The effects of arbuscular mycorrhizal fungi and root interaction on the competition between *Trifolium repens* and *Lolium perenne*

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Trifolium repens and *Lolium perenne* are two crucial forage grasses for grassland management. A pot experiment was conducted to test effects of root interaction and arbuscular mycorrhizal fungi (AMF) inoculation on the interspecies competition between *T. repens* and *L. perenne* under different proportions of mixed sowing by the combination treatment of two levels of AMF inoculation (inoculation and non- inoculation) and two levels of root interaction (root interaction and non- root interaction). The main results were as following: (1) the aboveground and belowground biomass of *T. repens* and *L.perenne* were insignificantly altered by AMF inoculation under different mixture ratio; (2) inter and intraspecies root interaction significantly decreased the aboveground biomass of *T. repens*, and therefore decreased the competitiveness of *T. repens* and the total belowground biomass; (3) inter and intraspecies root interaction significantly increased the belowground biomass of *L. perenne*, which contributed to the increase in the total underground biomass under root interaction treatment; (4) The interspecies root interaction increased the aboveground biomass of *L. perenne* but intraspecies root interaction reduced it. Our results indicate that mixed sowing suppress the intraspecies interaction root of *L. perenne,* increase the total aboveground biomass and make great contribution for transgressive overyielding.

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A running headline: Arbuscular mycorrhizal fungi and root interaction effects

Abstract

Trifolium repens and *Lolium perenne*are two crucial forage grasses for grassland management. A pot experiment was conducted to test effects of root interaction and arbuscular mycorrhizal fungi (AMF) inoculation on the interspecies competition between *T. repens* and *L. perenne* under different proportions of mixed sowing by the combination treatment of two levels of AMF inoculation (inoculation and non- inoculation) and two levels of root interaction (root interaction and non- root interaction). The main results were as following: (1) the aboveground and belowground biomass of *T. repens* and *L.perenne* were insignificantly altered by AMF inoculation under different mixture ratio; (2) inter and intraspecies root interaction significantly decreased the aboveground biomass of *T. repens*, and therefore decreased the competitiveness of *T. repens* and the total belowground biomass; (3) inter and intraspecies root interaction significantly increased the belowground biomass of *L. perenne*, which contributed to the increase in the total underground biomass under root interaction treatment; (4) The interspecies root interaction increased the aboveground biomass of *L. perenne* but intraspecies root interaction reduced it. Our results indicate that mixed sowing suppress the intraspecies interaction root of *L. perenne,* increase the total aboveground biomass and make great contribution for transgressive overyielding.

Key-words: Arbuscular mycorrhizal fungi; interspecific relationship; root interactions; competition; productivity, transgressive overyielding

Introduction

In productive agricultural grassland, mixture of highly productive grass species produces high forage yields and sustainability. Plant communities with more than one species are expected to optimize available resources due to positive interspecific interactions and species niche complementarities (Loreau and Hector 2001, Hooper et al. 2005). So far, modern coexistence theory and contemporary niche theory are most popular hypotheses for illustrating intra- and interspecific interactions (Letten et al. 2017). In comparing with monocultures, mixtures increases interspecific competition and reduce intraspecific competition (Nyfeler et al. 2009, Gallien 2017). Performance better can be achieved with grass–legume mixtures rather than monocultures, with less species across a wide range of species proportions(Nyfeler et al. 2009).

Legume *T.repens* and grasses *L.perenne* are two most important forage grasses in global temperate grassland and are commonly used in mixtures (Hebeisen et al. 1997, Carlsson and Huss-Danell 2003).Their coexistence links to intra- and interspecific competitive interactions (Ulrich et al. 2017). *T.repens* and *L.perenne* compete for light, water and soil nutrient resources and also compensate for physiological traits and nutrient distribution from each other (Schwinning and Parsons 1996, del Río et al. 2016). Several studies found that *L.perenne* had higher competitive root than *T.repens*, resulting from its fast-growing root and higher utilization efficiency in light (Luescher and Jacquard 1991, Kleen et al. 2011). Besides, the fix nitrogen trait of *T.repens* could help *L.perenne* for obtaining extra nitrogen. However, Goldberg and Landa (1991) had an inversely conclusion, which considered that *T.repens* could perform better in related to its root trait and soil nutrient elements (e.g. soil phosphorus) and concentration. The previous study verify that adding nitrogen fertilizer could enhance the competence of *L.perenne*;

