PHOSPHORUS FERTILIZATION OF A GRASS-LEGUME MIXTURE: EFFECT ON PLANT GROWTH, NUTRIENTS ACQUISITION AND SYMBIOTIC ASSOCIATIONS WITH SOIL MICROORGANISMS

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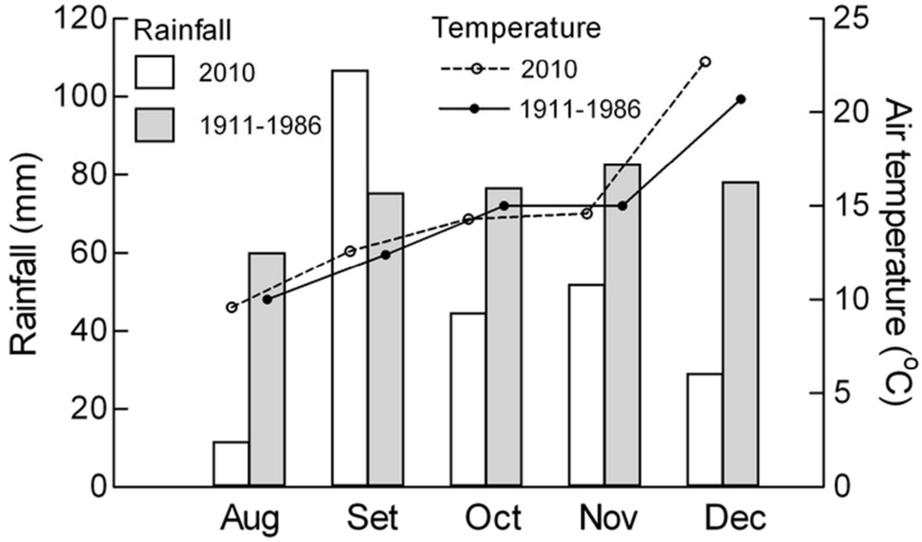

Figure 1. Rainfall and air temperature registered at the experimental location during a historical period of 75 years (1911-1986) and during the period of the present experiment (2010) from August to December.
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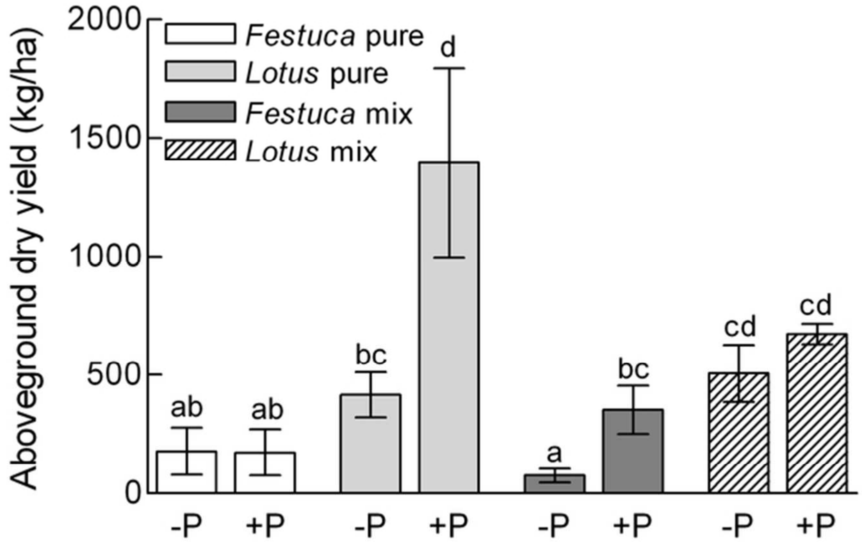

Figure 2. Aboveground dry yield of biomass harvested from Festuca and Lotus grown in monoculture, Festuca mix and Lotus mix for non-fertilized and fertilized soils with TSP. Different letters among columns indicate significant differences by LSD method at a level of P<0.05. Data were logarithm-transformed for this analysis and plotted back as original data. Bars show the standard error of the mean of three replicates for each treatment.
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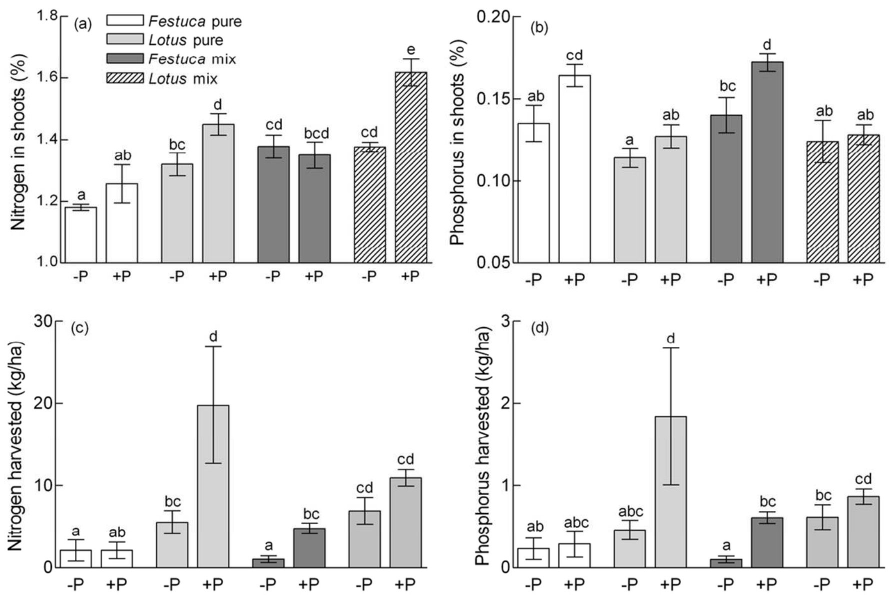

