Responses of Trifolium repens L. root structure and function to shading and phosphorus shortage: limits to adaptative plasticity during establishment?

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Research Article

Keywords: Shoot to root ratio, taproot biomass, coarse and fine fibrous roots, specific taproot length, root specialization

Posted Date: March 23rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2715779/v1

Version of Record: A version of this preprint was published at Agroforestry Systems on July 10th, 2023. See the published version at https://doi.org/10.1007/s10457-023-00867-7.

Abstract

Shading and low soil phosphorus availability may limit root growth of forage species in humid-temperate silvopastoral systems. However, plants are able to cope with such constraints by modifying root structure to improve the establishment and survival. The aim of this work was to evaluate the plasticity of different types of roots of *Trifolium repens* L. and its functional impact in the first two years of the species. A pot trial designed in 3 randomized complete block was carried out in sub-subdivided plots: main plot was the shading treatment (4 levels: full sun = 0% and 30%, 60% and 90% of photosynthetically active radiation (PAR) reduction), sub-plots were 2 cultivars of large-leafed (*cv.* Junín and *cv.* El Lucero) and sub-sub-plots were 2 phosphorous conditions (without P- and with added P+). Whole plants were harvested and the root system was divided into seminal taproot and fibrous roots: coarse roots (1 to ≤ 2 mm of diameter) and fine roots (≤ 1 mm of diameter). Even under 60% of shading, both cultivars were able to maintain root soil penetration and water and nutrients acquisition, regardless of the level of P. These functions were associated with the length and taproot diameter, specific taproot length and fine root biomass during establishment. Instead 90% of shading was a clear limit to plasticity and survival of the plants during the second year. Under mean PAR radiation > 212 µmol $m^{-2}.s^{-1}$ and P shortage, it is expected that the enrichment with large-leafed cultivars could be successful in these systems.

Introduction

In humid-temperate silvopastoral systems (SPS) in South America, the enrichment with perennial legumes could fulfill a clear ecological and productive function. This is linked to the sustainability of these fragile environments, as well as protein contribution to grazing animals. However, shading by trees and medium-low availability of phosphorus, typical of the soils, could limit the adaptation of the species. The functional balance or optimal partition theory establishes a greater partition of biomass to the aerial (shoot: S) compartment with respect to root (R) compartment in shaded conditions (< light ; > S/R) and a decrease, in the case of soil nutrient limitation (< edaphic phosphorus (P) ; < P; < S/R) (Brouwer, 1963; Ryle and Powell, 1976; Wilson, 1988; among others). However, in environments that are simultaneously limited in light and P, a compromise would be established between competing for light and for soil resources, and therefore, in the biomass partition between the aerial and root compartments. So, if the plant allocates less biomass to its root system, the establishment and survival of plants can be severely constrained because both stages depend on root structure and function, particularly in legumes. Various studies have documented that the traits associated with root growth are more important than those associated with aerial growth during the critical stage of establishment (Whalley et al, 1966; Sanderson et al., 2002).

However, in general, humid-temperate silvopastoral systems have more than one limiting resource. There are few studies that evaluate the growth of forage legumes under simultaneous light and P shortage. Cheng et al. (2014) found an interaction response between light intensity and phosphorus nutrition in *Lupinus albus* L. Shoot and root dry weights were more than tripled when the light intensity was increased from 200 to 600 µmol $m^{-2}s^{-1}$ in plants supplied with sufficient (50 µM) P, but were not

significantly affected by light intensity when plants were grown at a low P supply (1 μM). As a consequence, the S/ R was higher with shading regardless of the addition of P (3.5 $g.g^{-1}$) and lower with light and P availability (2.1 $g\text{-}g^{-1}$). Also, with adequate P supply, total root length and root surface area increased significantly under increasing light intensity but was lesser when plants were grown in shaded conditions.

Trifolium repens (white clover; TR) is a temperate perennial legume, quite different from *Lupinus albus.* It would be of potential importance in SPS because has mechanisms of shade and grazing tolerance and avoidance, is a good nitrogen fixer and provides quality forage for animals (Robin et al., 1992; Whitehead, 1995; Gautier et al., 1998; Lemaire and Millard, 1999; Brock and Hay, 2001; Carlsson and Huss-Danell, 2003; Christophe et al., 2006; Weijschedé et al., 2006; Black et al., 2009). In general, the seedling forms a primary stem, 1–3 months during the seedling phase of establishment, and shows a tap-rooted phase during 15–24 months. Those die and then it is initiated its clonal form with a stoloniferous growth habit (Brock et al., 2000; Brock and Tilbrook, 2000; Brock and Hill, 2001; Black et al., 2009). In this third phase of growth, TR has a shallow fibrous in its nodal root system (Caradus and Woodfield, 1998; Brock and Tilbrook, 2000). Therefore, TR has its 90% of nutrient absorption in the top 8 cm but with high assimilable P requirements - ≥ 20 ppm-. This root system could condition the absorption of immobile P (Boggie and Knight, 1960; Caradus and Woodfield, 1998; Scheneiter, 2001). But, there would be a number of mechanisms that allow plants to conserve or enhance the exploration of the soil volume and the acquisition of water and nutrient (Roumet et al., 2006). As most of these mechanisms rely on root growth (Evans, 1977), the negative effect of shading could reduce the acquisition of the inmobile P and the successful establishment of TR when P is limiting.