while adding phosphorous fertilizer could enhance the competence of *T.repens* in their mixtures (Dennis and Woledge 1987, Davidson and Robson 1990). The persistence and stability of these two species mixed grassland depend on fertilizer type, growth period, outer press frequency and time (e.g. mowing) and so on (Elgersma et al. 1998, Nassiri and Elgersma 1998, Elgersma et al. 2000). In addition, Cernoch and Houdek (1994) found that soil moisture had strong effect on the nitrogen utilization of *T.repens* and *L.perenne*. *L.perenne* increased its nitrogen absorbing along with increased soil moisture and further enhanced its competitive ability. Although many studies have explored the mechanisms of interspecific competition between *T.repens* and *L.perenne*, their works more focused on aboveground biomass, light competition and so on, less is done with regard to their root interactions and soil microorganism effects.

Arbuscular mycorrhizal fungi (AMF) are considered as key soil microorganisms and can colonize 80% terrestrial plants (Smith and Read 2008, Brundrett 2009). AMF can form symbiotic associations with plants and affect interspecific interactions by enlarging nutrient absorbing area of plant species root systems, and further promote plant nutrient uptake and resistance to stress (Smith and Read 2008). It produce a large number of extraradical hyphae radiating from plant roots for plant-plant interaction, nutrient uptake and redistribution (Wilson et al. 2009, Rillig et al. 2010, Horton 2015, Kohler et al. 2017, Lin et al. 2017). Wagg et al. (2011) found that in mixtures of *T.repens*and *L.perenne*, AMF could reduce their differences in competence ability by enhancing the competitive power of *T.repens*. Moreover, higher diversity of AMF could improve species coexistence (van der Putten 2017). AMF inoculation helps some species for nitrogen absorbing by changing their AMF colonization (van der Heijden et al. 2008).

AMF could also enhance intraspecific competition and promote interspecific coexistence (Teste et al. 2017). However, how does AMF, root and their interactions affect *T.repens* and *L.perenne* in their mixtures need to be further tested.

In this study, AMF inoculation and root interaction of legume *T.repens* and grasses *L.perenne* among their different mixture ratio were investigated. We aim to test the effects of AMF inoculation, root interaction, mixture ratio and their interactions on these two valuable forage grasses. It would help us deeply understand their survival strategies and effectively guide grassland management.

Materials and Methods

Experimental design

A greenhouse experiment was conducted in the laboratory of Prataculture Science, Nanjing Agriculture University, with 20 treatments and 5 replications each. The 20 treatments included 3 factors with 5 levels, 2 levels and 2 levels, respectively. We employed a randomized design consisted of factorial combinations of 5 mixed sowing ratio from *T.repens* (T) and *L.perenne* (L) (T8L0, T6L2, T4L4, T2L6, T0L8 mean mixture ratio: 8:0, 6:2, 4:4, 2:6, 0:8, respectively), AMF inoculation (+AMF and –AMF) and root interaction (+root and –root) treatments. In total, 20 treatments were randomly assigned with 5 replications, resulting in 100 pots. Each pot was 1.5 height × 1.9 cm diameters in size and separated from the others by 25 μm mesh (to keep root from competitive interaction).

The seed were sterilized firstly and germinated in lighting incubator (20℃) and transplanted into pots (height 15 cm, diameter 19 cm). Each pot was filled with 900 g of soil (dry weight equivalent) and 4989 g soil in meshes and gaps (See Figure 1 for the experimental pot). The soil was evenly mixed with 5:4:1 (w/w) loess, sand and peat, then sterilized with γ-ray（Nanjing University of Aeronautics and Astronautics, the radiant quantity equals to 25 kGy）and applied to each pot. The characteristics of the soil were measured, which contained 5.6% soil water content, 7.7 g/kg soil organic C, 18.49 mg/kg available P, 36.24 mg/kg nitrate-N, 19.61 mg/kg ammonium-N, and pH = 7.4.