Figure 3. Concentration of nitrogen (a) and phosphorus (b) in shoots tissue of Festuca and Lotus grown in monoculture and in mixed stands for non-fertilized (-P) and fertilized plots with TSP (+P); and nitrogen (c) and phosphorus (d) harvested (kg/ha) in shoots of Festuca and Lotus grown in monoculture and in mixed stands for non-fertilized (-P) and fertilized plots with TSP (+P). Different letters among columns indicate significant differences by the LSD method at a level of P<0.05. Data in figures 2c-2d were logarithm-transformed for this analysis and plotted back as original data. Bars show the standard error of the mean of three replicates of each treatment.
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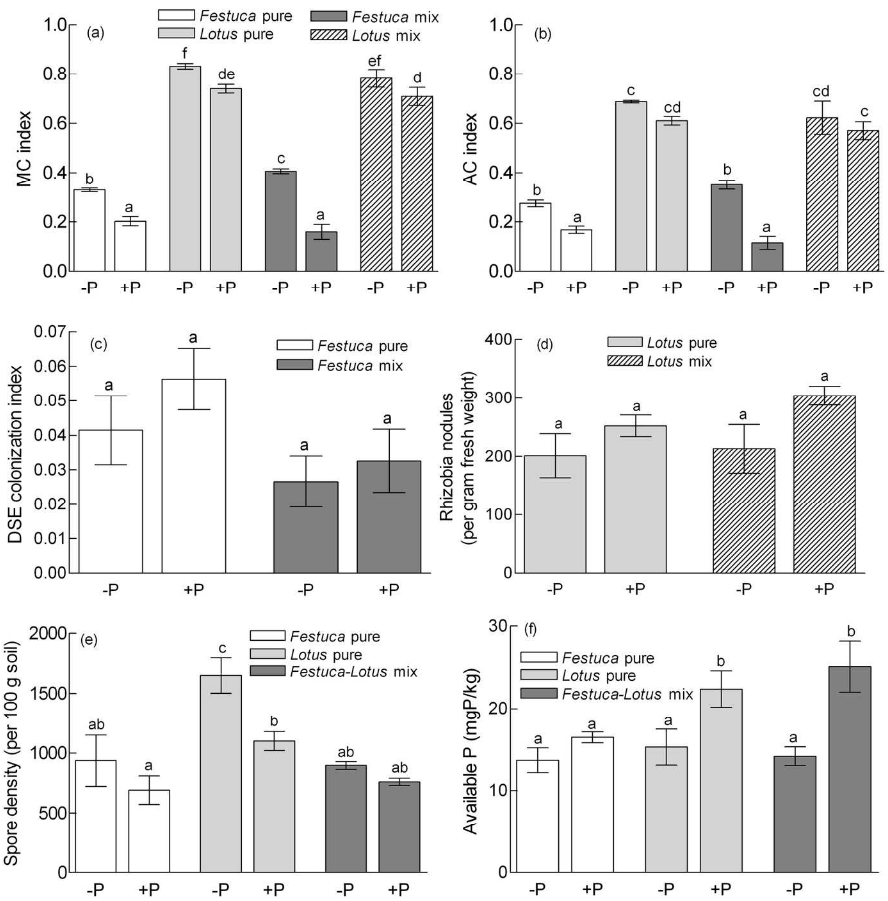

Figure 4. Mycorrhizal colonization (a) and arbuscular colonization (b) indexes in roots of Festuca or Lotus grown in monoculture or mixed; and DSE colonization (c) index in roots of Festuca, number of rhizobia nodules per gram of fresh roots (d), AM spore density (d) and available P in soil (Bray 1) in the rhizospheric soil of Festuca or Lotus in monoculture or mixed for non-fertilized (-P) and fertilized soils with TSP (+P). Different letters among columns in each graph indicate significant differences by LSD method at a level of P<0.05. AM spore density data were arcsine-transformed for this analysis and plotted back as original data. Bars show the standard error of the mean of three replicates of each treatment.
163x157mm (300 x 300 DPI)

Table 1. Dry yield of aboveground biomass, nitrogen and phosphorus harvested in shoot tissue of *L. tenuis* and *F. arundinacea* from mixed stands non-fertilized and fertilized with TSP expressed in kg/ha and as a percentage of the total dry yield and total nitrogen or total phosphorus harvested by both plants.

