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REGULAR ARTICLE

The free-living rhizosphere fungus *Trichoderma hamatum* GD12 enhances clover productivity in clover-ryegrass mixtures

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Abstract
Aim A principal goal of grassland management is to minimize the use of artificial fertilizers by maximising the productivity of nitrogen-fixing leguminous plants such as clovers. The objective of this study was to investigate whether a plant-growth-promoting strain of the free-living rhizosphere fungus *Trichoderma hamatum* (GD12) could be used as a natural and sustainable means of enhancing the competitiveness of white clover (*Trifolium repens*) while allowing increased productivity of both clover and ryegrass (*Lolium perenne*) in mixed species systems.

Methods An assay was conducted in rhizotrons with white clover and ryegrass sown alone and in mixture and in soils inoculated and non-inoculated with GD12. Plant height, growing rate, phenological stage, number of *Rhizobium* nodules and biomass were assessed. A histological study of *Rhizobium* nodules and a stable isotopes analysis was conducted to determine the N fixation capacity of white clover.
Results When introduced as a soil inoculant, the fungus increased biomass production of both plant species and shortened their phenological cycles. Furthermore, in clover, GD12 enhanced plant height and growth rate and stimulated *Rhizobium* nodulation, while ^{15}N stable isotope analysis demonstrated increased N_2 fixation.
Conclusion This shows that soil amendment with a beneficial strain of saprotrophic fungus bestows a competitive advantage to white clover in clover-ryegrass mixtures and provides a sustainable mechanism for improving the mixture productivity.

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Introduction

Managed grassland systems rely on a number of sources for their nitrogen, including (i) industrially produced nitrogen fertilizer, (ii) biologically fixed nitrogen via the symbiotic relationships of Rhizobia with leguminous plants, and (iii) nitrogen contained in animal

manures and urine. One of the major aims of managing sustainable grassland systems is to minimise the use of industrially produced fertiliser, due the large amount of energy required for its production and the environmental and human health impacts arising from leaching into watercourses (Frankow-Lindberg and Frame 1996; Peeters 2009). Therefore, the introduction of legumes and the management of the composition of grassland sward to maximize atmospheric N-fixation is one of the pillars in these production systems worldwide (Peeters 2009).

White clover (*Trifolium repens* L.) is the dominant forage legume species in north-west Europe (Gierus et al. 2012) and is an integral part of many grassland systems in mixtures with perennial ryegrass (*Lolium perenne* L.). It is valued not only for its N-fixation ability, but also for its feeding quality (Gierus et al. 2012) for grazing animals, especially sheep (Penning et al. 1997; Harvey et al. 2000; Rook et al. 2002). However, the establishment and maintenance of clover to enable an adequate balance in the sward for optimum feeding quality, is often difficult. White clover is sensitive to competition for light and nutrients and often does not thrive (Haynes 1988). This is especially true when grown with grasses of high forage yielding ability that show faster growth rate and shade the clover. Also, intensive management based on mowing or rotational grazing has a depressive effect and results in the defoliation of clover in mixtures (Frame and Newbould 1986). A number of strategies have been proposed to enhance the competitive ability of white clover in mixtures including combining N fertilization, wide spacing of drilled grass rows (Annicchiarico and Tomasoni 2010), the use of white clover varieties selected for their competitive ability and compatibility with grass (Annicchiarico and Proietti 2010) and in compound mixture with other legumes, delaying the seeding of companion grasses (Walker and King 2010). Because clover leaves do not completely develop their photosynthetic capacity until the leaflets have been carried to the top of the canopy where there is sufficient light (Parsons and Chapman 2000) one strategy to improve competitiveness over grass would be to enhance its growth during the early stages of development.

Species in the fungal genus *Trichoderma* are ubiquitous saprotrophs found in a wide range of temperate and tropical ecosystems, and present in root, soil and foliar environments (Harman et al. 2004). The fungi are well characterised biocontrol agents of plant diseases, displaying hyperparasitic and antimicrobial activities (Viterbo and Horwitz 2010). They are regarded as a beneficial component of the rhizosphere microbiota due to their abilities to colonize root surfaces and, in certain instances, to form advantageous endophytic associations with plant tissues (Romão-Dumaresq et al. 2012). The beneficial effects of *Trichoderma* on plant productivity are broad, including nutrient mineralisation and plant nutrient acquisition, the production of water-soluble plant growth promoting compounds, niche exclusion of root pathogens, induction of systemic and localized resistance to a variety of foliar pathogens (Harman et al. 2004), and improved tolerance to abiotic stresses such as drought (Shoresh et al. 2010). However, the characteristic of *Trichoderma* that is currently attracting renewed interest for sustainable agriculture, is the capacity of certain strains to promote growth of plants observed both under field (Harman 2000) and controlled conditions (Yedidia et al. 2001) and in a wide range of crops. Growth promotion by *Trichoderma* has been shown in horticultural crops such as pepper (*Capsicum* spp.), tomato (*Solanum lycopersicum*), melon (*Cucumis* spp.) and radish (*Raphanus sativus*) (Baker et al. 1984; Chang et al. 1986) and also in crops such as maize (*Zea mays*) (Harman 2000; Vargas et al. 2009) and rapeseed (*Brassica napus*) (Viterbo and Horwitz 2010). In legume species, the effect of *Trichoderma* on growth promotion, disease control and interaction with *Rhizobium* have been described in black gram (*Vigna mungo*), chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), lupin (*Lupinus* spp.) and pea (*Pisum sativum*) (Naseby et al. 2000; Shaban and El-Bramawy 2011; Badar and Qureshi 2012). However, to the best of our knowledge, the effects of *Trichoderma* spp. on forage legume species and, more specifically, on white clover has not been studied.

Recently, Ryder et al. (2012) and Studholme et al. (2013) described the dual biocontrol and plant growth promoting properties of *T. hamatum* strain GD12 (hereafter referred to as GD12) that stimulates growth of a wide range of dicotyledonous plant species including brassicas (cabbage and brussels sprouts (*Brassica oleracea*) and thale cress (*Arabidopsis thaliana*)) and lettuce (*Lactuca sativa*), and controls root and leaf diseases both of dicotyledonous and monocotyledonous plants. In this study, we set out to investigate whether strain GD12 could bestow a growth advantage to white clover both alone and in mixed species microcosms with perennial ryegrass. We show that GD12 provides a

competitive advantage to white clover in clover-ryegrass mixtures by stimulating clover biomass production and shortening the phenological cycle. The fungus enhances the height and growth rate of clover and increases *Rhizobium* nodulation and N_2 fixation. The fungus therefore represents a sustainable option for increasing clover productivity in clover-ryegrass ecosystems.

Materials and methods

Experiments were conducted using thin-plate rhizotrons under controlled conditions. Each rhizotron was constructed from two plates, one glass and one plastic (each 120×240 mm) separated by 5 mm with 10 mm wide plastic strips. The top surface of each rhizotron was left open to allow plant growth. The rhizotrons were filled with 100 g of air-dried, sieved (to 2 mm) and re-wetted grassland soil or the same soil mixed with GD12 inoculum (GenBank accession no. AY247559). Bran inoculum was prepared using the method detailed in Ryder et al. (2012). In brief, a bran and dH_20 mixture was sterilised by autoclaving at 121 °C for 15 min in conical flasks. Five plugs taken from the leading edge of 3 day old GD12 cultures propagated on Potato Dextrose agar (Sigma) were added per 40 g wet bran, mixed and the resulting inoculum was incubated for 5 days under a 16 h light regime. Bran inoculum was added at 2.67 % (*w*/*w*) of rewetted soil immediately before sowing seeds. The soil was of the Hallsworth series and was collected from a permanent grassland site in south west England (50° 46′ 54.55″ N, 3° 55′ 1.03″ W), described by Harrod and Hogan (2008). The soil had received no inorganic-N fertiliser for over 30 years and had a total N content of 0.6 %, total C content of 6.6 %, pH of 5.7 and an Olsen P of 23.9 mg kg^{-1} (Crotty et al. 2012). Although it may be correct to use sterile bran for the control rhizotrons, it was felt that adding this would provide a substrate for other soil-borne fungi to proliferate and could confound the effect of the *Trichoderma*.

