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A physiological approach to study the competition ability of the grassland species *Trifolium pratense* and *Agrostis capillaris*

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Summary

The response of plant species to external factors depends partly on the interaction with the environment and with the other species that coexist in the same ecosystem. Several studies have investigated the main traits that determine the competitive capacity of plant species, and although the relevance of the traits is not clear, traits both from belowground and aboveground have been observed. In this paper, we grew *Trifolium pratense* and *Agrostis capillaris* in intra- and interspecific competition, analyzing the photosynthetic metabolism and nitrogen uptake, among other variables. The results indicated that *T. pratense* possesses better competition ability due to the higher competitive performance for soil resources compared to *A. capillaris*, explained by a higher root biomass and a higher nitrogen uptake rate in the former than in the latter.

These traits permitted *T. pratense* to show higher photosynthetic rate than *A. capillaris* when both species were grown in mixture. Furthermore, the interspecific competition provoked *A. capillaris* to activate its antioxidant metabolism, through SOD activity, to detoxify the reactive oxygen species generated due to its lower capacity for using the photochemical energy absorbed. In this experiment, we conclude that the competitiveness seems to be more related with soil resources competition than with light competition, and that the photosynthetic rate decline in *A. capillaris* is more a secondary effect as a consequence of nitrogen limitation.

Keywords: *Agrostis capillaris*, competition, grasslands, nitrogen uptake, photosynthesis, *Trifolium pratense*.

1. Introduction

In natural ecosystems, plants coexist with other plants of the same and different species. This coexistence involves competition between them for the available resources such as water, nutrients or light (Van der Werf et al., 1993; Warwick et al., 1998); therefore, competition could be localized aboveground and belowground.

Aboveground competition mainly consists of light competition. Plant architecture is an important trait that determines plants capacity for light uptake, so the ability of a plant to place foliage in upper, well-lit canopy layers should be an important structural trait contributing to competitive ability (Barnes et al., 1990 and literature therein). Pierik et al. (2013) and Ye et al. (2019) claimed that stem elongation is one of the mechanisms that plants exhibit to outcompete other species, avoiding shade or even prevailing over the other species. Species shadowed by taller or dominant species usually show a

change in leaf chlorophyll content with a trend to decrease chlorophyll *a*/*b* ratio and to show lower photosynthetic rates (Lichtenthaler et al., 2007).

If a reduction in photosynthetic capacity is not accompanied by a decrease in energy absorption through decreases in pigment composition and/or fluorescence emission, ROS formation could occur. Moreover, if the plant does not induce its antioxidant metabolism to cope with the aforementioned oxidative burst, even higher growth suppression could be observed (Matsubara et al., 2016).

Regarding belowground competition, the presence of multiple plants in a given volume of soil can induce nutrient stress in a given plant as neighboring plants acquire limiting resources (Craine and Dybzinski, 2013). These authors stated that plants do not outcompete others by reducing the concentration of resources in the environment, but instead by pre-empting the resource supplies from coming in contact with other species, according to the pre-emption hypothesis. Various studies determined that, in competition, the nutrient acquisition is proportional to the root length density of different individuals (Reich et al., 2003; Raynaud and Leadley, 2004; Craine et al., 2005).

In a review, Wilson (1988) reported that, in general, belowground resources competition had a greater effect than aboveground resources competition being water and nutrients the main factors involved. Wilson (1988) also stated that the relative importance of root competition increases with time, though in some situations the reverse may be true, and competitive imbalance is often greater at higher resource levels.

Additionally, Kiær et al (2013) in a meta-analysis stated that despite large variation among experiments, the effects of root competition are generally stronger than

shoot competition, and whereas root competition may be the primary limitation on mean plant performance, shoot competition will modify this influence and may determine which individuals or species dominate.

Although the literature is limited, from these studies we can conclude that there is no consensus either on plant traits that are best to compete for resources or on the physiological mechanisms that rely on this ability. Nevertheless, what is clear is that both the constitutive traits of the species and their plasticity are important to compete for light and nutrients (Díaz et al., 2013; Pierik et al., 2013).

One of the main ecosystems where plant competition plays a key role is grasslands, where a competitive advantage of one species over another would imply changes in the biomass structure of the ecosystem and this shift in species or functional groups could imply changes in forage quality (Odriozola et al., 2014). Additionally, grasslands represent approximately 25% of land cover (FAO, 2010) being one of the largest ecosystems. The study was done in two grassland species, *Trifolium pratense* and *Agrostis capillaris*. In a previous work we already analyzed how these two species will address future climatic conditions when grown in intraspecific competition (Miranda-Apodaca et al., 2018a), but so far no studies have been done analyzing the interspecific competition ability of these two species when grown together. In works done with similar species (clovers*)* and (ryegrass) in competition, Lucero et al. (1999) and Munoz and Weaver (1999) observed that the higher ability of belowground nutrients uptake of the grass due to its higher root biomass provided an advantage over the clover in biomass production. Zhang et al. (2008) in an experiment with *Trifolium pratense* and *Festuca rubra* detected that in mixture the light competition was decisive to determine the photosynthetic capacity and biomass production.

With all this in mind, the objectives of this work were: (1) to examine the dynamic of each species regarding above and belowground traits when they grow in intraspecific competition; (2) to study the competition ability of the two species, analyzing the plasticity of the traits that make one species to be more competitive than the other and the physiological mechanisms that rely on this plasticity and; (3) to evaluate if oxidative metabolism is induced in this situation as a consequence of a reduction in photosynthesis in competition.