Plant species	Dry yield				N harvested				P harvested			
	-P		+P		-P		+P		-P		+P	
	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)
Lotus	505	87	672	66	6.92	87	10.91	69	0.62	85	0.86	58
Festuca	75	13	352	34	1.03	13	4.78	31	0.11	15	0.61	42
Total	580	100	1024	100	7.95	100	15.69	100	0.73	100	1.47	100

PHOSPHORUS FERTILIZATION OF A GRASS-LEGUME MIXTURE: EFFECT ON PLANT GROWTH, NUTRIENTS ACQUISITION AND SYMBIOTIC ASSOCIATIONS WITH SOIL MICROORGANISMS

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Running head: Phosphorus fertilization of a grass-legume mixture

ABSTRACT

Adding P on *Lotus tenuis* and *Festuca arundinacea*, pure or mixed, on growth, nitrogen (N) and phosphorus (P) acquisition and associations with soil microorganisms was studied to investigate the establishment of *Lotus* for competing with *Festuca*. Triple-superphosphate was applied on a Typic Natraquoll where *Lotus* grows spontaneously. Biomass, N-P uptake, arbuscular mycorrhizal colonization and rhizobia nodulation were measured. *Lotus* achieved the highest biomass, N-P uptake in fertilized stands and *Festuca* the lowest in fertilized and non-fertilized stands. Mycorrhizal colonization decreased with P-fertilization in both plants. Rhizobia nodules in *Lotus* showed little changes with P-fertilization. In mixed fertilized-stands, *Lotus* promoted the growth, N-P uptake of *Festuca.* P-fertilization increases the ability of *Festuca* to compete with *Lotus* for available-P in soil. *Lotus* improves nutrient cycling, maintains high level of rhizobia nodules and arbuscular mycorrhizal colonization in roots. Adding P to limited N-P environments depress grasses growth to compete with legumes for resources.

keywords: N-P limited environment, *Lotus-Festuca* mix, arbuscular mycorrhizae-rhizobia-dark septate endophyte.

INTRODUCTION

In temperate latitudes, native grasslands are dominated by a range of perennial grasses associated with a decline or absence of native legumes because of land use and/or selective grazing (Muir et al., 2011). Grasses have fibrous root systems that give them an advantage when competing for shallow moisture and soil nutrients. They also tend to establish more easily, grow more rapidly and recover from grazing more quickly than legumes (Muir et al., 2011). Grasses are at a disadvantage relative to legumes only when soil N is low because legumes are able to fix atmospheric N in poor-N ecosystems such as native grasslands (Temperton, 2007). Legumes have an additional advantage over grasses in poor-N ecosystems when available P is relatively high. It has been reported that increasing P supply increases the competitive power of white clover growing in association with ryegrass (Høgh-Jensen and Schjoerring, 2010). Mixing grasses with legumes is one of the effective and economical management options to improve the quantity and/or quality of grasslands in regions where both temperate grasses and temperate legumes are adapted (Cahuépé, 2004; Høgh-Jensen and Schjoerring, 2010; Nyfeler et al., 2011).

Species of the genus *Lotus* are increasingly used in both natural and artificial pastures over the world because of their plasticity and productivity in a wide range of soils (Escaray et al., 2012). *Lotus tenuis* Waldst. & Kit., is a growing winter-spring-summer legume widely spread which grows naturally in the lowlands of the Pampas of Argentina. This legume produces a high quality of nutritive forage for beef and dairy cattle in nutrient deficient soils which are poor for agricultural purposes (Cahuépé, 2004). In addition, prevents pasture bloat in ruminants when the gas produced during fermentation of feeds is trapped within the rumen (McMahon et al., 2000). The forage species of the genus *Lotus* do not produce pasture boat and are thus recommended for monoculture pasture to reduce the incidence of this disorder in animals that consume high risk plants, such as alfalfa, white clover and red clover (Berard et al., 2011; Escaray et al., 2012).

The productivity of the lowlands of the Pampas is strongly affected by the topography that determines a range of salinity, alkalinity, excess or deficit of water in the soil and low availability of P and other nutrients for plant growth (Escudero and Mendoza, 2005). One of the plant strategies to grow under adverse soil environment is the association with soil microorganisms, such as arbuscular mycorrhizae (Smith and Read, 2008), dark septate endophyte (Newsham, 2011) and/or *Rhizobium* in *Lotus tenuis* plants (García et al., 2008; Estrella et al., 2009).

However, *Lotus tenuis* can grow, become nodulated by *Rhizobium* and colonized by arbuscular mycorrhizae (AM fungi) under deficit or excess of water in the soil by using a different strategy to invest its available resources (Mendoza et al., 2005; García et al., 2008). Under water excess, AM fungi reduced nutrient transfer structures (arbuscules), the number of entry points and spore, and hyphal densities in soil, but increased resistance structures (vesicles). At water deficit, AM fungi reduced external hyphae and arbuscules, investing more in maintaining a similar proportion of vesicles in roots and spores in soil compared to control treatment.

Lotus strongly responds to the addition of P in nutrient deficient soils (Mendoza and Pagani, 1997) and adult plants can tolerate waterlogging or drought conditions for more than one month (Vignolio et al., 1999; Mendoza et al., 2005; García et al., 2008).

Festuca arundinacea Schreb., is a growing perennial fall-winter-spring grass, which produces excellent quality pasture (Frame, 1990). Tolerate moisture excess in soil, salinity and some resistant to drought (Bañuelos and Beuselinck, 2003).

Fertilizations with N and P, seeding legume plants and mixing legume with grasses have been the alternatives used to improve forage yield (Muir et al., 2011). It has been shown that the presence of *L. tenuis* in mixing communities is crucial for improving the quality of the natural grassland for beef production (Cahuépé 2004). In lowlands, where low P availability and salinity are common in soils, *Festuca-Lotus* mixes can be a good alternative to improve the quantity and quality of the forage, since both species have shown good adaptability to grow in these soils (Bañuelos and Beuselinck, 2003; Cahuépé, 2004).