The plant species used in the study were perennial ryegrass (cv. AberMagic) and white clover (cv. AberDai). These were grown alone and in mixtures. Several seeds were sown in each rhizotron and covered with a thin layer of soil. After germination, two seedlings among the best developed per rhizotron were selected and were allowed to develop, while the remainder were removed. The treatments were GR (ryegrass+ryegrass), CL (clover+clover) and MX (ryegrass+clover) in soil inoculated with *T. hamatum* (+T) or un-inoculated (−T). (Clover plants in the in the MX treatment are referred to as MX CL and ryegrass plants as MX GR).

The rhizotrons were wrapped in aluminium foil to keep the roots in the dark and were stood in water filled trays with approximately 20 mm depth of water that was periodically replaced. The treatments (+T) and (−T) were placed in separate trays in order to avoid cross-contamination between inoculated and un-inoculated rhizotrons. The trays were placed in three separate growth cabinets (Sanyo 350HT, Sanyo Corporation, Japan) each with constant temperature (20 °C) and 18:6 h light:dark regime. Two replicate trays of rhizotrons of each treatment were placed in each cabinet and they were periodically rotated to different shelves. The experimental design was factorial consisting of three randomized complete blocks, with each cabinet as a different block and the shelf position as a sub-block. Because of the difficulty in separating the two plants, especially in the single species rhizotrons, the data captured is of mean plant variable in each rhizotron. Where there were the two plant species we took great care to disentangle the roots which was easier due to the differing root morphologies.

Plant height from the soil surface was periodically measured in both species. Due to the initial growth of clover being slower than ryegrass, height was evaluated when the first true leaf appeared in the clover plants,that is from 18 days after sowing (DAS), until 36 DAS.

The growth rate (GR) in period 1 (0–18 DAS) and period 2 (18–36 DAS), expressed as growth per day (mm), was calculated by the equation:

$$GR = (H_2 - H_1)/t_2 - t_1$$

Where H_1 and H_2 are the plant heights reached on the first and last day respectively of the period evaluated and t1 and t2 are the first and last DAS of the period studied.

Phenological stages of clover and ryegrass plants in all treatments were monitored from sowing, and the time to the main development stages were recorded following the BBCH scale (Lancashire et al. 1991). These main stages were: 1 leaf, 3 leaves, 5 leaves and tillering in ryegrass plants; and cotyledons, spade leaf, one true leaf and three true leaves in clover plants.

The numbers of *Rhizobium* nodules on the roots of the clover plants were counted periodically from 26 DAS until the end of the experiment. Those which were pigmented were assumed to be active. A sample of root with attached nodules was excised from one clover plant per rhizotron per treatment. Active *Rhizobium* nodules were fixed for 48 h in FAA (ethanol 50 %, formaldehyde 5 % and glacial acetic acid 10 % in water). The fixed samples were subsequently embedded in synthetic resin using the Leica Historesin© kit according to the protocols recommended by the manufacturer (Leica Microsystems GmbH, Wetzlar, Germany). 2 μm were cut using a Leica RM 2245 Microtome (Leica Microsystems GmbH, Wetzlar, Germany) with carbon-tungsten knives Leica TC-65 (Leica BiosystemsNusloch GmbH; Geschäftsfürher, Germany). Sections were stained (Ruzin, 1999) using a 0.1 % toluidine blue-O (TBO) solution in citrate buffer (pH 5) and mounted with Entellan© synthetic resin for quick assembly (Merck KGaA, Darmstadt, Germany). Sections were observed using a Nikon Eclipse 50i optic microscope and images were obtained using a Nikon DS-Fi1 digital optic device connected to a PC through the Nikon DS-U2 control unit (Nikon Instruments Inc., Melville, NY, USA) using NIS-Elements F3.22.00 Build 710 computer software (Nikon Instruments Inc., 2008).

After 40 days, the rhizotrons were disassembled and the roots and aboveground components of each plant were separated, dried and weighed. The aerial parts of clover (leaves, petioles and stolons) were assessed separately. Dry weights of biomass were determined after drying for 48 h in an oven at 80 °C. Samples were finely ground and weighed using a high (1 μg) precision microbalance (MX5, Mettler Toledo, Leicester, UK) and placed in tin capsules (8×5 mm, pressed, standard weight; Elemental Microanalysis Ltd., Okehampton, UK) in preparation for C, N and stable isotope analysis (Murray et al. 2009). The C and N content, together with the stable isotope concentration ($\delta^{13}C$ and $\delta^{15}N$) was determined using an elemental analyser (N1500, Carlo Erba, Milan, Italy) linked to an isotope ratio mass spectrometer (20/22, Sercon, Crewe, UK).

Soil samples close to the roots were taken carefully with a lab small spoon from each rhizotron and kept in 1.5 ml eppendorf tubes (Eppendorf, Hamburg, Germany) at 4 °C. Fungal strains were cultured from soil samples by spreading 1 g preserved soil onto Potato Dextose Agar(Sigma), amended with rifampicin (Sigma) at 0.01 μg / ml. Emerging fungal hyphae were transferred onto fresh PDA plates with the tip of a sterile scalpel and subcultured until in axenic culture. Isolated fungal strains were identified by amplification and sequencing of the internally transcribed spacer regions ITS1–5.8S–ITS2 of the rRNA encoding unit as described by White et al. 1990, and adapted by Sreenivasaprasad et al. 1996. DNA was extracted from 3 day old mycelium following the cTAB method as described by Talbot et al. 1993, and PCR was carried out in 25 μl reactions each containing 12.5 μlGo Taq® Green master mix (Promega), 10μMPrimer ITS1ext (5′-GTAACAAGGTTTCCGTAGGTG-3′) and 10μMITS4ext (5′-TTCTTTTCCTCCGCTTATTGATATGC-3′), and 25–50 ng genomic DNA. PCR was carried out under the following cycling conditions; 2 min at 94 °C, 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, with a final extension of 5 min at 72 °C. PCR products were separated on a 0.8 % agarose gel and DNA was extracted from the gel using Wizard® SV Gel and PCR clean up system (Promega) following the manufacturer's instructions. The resulting amplicon containing both ITS regions and the 5.8S gene of the rRNA encoding gene unit was sequenced using primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) by MWG biotech AG (Ebersberg, Germany). Sequences of re-isolated fungi were compared to the same region in *T. hamatum* GD12 using NCBI BLAST. The sequence identity percentage was used to indicate the likelihood of the isolated fungus positively identified as *Trichoderma hamatum* GD12 (100 % match).

Statistical analysis

In the experimental design, to minimize the risk of cross-contamination, we sacrificed a small degree of independence (i.e., full randomization within each cabinet) for greater separation between control rhizotrons and those inoculated with the fungus. We used a two factor ANOVA with sub-block nested within the block structure to take into account the positioning of trays within the cabinets. This was used for ryegrass and clover separately, for all variables except the C and N data where the plants from the same treatment in the same cabinet were ground jointly to provide enough material for analysis. In this case the data were analysed without the sub-plot structure. The factors analyzed were: (1) the effect of the species composition (alone or in mixture) and (2) the effect of *Trichoderma*

inoculation, and the interaction between factors on plant growth. The normality of the data of each variable was evaluated by Shapiro-Wilt test. The growing rate variable in period 1 and 2 was subjected to an $x^{1/2}$ transformation to normalise the data and stabilise variance throughout the data range.

Height and number of active Rhizobium nodules, which were measured at several time points during the experiment, were analyzed for the full dataset by a two way ANOVA. The value of the full data set for each treatment was determined by calculating the area under the curve by the equation:

$$\text{Area} = \Sigma\,(V_n + V_{n+1})/2 \times \Delta t$$

Where, n is the day of evaluation, V_n and V_{n+1} are the values of the variables measured (height or number of active *Rhizobium* nodules) in the days of evaluation n and n+1, and Δt is the difference of days between both evaluations.

This enabled us to analyze both variables for each factor and define the interaction between them for the full dataset, however the trend of the variables evaluated along the time in each treatment and factor studied was determined by the adjustment of the data to a linear regression and the comparison of their slopes and intercepts.

All analyses were performed with the Statistix 9 statistical program (Analytical Software, Tallahassee).