The seedlings were grown under glasshouse conditions at 50-70% relative humidity and a temperature regime of 20-25℃ during day and night. Pots were watered to maintain soil water content between 15 and 20%. Two months later, all seedlings were mowed to 2 cm height, and 80 days later, all plant were destructively harvested, separated into shoots and roots, their biomass measured, and were examined for nitrogen content by using alkaline-hydrolysable diffusion method with Kjeldahl apparatus（Kjeltec Analyzer Unit 8400, FOSS, Hillerod, Sweden）(Bremner et al. 1996).

AMF colonization analysis

With AMF adding treatment, in total 48 g AMF inoculums (mixed with 5 strains: *Glomus etunicatum.* Ge.; *Glomus tortuosum.* Gt.; *Glomus intraradices.* Gi.; *Glomus versiforme.* Gv.; *Glomus aggregatum.* Ga., spore density is around 48/g) was applied in each pot. The control with –AMF treatment added 48 g sterilized AMF inoculums (110℃ for 120 min). To compensate other lost soil microbe because of sterilization, 80 ml filtrate from native soil mud excluding AMF by 25 μm mesh was applied to each pot. Because the root of *T.repens* is easily colonized by AMF, 24 ml mixed rhizobium inoculants (Rhizobium sp. WYCCWR R10051, Rhizobium sp. WYCCWR R10062) was added to each pot.

AMF colonization was measured using the membrane filter technique (Jakobsen and Robson 1993). Briefly, 5 g of soil was blended in 250 ml deionized water. Hyphae in 5-ml suspension were collected on a 25-mm membrane filter (1.2 μm pore size) and then were stained with Trypan Blue. Quantification of AMF colonization was measured under a microscope at 200 magnification using the gridline intercept method.

Statistical analysis

A multi-way ANOVA and two-way ANOVA were applied to analyze the effects of AMF inoculation, mixture ration, root interaction and their interactions on plant aboveground biomass, belowground biomass, nitrogen content and AMF colonization. T-tests was used to compare the variations between treatments –AMF/ +AMF and –Root/ +Root, *P* The level of significance was $P < 0.05$. LSD multiple-range test was applied to analyses the differences among mixture ratio treatments. All ANOVAs were analyzed using the SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA), and all figures were made by using Sigma plot 12.0 (Systat Software Inc., San Jose, CA, USA).

The net biodiversity effect is the extra yield produced by mixtures relative to the mean yield of the monocultures of the mixed species. A mixture is overyielding when its net biodiversity effect shows positive. Overyielding is transgressive when the real yield of a mixture is higher than the estimated yield of the most productive (Niklaus et al. 2017). Transgressive overyielding was calculated following these equations:

$$\text{Estimated productivity} = L_{Tp} \times B_{Tm} + G_{Lp} \times B_{Lm}, \quad (1)$$

Where L_{Tp} is the proportion of legume *T.repens* (T), G_{Lp} is the proportion of grass *L.perenne* (L). B_{Tm} and B_{Lm} mean the monoculture biomass of *T.repens* and *L.perenne*.

$$\text{Transgressive overyielding} = \text{Real productivity} - \text{Estimated productivity} \quad (2)$$

Results

AMF root colonization in *T. repens* and *L. perenne*

As Fig.2 shown, AMF root colonization of *T.repens* and *L.perenne* were not affected by either root interaction, mixture ratio or their interactions, except *L.perenne*, which was affected by mixture ratio. When mixture ratio between *T.repens* and *L.perenne* was ranged from 2:6 to 6:2, the proportion of AMF root colonization showed inversely response to root interaction: lower with root interaction in T2L6 ratio but higher in T6L2. With the increased proportion of *T.repens* in mixtures, AMF root colonization of *L.perenne* increased significantly. The nitrogen content of both *T.repens* and *L.perenne*were not affected by AMF inoculation, root interaction, mixture ratio or their interactions (Table 1 and Table 2). The exception was *L.perenne*, which nitrogen content significantly decreased when its mixture ratio changed from monocultures to mixtures in any ratio with *T.repens* (Table 3).