The objective of the present field experiment was to study the effects of adding moderate amounts of P by fertilization on *Lotus tenuis* and *Festuca arundinacea* in monoculture or *Festuca-Lotus* mixed stands on plant growth, N and P uptake and the symbiotic associations with arbuscular mycorrhizae and rhizobia nodulation. We were especially interested in knowing the establishment of the pasture at the first cutting in late spring, as it is crucial to achieve a fast and good development since grasses tend to establish more easily, grow more rapidly and compete much better with forbs than legumes.

MATERIALS AND METHODS

Location and soil site

The experimental area was located in the lowlands Pampas, 140 km south of Buenos Aires (Chascomús, Argentina), in the Experimental Station "Manantiales" (INTA-Ministry of Agricultural Affairs of the Province of Buenos Aires), S 35^{o} 44` 45.54" – O 58^{o} 03' 02.76". Historical air temperature and rainfall over 75 years (1911-1986) from Chascomús provided by the Biometeorological Research Centre (CIBIOM-CONICET) was used to compare the temperature and rainfall recorded during the experimental period. The climate of the region and the presence of saline-sodic and nutrient deficient soils, submit the plants to different types of stress. It is an area of cattle breeding for beef production mainly sustained by natural pastures dominated by perennial and seasonal grasses with a lack of legumes, which decline the quantity and quality of the pasture. The experiment was conducted in a soil classified as Typical Natraquoll limited in available N and P for plant growth. The main soil properties of the 0.20 m of the upper A1 horizon were: pH 6.7, EC (electrical conductivity) 2.4 dS/m, C (total carbon) 2.2 %, N (total nitrogen) 0.23 %, OM (organic matter) 3.50 %, P-availability (Bray I) 12.0 mgP/kg, 21 mgN (NO_3^-)/kg, ESP (exchangeable sodium percentage) 13.2%, clay content 20%, loam 55% and sand 25%. The field water capacity of the top horizon was 31% and the water content at 1500 KPa was 10%. The top horizon was followed by 23 cm of a B2t horizon of soil pH: 9.2, EC: 1.7 dS/m, C: 0.36%, N: 0.04%, OM: 0.62%, P Bray: 3.5 mgP/kg and ESP: 51%.

Experimental design

Glyphosate was applied twice on the experimental area 40 days before sowing and after sowing to eliminate weeds. Flumetsulam (Preside) was additionally applied after sowing and before emergence to eliminate weeds. Before sowing, the land was leveled by a disc harrow to homogenize sowing depth. *Lotus tenuis* cv. Esmeralda and *Festuca arundinacea* cv. Maximize were direct seeded pure or mixed in late August (winter) in plots of 9 m × 3 m in rows separated 17.5 cm at a rate of 13 kg/ha and 16 kg/ha respectively. The *Lotus*, *Festuca* and *Lotus-Festuca* stands were P-fertilized with granulated triple superphosphate (TSP) containing 20% of P at a rate of 90 kg/ha, equivalent to 18 kgP/ha, or not fertilized. The experimental design consisted in three stands (*Festuca* pure, *Lotus* pure and *Festuca-Lotus* mix) combined with two levels of P-fertilization (-P and +P) replicated three times and distributed in randomized complete blocks design.

Aboveground biomass, plant and soil analyses

An area of 6 m^2 placed centrally in the plots was harvested in late spring, 4 months after sowing by a 1 m-wide scythe mower, leaving a stubble height of 3 cm. The green material of the *Festuca-Lotus* treatments was separated into *Lotus* and *Festuca*, dried at 70 ^oC for 48h, weighed and analyzed for N and P. The shoot dry material was digested separately in a nitric-perchloric acid mixture (3:2) to determine P by the vanadomolibdophosphoric method, and in sulphuric acid to determine N by the Kjeldahl method (Jackson, 1964).

Individuals of similar size of *Lotus* and *Festuca* plants together with their respective roots and rhizospheric soil were randomly sampled in each plot. Rhizospheric soil samples (250 g) were taken from the centre of each root system and divided into two portions; one to measure soil properties and one to measure AM fungal spore density. Soil samples were kept in plastic bags at 4 ^oC until processed. The roots were washed with tap water and then twice with deionized water.

The following measurements were performed on soil samples: pH (1:2.5 water), electrical conductivity, available P (Bray and Kurts, 1945), total carbon (Richter and von Wistinghausen, 1981), and total nitrogen (Bremmer and Mulvaney, 1982) and cation exchangeable capacity (Jackson, 1964).

Arbuscular mycorrhizae and DSE colonization and root nodulation

Mycorrhizal root colonization and dark septate endophyte (DSE) were measured in fresh roots cleared in 10% KOH for 12 min at 90 ^oC, and stained in 0.05% lactic acid–glycerol Trypan blue (Phillips and Hayman, 1970). Twenty-five root segments per plant sample were examined under a microscope at x200 magnification. The fraction of root length colonized by mycorrhizae (MC), containing arbuscules (AC), vesicles (VC), hyphae only (HO) and DSE was determined following McGonigle et al., (1990). Rhizobia nodules were counted in the fresh root system under a binocular stereomicroscope (×7.5).