2. Materials and Methods

Plant material and experimental design

Seedlings of two grassland species, *Agrostis capillaris* L. (Ac; grass) and *Trifolium pratense* L. (Tp; legume), were grown in a mixture of peat/vermiculite (1/1 v/v) in 6 L pots. This study is focused on nutrients competition rather than in water competition, so plants were watered reaching field capacity. Pots were watered every other day with deionized water for 4 days till the plants germinated. From here, and for the whole experiment that lasted 37 days, seedlings were watered two days a week with 250 mL of Hoagland's solution and plants were also watered with deionized water between each application of Hoagland's solution to reach field capacity. The nutrient solution contained 6 mM KNO_3, 4mM $Ca(NO_3)_2$, 1mM $NH_4H_2PO_4$, 2mM $MgSO_4$, 9 µM $MnCl_2$, 46 µM H_3BO_3, 0.8 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.1 µM Na_2MoO_4 and 0.01 g l^{-1} Fe chelate (LibFer SP, Allied Colloids) with the pH adjusted to 5.5. The nutrient solution was made up from deionized water. Fourteen days after sowing, the twelve more uniform plants were selected, reaching a final density of 315 plant m^{-2}. The plants were

grown in two conditions of competition: monoculture (intraspecific competition) and mixture (intra- and interspecific competition). In monoculture, all the plants in each pot were from the same species, whereas in mixture, 6 plants per species were equally distributed.

Plants were grown in a Conviron PGR15 controlled environment growth chamber (Conviron, Manitoba, Canada) with a daily 14 h light regimen, an average day/night temperature of 24/20 °C and a relative day/night humidity of 70/80%. During the light period, the photosynthetic photon flux density in the chamber was 400 μmol m^{-2} s^{-1}. Light was provided by a combination of incandescent bulbs and warm-white fluorescent lamps. To minimize the intra-chamber effects, plants were randomly repositioned within the chamber each week.

Photosynthesis and related parameters

Gas exchange parameters

The determination of gas exchange parameters was performed by the gas analyzer in the infrared (IRGA) in open system Li-6400 (Li-Cor Inc., Lincoln, NE, USA). Measurements were performed 3 h after dawn with a cuvette at a stable temperature of 24 °C and a relative humidity of 60% (Miranda-Apodaca et al., 2018b). The photosynthetic photon flux density was 400 μmol m^{-2} s^{-1}, provided by a red/blue LED light source (model Li 6400-02B, Li-Cor Inc.). The CO_2 concentration of the cuvette was the same as in the growth conditions, 400 μmol mol^{-1}. The measurement record was made when the equilibrium of water and CO_2 exchange (steady state) was reached, a condition that was obtained after 15 minutes. CO_2 assimilation rate (*A*), stomatal conductance (*gs*), intercellular CO_2 concentration (*Ci*) and the instantaneous

transpiration rate (*E*) were determined according to the method of von Caemmerer and Farquhar (1981).

Chlorophyll *a* fluorescence determination

Chlorophyll *a* fluorescence was determined using the open gas exchange system, Li-6400 (Li-Cor Inc., Lincoln, NE, USA), with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc.). Measurements were made before dawn and the leaves were exposed to different light pulses as described in Miranda-Apodaca et al. (2015), measuring the photochemical efficiency of PSII in light-adapted leaves (*Fv'*/*Fm'*), the photochemical quenching (*qP*), the non-photochemical quenching (*NPQ*) and the electron transport rate (*ETR*) as Genty et al. (1989).

Photosynthetic pigments determination

Chlorophylls and carotenoids were extracted in dimethylsulfoxide (DMSO) as Barnes et al. (1992). The samples were incubated in 2 mL of DMSO for 2 h in the dark, in an oven at 80 °C. Subsequently, the absorbance was measured at 665, 649 and 480 nm. The quantification was made following the formulas of Wellburn (1994).

Growth and nitrogen uptake determination

Growth analysis

Plant biomass was measured 28 days after sowing (DAS) and 37 DAS. The plants were harvested and the different plant organs (leaves, stems and roots) were separated and weighted to determine the fresh weight (FW). Besides, leaf area was determined using Winfolia software (Regent Instruments Inc., Canada) associated to a scanner (Epson expression 10000 XL). Afterwards, the samples were dried at 70 ºC for 48 h, and their dry weight (DW) was determined.

To determine competitiveness of the species, the relative neighbour effect (*RNE*) was calculated (Markham and Chanway, 1996): *RNE*= (Bmono-Bmix)/X; where, B stands for biomass in monoculture and in mixture and X is Bmono when Bmono is greater than Bmixture and X is Bmixture when Bmixture is greater than Bmono.

Nitrogen uptake

Nitrogen content (N) was determined in dry and ground pooled samples of leaf, stem and root (2 mg DW for each organ) from each experiment, using an elemental analyzer (FlashEA 1112; ThermoFinnigan, Germany). The whole plant nitrogen uptake (NU) was calculated multiplying N concentration of each organ by plant biomass of each organ as proposed by Subedi and Ma (2005).

The nitrogen uptake rate (NUR) was calculated based on equations described by Franklin and Zwiazek (2004). The NUR was calculated as: $[(Ntot2-Ntot1)\times(\ln(Wr2/Wr1))]/[(T2-T1)\times(Wr2-Wr1)]$; where Ntot was the average content of N per plant (mg) at T1=28 days (Ntot1) and at T2=37 days (Ntot2); and Wr was the mean root dry weight (g) at day 28 (Wr1) and day 37 (Wr2).

Total soluble proteins were extracted as proposed by Pérez-López et al. (2015), and measured by the method of Bradford (1976).

Antioxidant metabolism

Superoxide dismutase (SOD; EC 1.15.1.1) activity determination

SOD enzyme was extracted according to McCord and Fridovich (1969) with some modifications proposed by Pérez-López et al. (2009). SOD activity was assayed by the ferricytochrome-c reduction spectrophotometric test, using xanthine/xanthine oxidase as the source of superoxide. The reaction mixture (280 µL) contained 50mM potassium

phosphate (pH 7.8), 0.1mM EDTA, 1 mM cytochrome-c (cyt-c) and 1 mM xanthine. Then, 10 µL of xanthine oxidase (XOD) was added to the reaction mixture and the reduction rate of cyt-c in the absence of supernatant was monitored at 550 nm. This value was circa 0.025 ΔAbs550 min^{-1} and served as a control (Pc). Later, 10 µL of XOD and 10 µL of the extract were added to the reaction mixture and the reduction rate of cyt-c was monitored at 550 nm (Ps). One unit of SOD (U) was defined as the amount of enzyme that inhibited the rate of cyt-c reduction by 50%. The inhibition was calculated by the following formula: 100 x ((Pc-Ps)/Pc).