Results

Statistical analysis showed there was no interaction between factors in any variable evaluated except for the number of active *Rhizobium* nodules (Table 1). In the remaining variables the effect of GD12 inoculation on both clover and ryegrass was independent of whether the species were grown in monoculture or as mixtures. For clarity, the results have been represented for each factor separately in the figures.

Plant biomass

There were no significant differences in total above-ground biomass or of individual plant components (leaves, petioles and stolons), between clover alone (CL) and clover sown with ryegrass (MX CL). However, there were significant differences (F=6.22, p <0.05) in root biomass with the CL plants having more than twice the root biomass compared to the MX CL plants (Fig. 1a). In contrast, the above-ground biomass of the CL plants sown in GD12 inoculated soil (CL+T) was significantly (F=8.94, p <0.05) greater than those growing in un-inoculated soil (CL-T) (Fig. 1b). These differences between treatments were observed in leaves and petioles but not in stolons. The effect of GD12 on clover root biomass was not significant.

For the ryegrass plants, the MX GR plants had significantly (F=17.14, p <0.001) greater biomass than GR plants (Fig. 1c). Also, GR plants grown in inoculated soil (+T) had significantly (F=21.54, p <0.001) greater biomass than those in un-inoculated soil (−T) (Fig. 1d). The growth promoting effects of GD12 was also observed in total biomass per rhizotron regardless of treatment (CL, GR or MX) (Fig. 2).

Plant height and plant growth

Analysis of the area under the curve for the clover showed significant differences (F=16.27, p <0.01) between clover sown in soils inoculated (+T) and not inoculated (−T). However, there were no differences in clover sown in monoculture (CL) and clover in mixture (MX CL). Interactions between factors were not significant (Table 1). Linear regression confirmed these results with R^2 varying between 0.93 and 0.98 (Fig. 3a and b). The trends of plant height when comparing CL+T and CL-T were clearly different, with a greater line elevation and slope in the+T treatment (Fig. 3b). In contrast, ryegrass plants grown in mixtures (MX GR) were significantly (F=48.71, p <0.001) taller than plants grown in monoculture (GR) and although there were significant differences in height between ryegrass plants sown in inoculated soils (+T) and in un-inoculated soils (−T) (F=6.41, p <0.05), these were much more lower compared with those displayed in clover. There was not also an interaction between factors (Table 1). In this case the linear regression had an R^2 between 0.91 and 0.95 (Fig. 3c and d). Plants grown in mixtures were consistently taller than those grown in monoculture and grew faster (Fig. 3d) while plants in soil inoculated with GD12 were slightly taller than in un-inoculated soils but did not grow faster (Fig. 3d).

The relative growth rate (mm day^{-1}) of the clover was lower in period 1 (0–18 DAS) than the period 2 (18–36 DAS) and the inoculation with GD12 significantly increased the growth rate of the clover plants in

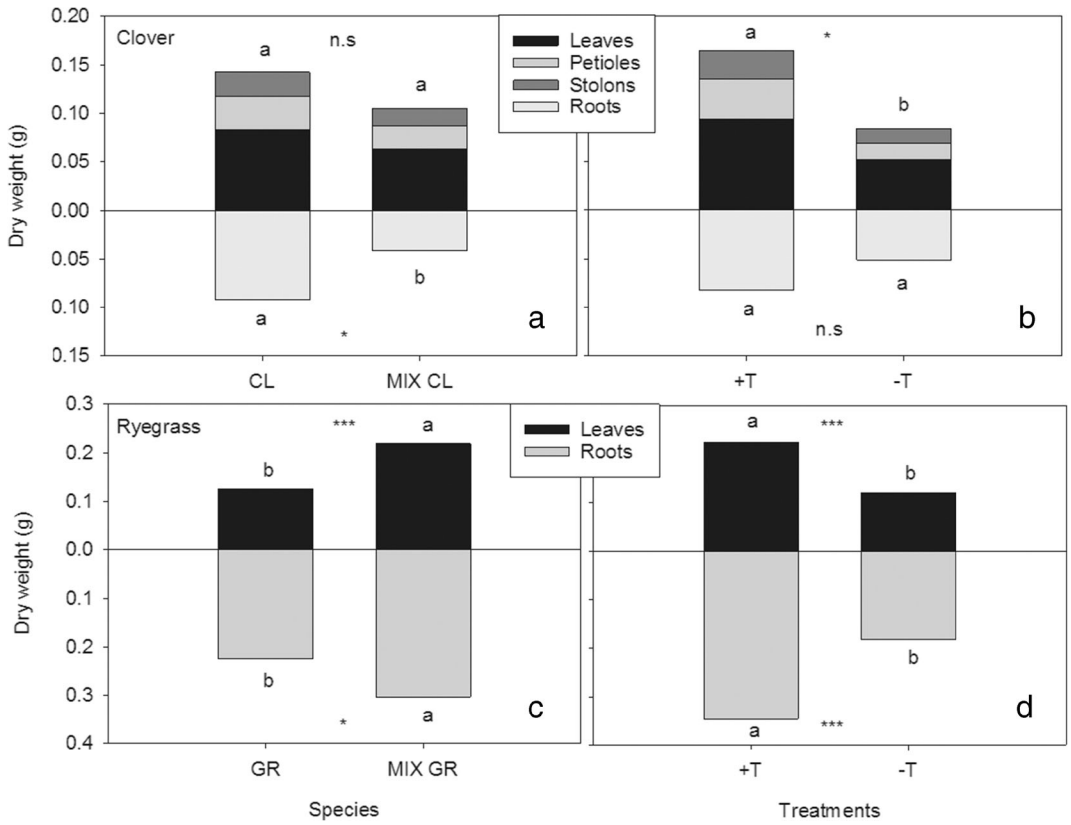

Fig. 1 Dry weight of white clover (CL) and ryegrass (GR) plants grown alone or in mixture (MX) (**a**, **c**) in soil inoculated with *T. hamatum* GD12 strain (+T) or un-inoculated (−T) (**b**, **d**). Different letters between treatments represent significant differences. The significance is shown as: n.s. no significant, *p <0.05, **p <0.01, ***p <0.001

both periods (F=33.82, p <0.001; F=6.19 p <0.05) (Fig. 4a). No differences in growth rate were found between clover in monoculture and as mixtures with ryegrass. In contrast, the faster growth rate in ryegrass occurred in the first period, between 0 and 18 days, with lower growth rate between 18 and 36 days (Fig. 4b). In

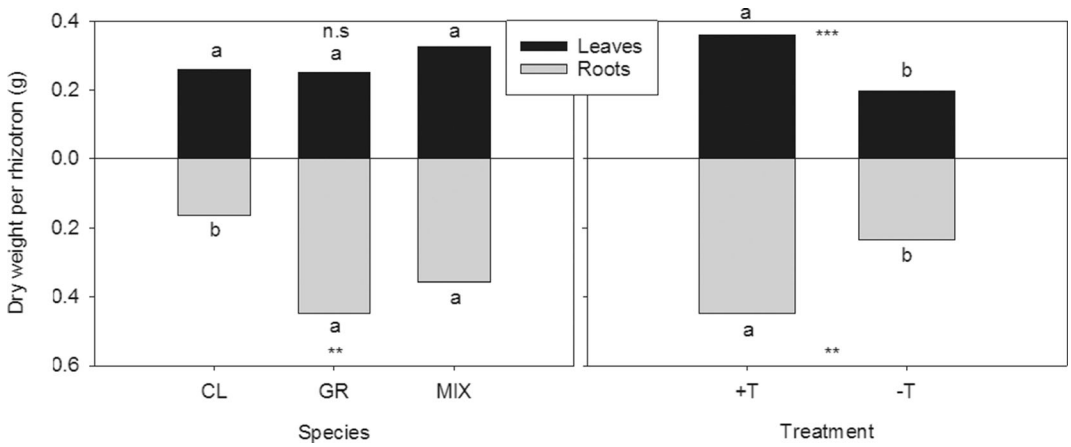

Fig. 2 Total dry weight per rhizotron of white clover (CL), ryegrass (GR), and mixture (MX) in soil inoculated with *T. hamatum* GD12 strain (+T) or un-inoculated (−T). Different letters between treatments represent significant differences. The significance is shown as: n.s. no significant, *p <0.05, **p <0.01, ***p <0.001