Arbuscular mycorrhizal spore density

AM fungal spores were isolated from a fraction of soil by a modification of the sucrose gradient centrifugation technique (Daniels and Skipper, 1982), and counted under ×35 magnification using a dissecting microscope. Spore density was expressed as the number of spores per 100 g of dry soil. In temperate grasslands, plants grow close each other and their root systems overlap occupying the same soil portion. Hence, separating the rhizospheric soil corresponding to one plant from that corresponding to other plant is not possible or reliable. Therefore, in *Festuca-Lotus* mix plots a same sample of rhizospheric soil was used to measure the spore density.

Statistical analyses

Data sets were tested for normality and variance heteroscedasticity with standard methods. Non-normal data were transformed for comparing treatment means. Analyses of variance were used to test the equality of treatment means. Mean separation was performed by the LSD test. Statgraphic 5.0 plus software was used for statistical analyses.

RESULTS

Climate, soil characteristics and aboveground biomass

The soil represents one of the typical conditions of the area with a top horizon close to neutrality followed by a B2t horizon very poor in nutrients and organic matter, with 51% of ESP and 45% of clay plus fine silt (Wermbter and Ramallo, 1980). The soil is limited in drainage capacity and is not suitable for crop production. During the experimental period, the air mean temperature was 14.8 ^{o}C and the accumulated rainfall 231 mm. The air temperature was quite similar to historical temperature recorded (14.6 ^{o}C) at the experimental location along 75 years (1911-1986), but the rainfall was around 35% lower in August-December period (Fig. 1), which may led the plants to suffer a dry period before harvesting.

The soil pH, electrical conductivity, total C, total N, cation exchangeable capacity and moisture content in soil did not change significantly with P-fertilization and plants species sown in monoculture or mixed (data not shown). The overall mean value of the moisture content in soil at sampling was 13%.

Aboveground dry yields ranged from 75 kg/ha of *Festuca* biomass in non-fertilized mixed plots to 1395 kg/ha of *Lotus* biomass in P-fertilized monoculture plots (Fig. 2). *Festuca* grew better when it was mixed with *Lotus* and fertilized with TSP (352 kg/ha) producing the 34% of the total biomass than when it was mixed but not fertilized (75 kg/ha) producing 13% of the total biomass (Table 1). *Lotus* in pure stands strongly responded to P-fertilization (1395 kg/ha) compared to its production in non-fertilized stands (415 kg/ha). However, in mixed stands *Lotus* produced statistically the same yield in non- fertilized (505 kg/ha) and P-fertilized stands (672 kg/ha).

N and P acquisition

In *Festuca* pure and in *Festuca* mixed stands separately, the concentration of N in shoots was not affected by P-fertilization, but the concentration P increased with P-fertilization in both stands (Fig. 3a, 3b). In *Lotus* pure stands, the concentration of N in tissue increased with P-fertilization, whereas in mixed stands the concentration of N increased but the concentration of P did not change with P-fertilization (Fig. 3a, 3b). The amounts of N and P harvested in shoot tissue followed similar patterns to those of the aboveground biomass in both pure and mixed stands (Fig. 3c, 3d). However in mixed stands, the amount of N and P harvested in shoot tissue of each plant species differed between nutrients and P-fertilization

(Fig. 3c, 3d, Table 1). Regarding N in non-fertilized mixed stands, the 87% of N harvested by both species in shoot tissue was harvested in *Lotus* (6.92 kg/ha) and only 13% by *Festuca* tissue (1.03 kg/ha), whereas in fertilized stands, 69% of N was harvested in *Lotus* shoots (10.91 kg/ha) and 31% in *Festuca* shoots (4.78 kg/ha) (Fig. 2c, Table 1). Regarding P in non-fertilized stands, the amount of P removed by *Lotus* shoots (0.62 kg/ha) was the 85% of the amount harvested by both species and only 15% by *Festuca* shoots (0.11 kg/ha), whereas in P-fertilized stands, the amount of P harvested decreased to 58% in *Lotus* (0.86 kg/ha) and increased to 42% in *Festuca* shoot biomass (0.61 kg/ha) (Fig. 3d, Table 1).

Symbiotic soil microorganisms

The percentage of root length colonized by AM fungi differed between plant species, P-fertilized and non-fertilized plants, and pure and mixed stands (Fig. 4). The MC and AC indexes were always significantly higher in *Lotus* roots than in *Festuca* roots (Fig. 4a, 4b). The overall mean value of the MC index was 0.77 in *Lotus* roots and 0.28 in *Festuca* roots. The MC index decreased with P-fertilization in roots of both species, pure or mixed (Fig.4a) . The AC index decreased in *Festuca* roots but did not change significantly with P-fertilization in *Lotus* roots (Fig. 4b). In addition, the roots of *Festuca* in non-fertilized stands were much more colonized by AM fungi (MC index) when grown mixed with *Lotus* that when grown in monoculture (Fig. 4a).

The roots of *Festuca* were co-colonized by DSE but in *Lotus* roots DSE colonization was not detected (Fig. 4c). DSE colonization in *Festuca* roots was not statistically affected by P-fertilization and between pure and mixed stands.

The number of rhizobia nodules in *Lotus* roots was not affected by P-fertilization in either pure or mixed (Fig. 4d).

The spore count from the rhizospheric soils of *Lotus* was highest (1650 spores/g soil) in pure stands non-fertilized and statistically different to the soil samples collected from the other treatments (Fig. 4e). In *Festuca* pure and in *Lotus-Festuca* mixed stands, P-fertilization did not affect the spore density in soil (Fig. 4e).