Lipid peroxidation determination

Lipid peroxidation was determined by measuring malondialdehyde (Mda) formed using the thiobarbituric acid method as described by Buege and Aust (1978) with some modifications by Hodges et al. (1999).

Statistical analysis

Results are reported of four experiments. In each experiment, we measured at least three different replicates (plants). Each of the measured physiological parameters remained statistically similar between the four independent experiments; therefore, we pooled the measurements from the four experiments. Data analyses were performed using the SPSS 23.0 software package (Chicago, IL). Two-way analysis of variance (ANOVA) was used to evaluate the main effects of species and competition and their interaction. Means were compared using Duncan's multiple range test. P-values ≤ 0.05 were considered statistically significant. Prior to analyses, we tested whether the assumptions of an ANOVA, homogeneity of variances and normally distributed residuals were achieved. The homogeneity of variances for all the studied parameters was evaluated by Levene's test and the distribution of the residuals was assessed by

211 Kolmogorov–Smirnov test. When necessary, transformation was applied before
212 statistical analysis was performed.

213

214 **3. Results**

215 **Gas exchange parameters**

216 In monoculture, CO_2 assimilation rate (*A*) was 37% higher in *Trifolium pratense*
217 (Tp) than in *Agrostis capillaris* (Ac), 17 *vs* 12.5 μmol CO_2 m^{-2} s^{-1}. In mixture this
218 difference was higher, reaching up to 80% (Fig. 1A), because the interspecific
219 competition provoked a decrease of 25% in *A* in Ac plants whereas it did not change in
220 Tp. Stomatal conductance (*gs*) was also higher in Tp (0.43 mol H_2O m^{-2} s^{-1}) than in Ac,
221 74% in monoculture and 130% in mixture (Fig. 1B). The interspecific competition
222 decreased *gs* in Ac by 20%. There were no differences in intercellular CO_2
223 concentrations (*Ci*) either between species or between types of competition (Fig. 1C).
224 The instantaneous transpiration rate (*E*) was 44% higher in Tp than in Ac in
225 monoculture (Fig. 1D). The interspecific competition decreased *E* by 10% in Ac,
226 although the decrease was not statistically significant.

227 **Photosynthetic pigments**

228 The concentration of chlorophyll *a*, *b* and carotenoids were higher in Tp than in
229 Ac, 48%, 38% and 69%, respectively (Fig. 2A-C). The interspecific competition did not
230 alter the pigments concentration in any species. The chlorophyll *a*/*b* ratio was neither
231 modified in any treatment (Fig. 2D).

Chlorophyll *a* fluorescence

The values of *Fv'/Fm'*, *qP* and *ETR* of Tp were higher than the ones of Ac, *circa* 15%, 40% and 30%, respectively (Fig. 3A-C). In the case of *Fv'/Fm'*, the type of competition did not provoke any difference in any species in this parameter. However, in *qP* and *ETR*, the interspecific competition provoked a decrease of around 25% in these variables in Ac, while in Tp no differences were detected. Conversely, *NPQ* was higher in Ac than in Tp, 74% in monoculture and 113% in mixture (Fig. 3D).

Growth analysis

Total leaf area in monoculture was higher in Tp (335.4 cm^2 $plant^{-1}$) than in Ac (120.9 cm^2 $plant^{-1}$) (Fig. 4A). However, the response of each species under mixture compared to monoculture was the opposite. Tp, increased total leaf area in mixture by 22%, whereas in Ac, it decreased by 65%. Regarding root biomass, we detected a similar trend to the one observed for leaf area. Root biomass was higher in Tp than in Ac, regardless of the type of competition (Fig. 4B). In mixture, Tp showed a 29% higher root biomass than the one detected in monoculture, while Ac showed a 65% lower root biomass.

Finally, these changes in each species' traits were traduced in different relative neighbour effect (*RNE*) which indicates the competitive capacity of each species (Fig. 4C). When plants grew together in mixture, the interspecific competition turned out facilitation for Tp (because *RNE* value was negative) and competition for Ac (because *RNE* value was positive).

253 **Nitrogen uptake and protein concentration**

254 The total nitrogen uptake (NU) of Tp in monoculture was 39.6 mg N $plant^{-1}$, while
255 in Ac was 15.4 mg N $plant^{-1}$, around 60% lower. In mixture the differences were much
256 greater, increasing in the case of Tp by 44% and decreasing in Ac by 79% (Fig. 5A). If
257 we express the nitrogen uptake per unit of root biomass (nitrogen uptake rate; NUR),
258 the differences between species were smaller and, in monoculture, the NUR of Ac was
259 even 55% higher than the one of Tp (Fig. 5B). However, in mixture, when both species
260 compete for the same soil resources, the NUR of Tp was twice of the one of Ac, caused
261 by an increase in Tp and a decrease in Ac compared to plants grown in monoculture.

262 Protein concentration was 10% higher in Tp (275 mg g^{-1} DW) than in Ac (250 mg
263 g^{-1} DW) in monoculture (Fig. 5C). The interspecific competition increased the protein
264 concentration in Tp by 10%, but in the case of Ac, it decreased protein concentration by
265 17%, to 210 mg g^{-1} DW.

266 **Antioxidant metabolism**

267 Both species showed a similar superoxide dismutase (SOD) activity in
268 monoculture (Fig. 6A), *circa* 65 U mg^{-1} prot. The interspecific competition provoked an
269 increase of 32% in Ac to 95 U mg^{-1} prot, while in the case of Tp no differences were
270 detected. The lipid peroxidation, measured as malondialdehyde content, showed no
271 differences between species and competition treatments (Fig. 6B).