Trifolium repens also presents different plant types resulting from the combination of shoot (based on leaf size) and root-types (based on taproutedness). Large-leafed cultivars are more efficient in radiation interception and more deeply rooted, with thicker seminal and nodal taproots and more drought tolerant than smaller leaved shallower rooting types (Caradus and Woodfield, 1998). For this reason, large-leafed cultivars would be most suitable to persist in these SPS. Also, there are other differences between cultivars. Cultivars selected on fertile soils usually present thicker and less dense roots than genotypes selected in less fertile environments (Martín-Robles et al., 2019). Caradus (1991) evaluated different *Trifolium repens* populations collected at low and high-soil P. While there was no difference between low-P and high-P populations for S/R when growth at high levels of P supply, populations from low-P soils had a lower S/R, longer roots and a higher proportion of fines roots than populations from high-P soils when grown at low levels of P supply. However, root growth and root traits under simultaneous light and P deficit have not yet been evaluated in TR. Especially the tolerance or plasticity of the different types of roots -taproot and fibrous, coarse and fine ones-, in various large-leafed cultivars and their impact on establishment and survival in the first two years of the crop.

The aim of this work was to evaluate how the biomass partitioning, root growth and a suit of associated root traits, are modified in two large-leafed TR cultivars under simultaneous light and phosphorus shortage during establishment. The study attempted to answer three main questions: 1) what relative

importance do both stresses have on root biomass partitioning and on associated traits?, 2) how does phosphorus shortage influence these responses?; i.e. is there a light threshold to maintain root growth when edaphic P is limiting? and 3) are these responses cultivar-specific?

Materials And Methods

Environmental conditions

The experiment was carried out in the experimental field of the School of Agronomy, University of Buenos Aires (FAUBA, 34°35 S, 58°29 W). It lasted two years. In the first year, 48 pots (5 liters each) containing soil from the lower Delta area of the Paraná River were used and harvested; in the second year, 36 pots. The soil used corresponded to serie number 16 (Nueva Esperanza), belonging to the Entisols Order; Suborder Acuentes; Great group Endoaquents; Oxyacuent subgroup and without any degree of development of the genetic profile. They are gently sloping soils; with a very slow runoff, slow permeability and poor drainage; these traits lead to a great facility to flood and puddle frequently is characteristics: alkaline (grade 2), not saline and of a clay loam to sandy clay loam textural class (INTA Castelar, 2006). In general, chemical analysis of the top soil (0–15 cm) indicated a pH (1:H_2O): ≈5.10–5.60. Total Nitrogen (%) 0.30 ± 0.009 and Equivalent Humidity (%) = 32.8 ± 0.70. In particular, the soil was harvested in the first horizons (0–15 cm) of a field in the area under study (34°10'25.7"S, 58°51'58.1"W).

Plant material

Two cultivars were used: El Lucero MAG and Junín. El Lucero MAG is a highly used and widely published cultivar. It grows during autumn and winter but its maximum production takes place in spring. Scheneiter and Pagano (1995) and Randazzo et al. (2013) classify it as having large-leafed with thick stolons, robust roots and erect growth. It has wide adaptation and is productive with P availability.

Junín is a new FAUBA research development. It was registered in the Cultivar Own and National Cultivar Registries of the INASE, Argentina (Registration No. 9547). It is a synthetic variety evaluated in pure plots and under competition with *Festuca arundinacea* cv. Federation; this would give it better behavior in shading situations. It adapts to water saturation and low phosphorus content soils. It has exuberant growth with very large-leafed, longer half-life of its leaflets and high digestibility. For the purposes of the present study Junín cultivar, selected in sites with low P content, was tested vs. El Lucero.

Experimental layout

On April 20th, 2018, approximately 15 seeds per pot were sown. The fifty percent of the seedlings had emerged seven days later. Due to the high nutrient demand of the legume, half of the pots were fertilized. We enhanced phosphorus level to ≈ 35 ppm so plants were fertilized with 129 kg P.ha^{-1}, in agreement with Rubio et al. (2012). Fertilization coincided with the appearance of the first trifoliate leaf on May 20th. Then, the pots were placed under wooden structures that simulated shading (Peri et al., 2007). A completely randomized three blocks design in sub-subdivided plots was established. The main plot

corresponded to the shading factor (S) and had 4 levels: 0, 30, 60, 90% of PAR light reduction or shading (0% -control treatment in full sun- 30%, 60% and 90% S). The two fixed factors were arranged in a factorial design within each main plot; the two cultivars (Junín and El Lucero) in the subplot and the P content (with added P+ -control- and without added P-) in the sub-subplot.