Available P in soil

The available-P in soil measured by Bray I test increased with P-fertilization in soil samples collected from *Lotus* pure or mixed with *Festuca* (Fig. 4f), but in soils from *Festuca* in monoculture the available P did not statistically change with P-fertilization (Fig. 4f). The mean values of the overall data were 14.7 mgP/kg in non-fertilized soils and 21.5 mgP/kg in P-fertilized soils (Fig. 4f). In P-fertilized soils, the highest values of available P were 22.4 mgP/kg in *Lotus* pure and 25.1 mgP/kg in *Lotus-Festuca* mixed. The lowest values were quite similar between non-fertilized soils ranged from 13.7 mgP/kg to 15.3 mgP/kg (Fig. 4f).

DISCUSSION

The aboveground biomass, N and P in shoot tissue and the association between the two studied species with symbiotic soil microorganisms differed between plant species, P-fertilization and pure or mixed stands. As previously reported, *Lotus tenuis* responds strongly to the addition of P in monocultures and in grassland communities when grown in P deficient soils of the lowlands of the Argentine Pampas (Ginzo et al., 1982; Mendoza and Pagani 1997; Bailleres and Sarena, 2010). In the present experiment, we found strong response of *Lotus* to a moderate rate of 90 kg of TSP compared to the higher rates (100-200 kg TSP/ha) commonly used in the region (Ginzo et al., 1982; Bailleres and Pirodi 2000). Low rates of P-fertilizer application are more economically and ecologically feasible for managing agricultural systems and preventing soil and water contamination.

Lotus in pure stands fertilized with TSP produced around 3.4 and 2.8 times more biomass that in non-fertilized pure or mixed stands. Besides, *Lotus* P-fertilized in monoculture produced between 2.0 times more biomass in the present experiment compared to a field experiment of a natural grassland, where *Lotus* grows spontaneously, in the same soil fertilized with 200 kg/ha urea and 100 kg/ha TSP (Bailleres and Pirodi, 2000). Our results are quite similar to the aboveground produced by natural grassland fertilized with 90 kg/ha of TSP where *Lotus* was favored when eliminating the accompanying grasses by applying herbicides (Bailleres and Sarena, 2010). These two previous experiments and the present results may justify cultivating *Lotus* in monoculture and fertilizing it with moderate amounts of TSP, since *Lotus* does not produce meteorism in ruminants (Berard et al*.,* 2011).

The concentration of N and P in shoot tissue differed due plant species, P-fertilization and plant stands. In *Lotus* tissue*,* the concentration of N increased with P-fertilization but P concentration did not change. However, in *Festuca* the concentration of N in shoot was not affected by P-fertilization whereas that of P increased. These results suggest that one or more limiting factors may inhibit differently the growth of each plant and/or that the two species use the available resources to grow differently. In limited-N environments, as in the present case, grasses are restricted to grow (Høgh-Jensen and Schjoerring, 2010; Muir et al*.,* 2011) and this can be one of the limiting factors that affect the growth of *Festuca* in pure stands. Grasses have fibrous and shallow root systems that can give them an advantage when competing with other plants for shallow moisture and soil nutrients (Muir et al*.,* 2011). However, this type of root system can be a disadvantage in dry periods to access soil moisture in depth (Pate 1994). In the present experiment, the low amount of rainfall during August-December, of only 231 mm, was almost 35% lower than the 370 mm of the rain historically recorded in the region (Fig. 1). Further, the soil moisture at harvest was 12%, very close to the 10% of soil moisture measured in laboratory at 1500 KPa, which is commonly associated with permanent wilting point. Probably the insufficient amount of rainfall and soil moisture for plants affected the growth of *Festuca* more than that of *Lotus* and can be another factor that should be considered in the evaluation of *Festuca* growth in pure stands. We concluded that the growth of *Festuca* in pure stands P-fertilized and non-fertilized was restricted by a deficit in both N availability and moisture content in soil.

Despite this deficit of water in soil, *Lotus* plants in pure stands responded to P-fertilization accumulating more N and P in tissue to grow. *Lotus* plants can have an advantage over *Festuca* plants in dry periods since they have taproots that allow them to penetrate deeper into the soil profile in searching moisture and nutrients to grow (Kirkbride, 1999; Paz et al., 2012). In addition, *Lotus* roots were heavily colonized by AM fungi (77%), which could be another advantage over the less colonized *Festuca* roots (28 %) for accessing moisture and nutrients in the soil (García and Mendoza, 2008). AM symbiosis is widely believed to protect host plants from the detrimental effects of drought (Subramanian et al., 2006) by improving the absorption of water and P in the soil (Augé et al., 2003). *Lotus tenuis* plants could grow, be nodulated by *Rhizobium* and maintain a functional symbiotic association with AM fungi under more than one month of water deficit in the soil (García et al., 2008). In the present experiment, MC and AC colonization in *Lotus* roots were little affected (around 10%) by the added P, but in *Festuca* roots both indexes decreased markedly after P-fertilization to 39% in pure and 61% in mixed stands. These abilities may additionally explain why *Lotus* grew better than *Festuca* when soil P-fertility increases under the drought stress of the present experiment.