272

4. Discussion

Differential traits between *Trifolium pratense* and *Agrostis capillaris*

The constitutive traits of a plant species determine the ability to be competitive (Díaz et al., 2013). So, in this first section of the discussion we will analyze some important above and belowground traits of *Trifolium pratense* and *Agrostis capillaris*.

Differences between species were detected in the photosynthetic metabolism. Tp showed a higher *A* and *gs* than Ac (Fig. 1A-B). However, the higher *gs* was not responsible to explain the higher *A* in Tp, as *Ci* was similar for both species (Fig. 1C); i.e., for the same *Ci* Tp showed higher *A* than Ac. The lower values of *A* in Ac compared to Tp could be provoked by a limitation in the light phase (photochemical phase) and/or in the dark one (biochemical phase). Regarding the limitation in the light phase, the concentration of pigments was lower in Ac than in Tp (Fig. 2A-C) and it could provoke a smaller light absorption. Additionally, the photochemical efficiency was lower in Ac than in Tp (*Fv'Fm'*, *qP* and *ETR*, Fig. 3A-C), showing that a smaller fraction of electrons was derived to photochemical processes. Other authors also detected a higher photochemical efficiency in one species over another that could be related to competitive advantage permitting a higher photosynthetic capacity (Campoy et al., 2019). Besides, we detected an increase in *NPQ* (Fig. 3D) which would indicate that the energy of the photons was dissipated as heat. However, as the concentration of carotenoids did not increase in Ac, the higher *NPQ* could be provoked by a higher VAZ cycle activity (Müller et al., 2001), but without changing total carotenoids concentration, as Abadía et al. (1999) also detected in barley.

The fact that Ac diverted part of the energy absorbed as heat could indicate that the light requirement of Ac is lower than the one of Tp, and under 400 µmol m^{-2} s^{-1} light

297 intensity, Ac receives higher energy than the one it is able to use in photochemical
298 processes. This lower capacity to use the energy in photochemical processes could be
299 related to a lower Calvin cycle activity, as we have detected lower Rubisco activity in
300 these conditions (data not shown).

301 This lower *A* in Ac could explain the lower leaf area and root biomass (Fig. 4),
302 compared to Tp, since the lower *A* in Ac could imply lower carbohydrate availability to
303 perform growth. However, it is important to highlight that although root biomass was
304 lower in Ac than in Tp, the NUR was higher in Ac than in Tp (Fig. 5B). This fact
305 indicates that Ac, in monoculture, is able to compensate the lower root biomass with
306 higher uptake capacity of the root in order to maintain nitrogen uptake and provide the
307 plant with enough nitrogen to synthesize proteins (Fig. 5C). Evans (1977) detected
308 higher root hair length and higher percentage of roots with root hairs in Ac than in Tp,
309 which could explain, in part, the higher NUR since root hairs are responsible for
310 nutrient uptake.

311 **Competition effects and consequences in *Trifolium pratense* and *Agrostis***
312 ***capillaris***

313 Besides analyzing the constitutive traits of each species, it is also important to
314 study their plasticity in order to adapt to the new environment were competition
315 between species for light and nutrients occurs (Pierik et al., 2013). When plants grew
316 together in mixture, the interspecific competition turned out facilitation for Tp (Fig. 4C)
317 and competition for Ac. Zhang et al. (2008) in a study with *Trifolium pratense* and
318 *Festuca rubra* detected that the interspecific competition also benefitted the legume;
319 however, Lucero et al. (1999) and Munoz and Weaver (1999) discovered the opposite
320 effect, being ryegrass benefitted from the competition with clovers. Looking for the

321 reasons for the dominance of one species over the other, we analyzed several key
322 processes of the metabolism of each species.

323 Competition between species did not affect the photosynthetic metabolism of Tp,
324 but it did in the case of Ac; where *A* was 25% lower in mixture than in monoculture
325 (Fig. 1A). Some authors have found that the lower *A* under interspecific competition is
326 due to a shading effect provoked by dominant species by being taller or with more leaf
327 area (Lichtenthaler et al., 2007). Usually, plants growing in these conditions tend to
328 develop the so-called shade leaves and, remarkably, the chlorophyll *a*/*b* ratio decreases
329 by a higher invest in the antenna system to increase the light harvest (Lichtenthaler et
330 al., 2007). However, we think that the decrease in *A* in Ac was not caused by a shading
331 effect, as the chlorophyll *a*/*b* ratio was not modified (Fig. 2D). Furthermore, neither *gs*
332 nor *Ci* were altered, so stomatal limitation should be also discarded (Fig. 1B-C). In
333 contrast, a decrease in *qP* and *ETR* were detected (Fig. 3B-C). The electron transport is,
334 partly, dependent on protein concentration in the thylakoids since electron transport is
335 carried among different proteins (Taiz and Zeiger, 2014). So, the decrease of the
336 aforementioned parameters could be related to the lower protein concentration in
337 mixture than in monoculture of Ac (Fig. 5C); Campoy et al. (2019) related
338 photochemical efficiency with nutrional status. Specifically, the association of lower
339 protein concentration and decreases in *qP* and *ETR* was also detected by Lu et al. (2001)
340 and Kumagai et al. (2007) in other *Poaceae* species such as wheat and maize, and rice
341 plants, respectively.