Table 1 Summary of 2-way ANOVA for testing the effect of combination of species (in mixture vs. in monoculture), effect of the *Trichoderma* inoculation (+T vs. –T) and interactions between factors on the different evaluated variables in clover and grass

Clover						
Variables evaluated	[1]Species (Sp)		[2]Treatments (Tr)		Sp vs. Tr	
	d.f.	*F*	d.f.	*F*	d.f.	*F*
Total dry weight						
Leaves	1	1.08[n.s.3]	1	8.94*	1	0.11[n.s.]
Roots	1	6.22*	1	3.83[n.s.]	1	1.18[n.s.]
[4]Height	1	0.36[n.s.]	1	16.27**	1	0.39[n.s.]
Growth rate						
18 Days	1	0.28[n.s.]	1	33.82***	1	3.85[n.s.]
36 Days	1	1.66[n.s.]	1	6.19*	1	0.00[n.s.]
Phenological stage						
Cotyledons	1	0.00[n.s.]	1	0.37[n.s.]	1	3.29[n.s.]
Spade leaf	1	0.47[n.s.]	1	16.26*	1	0.50[n.s.]
1 true leaf	1	0.46[n.s.]	1	3.57[n.s.]	1	0.47[n.s.]
3 true leaves	1	12.00**	1	9.96*	1	0.05[n.s.]
[5]Active *Rhizobium* nodules	1	6.48*	1	139.22***	1	6.90*
C content						
Leaves	1	22.55**	1	11.00*	1	0.02[n.s.]
Roots	1	26.05**	1	2.47[n.s.]	1	0.13[n.s.]
N content						
Leaves	1	30.36**	1	12.43*	1	0.59[n.s.]
Roots	1	37.26**	1	1.36[n.s.]	1	0.08[n.s.]
δ ^{15}N content						
Leaves	1	0.02[n.s.]	1	6.08*	1	0.01[n.s.]
Roots	1	2.49[n.s.]	1	0.80[n.s.]	1	0.08[n.s.]
Grass						
Variables evaluated	[6]Species (Sp)		Treatments (Tr)		Sp vs. Tr	
	d.f.	*F*	d.f.	*F*	d.f.	*F*
Total dry weight						
Leaves	1	17.14***	1	21.54***	1	2.31[n.s]
Roots	1	4.14*	1	17.71***	1	0.867[n.s]
[7]Height	1	48.71***	1	6.41*	1	0.00[n.s]
Growth rate						
18 Days	1	1.72[n.s.]	1	0.36[n.s.]	1	0.52[n.s.]
36 Days	1	10.39*	1	0.05[n.s.]	1	1.42[n.s.]
Phenological stage						
1 leaf	1	0.05[n.s.]	1	0.45[n.s.]	1	0.45[n.s.]
3 leaves	1	0.86[n.s.]	1	1.69[n.s.]	1	0.03[n.s.]
5 leaves	1	8.00**	1	1.20	1	0.63[n.s.]
Tillering	1	7.25*	1	4.49*	1	0.03[n.s.]
C content						
Leaves	1	0.18[n.s.]	1	13.02*	1	0.69[n.s.]
Roots	1	0.84[n.s.]	1	2.03[n.s.]	1	0.22[n.s.]
N content						
Leaves	1	0.03[n.s.]	1	6.43*	1	0.24[n.s.]
Roots	1	0.91[n.s.]	1	3.73[n.s.]	1	0.00[n.s.]
δ ^{15}N content						
Leaves	1	0.03[n.s.]	1	2.14[n.s.]	1	0.02[n.s.]
Roots	1	0.42[n.s.]	1	2.55[n.s.]	1	0.14[n.s.]
Variables evaluated	[8]Species (Sp)		Treatments (Tr)		Sp vs. Tr	
	d.f.	*F*	d.f.	*F*	d.f.	*F*
Dry weight per rhizotron						
Leaves	2	4.02[n.s.]	1	49.37***	1	0.92[n.s.]
Roots	2	18.27**	1	33.75**	1	4.18[n.s.]

[1] Species: CL, MX CL

[2] Treatments: +T, −T

[3] p <0.05*, p <0.01**, p <0.001***, n.s: not significant

[4] Height of clover for all data set (from 18 to 36 days after sowing) calculated as the area under the curve by the equation $Area_H = \Sigma (H_n + H_{n+1})/2 \times \Delta t$

[5] Number of active *Rhizobium* nodules for all data set (from 26 to 39 days after sowing) calculated as the area under the curve by the equation $Area_{RN} = \Sigma (RN_n + RN_{n+1})/2 \times \Delta t$

[6] Species: GR, MX GR

[7] Height of ryegrass for all data set (from 18 to 36 days after sowing) calculated as the area under the curve by the equation $Area_H = \Sigma (H_n + H_{n+1})/2 \times \Delta t$

[8] Species: CL, GR, MX

the latter period, a significant (F=10.39 p <0.05) difference in growth rate was observed between ryegrass sown in the mixture with clover and ryegrass sown in isolation. No differences in growth rate were observed between ryegrass in soil inoculated with GD12 and uninoculated soils.

Phenological stage

There were no differences in plant phenology in the first developmental stages (cotyledons, spade leaf and one true leaf) between clover grown alone or in mixture with ryegrass (Fig. 5a). However, clover grown in mixtures showed a 2 day lag in development to the three leaf stage (F=12.00, p <0.01). Inoculation with GD12 shortened the clover phenological cycle so that plants in inoculated soil were developmentally further advanced by the time of sampling (F=9.96, p <0.05) (Fig. 5b).

Ryegrass plants growing in mixtures reached the 5-leaf and tillering stage 3 days before those sown with ryegrass. The interaction with clover was significant (F=8.00 p <0.01, F=7.25 p <0.05), with ryegrass in mixtures being more developmentally advanced (Fig. 5c). The effect of GD12 on the ryegrass

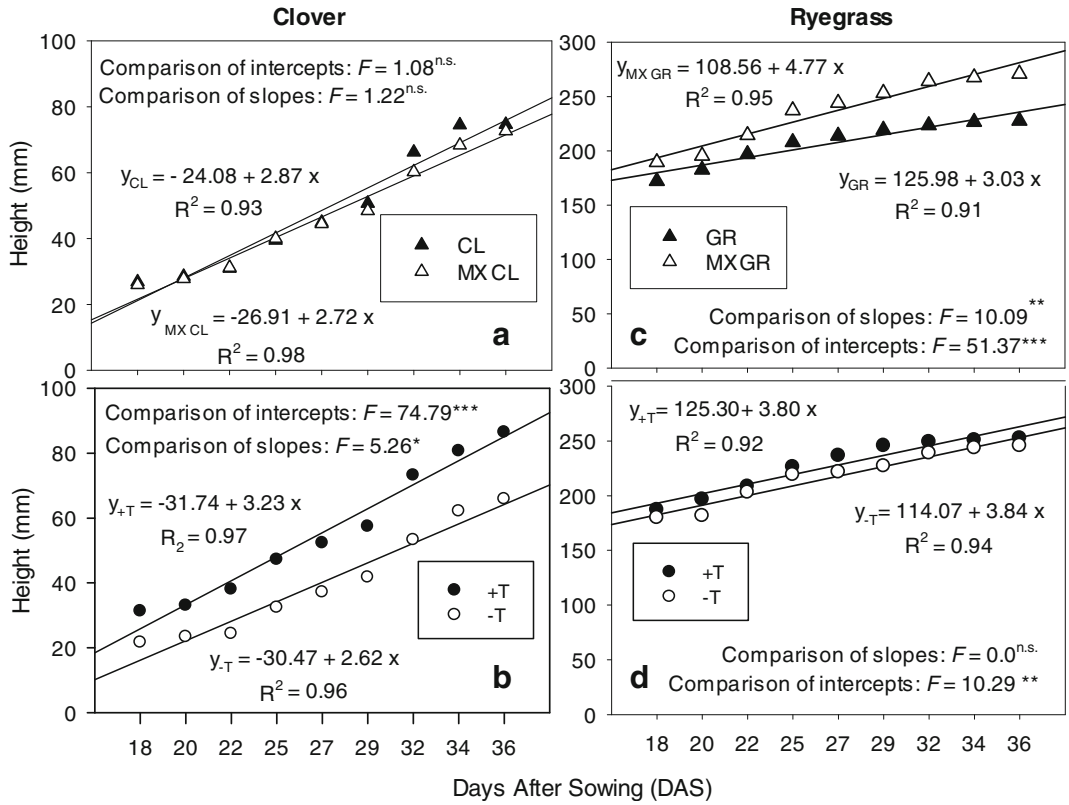

Fig. 3 Evolution of the height along the time and adjustments to linear regression of white clover (CL) and ryegrass (GR) grown alone or in mixture (MX) (**a**, **c**) in soil inoculated with *T. hamatum* GD12 strain (+T) or un-inoculated (−T) (**b**, **d**). Differences between lines slope and elevation are shown as: n.s. no significant, *p <0.05, **p <0.01, ***p <0.001

phenological stage was significant and clear differences were observed between treatments at the tillering stage (F=4.49 p <0.05) (Fig. 5d). Plants in inoculated soil reached this stages 3 days before plants in un-inoculated soil.