Root growth evaluation was carried out during two consecutive years. The evaluation covered the stages of seedling and seminal tap-rooted phase and clonal growth form; which corresponded to the crop establishment and implantation stages, respectively (Brock and Hay, 2001). The potential persistence of the perennial species requires the inclusion of the clonal growth phase of *Trifolium repens*. The experimental period till the end of the first year, lasted from the moment the pots were placed under the structures -May 22th - to the final harvest -September 10th -, was 111 days long. The experimental period for the pots harvested at the end of the second year was, 507 days (till October 11st 2019; implantation stage).

Climatic factors

Data obtained at the FAUBA meteorological station showed that precipitation was 813 mm in 2018 and 555 mm in 2019. Records of temperature and PAR radiation were taken during the entire experimental period at full sun and PAR reduction under wooden structures with a sensor with datalogger. Temperatures were less variable than radiation among treatments (Fig. 1 vs. Figure 2 of Supplementary Material 1).

Light conditions changed seasonally; autumn-winter (April to August) and spring (September to November) sub-periods (Supplementary Material 1, Fig. 2a and b), resulting markedly different between the three levels of shading and full sun. On average during the experimental period, the incident photosynthetically active radiation (PAR) was 556 µmol $m^{-2}.s^{-1}$ in full sun and PAR reduction under wooden structures was 396, 212 and 54 µmol $m^{-2}.s^{-1}$ for the treatments of 30%, 60% and 90% S levels, respectively or PAR reduction (%) (Table 1 of Supplementary Material 1). The values recorded during the first year were similar to those of the second year so only the data for 2018 are presented. During the two years, manual control of weeds was carried out and irrigations were given in order to avoid water deficits.

Data collection

On May 20th, as soon as the first trifoliate leaves have appeared, plants were thinned to only 5 per pot in order to minimize competition. Three of them, were ringed with colour rings. Whole plants were harvested at the end of the growing season maintaining the identity of the marked plants until the end of the first year of the experiment (September 10th ; 143 days from sowing). This period corresponded to the establishment stage and is especially relevant for the success of the species in an environment. Plant root system was manually removed; the soil surrounding was carefully extracted and then the root was rinsed with water (Fang et al., 2012). The harvested material was separated into root and aerial fractions. By examining young plants the problem of death an decay of roots has been avoided (Evans, 1977). At harvest time, rooting from nodes was insignificant perhaps as an expression of taproot dominance

(Brock and Hay, 2001). Plant root system was divided into compartments of seminal taproot (true taproot) and fibrous roots, mainly as lateral from taproot. Taproot length (cm. $plant^{-1}$) was measured in fresh with a graduated ruler. Also and from digital images obtained with a scanner, and later processed with Scion Image software (O'Neal et al., 2002), the taproot base diameter was determined and the fibrous roots were classified. The taproot base diameter ($mm.plant^{-1}$) was the measure of the greatest width of the taproot (Caradus, 1991; Caradus and Woodfield, 1998). Fibrous roots were divided into coarse roots (1 to 2 mm of diameter) and fine roots (≤ 1 mm of diameter) (Caradus, 1991). The nomenclature proposed by Caradus (1991) was followed, since both categories are included within fine roots by other authors (with diameters ≤ 2 mm- but with a distinction between root orders *sensu* McCormack et al., 2015 and Freschet and Roumet, 2017). Then, all root fractions were oven dried at 50 ºC until constant weight and weighed. At harvest time, the soil was sampled to establish the remaining P content (ppm).

The harvest of the second growth cycle was carried out in October 11st, 2019. Previously, 5 cuts of the aerial material had been made above 8 cm from the ground (two in December 2018 and then in March, May and August of 2019). This second experimental period corresponded to the implantation stage of the crop and the transition between the tap-rooted phase and the clonal phase of TR (Brock and Hay, 2001). At harvest time (539 days from seedling), the main taproot of the plants had died. The identity of each plant could not be established due to the stolinification process and the strong interweaving between the roots, so total root biomass was expressed at pot scale ($g.pot^{-1}$). Only fibrous roots - adventicious nodal roots- were harvested (Caradus and Woodfield, 1998). Eighty percent of plants assigned to the 90% S treatments had died, so it was not possible to determine the root biomass in that treatment. The soil in the pots was also sampled at the time of the second harvest to establish the remaining P content (ppm).