342 The reason to explain the lower protein content could lie on a decreased capacity
343 for nitrogen uptake in Ac in mixture compared to the one detected in monoculture (Fig.
344 5A). Hu et al. (2015) also detected a smaller nitrogen concentration in trembling aspen

345 and white spruce caused by a lower uptake when these plants grew in competition with
346 *Hordeum jubatum* compared to plants grown in monoculture. In several studies it has
347 been demonstrated that the mineral uptake depends on root biomass (Shinano et al.,
348 2007). Tp showed greater root biomass than Ac (0.164 g DW plant^{-1} *vs* 0.012 g DW
349 plant^{-1}), which would lead Tp to increase soil exploration and to increase soil resources
350 uptake (Aerts et al., 1991; Lucero et al., 1999; Acciaresi and Guiamet, 2010), while in
351 the case of Ac, the opposite response would occur. Craine et al. (2005) showed that the
352 plant that possessed higher root biomass displaced competitors, capturing the largest
353 proportion of the nutrient supply by ensuring nutrients contact its roots before those of a
354 competitor.

355 However, mineral uptake capacity not only depends on root biomass, as it was
356 demonstrated by Caldwell et al. (1985). If we eliminate the effect of the higher root
357 biomass using the nitrogen uptake rate, *i.e.* per unit of root biomass, NUR was still
358 higher in mixture than in monoculture in the case of Tp and the opposite was true for
359 Ac, being the values lower in mixture than in monoculture (Fig. 5B). Some authors have
360 detected that higher photosynthesis could lead to greater energy available, on one hand,
361 to increase mineral uptake (BassiriRad et al., 1996; Pérez-López et al., 2014) and, on
362 the other hand, to prevent the nutrient uptake of the other species following the pre-
363 empty theory (Craine et al., 2005; McNickle et al., 2013). In this case, the NUR of Tp in
364 mixture was higher than the one of Ac in mixture, because in Ac, the interspecific
365 competition provoked a decrease in NUR. Therefore, according to the data, initially the
366 higher root biomass of Tp would provoke a decrease in the competitive ability of Ac to
367 uptake minerals, leading to a decrease in proteins as aforementioned and, ultimately, a
368 decline in *A* in Ac in mixture as a secondary effect. As time goes by, the decrease of *A*

would imply a lower energy availability for mineral uptake, reducing even more the mineral uptake capacity, as it was detected in NUR, where the effect of root biomass is removed.

The decrease in proteins (Fig. 5C) could lead to a smaller amount of proteins related to energy transport processes and Calvin cycle activity, complicating the correct transduction of energy in the photosystems (Pao et al., 2019). The lower proportion of electrons derived to photochemical processes could imply a lower NADPH and ATP synthesis, diminishing the Calvin cycle activity and, therefore, photosynthesis.

As we have stated, as a result of this competition for nitrogen uptake between species, we observed a decrease of photosynthetic rate in Ac. This lower *A* in Ac in mixture could imply an imbalance between energy absorbed and energy used. The chlorophyll *a+b* levels were not altered between monoculture and mixture (Fig. 2A-B), so the energy absorbed would be similar to the one in monoculture; besides, we detected lower values of *qP* and *ETR* (Fig. 3B-C) which could be related to a decrease in photochemistry reactions since an increase in *NPQ* was not detected (Fig. 3D). All these results could indicate an accumulation of energy in the chlorophylls (Krieger-Liszkay, 2005). In these conditions, the chlorophyll photoactivated would transfer its energy to oxygen, generating singlet oxygen ($^1O_2$) (Sgherri and Pérez-López, 2013). $^1O_2$ is highly reactive and the plant must detoxify as quickly as possible. There are two mechanisms, on one hand the physical quenching and, on the other hand, the chemical quenching, performed by lipophilic and hydrophilic antioxidants, respectively (Triantaphylidès and Havaux, 2009). In some of the processes of $^1O_2$ quenching it has been detected a superoxide radical ($O_2^{\cdot -}$) formation (Saito et al., 1981; Inoue et al., 1985). The higher SOD activity detected in mixture than in monoculture in Ac (Fig. 6A) could support this

idea because the increase in SOD activity would permit the plant to detoxify $O_2^{\cdot-}$ to avoid membrane damage. Other studies have shown that SOD could even prevent directly the deleterious action of singlet oxygen (Weser et al., 1975; Rao et al., 1988). As no membrane damage was detected (Fig. 6B), it reinforces even more our idea.

Therefore, from our results, we can state that the competitiveness seems to be related with soil resources competition rather than with light resources. However, Zhang et al. (2008) in a study done in *Trifolium pratense* and *Festuca rubra* argued that light competition determines the competitiveness in mixtures of grass and legumes when the nitrogen supply is sufficient as in our experiment. The reason for this discrepancy could lie on the experiment duration, as our experiment lasted 37 days and the one of Zhang et al. (2008) lasted 60 days. Reekie and Bazzaz (1989) stated that the competitive performance of plants in early stages of development is associated with competition for soil resources, and later, when the canopy closes in, the competition for light becomes the overriding factor determining competitive success. Following this model, our experiment would be in early stage, as the main competitive advantage of Tp (related to negative *RNE* value, Fig. 4C) seems to be associated with its higher nutrient uptake (*e.g.* NUR) when grown in competition with Ac. It is also important to note that this experiment was done under ample nutrient concentration. If the nutrient availability had been lower, *T. pratense* could not have overgrown *A. capillaris* and the higher root hair length and percentage of root with root hairs would have permitted *A. capillaris* to obtain most of the nutrients. However, this idea should be tested in further research studies.

In conclusion, due to the higher competitive performance of Tp for soil resources –explained by a higher root biomass and higher NUR than Ac–, Tp showed higher

photosynthetic rate than Ac when both species were competing in mixture. Furthermore, the interspecific competition provoked Ac to activate its antioxidant metabolism, through SOD activity, to detoxify the reactive oxygen species generated due to its lower capacity of use of the photochemical energy absorbed. In this experiment we show that the competitiveness seems to be more related with soil resources competition than with light competition and that the photosynthetic rate decline in Ac is more a secondary effect as a consequence of nitrogen limitation.