Clover nodulation

In all treatments the development of active and total *Rhizobium* nodules showed similar trends and there were no significant differences among treatments in the proportion of active nodules compared to the total *Rhizobium* nodules. There were significant (F=139.22 p <0.001) differences between clover plants sown in soil with GD12 and those in un-inoculated soils. Differences were also observed between clover plants sown in mixtures and those sown in monoculture (F=6.48, p <0.05). The presence of GD12 increased the number of nodules compared to the un-inoculated treatment with a significant difference in slope and elevation between treatments (Fig. 6). Here we found that there was significant (F=6.90, p <0.05) interaction between factors (Table 1) and the nodulation in clover was greater in the mixture than in monoculture when the soil was inoculated.

Cross-sections of *Rhizobium* nodules from clover roots are shown in Fig. 1 of the supplementary material. Under low magnification (×100) there were no visible differences between plants grown in un-inoculated soil (Fig. 1a) and plants in GD12 inoculated soil (Fig. 1f). The nodules displayed typical characteristics of normal healthy growth, and several zones could be differentiated. Firstly, a bacteria-free meristematic zone in the distal part, followed by an infection zone where cells start to differentiate. Subsequently, the nitrogen-fixing zone appeared with a profusion of differentiated cells containing bacteroids (strongly stained dark blue). It is important to note that Toluidine Blue O is a dye for general tissue staining, so no differences in activity regarding N fixation can be detected using this method. Finally, near the

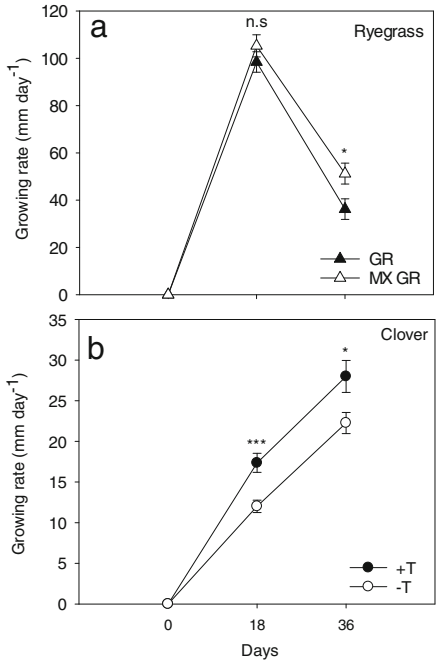

Fig. 4 Growth rate of Ryegrass growing alone (GR) or in mixture with clover (MX GR) (**a**) and growth rate of Clover in soil inoculated with *T. hamatum* GD12 strain (+T) or un-inoculated (−T) (**b**). *Vertical bars* represent the standard errors of the means across 3 replicates. Differences and significance between treatments are represented as: n.s. no significant, **p* <0.05, ***p* <0.01, ****p* <0.001

proximal part of the nodule, an area of senescence was visible, in which the differentiated cells stains less intensely.

Under higher magnification (×400 to 1000) it was possible to identify the bacteroids inside the cells and to detect differences in distribution inside the nodules. At the infection zone, the cells were densely filled with the bacteroids showing a rod-shaped form (Figs. 1b and 1G). The density slightly diminishes at the nitrogen-fixing zone (Figs. 1c and 1h) but the bacteroids reached their maximum size, between 4 and 5 μm length (Figs. 1e and 1j). In the senescence zone, the bacteroids appeared with a circular shape (Figs. 1d and 1i) and were less intensely stained. No significant differences in number of bacteroids were observed between plants grown in un-inoculated soil (Figs. 1a and 1e), and plants in GD12 inoculated soil (Figs. 1f and 1j). However, a striking characteristic was evident when comparing both types of nodules: the presence of starch and amyloplasts. Cells containing amyloplasts were present consistently within the nitrogen-fixing zone in plants sown in inoculated soil (Figs. 1h and 1j), whereas such cells were absent from plants in un-inoculated soil (Fig. 1c and 1e). No differences between nodules from clover in mixture or in monoculture were found.

Carbon and nitrogen contents

Significant differences between treatments were observed in the content of C and N in clover leaves and roots and ryegrass leaves as well as in the $\delta^{15}N$ values of clover leaves. Clover plants grown in isolation had significantly greater N and C contents in leaves and roots than those that were sown as mixtures with ryegrass (Fig. 7). Also were greater N and C contents in clover and ryegrass plants grown in GD12 inoculated soil compared to those grown in un-inoculated soil, however, significant differences were only observed in leaves (Fig. 8). In contrast, the $\delta^{15}N$ of clover leaves was significantly greater (F=6.08 p <0.05) in plants sown in un-inoculated soils compared to those grown in GD12 inoculated soil ($\delta^{15}N$ 5.4 ‰ and 1.7 ‰ respectively), with the content of GD12 untreated plants more closely aligned to the $\delta^{15}N$ ratio of the soil (3.49±0.169 ‰).

ITS sequencing of fungal strains isolated from soil microcosms

The similarity of Trichoderma sequences and their match with GD12 is shown in Table 2. Here we show that there is a Trichoderma species present in nearly all of the rhizotrons, with a 90 % similarity to GD12, and there is a *T hamatum* in nearly all the soils, (99 %) but generally GD12 (100 % sequence match) occurs mostly in the inoculated soils. However, only those microcosms supplemented with *T. hamatum* GD12 displayed the reported growth promotion of clover and ryegrass despite the presence of other *Trichoderma* species.

Discussion

Trichoderma hamatum GD12 promotes growth of clover and ryegrass, C and N leaf contents, *Rhizobium* nodulation and N fixation

The ability of certain strains of *Trichoderma* spp. to promote growth has been reported in different plant species, with strain-specific effects on aboveground biomass and root matter (Chang et al. 1986; Harman 2000; Naseby et al. 2000; Contreras-Cornejo et al. 2009).