The *Festuca* plants mixed with *Lotus* grew better in P-fertilized than in non-fertilized stands. The N harvested in *Festuca* shoot tissue from P-fertilized mixed stands was 30% of the amount of N harvested by both plant species compared to only 13% of N harvested from non-fertilized stands. In limited-N environments, the growth of grasses is restricted whereas legume plants that have a higher concentration of N in tissue can accelerate N cycling (Craine et al., 2002), improving the access and acquisition of N by the grasses. Improving N availability favors the grass component in mixtures with legume accumulating higher amounts of N and P in grass tissue than in pure stands (Høgh-Jensen and Schjoerring, 2010; Nyfeler et al., 2011). This may partly explain the better growth of *Festuca* plants when mixed with *Lotus* in P-fertilized stands.

Lotus in pure stands produced both higher biomass and N concentration in P-fertilized plots than in non-fertilized plots. This increase of the N concentration in *Lotus* tissue can be associated with the effect of rhizobia nodulation. When comparing together the roots of P-fertilized plants (pure and mixed) against the roots of non-fertilized plants (pure and mixed), the number of nodules per gram of fresh roots was 278 in fertilized plants and 207 in non-fertilized plants. The rhizobia nodules in P-fertilized roots could have been more efficient for fixing atmospheric-N than the nodules of non-fertlized roots. Bardin et al., (1996) has demonstrated that a phosphate transport system is required for *Rhizobium*-N fixation in legume plants. Then, the increase in availability of P by P-fertilization in the deficient soil of the present experiment can be one of reasons to justify the increase observed in the concentration of N in *Lotus* tissue from P-fertilized plants.

Lotus mixed with *Festuca* produced statistically the same biomass in P-fertilized and non-fertilized plots, which differed from *Lotus* in monoculture where it produced higher yield in P-fertilized than in non-fertilized plots. In these stands, *Festuca* showed both increases of growth and concentration of P in tissue with P-fertilization. As a result, the P harvested in *Festuca* tissue increased from 15% of the amount harvested by both species in non-fertilized stands to 42% in P-fertilized stands. Our hypothesis is that the decrease of *Lotus* growth in mixed and P-fertilized stands is due to the competition exerted by *Festuca* for the available P in soil. Høgh-Jensen and Schjoerring (2010) reported similar results for white clover cocultivated with rye grass, suggesting that the growth of clover in mixed stands was deprived of some of its P supply in the soil. These results are consistent with the present experiment and may partly explain why *Lotus* decreased the proportions of P removed by its shoots when mixed with *Festuca.*

Available soil P extracted by the Bray I soil test increased with P-fertilization in stands where *Lotus* grew alone or mixed but it did not change in soils from *Festuca* monoculture. Legume plants can provide benefits to the growth of grasses through exuded organic anions that may release P for growth of both plant species under drought and limited P availability in the soil (Suriyagoda et al., 2011). These observations suggest that in addition to the acceleration of N-cycling, *Lotus* plants can increase P availability in the soil and that *Lotus* is more efficient than *Festuca* to recycle P in grassland ecosystems. Yield, forage quality and nutrient cycling are generally lower for grass species than for legumes, while other agronomic factors such as longer stand life, more tolerance of grazing and trampling may make grasses more desirable (Paulson et al., 2008; Muir et al., 2011). Hence, establishing early and achieving good growth before the first cuttings are important factors for legume plants to have a better persistence and competence with grasses in grassland communities.

The colonization of *Festuca* roots by DSE was low and ranged from 2.7% in non-fertilized mixed stands to 5.6% in P-fertilized pure stands. In contrast, *Lotus* roots were not colonized by DSE. These are low values and could be considered insufficient to expect any positive effect of DSE on plant nutrition, as reported in previous studies where grasses in grasslands (*Cynodon dactylon, Poa annua, Digitaria cruciata*) were colonized by DSE up to 25% (Li et al., 2005).

Our results suggest that in limited-N and P environments, adding moderate amounts of P to the soil can be a good strategy to weaken the ability of grasses for competing with legumes for resources, maintaining a relative good proportion of legumes in the grassland and extending the quality of the pasture through time. In addition, our results justify the use of *Lotus* in monoculture with moderate amounts of P since *Lotus* improves nutrient cycling in the grassland and maintains high level of rhizobia nodules and arbuscular mycorrhizal colonization in roots.

Acknowledgments

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Table 1. Dry yield of aboveground biomass, nitrogen and phosphorus harvested in shoot tissue of *L. tenuis* and *F. arundinacea* from mixed stands non-fertilized and fertilized with TSP expressed in kg/ha and as a percentage of the total dry yield and total nitrogen or total phosphorus harvested by both plants.

Plant species	Dry yield				N harvested				P harvested			
	-P		+P		-P		+P		-P		+P	
	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)	(kg/ha)	(%)
Lotus	505	87	672	66	6.92	87	10.91	69	0.62	85	0.86	58
Festuca	75	13	352	34	1.03	13	4.78	31	0.11	15	0.61	42
Total	580	100	1024	100	7.95	100	15.69	100	0.73	100	1.47	100

Legends of figures

Fig. 1. Rainfall and air temperature registered at the experimental location during a historical period of 75 years (1911-1986) and during the period of the present experiment (2010) from August to December.

Fig. 2. Aboveground dry yield of biomass harvested from *Festuca* and *Lotus* grown in monoculture, *Festuca* mix and *Lotus* mix for non-fertilized and fertilized soils with TSP. Different letters among columns indicate significant differences by LSD method at a level of $P<0.05$. Data were logarithm-transformed for this analysis and plotted back as original data. Bars show the standard error of the mean of three replicates for each treatment.