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609

610 Legends for figures

611 Fig. 1. Scatter dot plot of the effect of competition on CO_2 assimilation rate (*A*,
612 µmol CO_2 m^{-2} s^{-1}, **A**), stomatal conductance (*gs*, mol H_2O m^{-2} s^{-1}, **B**), intercellular CO_2
613 concentration (*Ci*, µmol CO_2 mol^{-1} air, **C**) and instantaneous transpiration rate (*E*, mmol
614 H_2O m^{-2} s^{-1}, **D**) of *Trifolium pratense* (Tp) and *Agrostis capillaris* (Ac) species grown in
615 monoculture (Mono) and mixture (Mix). Individual values of each treatment are
616 represented plus the mean as a line (circles, Tp Mono; squares, Tp Mix; triangles, Ac
617 Mono; inverted triangles, Ac Mix). The same letter above the individual values
618 indicates no significant differences between species and competition treatment.

619 Fig. 2. Scatter dot plot of the effect of competition on chlorophyll *a* (Chl *a*, mg g^{-1}
620 DW, **A**), chlorophyll *b* (Chl *b*, mg g^{-1} DW, **B**), carotenoid (Car, mg g^{-1} DW, **C**)
621 concentration and chlorophyll *a* to *b* ratio (Chl *a*/*b*, **D**) of *Trifolium pratense* (Tp) and
622 *Agrostis capillaris* (Ac) species grown in monoculture (Mono) and mixture (Mix).
623 Statistical analysis and notations as explained in Fig. 1.

624 Fig. 3. Scatter dot plot of the effect of competition on photochemical efficiency of
625 PSII in light-adapted leaves (*Fv'/Fm'*, **A**), photochemical quenching (*qP*, **B**), electron
626 transport rate (*ETR*, **C**) and non-photochemical quenching (*NPQ*, **D**) of *Trifolium*
627 *pratense* (Tp) and *Agrostis capillaris* (Ac) species grown in monoculture (Mono) and
628 mixture (Mix). Statistical analysis and notations as explained in Fig. 1.

629 Fig. 4. Scatter dot plot of the effect of competition on leaf area (cm^2 $plant^{-1}$, **A**)
630 and root biomass (g DW $plant^{-1}$, **B**) of *Trifolium pratense* (Tp) and *Agrostis capillaris*
631 (Ac) species grown in monoculture (Mono) and mixture (Mix). Statistical analysis and
632 notations as explained in Fig. 1. Scatter dot plot of the Relative Neigbour Effect (*RNE*,

C) of *Trifolium pratense* (Tp, circles) and *Agrostis capillaris* (Ac, triangles). Statistical analysis as explained in Fig. 1.

Fig. 5. Scatter dot plot of the effect of competition on nitrogen uptake (NU, mg N plant^{-1}, **A**), nitrogen uptake rate (NUR, mg N g^{-1} DW root day^{-1}, **B**) and proteins (mg g^{-1} DW, **C**) of *Trifolium pratense* (Tp) and *Agrostis capillaris* (Ac) species grown in monoculture (Mono) and mixture (Mix). Statistical analysis and notations as explained in Fig. 1.

Fig. 6. Scatter dot plot of the effect of competition on superoxide dismutase (SOD, U mg^{-1} prot, **A**) and lipid peroxidation (Mda, µmol g^{-1} DW, **B**) of *Trifolium pratense* (Tp) and *Agrostis capillaris* (Ac) species grown in monoculture (Mono) and mixture (Mix). Statistical analysis and notations as explained in Fig. 1.

Figure 1

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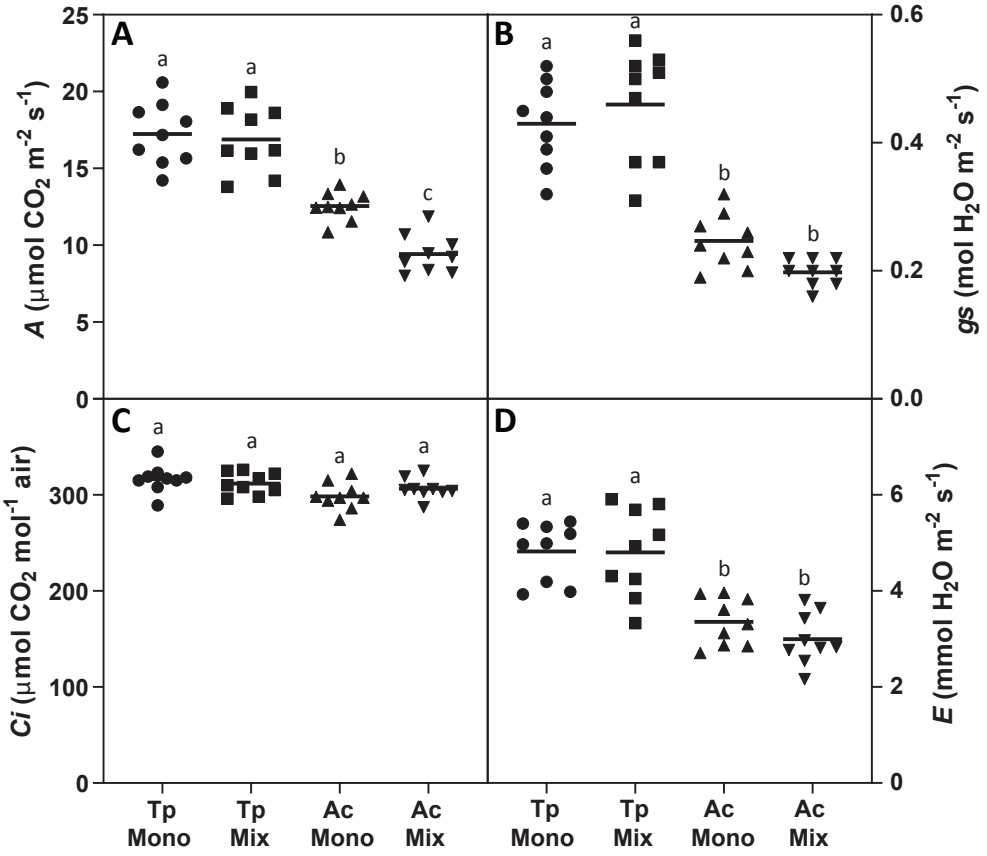