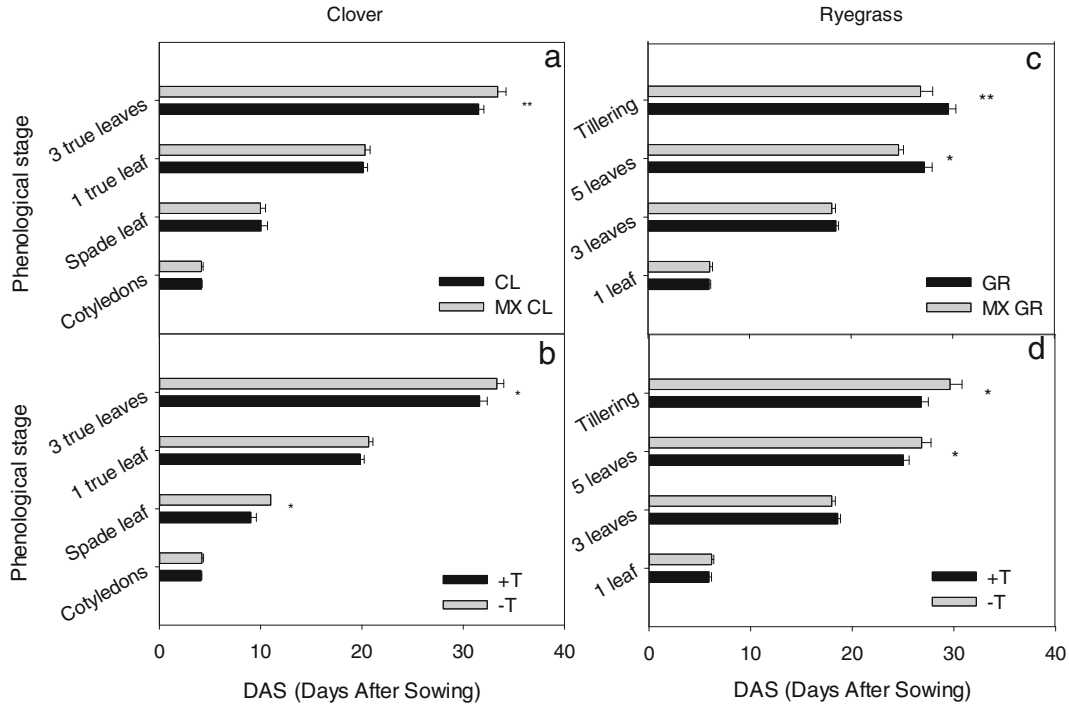

Fig. 5 Time taken to reach each phenological stage of white clover grown alone (CL) or in mix (MX CL) (**a**) and ryegrass grown alone (GR) or in mix (MX GR) (**c**) in a soil inoculated with *T. hamatum* GD12 strain (+T) or un-inoculated (−T) (**b**, **d**). *Bars* represent the standard errors of the means across 3 replicates. Differences between treatments only are represented when they were significant as: **p* <0.05, ***p* <0.01, ****p* <0.001

Previous studies have also reported the effect of *T. hamatum* GD12 on plant growth promotion (Ryder et al. 2012; Studholme et al. 2013). In our study, the amendment of soil with the strain GD12 had a significant beneficial effect on the clover growth and was independent of whether growing in a mixture or as a single species. Despite other *Trichoderma* spp. present in the soil, the growth promotion only was observed in rhizotrons inoculated with GD12 and its effects were very clear (Fig. 2 of the supplementary material). Clover plants grown in GD12 inoculated soil showed enhanced aboveground biomass, plant height and growth rate as well as faster development time than plants grown in un-inoculated soil. The ryegrass plants similarly had a higher biomass in GD12 inoculated soil compared to un-inoculated soil and a shorter phenological cycle, but did not have increased growth rate and little effect on the plant height compared with the effects on the clover plants. While clover root biomass in inoculated soils tended to be greater than in the un-inoculated controls, the differences in weights were not statistically different. Nevertheless, two striking differences in root morphology were apparent; firstly the architecture of the roots and, secondly, the numbers of active *Rhizobium* nodules. In GD12 inoculated soil, the plant roots formed a dense interwoven mat whereas, in control soil, root interaction was less pronounced and roots from the different plant species could be separated easily (Fig. 3 of the supplementary material). There was a greater abundance of *Rhizobium* nodules on *Trichoderma*-treated plants and nodulation was greater in clover plants grown in clover-ryegrass mixtures. Histological examinations of *Rhizobium* nodules showed typical development in both treatments and similar inter-nodule zones to those previously described in alfalfa (Vasse et al. 1990). The presence of bacteroids in *Rhizobium* nodules is indicative of normal activity of nodules (Vance et al. 1980; Oke and Long 1999; Melino et al. 2012) and the nodules in treatments were of comparable health and activity (N-fixation). However, there was a greater abundance of amyloplasts in cells sited in nitrogen-fixing zone of plants grown in GD12 inoculated soil.

Amyloplasts are non-pigmented organelles responsible for the storage of starch granules and its conversion into sugar when the plant requires energy. The presence of amyloplasts in the cells is an indicator of healthy

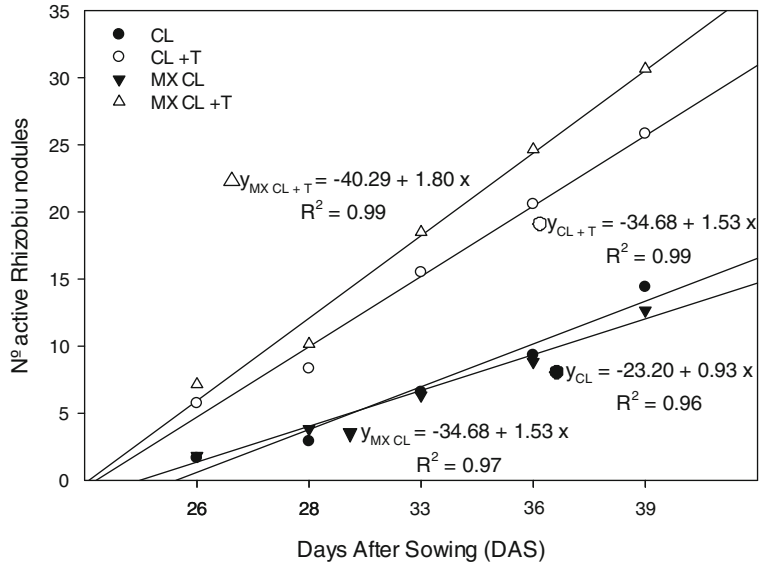

Fig. 6 Evolution of the number of active *Rhizobium* nodules along the time and adjustments to linear regression of white clover grown alone (CL) and in mixture (MX CL) in soil inoculated with *T. hamatum* GD12 strain (+T) or un-inoculated (−T). Differences between *lines slope* and *elevation* are shown as: n.s. no significant, **p* <0.05, ***p* <0.01, *** *p* <0.001

	Comparison of Slopes	Comparison of Intercepts
MX CL +T vs. MX CL	112.23***	35.32***
MX CL + T vs. CL +T	12.51*	26.09**
CL vs.MX CL	1.44$^{n.s.}$	0.18$^{n.s.}$
CL vs.CL + T	27.04**	44.64***

nodules (Vasse et al. 1990). In this case, the absence of starch could be an indication of senescence of the nodule or a lack of available nutrients. Previous studies with a root parasitic plant (*Orobanche crenata*) established that the absence of starch and amyloplasts in the infecting tubercles indicated a lack of reserves and death of

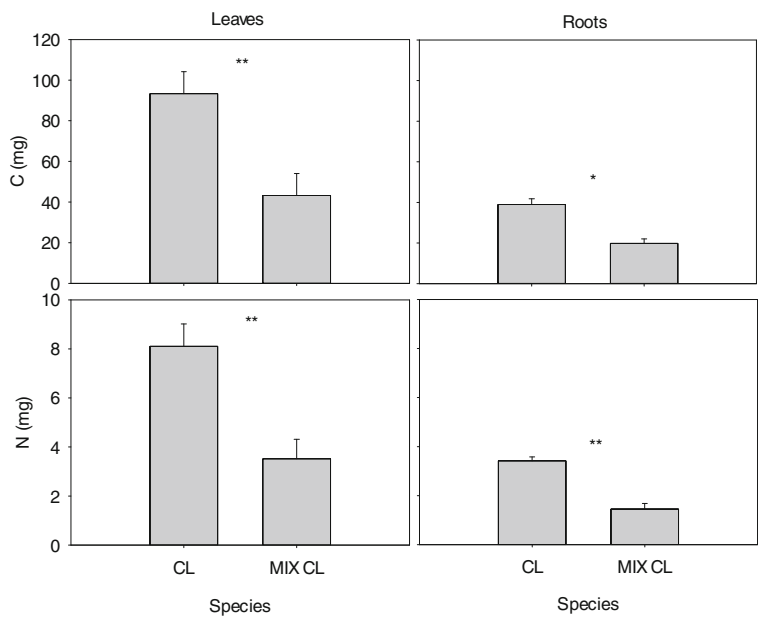

Fig. 7 C and N contents in leaves and roots of white clover grown alone (CL) and in mixture with ryegrass (MX CL). *Vertical bars* represent the standard errors of the means across 3 replicates. Differences and significance between treatments are represented as: n.s. no significant, **p* < 0.05, ***p* <0.01, *** *p* <0.001

