased shoot growth when grown in association. This result is also in disagreement with the second hypothesis, because P supply favoured the growth of the grass more than that of the legume when plants grew in mixed stands. Both N and P contents in the shoot tissue responded in similar directions to those of shoot growth in the two plant species; that is, the contents of both nutrients in the shoot tissues increased in *F. arundinacea* but the contents of both nutrients decreased in *L. tenuis* tissue when grown in mixed stands relative to pure stands. With P fertilisation in mixed stands, *F. arundinacea* contained 60% of the total P absorbed by the two species and *L. tenuis* the remaining 40%, but in the unfertilised control soil, *F. arundinacea* and *L. tenuis* contained almost the same proportion of the total absorbed P (48% and 52% respectively). These results suggest that in P-fertilised stands, *L. tenuis* plants can perform better when grown in pure stand than when competing in mixed stand with *F. arundinacea*, whereas *F. arundinacea* can grow better when mixed with *L. tenuis* plants than in pure stand. Furthermore, P fertilisation increased the number of rhizobial nodules per unit fresh weight in *L. tenuis* roots as had been reported for other legumes (Monaghan *et al*. 2007). The additional nodulation would increase the availability of inorganic N in soil, as was corroborated in this experiment after P fertilisation of the soil (Fig. 5*d*). For legumes, low availability of soluble P in soils is a critical limiting factor because P affects not only plant growth but also root nodulation and symbiotic N_2 fixation (Mosse 1978). The increase in nodulation in *L. tenuis* roots upon P supply may also have contributed to increased fixation of atmospheric N_2 for absorption and utilisation by both plants, especially by *F. arundinacea*. This is because grasses grow better when associated with legumes than when grown in monoculture, owing to the mutual stimulatory effect on N acquisition of both components (Nyfeler *et al*. 2011).

Positive interaction between N and P in stimulating plant growth in grasslands has been previously reported (Jouany *et al*. 2011). In agroecosystems, N and P enrichment produce strong synergism in crops as well as in grasslands (Loeppky *et al*. 1999; Aulach and Malhi 2005). In the present experiment, the yield of both plants either pure or in mixtures upon N + P supply was higher than that of the plants fertilised with P alone, except for *L. tenuis* in mixed stands where the biomasses of the shoots and roots were the same after fertilisation with P and N + P. This suggests that in mixed stands fertilised with P, the competition exerted by *F. arundinacea* over *L. tenuis* increased with addition of N. Under conditions of combined N and P sufficiency, *F. arundinacea* in mixed culture with the legume had the highest shoot and root growth per plant. This suggests that the development of the grass was even more favoured under N + P sufficiency than otherwise and that *F. arundinacea* was able to compete with *L. tenuis* much more efficiently under those nutritional conditions than under the sufficiency of P alone. Plants of *F. arundinacea* in mixed stands produced 64% of the total shoot biomass with N + P fertilisation, but this value dropped to 51% with P alone. Similarly but more marked, *F. arundinacea* produced 74% of the total root biomass with N + P fertilisation as opposed to 69% with P fertilisation alone. Furthermore, the total nutrients absorbed (shoots + roots contents) in tissues reflected trends similar to those observed for biomasses in mixed stands. Plants of *F. arundinacea* absorbed 54% of the total N absorbed by both plants with N + P supply; however, this value dropped to 39% under P alone. In a similar manner, *F. arundinacea* absorbed 65% of the total P with N + P fertilisation and 60% with P alone. These results indicate that the main competitive advantage of the grass over the legume for using resources is linked to the root system. Three hypotheses may explain the increases in nutrient absorption (N and P) and biomass yield (shoots and roots) following P or N + P supplies and the competitive edge of the grass over the legume. The first hypotheses is that improving P nutrition increases the shoot growth but especially the development of the root system in grasses, and thus improves the soil-exploration capacity and consequently encounter with mobile nutrients in soil, such as nitrate (Duru 1992). This hypothesis is consistent with the results of the present experiment. The root length and the root surface area in contact with the soil were much higher and the roots much finer in *F. arundinacea* than in *L. tenuis*. These morphologic properties improve soil exploration by the roots of the grass and increase the ability of *F. arundinacea* to take up nutrients from the soil over that of the *L. tenuis* plants. The second hypothesis concerns the effect of P on organic matter mineralisation. An increase in P supply leads to an enhancement in the recycling of soil N (mineralisation and subsequent nitrification) and/or an improvement in litter quality (Parfitt *et al*. 2005). The third is

simply the complementarity for the use of N between the associated plants.