Figure 2

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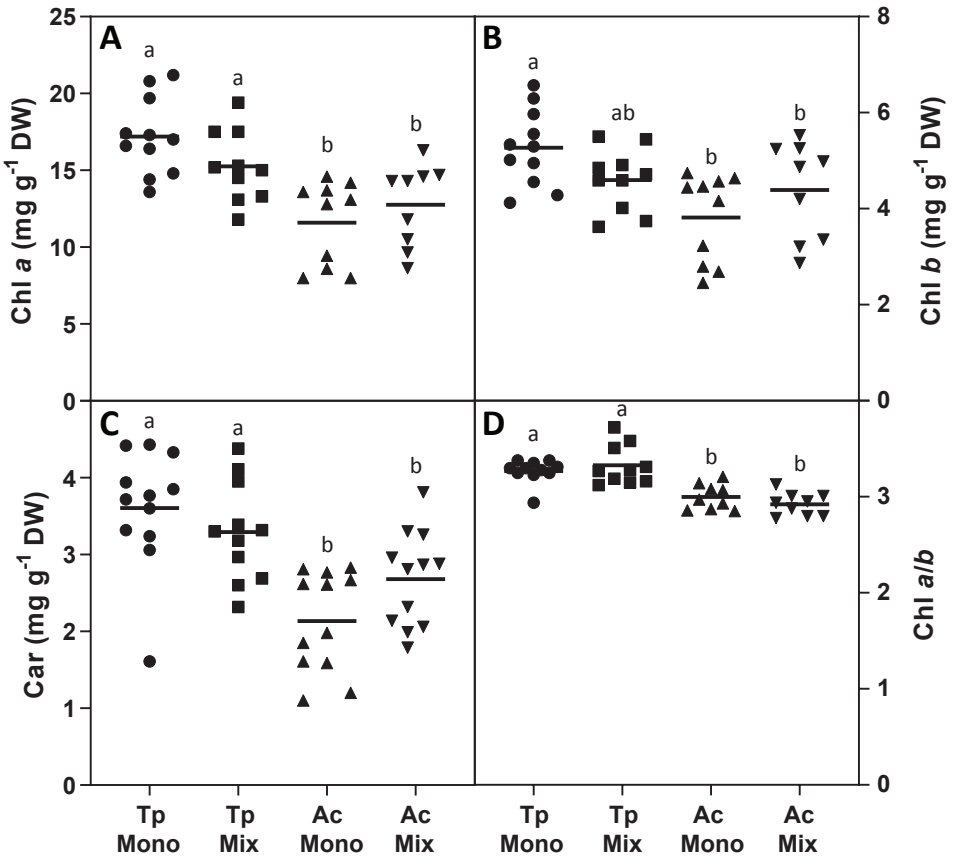

Figure 3

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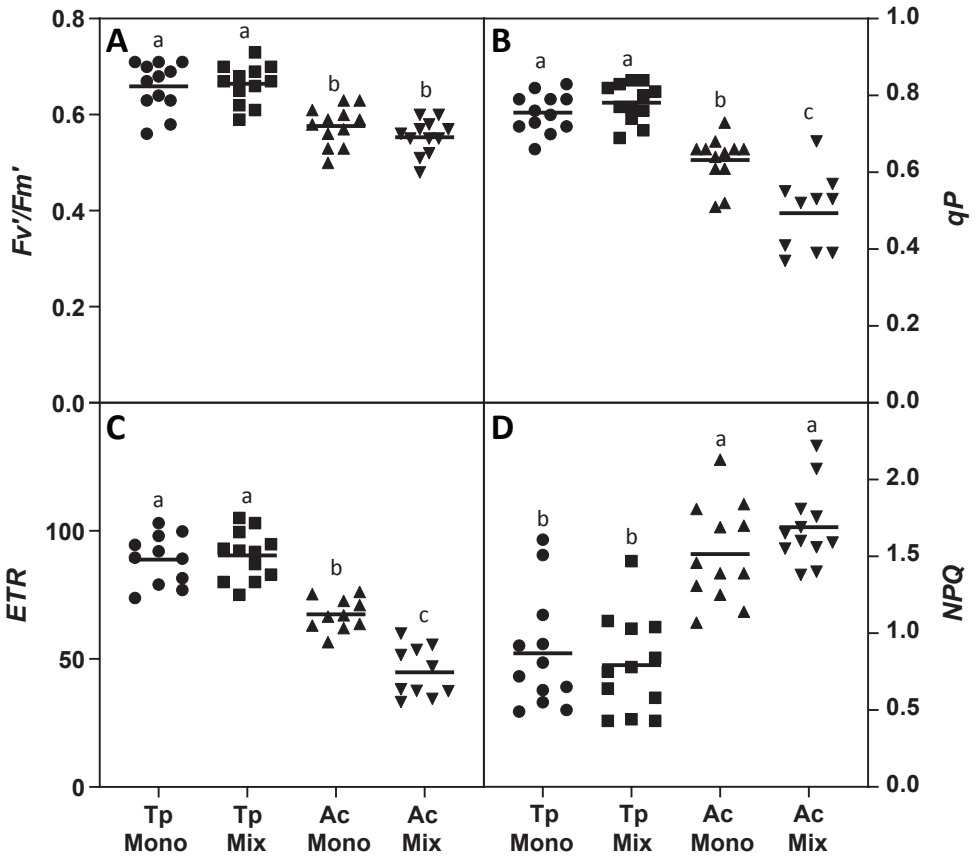