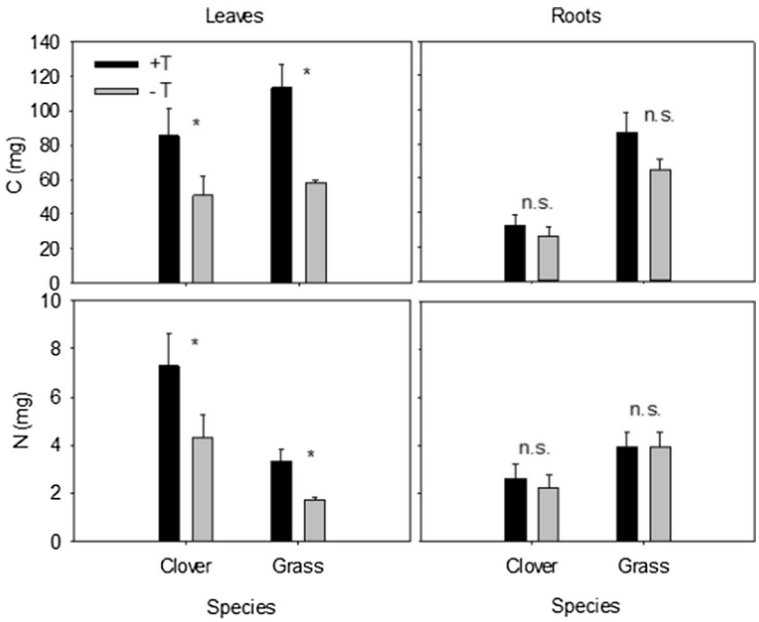

Fig. 8 C and N contents in leaves and roots of white clover and ryegrass in soil inoculated with *T. hamatum* GD12 strain (+T) and un-inoculated (−T). *Vertical bars* represent the standard errors of the means across 3 replicates. Differences and significance between treatments are represented as: n.s. no significant, **p* <0.05, ***p* <0.01, *** *p* <0.001

the parasite by nutrient depletion (Pérez-de-Luque et al. 2005). The presence of healthy nodules containing amyloplast-laden cells in GD12 treated plants implies an advantageous interaction between the bacterium *Rhizobium* and the fungus *Trichoderma* that facilitates the accumulation of carbohydrate reserves and might explain the differences in growth and development exhibited by clover plants grown in inoculated and un-inoculated soils. The positive interaction between *Rhizobium* spp. and *T. hamatum* has been previously reported in *Vigna mungo* (Badar and Qureshi 2012) in which the combination of both microorganisms improved root and shoot lengths and fresh biomass and increased the total chlorophyll, carbohydrate and crude protein contents as well as the amount of nitrogen and phosphorus in plant leaves. The effect of dual inoculation with *Rhizobium* and other *Trichoderma* species on yield components such as branches/plant, pods/plant, seed/pods and seed yield also has been found in bean (*Faba* spp.), chickpea (*Cicer arietinum*) and lupin (*Lupinus* spp.) (Shaban and El-Bramawy 2011).

Table 2 Number of replicates of each treatment where *Thrichoderma* spp. isolated from soil rhizotrons were identified by amplification and sequencing of the internally transcribed spacer regions ITS1–5.8S–ITS2 of the rRNA encoding unit

Species	Treatment	% Similarity to *T. hamatum* GD12			
		89	90	99	100
Grass	+T	1	3	1	2
Grass	-T	1	2	1	1
Clover	+T	1	5	1	2
Clover	-T	0	5	2	1
MX	+T	0	4	0	4
MX	-T	0	3	3	0

Table shows percentage similarities to *T. hamatum* GD12

In this study, *T. hamatum* GD12 enhanced clover and ryegrass utilization of carbon and nitrogen resources. Analysis of the C and N contents of leaves and roots revealed higher amounts in both organs in clover and ryegrass plants grown in GD12 inoculated soil compared to un-inoculated controls, although the differences were only significant for leaves. Previous work with different *Trichoderma* species and strains in dicotyledonous (Borrero et al. 2012; de Santiago et al. 2009; Yedidia et al. 2001) and monocotyledonous (Harman 2000; de Santiago et al. 2011) species have reported an increase in N and micronutrient (Fe, Mn, Zn and Cu) contents in leaves and roots although this effect was influenced by the nutritional status of the medium in which plants are grown.

The leaf $\delta^{15}N$ ratio of the clover plants grown in the presence of *Trichoderma* (1.7 ‰) compared to that of the soil (3.5‰) and clover grown in un-inoculated soil (5.4‰) indicate that there was a greater contribution

from fixation of atmospheric N in these plants ($\delta^{15}N$ of air=0‰). The increased N-fixation in clover plants grown in soil inoculated with GD12 might be explained by the increased number of nodules on these plants.

Trichoderma hamatum GD12 enhances clover competitiveness and ryegrass productivity in the mixture

It is widely accepted that growing legumes in association with grasses enhances grass productivity but does not lead to concomitant increases in clover growth (Rassmusen et al. 2012). The results of our work are consistent with this, since clover plants grown with ryegrass showed lower dry weights than when grown alone, although significant differences were only were observed in root biomass. However, clover grown alone was more phenologically advanced than when grown as a mixture with ryegrass. Furthermore, N and C contents of clover leaves and roots were greater in plants grown alone than in clover-ryegrass mixtures. Conversely, ryegrass grown with clover had a greater leaf and root biomass, height and growth rate and a shorter phenological cycle. It is important to highlight that the growth differences between treatments in ryegrass did not begin until 25 days after sowing (Fig. 3b), coincident with *Rhizobium* nodule appearance (see Fig. 7). These results indicate that clover provided an important source of nutrients for the companion ryegrass plants, but that clover development was unimpaired. Many studies have reported this positive effect of legumes on grass growth by means of both direct effects (legumes provision and transfer of N to grass species from the symbiotic N_2 fixation (Murray and Hatch 1994; Paynel et al. 2001; Rassmusen et al. 2007; Rassmusen et al. 2012; Høgh-Jensen and Schjoerring 2010) and mutual stimulation by multiple effects (Nyfeler et al. 2011). It is similarly well-documented that clover growth can be reduced in mixtures compared to clover monocultures (Carlsson et al. 2009; Nesheim and Boller 1991; Woledge et al. 1992).

Two aspects showed that *Trichoderma* provides a competitive advantage to clover in mixtures. The first is related to the growth differences between ryegrass and clover in *Trichoderma* inoculated soil, where the grass had greater biomass but no increment in growth rate, although this may be attributed to increased tillering in the inoculated rhizotrons. Clover in the inoculated soils, on the other hand, did show incremental growth in height compared to clover plants in un-inoculated soils. White clover is usually at a competitive disadvantage when growing in mixtures with ryegrass as it is sensitive to shading (Haynes 1988; Woledge et al. 1992). Our work demonstrates the potential of GD12 to enhance clover persistence in mixture with ryegrass by stimulating growth rate and plant height favoring a faster exposure to light of the clover leaf. Secondly, we demonstrated that growing clover in mixtures with ryegrass has a positive effect on clover nodulation, which is enhanced by *Trichoderma*.

The impact of the fungus on the root architecture may also be important in facilitating N-transfer and therefore have the potential to increase plant performance in the field. The increased branching and interweaving of roots in the inoculated soils, places the roots of the two species in closer association, thereby facilitating direct transfer of N between species. Growth and nutrient acquisition by clover and ryegrass interact in a complex manner involving different mechanisms such as competition, facilitation and complementarity (Høgh-Jensen and Schjoerring 2010). Other organisms such as arbuscular mycorrhizal fungi (Frey and Schüepp 1992; Johansen and Jensen 1996) and invertebrates such as the clover root weevil (*Sitona* spp.) (Murray and Hatch 1994; Murray and Clements 1998) may facilitate N-transfer. The dense intertwined root systems observed in GD12 inoculated soils indicate that the fungus may also act as a facilitator for nutrient transfer between the plant species.