Response variables

During the first year, several root responses were estimated at plant scale: the shoot to root ratio was the proportion of the aerial biomass with respect to the root biomass; $g.g.^{1}$. To calcule the total biomass, the total aerial material harvested during the year was added to the total root biomass. The total biomass of roots was composed of the biomass of taproots and biomass of coarse roots (> 1 and ≤ 2 mm of diameter) and fine roots (≤ 1 mm of diameter); all expressed in $g.plant^{-1}$. Also, the length of taproots ($cm.plant^{-1}$) and their diameter ($mm.plant^{-1}$) was measured. The specific taproot length ($cm.g^{-1}$) was the length of the taproot divided by its biomass (Cornelissen et al., 2003; Fort et al., 2015). For all the variables the average of the values corresponding to the three ringed plants per pot were calculated. In the second year, the shoot to root ratio and the root biomass were measured. In the latter case, the root biomass was the material contained in each pot ($g.pot^{-1}$).

Statistical analysis

Factorial analyzes of variance were performed following a randomized complete 3 block design in sub-subdivided plots (n = 3; 48 and 36 pots for the first and second year of evaluation, respectively). The 90% of shading treatment could not be evaluated in the second year. All traits were assessed for normality using the Shapiro-Wilk test. Means were compared using the Tukey test with α = 0.05. InfoStat Professional Package version 1.1 (Di Rienzo et al., 2020) was used to perform statistical analyses.

Results

a) Environmental variables

Soil phosphorous content

At the end of the first year, soil P content was differentially affected by shading according to the addition of P (shading x P interaction; p= 0.036). When no P was added (P-), shading did not affect the extractable P content of the soil (≈ 9.56 ppm Bray and Kurtz 1); being similar to the full sun value. However, when P was added (P+), the P content of the soil was higher in 90% S than in the other treatments (22.7 vs. 15.8 ppm). The cultivar did not influence the level of P in the soil (p = 0.71) (Fig. 1a of the Supplementary Material 2).

At the end of the second experimental period, the P level of the soil was affected by shading (p = 0.034) and the addition of P (p = 0.0029). The soil in the pots exposed to 60% shading conserved a greater amount of phosphorus (15.17 ppm); while that under 30% S, showed the lowest value (7.13 ppm) (Fig. 2b of Supplementary Material 2). P remnant in the soil was lower when it was not fertilized (P + = 14 vs. P-= 7 ppm; p = 0.029).

b) Morphological root traits

Shoot to root ratio

In the first growth cycle, total plant weight was reduced by 35% in 30 and 60% S and by 92% in 90% S with respect to the situation in full sun (3.94 $g.pl^{-1}$) and 29% with P- with respect to P+ (2.74 $g.pl^{-1}$; data not shown). Under shading, shoot to root ratio was similar to full sun and did not differ between low (P-) or high phosphorus availability (P+); 4.18 $g.g^{-1}$. However, the highest partition to aerial biomass occurred with 90% in P- (10.52 $g.g^{-1}$; interaction shading x P; p = 0.0348; Table 1).

In the second year, there were no differences in the shoot root ratio between shading levels, cultivars and phosphorus levels (p > 0.05). The shoot root ratio was on average 6.63 $g.g^{-1}$. Plants subjected to 90% S treatments did not survive the second experimental period.

Root biomass

Total root biomass

Total root biomass was differentially affected by shading between P levels (shading x P interaction; p= 0.027). In P+, the total biomass was higher and remained constant between full sun and with a light shade treatment – 30% S – up to a PAR radiation of 396 µmol $m^{-2}.s^{-1}$- (full sun P + ≈ 30% S P-=0.91 $g.pl^{-1}$). The root biomass was reduced by 55% with shading of 60% -PAR radiation < 212 µmol $m^{-2}.s^{-1}$-. Under P-, root biomass remained stable between full sun and 60% S (full sun ≈ 30% S ≈ 60% S at P- =0.52 $g.pl^{-1}$). The lowest total root mass was expressed with 90% P + and P- (0.06 $g.pl^{-1}$; data non shown).

During the second year, the total root biomass -nodal adventitious roots- was higher in full sun regardless of the P content of the soil. It was reduced ≈ 67% at 30 and 60% S (9.74 full sun vs. 30 and 60% S = 3.16 $g.pot^{-1}$; p = 0.01) associated with a decrease in the number of stolons (full sun = 15.3 vs. 60% S = 4.4 $stolons.pot^{-1}$; 30% S presented an intermediate number of stolons; p = 0.0134).

Taproot biomass

The taproot biomass was progressively reduced from 30% S in both cultivars (p < 0.0001; reductions of 29, 56 and 93% in 30% S, 60% S and 90% S, respectively, with respect to full sun (Fig. 1a; Table 1). Low P decreased the taproot biomass by 29% compared to P+ (p = 0.009). Cultivars did not differ in taproot biomass (p = 0.1709). However, the proportion of taproot on total biomass was similar among the factors analyzed and higher than the rest of the root types (p > 0.05), ≈ 63% of the total biomass.