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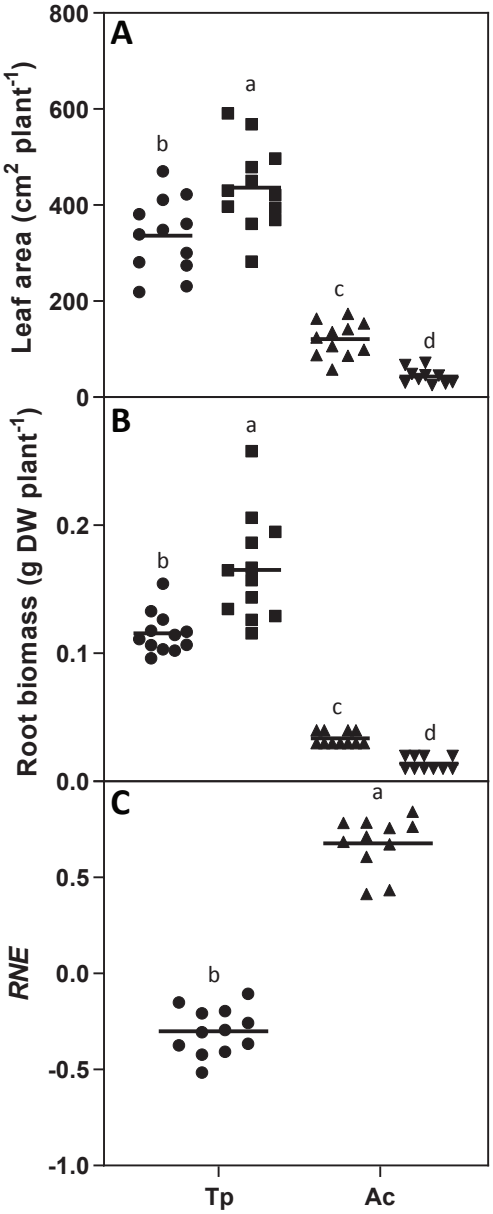

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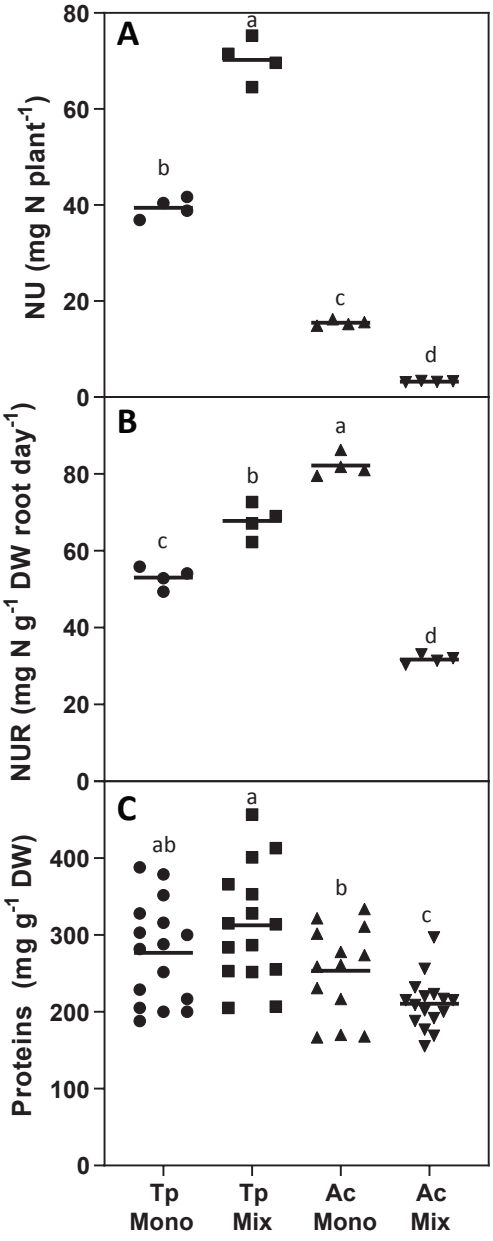

Figure 6

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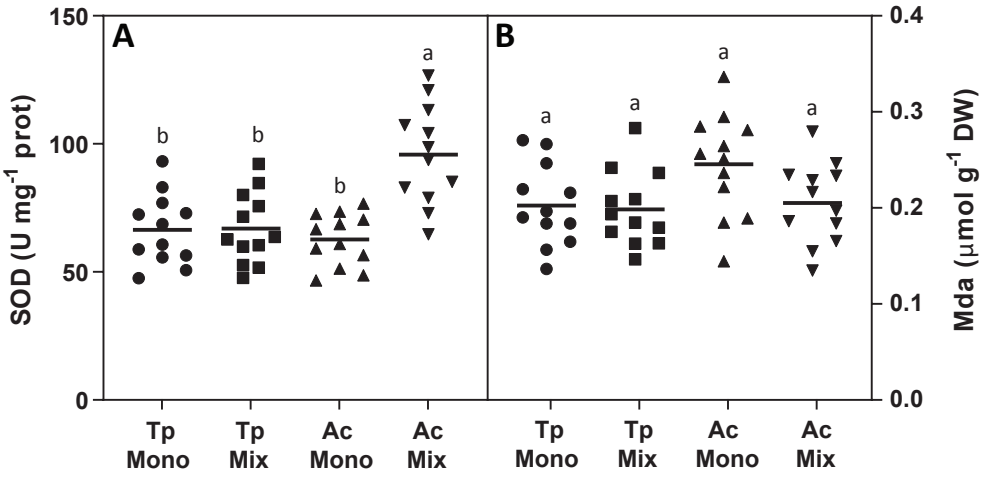

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Credit Author Statement

J.M-A: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing-Original Draft, Writing-Review & Editing, Visualization, Funding acquisition.

A.M-P: Methodology, Investigation, Writing-Review & Editing, Funding acquisition.

M.L: Writing-Review & Editing, Funding acquisition.

A.M-R: Conceptualization, Methodology, Investigation, Funding acquisition.

U.P-L: Conceptualization, Methodology, Investigation, Writing-Original Draft, Writing-Review & Editing, Supervision, Project administration, Funding acquisition.