Above- and belowground biomass of *T. repens* and *L. perenne*

The aboveground biomass of *T.repens* and *L.perenne* were all significantly affected by root interaction, mixture ratio and their interactions, as well as belowground biomass (Fig. 3 a and b). The exception was *T.repens*, which belowground biomass was not affected by root interaction. When mixture ratio between *T.repens* and *L.perenne* ranged from 2:6, 4:4, 6:2, to 8:0, the aboveground biomass of *T.repens* with root interaction decreased from 47%, 46%, 42% to 12% in comparing with no root interaction (Fig 3. a), but the aboveground biomass of *L.perenne* increased from 2%, 10%, to 30% and decreased 4% (Fig 3. b), respectively. Correspondingly, the belowground biomass of *T.repens* with root interaction decreased from 73%, 48% to 16%, and increased 21% in comparing with no root interaction (Fig 3. a); while the belowground biomass of *L.perenne* increased from 60%, 33%, 105% to 64% (Fig 3. b). These results showed that the root competition in intra- and interspecies all decreased the aboveground biomass of *T.repens* but increased the belowground biomass of *L.perenne*. The intraspecies interaction in root competition increased the belowground biomass of *T.repens* and decreased the aboveground biomass of *L.perenne*; Meanwhile the interspecies interaction in root competition decreased the belowground biomass of *T.repens* but increased both above- and belowground biomass of *L.perenne*.

Total biomass in mixtures and transgressive overyielding effects

Combining the above- and belowground biomass of *T.repens* and *L.perenne* in mixtures, the figure 3 showed that the total aboveground biomass was significantly affected by root interaction and mixture ratio, but not their interactions. The total belowground biomass was significantly affected by all three variables. When mixture ratio between *T.repens* and *L.perenne* ranged from 0:8, 2:6, 4:4, 6:2, to 8:0, the total aboveground biomass with root interaction decreased from 4%, 6%, 6%, 5% to 12% in comparing with no root interaction (Fig. 4), but the total belowground biomass increased from 64%, 45%, 14%, 26% to 21% (Fig. 4), respectively. From figure 3 and figure 4, it showed that the decrease in total aboveground biomass attributed to the decreased aboveground biomass of *T.repens*, and the increase in total belowground biomass was mainly contributed by the increased belowground biomass of *L.perenne*. In mixed sowing grassland, the real productivity was all higher than the estimated productivity in monocultures. In comparing with estimated productivity and real productivity in mixtures, mixture ratio of *T.repens* and *L.perenne* has no significant effect on transgressive overyielding regardless of root interaction (Fig. 5).

Discussion

No matter with or without root interaction, the mixtures of *T.repens* and *L.perenne* had no significant difference with the monocultures of *T.repens* in regarding to AMF colonization in root. This is not consistent with the study from Endlweber and Scheu (2007). It may attribute to the defect of short–term greenhouse experiment. However *L.perenne* showed higher AMF colonization in mixtures than in its monoculture, which indicates that mixed sowing grassland could enhance plant species' AMF colonization. Extraradical hyphae of AMF promote interspecific competition with root interaction in mixtures (Teste et al. 2017, van der Putten 2017). When comparing the legume *T.repens* and the grass *L.perenne*, our results showed that

T.repens had twofold higher AMF colonization than *L.perenne*, which resulted from the strong and deep root system of legume species than grasses.

Moreover, our findings showed that the total yield of mixtures decreased aboveground biomass majorly because of *T.repens*, and increased belowground biomass majorly because of *L.perenne*. Transgressive overyielding in mixtures was mainly contributed by the biomass of *L.perenne*. It indicates that *L.perenne* has much stronger root interaction than legume *T.repens*. The previous study from Mouat et al. (1987) also confirms that the root competitive capacity of *T.repens* is restricted by the concentration and types of soil nutrient elements. Since plant species absorbing soil nutrient largely rely on their root system, the different root interaction of *T.repens* and *L.perenne* thus affect their variable aboveground biomass. Besides, the mechanism of overcompensatory growthcould also verifies their root competitive capacity. *L.perenne* performs overcompensatory growth by decreasing root biomass or its shoot: root ratio, but *T.repens* is dependent on enhancing photosynthetic efficiency or symplastic growth of the shoot and root together (Faurie et al. 1996, Akmal and Janssens 2004). Our results challenge the conclusion that the resource competition of grass and legume in mixtures is only related to lighting absorbing rather than root interaction (Lantinga et al. 1999, Barillot et al. 2014). In fact, the mixed sowing of *T.repens* and *L.perenne* decreased the intraspecific root interaction of *L.perenne* and increased the total productivity in mixtures, and thus help for transgressive overyielding effect. It is worth mentioning that the competitive capacity of *T.repens* and *L.perenne* change along with their growth periods (Schenk et al. 1995, Coutts and Jones 2002). Although mixed sowing increase the yield of *L.perenne* and decrease it of *T.repens*, their competitive capabilities change in an inverse way and thus further enhance their compatibility (Elgersma et al. 2000, Nie et al. 2004). Therefore, they could sustainable coexistent in the long term.