Fibrous coarse and fine roots biomass

Responses to shading and P were different among the components of the fibrous root biomass. Only shading modified the biomass of coarse roots; they decreased by 88% from 60% S with respect to full sun and 30% S; p = 0.0012; Fig. 1b).

However, shading modified the biomass of fine roots differentially according to the availability of P (shading x P interaction; p= 0.0055) and followed a similar trend to that of the total biomass of roots. In P+, the biomass of fine roots expressed higher values than P- and decreased ≈ 60% with 60% S (Fig. 1c). Shading did not affect the biomass of fine roots in P-; its biomass was maintained up to 60% S. Then, the fine roots had a high light dependence to express their potential growth with P+ (full sun P + > 60% S with P + but 60% S P + ≈ full sun, 30 and 60% S under P-; Fig. 1c). The lowest fine root biomass occurred with 90% P + and P- (0.02 $g.pl^{-1}$).

c) Taproot allometry

Allometric measures were not affected by P shortage (p > 0.05), but showed differences among shading levels (Table 1).

Taproot length

Taproot length remained unchanged up to 60% of shading in both cultivars (p = 0.0011; 37.64 $cm.plant^{-1}$; Table 1). Extreme shading (90% S) reduced taproot length by 62%.

Specific taproot length

Inversely to taproot length, the taproot elongation with less biomass investment was only expressed at the extreme level of shading in both cultivars (p = 0.029). It was 266% higher in 90% S compared to the other treatments (full sun, 30% S and 60% S = 1.14 vs. 90% S = 4.17 $cm.g^{-1}$; Table 1)

Taproot diameter

Taproot diameter differed between cultivars according to the level of shading (cultivar*shading interaction = p = 0.016). Junín maintained its diameter up to 60% S. El Lucero cultivar maintained its taproot diameter only up to 30% S (full sun ≈ 30% S) and decreased by 27% with 60% of shading (4.7 $mm.plant^{-1}$). However, both cultivars presented similar taproot diameter in 60 and 90% shading (Table 1).

Discussion

In relation to our questions we found that:

1. Shade induces a biomass partition similar to that of full sun and independent of phosphorus availabitity. So, both stresses had similar importance in the biomass partition between aerial and root (shoot to root ratio; Table 1). The exception was the extreme level of shading – 90% S; average PAR radiation of 54 µmol $m^{-2}.s^{-1}$- with no added phosphorus (P-), that partitioned more biomass to the aerial compartment with respect to the root and greater to the rest of the treatments.
2. Under P-, root biomass was lower than in P + but remained with a similar growth between full sun and 60% S (full sun ≈ 30% S ≈ 60% S P-; Table 1). However, in general, the light threshold was between 30 and 60% of shading because of different sensitivity of root traits and a specialization function within the root system. Taproot biomass falls even at low shading level – 30% S-, especially in P^- (Fig. 1a). Course roots were maintained up to 60% S regardless of P level (Fig. 1b). Biomass of fine roots drop in 60% S con P + but were maintained up to 60% in P-(Fig. 1c). Allometric traits were a compensation mechanisms that maintained up to 60% of shading (Table 1). Extreme shading – 90%- caused plant death in the second year.
3. Only the diameter of the taproots differed between cultivars according to the level of shading (cultivar*shading interaction = p = 0.0160). Junín maintained its diameter up to 60% S while El Lucero, only up to 30% S (Table 1). No difference between cultivars was recorded in the second year of plants.

Plastic responses in root structure

In general, under shade, the shoot to root ratio was similar to full sun and indifferent to the addition of P (Table 1). These results differ from what was reported by Cheng et al. (2014) who found that shading of 200 µmol $m^{-2}.s^{-1}$ increased the S/R regardless of the P content of the soil. This would be because

Trifolium repens is a shade avoider species (Lemaire and Millard,1999) that maintained the S/R until a radiation of 212 µmol $m^{-2}.s^{-1}$-. This threshold is consistent with the photosynthetic asymptote located around 238 µmol.$m^{-2}.s^{-1}$ (Beinhart, 1962; Smetham, 1972). Below 200 µmol.$m^{-2}.s^{-1}$ the S/R increases; verified at extreme level of shading – 90%; 54 µmos $m^{-2}.s^{-1}$-, only under P-. Also, TR is tolerant to the shortage of P under shade.

However, under conditions of this trail, the functional balance was not able to capture differences between treatments. This agrees with what was postulated by Ryser and Eek (2000) that there are other traits that allow to overcome the constraint imposed by allocation pattern. In this sense, there were differential tolerance responses to the analyzed factors between the taproot biomass and length (Fig. 1a and Table 1, respectively) and between the types of roots evaluated (Fig. 1b and c). Taproot biomass was very sensitive to shading and P deficit; decreased with a light shading of 30%. These thickest roots might have a high growth cost for their low lifespan; high cost/benefit since they remain alive until the beginning of the second year (Brock et al., 2000; Pagès et al., 2011). However, the taproot length was a compensation mechanisms that maintained up to 60% S and irrespective of the presence of P (Table 1) ensuring a low carbon investment towards the taproot construction cost (length: taproot biomass) (Lambers et al., 2006).