We therefore conclude that, when P is not limiting, additional supply of N favours the competition of *F. arundinacea* over *L. tenuis* for resources. Furthermore, this advantage is mainly associated with the characteristics of the root system, because in mixed stands, N enrichment resulted in 74% of the total root biomass produced by *F. arundinacea* and the remaining 26% produced by *L. tenuis* in mixed stands. Nevertheless, we cannot discount the fact that the soil volume explored by the roots is limited in a pot experiment; the competition between plants would increase and this may not favour complementarity between roots for soil resources as they explore the same places in the soil.

The root length colonised by arbuscular mycorrhizae, arbuscular colonisation and the entry points per unit of root length decreased with P and N + P enrichments and they were always higher in *L. tenuis* roots than in *F. arundinacea* roots. Similar results were reported for other grasses in combination with legumes (Blanke *et al*. 2012; Mai 2013). The supply of N alone did not affect mycorrhizal colonisation in roots of the two plant species of the present experiment, in agreement with the findings of other authors (Treseder 2004; Chen *et al*. 2014). Plants control the degree of arbuscular mycorrhizae colonisation depending on their nutritional status, and it has been repeatedly reported that under high inorganic-P supply, mycorrhizal colony development is repressed (Braunberger *et al*. 1991; Breuillin *et al*. 2010). The mechanisms by which the plants control mycorrhizal colony development in roots by nutrient conditions are largely unknown (Carbonnel and Gutjahr 2014). Corkidi *et al*. (2002) and Johnson *et al*. (2003) reported no significant differences in the percentage of root length colonised by arbuscular mycorrhizae in N-fertilised plants compared with control plants. On the basis of the results of the present experiment, the more likely explanation is that N fertilisation in P-limited soils is mediated by availability of soil P, because that nutrient has been shown to increase the biomass of arbuscular mycorrhizae, whereas N enrichment of P-rich soil has the opposite effect (Johnson *et al*. 2003). At sufficient P supply, this increase in biomass might simply be absent (Carbonnel and Gutjahr 2014). Moreover, the reduction in the root length colonised by arbuscular mycorrhizae in the two species with increasing P supply could be explained by the interaction between the increases in root growth and root length, which dilutes the proportion of root colonised (Allen 2001) and the growth of the fungus within the root (Mendoza and Pagani 1997; Smith and Read 2008). This explanation is consistent with the inverse linear relationship found between the proportion of root length colonised by arbuscular mycorrhizae and the total root length of both plant species ($r = -0.81$; $P < 0.001$), especially in mixed stands fertilised with P or N + P, where the roots of *F. arundinacea* were seen to grow much better than those of *L. tenuis*.

In limited N and P environments, adding P to the soil can be a good strategy to weaken the ability of grasses to compete with legumes for resources, thus maintaining a good proportion of legumes in the grassland and extending the quality of the pasture over time. From an agronomic, ecologic and economic point of view, optimisation of P nutrition might help to maximise N inputs into grasslands through symbiotic N fixation. In turn, the decrease in inorganic-N inputs from fertilisation would decrease N_2O output to the atmosphere.

Acknowledgements

The authors thank technician Carlos Hernández (CONICET) and Ing. Agr. María Cristina Pomar for determining the nutrients in the plant tissues. Dr Donald F. Haggerty, a retired academic career investigator and native English speaker, edited the accepted version of the manuscript. This study was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

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