In this study, the mixture ratio of *T.repens* and *L.perenne* did not significantly affect their nitrogen content. *L.perenne* even had higher nitrogen content in monocultures than mixtures. This is not consistent with our common knowledge. Theoretically, legume *T.repens* could provide more nitrogen to *L.perenne* owing to the nitrogen fixing capacity itself. The reason may because that the pot experiment is limited by the intrinsic soil nitrogen in comparing with the field experiment. The increased belowground biomass of *T.repens* in monocultures had conversely trend in mixtures in our study confirm that legume monoculture could better promote their nutrient absorbing by root interaction.

Building productive and stable artificial grassland is a very important measure for meeting livestock - feeds balance (Zhou et al. 2006, Huang et al. 2017). Mixed sowing of legume *T.repens* and grass *L.perenne* could effectively improve grassland production and stability. Our findings provide better understanding of the root interaction and nutrient usage capabilities of these two most important forages, and have important implications for optimizing grassland measures according to the characteristic of component species.

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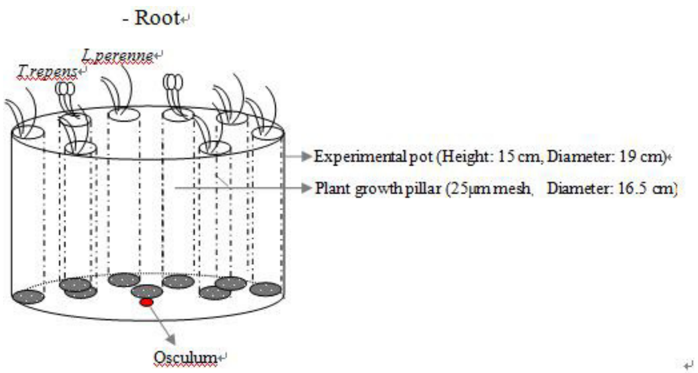
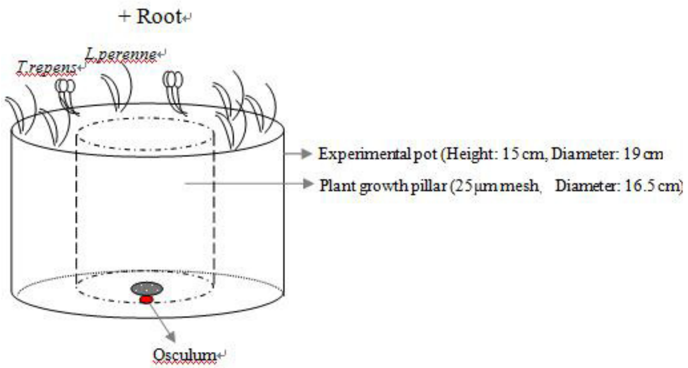
Figure 1(on next page)

Experimental pot drawing for root interaction treatment – root(a) and +root (b)



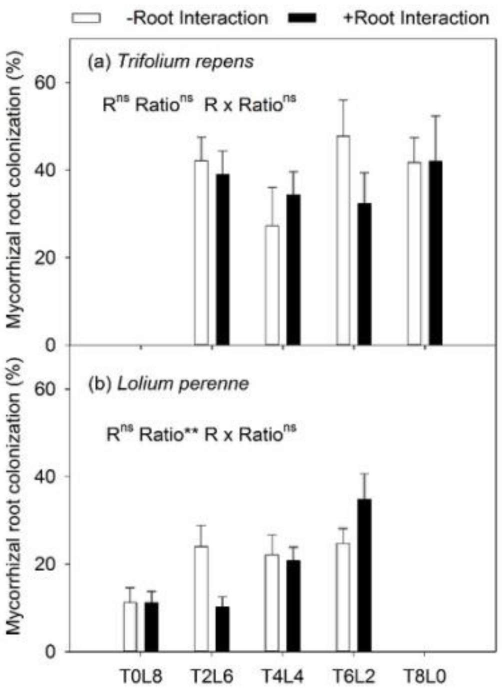
(a) Without root interaction



(b) With root interaction

Figure 2 (on next page)