The other kind of roots, coarse fibrous roots, also decreased their biomass from 60% S (Fig. 1b), regardless of the P content of the soil. This reduction may be due to low carbohydrate availability also demonstrated in *Trifolium respens* growing in competition for light with *Lolium perenne* (Jouany et al., 2004).

Fine roots could only express their maximum growth with light and P+ (mean PAR radiation of 556 µmol $m^{-2}.s^{-1}$). They were sensitive to shading > 60% S with P + and P- shortage even in full sun (Fig. 1c). Fine roots were also affected by located nutrient P- supply in soybean (Zhou et al., 2019). Shading of 90%, PAR radiation of around 54 µmos $m^{-2}.s^{-1}$, is clearly an extreme value that limits root growth in the first year. The plants tried to compensate by adjusting the taproot length (Table 1) but did not survive the second year. So, in general, the light tolerance threshold was around 60% of shading (212 µmol. $m^{-2}.s^{-1}$).

The differences between cultivars were scarce except in the taproot diameter (Table 1). This could be due to the low genetic variability between both cultivars.

At whole-plant level the balance in partitioned biomass between root categories was an efficient mechanism to adapt the root system sink to the overall availability of assimilates (Pagès et al., 2020) and to detect the plasticity between shading and P levels.

Impacts of root responses on root system functions and on establishment and implantation of Trifolium repens **under simultaneous light and P shortage**

The establishment of *Trifolium repens* depends on the ability to colonize the soil volume as quickly as possible (Pagès et al., 2020). Simultaneous light and P shortage maintained total root biomass up to 60% S in P- (Table 1). This would grant a benefit since higher root biomass of the entire root system would not limit whole-plant functioning; soil exploration and exploitation, acquisition of nutrients and water from soil (Ryser and Eek 2000; Ryser, 2006; Freschet and Roumet, 2017). However, there was a differential sensitivity to shade and phosphorus of the different types of roots evaluated. These shifts had consequences on the functions of the root system as a whole and are consistent with the existence of a specialization within the root system (McCormick et al., 2015; Freschet and Roumet, 2017).

Taproot biomass decreased with the least shading – 30% S-; this would condition the penetration and fixing point of the main roots into the soil (Roumet et al., 2006). However, plants maintained taproot length and diameter up to 60% S (Table 1). Taproot length impacted on the specific taproot length that is positively associated with the rate of water and nutrient uptake (Ryser, 1998; Roumet et al., 2006). Also, the maintenance of taproot diameter gave the differential ability to penetrate deeply into the soil (Caradus and Woodfield, 1998; Ryser, 1998; Roumet et al., 2006) (Table 1). Fine roots biomass was a stable trait between full sun and 60% with P-, because they develop an essential function of capturing the immobile P during the establishment of *Trifolium repens* (Fig. 1c). Cells of fine fibrous roots deal with absorption because they do not have secondary growth (Freschet and Roumet, 2017; Pagès et al., 2020). These responses would reflect the ability of TR to establish, survive and to monopolize space, water and nutrients in environments where competition may be strong (Caradus and Woodfield, 1998; Schenk and Jackson, 2002; Roumet et al., 2006). However, the greatest limitation in 60% S could be related to coarse root biomass during the first year. Coarse roots (Fig. 1b) are responsible for water and nutrients transportation because they have vascular structure and secondary development (Pagès et al., 2020).

In implantation (after the first year of the plants), the sensitivity of the total adventitious root was similar between 30 and 60% of shading and independent of P addition. This occurred even though there was still P in the soil (Supplementary Material 2, Fig. 1b). The effect of fertilization with P was not manifested in root biomass in the second year. Thus, fertilization with P + would have a limited scope in systems with < 396 µmoles $m^{-2}.s^{-1}$-. 30%S-.

Conclusions

The functional equilibrium (shoot to root ratio) was not able to capture differences, but biomass allocation between root categories -taproot and fibrous roots- allowed to establish the plasticity, the differential sensitivity to the analyzed factors and to demonstrate a specialization within the root system.

During establishment *Trifolium repens* was able to elongate and grow as deeply as needed to fulfill plant resource requirements and to establish an adequate exploratory capacity of the soil up to 60% S. These functions were associated with taproot length and diameter, specific taproot length, and fine root biomass. Large-leafed cultivars -Junín or El Lucero- would be equally convenient. At implantation, the total adventitious root was similar between 30 and 60% of shading -≈ between 396 and 212 µmol $m^{-2}.s^{-}$

^{1}- and independent of the addition of P. The addition of P to these SPSs would not be a recommended practice. Shading of 90%; PAR radiation of 54 μmos $m^{-2}.s^{-1}$- is a clear limit to the plasticity and survival of the plants in the second year.