Fig. 3. Concentration of nitrogen (a) and phosphorus (b) in shoots tissue of *Festuca* and *Lotus* grown in monoculture and in mixed stands for non-fertilized (-P) and fertilized plots with TSP (+P); and nitrogen (c) and phosphorus (d) harvested (kg/ha) in shoots of *Festuca* and *Lotus* grown in monoculture and in mixed stands for non-fertilized (-P) and fertilized plots with TSP (+P). Different letters among columns indicate significant differences by the LSD method at a level of $P<0.05$. Data in figures 2c-2d were logarithm-transformed for this analysis and plotted back as original data. Bars show the standard error of the mean of three replicates of each treatment.

Fig. 4. Mycorrhizal colonization (a) and arbuscular colonization (b) indexes in roots of *Festuca* or *Lotus* grown in monoculture or mixed; and DSE colonization (c) index in roots of

Festuca, number of rhizobia nodules per gram of fresh roots (d), AM spore density (d) and available P in soil (Bray 1) in the rhizospheric soil of *Festuca* or *Lotus* in monoculture or mixed for non-fertilized (-P) and fertilized soils with TSP (+P). Different letters among columns in each graph indicate significant differences by LSD method at a level of $P<0.05$. AM spore density data were arcsine-transformed for this analysis and plotted back as original data. Bars show the standard error of the mean of three replicates of each treatment.

Buenos Aires, 26th February 2014

Response to Editor comments
LPLA-2013-0230.R1 "Phosphorus fertilization of a grass-legume mixture: effect on plant growth, nutrient acquisition and symbiotic associations with soil microorganisms"

Comment 1: The manuscript is well structured and organized.
Response 1: Thank you.

Comment 2: Abstract: There is no rationale or justification statement in abstract to define the purpose of this work. It needs to be added.
Response 2: The purpose of the work was defined. See Pag 1 Line 19-22.
"Adding P on *Lotus tenuis* and *Festuca arundinacea*, pure or mixed, on growth, nitrogen (N) and phosphorus (P) acquisition and associations with soil microorganisms was studied to investigate the establishment of *Lotus* for competing with *Festuca*. Triple-superphosphate was applied on a Typic Natraquoll where *Lotus* grows spontaneously."

Comment 3: N and P should be spelled out in Abstract when they are mentioned the first time. It applies to the other abbreviations throughout the manuscript.
Response 3: done.

Comment 4: Materials and Methods: Treatments need to be clearly defined in Experimental Design sub section.
Response 4: done. See Pag 6 Line 130-133.
"The experimental design consisted in three stands (*Festuca* pure, *Lotus* pure and *Festuca-Lotus* mix) combined with two levels of P-fertilization (-P and +P) replicated three times and distributed in randomized complete blocks design.

Comment 5: In N and P acquisition; the second sentence is not true based on the Fig. 3a and b. Because the statistical lettering was indicated as cd and bcd, and a and ab for festuca. It was affected. Please check the numbers or statistics.
Response 5: The statistical lettering in fig 3a and 3b is correct. The statistical comparison has been done separately for Festuca pure and Festuca mix. The text clarified. See Pag 9 Line 203-205.
"In *Festuca* pure and in *Festuca* mixed stands separately, the concentration of N in shoots was not affected by P-fertilization, but the concentration P increased with P-fertilization in both stands (Fig. 3a, 3b)."

Comment 6: Please eliminate the one or two-sentence paragraphs.
Response 6: It is not clear which sentences is referred to. We have assumed that is referred to the first sentence of the N and P acquisition subsection in Pag 9.
The first sentence of the previous version "Shoot N and P concentrations differed due to plant species, P-fertilization and plant mixtures (Fig. 3).", was eliminated.

Comment 7: Soil Properties subsection need to be given at the beginning of the Results section.
Response 7: we suggest rearranging the results section as follow.
Part of the soil properties subsection was transferred to the subsection "Climate, soil characteristics and aboveground biomass", See Pag 9 Line 189-192.

"The soil pH, electrical conductivity, total C, total N, cation exchangeable capacity and moisture content in soil did not change significantly with P-fertilization and plants species sown in monoculture or mixed (data not shown). The overall mean value of the moisture content in soil at sampling was 13%."
Moreover the paragraph referred to Available P in soil was kept in the last subsection of the results because the plot of Available P in part of the fig 4. Otherwise, we may have two problems. One is that fig 4 will have 5 figures rather than 6, which much better in terms of distribution. The other, is that another figure should be included separately to describe the available P in soil. See Pag 11 Line 240-247.
"Available P in soil
The available-P in soil measured by Bray I test increased with P-fertilization in soil samples collected from *Lotus* pure or mixed with *Festuca* (Fig. 4f), but in soils from *Festuca* in monoculture the available P did not statistically change with P-fertilization (Fig. 4f). The mean values of the overall data were 14.7 mgP/kg in non-fertilized soils and 21.5 mgP/kg in P-fertilized soils (Fig. 4f). In P-fertilized soils, the highest values of available P were 22.4 mgP/kg in *Lotus* pure and 25.1 mgP/kg in *Lotus-Festuca* mixed. The lowest values were quite similar between non-fertilized soils ranged from 13.7 mgP/kg to 15.3 mgP/kg (Fig. 4f)."

Comment 8: References: This section needs to be carefully checked for format of the Journal.
Response 8: done.

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Author(s): **Rodolfo Mendoza, Matías Bailleres, Ileana García, and Oscar Ruiz**

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