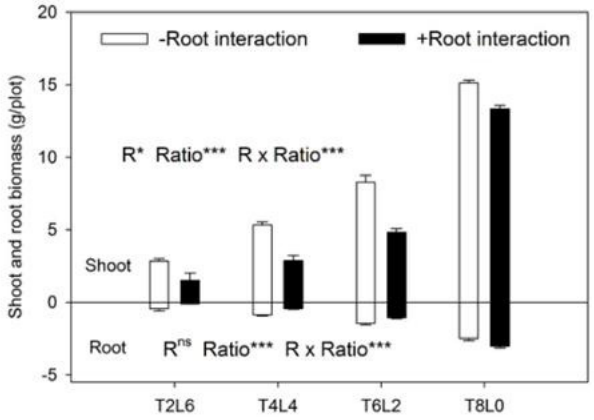
Effects of root interaction (R), mixture ratio (Ratio) and their interactions (R × Ratio) on AMF root colonization of T.repens (T) (a) and L.perenne (L) (b)



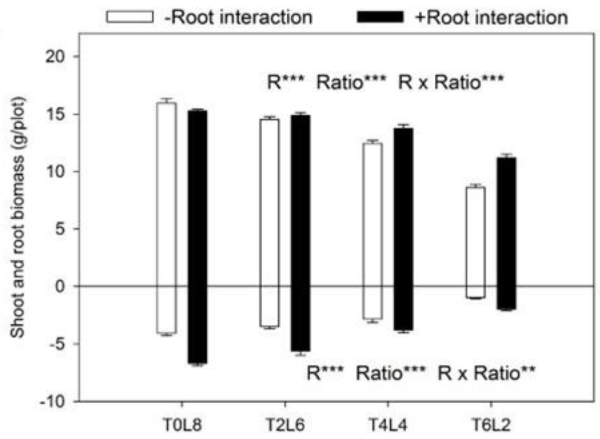
-Root Interaction
+Root Interaction
(a) Trifolium repens
R^ns Ratio^ns R x Ratio^ns
(b) Lolium perenne
R^ns Ratio** R x Ratio^ns
Mycorrhizal root colonization (%)
0
20
40
60
T0L8
T2L6
T4L4
T6L2
T8L0

Figure 3(on next page)

Effects of root interaction (R), mixture ratio (Ratio) and their interactions (R × Ratio) on above- and belowground biomass of T.repens (T) (a) and L.perenne (L) (b)



(a) *T.repens*



(b) *L.perenne*

Figure 4(on next page)

Effects of root interaction (R), mixture ratio (Ratio) and their interactions (R × Ratio) on total biomass of mixture of *T.repens* (T) and *L.perenne* (L)

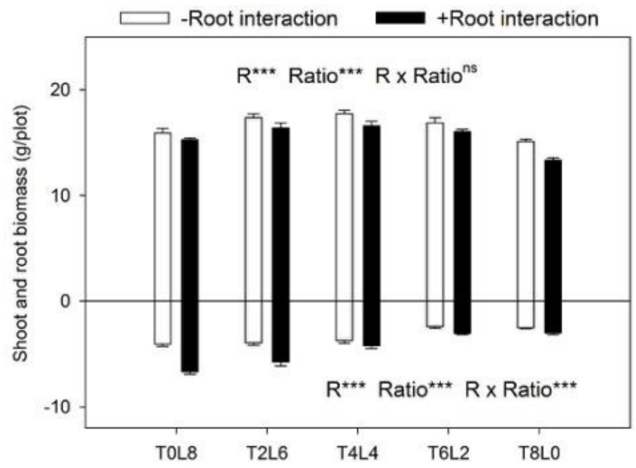

-Root interaction
+Root interaction
R*** Ratio*** R x Ratio^ns
R*** Ratio*** R x Ratio***
Shoot and root biomass (g/plot)
20
10
0
-10
T0L8
T2L6
T4L4
T6L2
T8L0