Under the conditions of the present trial, simultaneous light and P shortage, it is expected that the enrichment with *Trifolium repens* in humid-temperate silvopastoral systems will be successful.

Declarations

Funding

This study was funded by Universidad de Buenos Aires (G144, 247BA) and Ministerio de Agricultura Ganadería y Pesca de la Nación Argentina and Proyecto de Manejo Sustentable de Recursos Naturales (BIRF LN 7520 AR: PIA 10104).

Consent for publication

All authors have consented to the publication of this manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Mario Suárez (RIP) for his help with field work and Dr. Rodolfo Golluscio who has provided valuable suggestions that allowed improving the present manuscript.

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Table

Table 1. Results of the analysis of variance resulting from comparing the effect of the cultivar: cv. Junin or cv. El Lucero, the shading levels: 30%, 60% and 90% of shading (S) + Full sun (Non shade), the phosphorus level (P; P- or P+) and their interactions for the response variables of the first year of the

plants (establishment phase): shoot to root ratio ($g.g^{-1}$), root biomass ($g.plant^{-1}$), taproot biomass ($g.plant^{-1}$), taproot length ($cm.plant^{-1}$), specific taproot length ($cm.g^{-1}$) and taproot diameter ($mm.plant^{-1}$) during establishment phase (1^{st} evaluation year). The data correspond to mean and the F and p values: ** $P< 0.01$; * $P< 0.05$ and ns = non-significant effect. Tukey test was performed; Capital letters indicate significant differences between levels of shading and lower case letters, between levels of P or interactions.

Variables	*Cultivar effect* (C)		*Shading effect* (S)		*Phosphorus effect (P)*		*Interactions*
	Mean values	**F and *p***	**Mean values**	**F and *p***	**Mean values**	**F and *p***	**Mean values** **F and *p***
Shoot to root ratio **(g.g^{-1})**	*cv.* Junín= 4.04	ns	Full sun= 3.82 A	ns	P-= 5.69 b	4.94 *	Shade*P 3.67 *
			30 % S= 3.72 A				
	cv. El Lucero= 5.90		60 % S= 4.38 A		P+= 4.25 a		90%S P-= 10.52 b
			90 % S= 9.96 A				Rest = 4.17 a
Root biomass **(g.plant^{-1})**	*cv.* Junín= 0.53	ns	Full sun=0.85 D	70.17 **	P-= 0.40 a	13.28 **	Shade*P 3.94 *
							Full sun P+ = 1.05 e
			30 % S=0.65 C				30 % S P+ = 0.78 de
							Full sun P- = 0.65 cd
	cv. El Lucero= 0.45		60 % S=0.40 B		P+= 0.58 b		30 % S P- = 0.51 cd
							60 % S P+ = 0.41 bc
			90 % S=0.06 A				60 % S P- = 0.40 c
							90 % S P+ = 0.07 ab
							90 % S P- = 0.05 a
Taproot biomass (g. plant^{-1})	*cv.* Junín= 0.33	ns	Full sun= 0.54 D	71.33 **	P-= 0.25 a	9.75 **	ns
			30 % S= 0.38 C				
	cv. El Lucero= 0.27		60 % S= 0.24 B		P+= 0.35b		
			90 % S=				

			0.04 A				
Taproot lenght (cm.plant^{-1})	*cv.* Junín= 32.4	ns	Full sun= 39.8 B	22.66 **	P-= 29.8 a	ns	ns
			30 % S= 38.4 B				
	cv. El Lucero= 31.2		60 % S= 34.7 B		P+= 33.9 a		
			90 % S= 14.3 A				
Specific taproot length (cm.g^{-1})	*cv.* Junín= 1.77	ns	Full sun= 0.81 A	15.90 **	P-= 1.98 a	ns	ns
			30 % S= 1.12 A				
	cv. El Lucero= 2.02		60 % S= 1.49 A		P+= 1.98 a		
			90 % S= 4.17 B				
Taproot diameter (mm.plant^{-1})	*cv.* Junín= 0.49	ns	Full sun= 6.4 B	17.24 **	P-= 4.7 a	ns	Cultivar * Shade 6.42 *
							El Lucero full sun= 6.4 d
	cv. El Lucero= 0.49		30 % S= 5.8 B		P+= 5.1 a		El Lucero 30% S= 6.4 cd
							Junín full sun= 6.3 cd
			60 % S= 4.9 B				Junín 30% S= 5.2 bc
			90 % S= 2.6 A				Junín 60% S= 5.1 bc
							El Lucero 60% S= 4.7 b
							Junín 90% S= 2.8 a
							El Lucero 90% S= 2.3 a

Supplementary Materials

Supplementary Materials 1 and 2 are not available with this version.

Figures

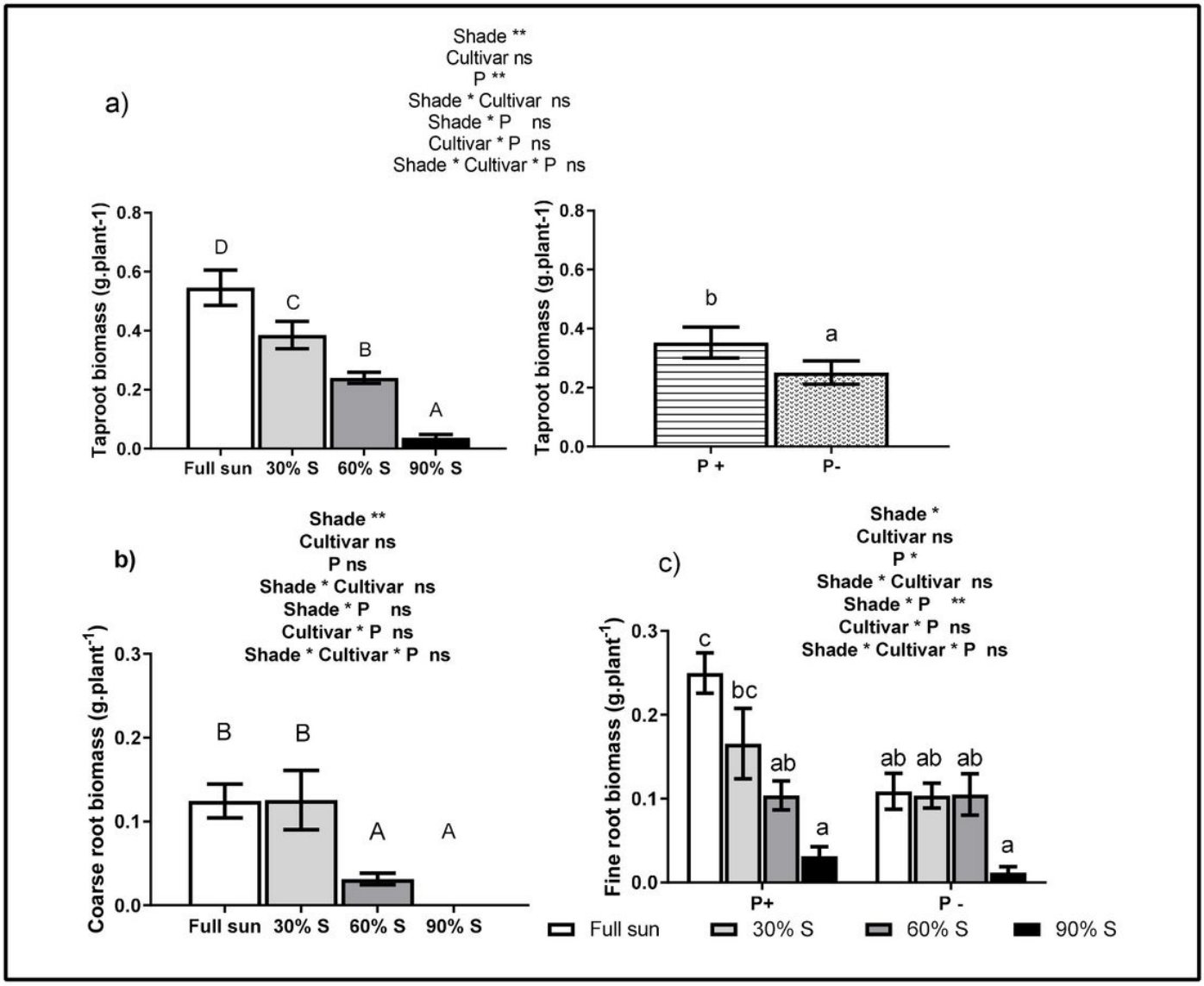

Figure 1

a) Taproot biomass (g.plant^{-1}) in two large-leafed cultivars of *Trifolium repens* L. There were independent effects of shading and P. Left panel) effect of shading: 30%, 60% and 90% of shading (S) + Full sun (Non shade) and right panel) effect of phosphorus (P); P+ or P-. Capital letters indicate differences between shading levels; lower case, between phosphorus ones.

Types of fibrous roots, b) coarse root biomass (g.plant^{-1}) and c) fine root biomass (g.plant^{-1}). In b) there was only an effect of shading and in c) there was an interaction between shading x phosphorus levels (P). Data correspond to mean values ± standard error and P values: ** $P < 0.01$; * $P < 0.05$ and ns = non-significant effect.