Figure 5 (on next page)

Transgressive overyielding effects in different mixture ratio of *T.repens* (T) and *L.perenne* (L) without root interaction (a) and with root interaction (b)

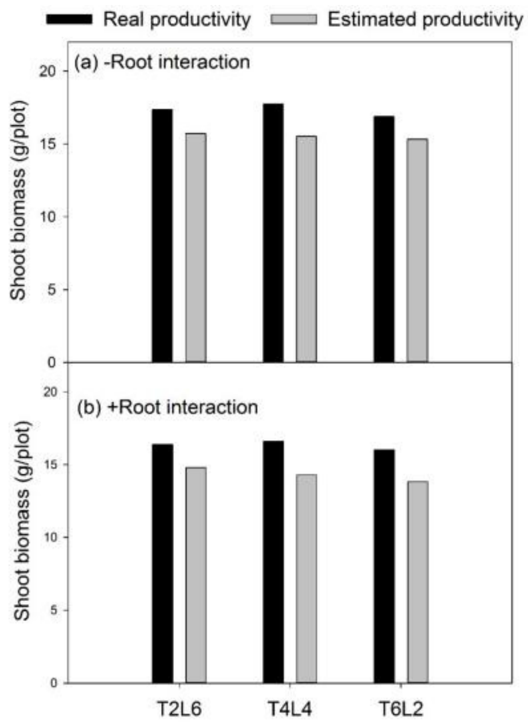

Real productivity
Estimated productivity
(a) -Root interaction
Shoot biomass (g/plot)
20
15
10
5
0
(b) +Root interaction
Shoot biomass (g/plot)
20
15
10
5
0
T2L6
T4L4
T6L2

Table 1(on next page)

ANOVA analysis of effects of AMF inoculation (AMF) root interaction (R), mixture ratio (Ratio) and their interactions on nitrogen content of T.repens *(T) (a) and* L.perenne *(L)*

Table 1 ANOVA analysis of effects of AMF inoculation (AMF) root interaction (R), mixture ratio (Ratio) and their interactions on nitrogen content of *T.repens*.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AMF	1	0.55538462	0.55538462	1.99	0.1618
R	1	0.05138087	0.05138087	0.18	0.6689
Ratio	3	0.40385769	0.13461923	0.48	0.6954
AMF*R	1	0.10967538	0.10967538	0.39	0.5323
AMF*Ratio	3	5.10741538	1.70247179	6.1	0.0008
R*Ratio	3	1.55857856	0.51952619	1.86	0.1419
AMF*R*Ratio	3	1.27638212	0.42546071	1.52	0.2137

Table 2(on next page)

ANOVA analysis of effects of AMF inoculation (AMF) root interaction(R), mixture ratio (Ratio) and their interactions on nitrogen content of *L.perenne* .

Table 2 ANOVA analysis of effects of AMF inoculation (AMF) root interaction (R), mixture ratio (Ratio) and their interactions on nitrogen content of *L.perenne*.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AMF	1	0.05612765	0.05612765	0.15	0.6979
R	1	0.05187185	0.05187185	0.14	0.709
Ratio	3	15.76142	5.25380667	14.21	**<.0001**
AMF*R	1	1.26217595	1.26217595	3.41	0.0683
AMF*Ratio	3	2.8395535	0.94651783	2.56	0.0607
R*Ratio	3	0.2666289	0.0888763	0.24	0.868
AMF*R*Ratio	3	1.28053644	0.42684548	1.15	0.3325

Table 3 (on next page)

Mixture ratio effects on nitrogen content of *L.perenne* (Mean ± SE)

Table 3 Mixture ratio effects on nitrogen content of *L.perenne* (Mean ± SE).

Treatment	T0L8	T6L2	T4L4	T2L6
Value	3.20 ± 0.11^{A}	2.41 ± 0.13^{B}	2.15 ± 0.13^{B}	2.15 ± 0.12^{B}

Different uppercases represent significant differences among mixture ratio of *T.repens* (T) and *L.perenne* (L) (LSD multiple-range tests, $P < 0.05$).