Two main conclusions can be drawn from our results; i) *Trichoderma* had a positive effect on the growth of white clover and favoured *Rhizobium* nodulation in clover, both in terms of number of nodules and their health. The presence of nodules containing amyloplasts in plants grown in soil inoculated with *Trichoderma* was an indication of active and healthy nodules. Furthermore, assessments of ^{15}N stable isotope confirmed a higher N_2 fixation in white clover grown in soil inoculated with *Trichoderma*. Both aspects demonstrate a positive interaction between *Rhizobium* and *Trichoderma* that deserves further investigations. ii) *Trichoderma* showed an important role as agent facilitator of the interrelation between clover and ryegrass showing positive effects for both species in the mixture. Several characteristics observed in the mixture in inoculated soils, such as root architecture, the higher number of *Rhizobium* nodules, and plant height and plant growth in white clover, demonstrate that *Trichoderma*

confers white clover a competitive advantage in clover-ryegrass mixtures.

These findings hold enormous potential for grassland management allowing increases in profitability (yields) and sustainability (reduced reliance on artificial fertilizers). However results obtained from experiments in rhizotrons in controlled conditions should be taken with caution and to be validated in field trials. Field-scale evaluations of *T. hamatum* GD12 are needed to determine the feasibility of using the fungus as a soil inoculant for improved clover-ryegrass productivity and the optimum conditions for inoculation and survival of the fungus in the field as well as how to optimize the application to large scale.

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References

Annicchiarico P, Proietti S (2010) White clover selected for enhanced competitive ability widens the compatibility with grasses and favours the optimization of legume content and forage yield in mown clover-grass mixture. Grass Forage Sci 65:318–324. doi:10.1111/j.1365-2494.2010.00749.x

Annicchiarico P, Tomasoni C (2010) Optimizing legume content and forage yield of mown white clover-Italian ryegrass mixtures through nitrogen fertilization and grass row spacing. Grass Forage Sci 65:220–226. doi:10.1111/j.1365-2494.2010.00740.x

Badar R, Qureshi SA (2012) Comparative effect of *Trichoderma hamatum* and host-specific *Rhizobium* species on growth of *Vigna mungo*. J Appl Pharm Sci 2(4):128–132. doi:10.7324/JAPS.2012.2409

Baker R, Elad Y, Chet I (1984) The controlled experiment in the scientific method with special emphasis on biological control. Phytopathology 74:1019–1021. doi:10.1094/Phyto-74-1019

Borrero C, Trillas MI, Delgado A, Avilés M (2012) Effect of ammonium/nitrate ratio in nutrients solution on control of Fusarium wilt of tomato by *Trichoderma asperellum* T34. Plant Pathol 61:132–139. doi:10.1111/j.1365-3059.2011.02490.x

Carlsson G, Palmborg C, Jummpponen A, Scherer-Lorenzen M, Högberg P, Huss-Danell K (2009) N2 fixation in three perennial *Trifolium* species in experimental grasslands of varied plant species richness and composition. Plant Ecol 205:87–104. doi:10.1007/s11258-009-9600-9

Chang YC, Chang YC, Baker R, Kleifeld O, Chet I (1986) Increased growth of plants in the presence of the biological-control agent *Trichoderma harzianum*. Plant Dis 70(2):145–148. doi:10.1094/PD-70-145

Contreras-Cornejo HA, Macías-Rodríguez L, Cortés-Penagos C, López-Bucio J (2009) *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. Plant Physiol 149:1579–1592. doi:10.1104/pp.108.130369

Crotty FV, Adi SM, Blackshaw RP, Murray PJ (2012) Using stable isotopes to differentiate trophic feeding channels within soil food webs. J Eukaryot Microbiol 59(6):520–526. doi:10.1111/j.1550-7408.2011.00608.x

de Santiago A, Quintero JM, Avilés M, Delgado A (2009) Effect of *Trichoderma asperellum* strain T34 on iron nutrition in white lupin. Soil Biol Biochem 41:2453–2459. doi:10.1016/j.soilbio.2009.07.033

de Santiago A, Quintero JM, Avilés M, Delgado A (2011) Effect of *Trichoderma asperellum* strain T34 on iron, copper, manganese, and zinc uptake by wheat grown on a calcareous medium. Plant Soil 342:97–104. doi:10.1007/s11104-010-0670-1

Frame J, Newbould P (1986) Agronomy of white clover. Adv Agron 40:1–88. doi:10.1016/S0065-2113(08)60280-1

Frankow-Lindberg BE, Frame J (1996) Developments and challenges in sustainable grassland production. Grassland Sci Eur 1:337–345

Frey B, Schüepp H (1992) Transfer of symbiotically fixed nitrogen from berseem (*Trifolium alexandrinum* L.) to maize via vesicular-arbuscularmycorrhizal hyphae. New Phytol 122:447–454. doi:10.1111/j.1469-8137.1992.tb00072.x

Gierus M, Kleen J, Loges R, Taube F (2012) Forage legume species determine the nutritional quality of binary mixture with perennial ryegrass in the first production year. An Feed Sci Technol 172:150–161. doi:10.1016/j.anifeedsci.2011.12.026

Harman GE (2000) Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T-22. Plant Dis 84(4):377–393. doi:10.1094/PDIS.2000.84.4.377

Harman GE, Howell CR, Viterbo A, Chat I, Lorito M (2004) Trichoderma species - opportunistic, avirulent plant symbionts. Nature Rev Microbiol 2:43–56

Harrod TR, Hogan DV (2008) The soils of North Wyke and Rowden. Soil survey of England and Wales. Rothamsted Research, Okehampton, UK http://www.rothamsted.ac.uk/sites/default/files/SoilsNWRowden.pdf Accessed 22 July 2014

Harvey A, Parsons AJ, Rook AJ, Penning PD, Orr RJ (2000) Dietary preference of sheep for perennial ryegrass and white clover at contrasting sward surface heights. Grass Forage Sci 55:242–252

Haynes RJ (1988) Competitive aspects of the grass-legume association. Adv Agron 33:227–262

Høgh-Jensen H, Schjoerring JK (2010) Interactions between nitrogen, phosphorus and potassium determine growth and N_2-fixation in white clover and ryegrass leys. Nutr Cycl Agroecosyst 87:327–338. doi:10.1007/s10705-009-9341-0

Johansen A, Jensen ES (1996) Transfer of N and P from intact or decomposing roots of pea to barley interconnected by an

arbuscular mycorrhizal fungus. Soil Biol Biochem 28:73–81. doi:10.1016/0038-0717(95)00117-4

Lancashire PD, Bleiholder H, Van Den Boom T, Langeluddeke P, Stauss R, Weber E, Witzenberger A (1991) An uniform decimal code for growth stage crops and weeds. Ann Appl Biol 119:561–601. doi:10.1111/j.1744-7348.1991.tb04895.x

Melino VJ, Drew EA, Ballard RA, Reeve WG, Thomson G, White RG, O'Hara GW (2012) Identifying abnormalities in symbiotic development between *Trifolium* spp. and *Rhizobium leguminosarum* v. *trifolii* leading to sub-optimal and ineffective nodule phenotypes. Ann Bot 110(1559–15):55

Murray PJ, Clements RO (1998) Transfer of nitrogen between clover and wheat: effect of root herbivory. Eur J Soil Biol 34: 25–30. doi:10.1016/S1164-5563(99)80003-X

Murray PJ, Hatch DJ (1994) Sitona weevils (Coleoptera, Curculionidae) as agents for rapid transfer of nitrogen from white clover (*Trifolium repens* L) to perennial ryegrass (*Lolium perenne* L) Ann. Appl Biol 125:29–33. doi:10.1111/j.1744-7348.1994.tb04943.x

Murray PJ, Clegg CD, Crotty FV, de la Fuente-Martínez N, Williams JK, Blackshaw RP (2009) Dissipation of bacterially derived C and N through the meso and macrofauna of a grassland soil. Soil Biol Biochem 41:1146–1150. doi:10.1016/j.soilbio.2009.02.021

Naseby DC, Pascual JA, Lynch JM (2000) Effect of biocontrol strains of *Trichoderma* on plant growth, *Pythium ultimum* populations, soil microbial communities and soil enzyme activities. J Appl Microbiol 88:161–169. doi:10.1046/j.1365-2672.2000.00939.x

Nesheim L, Boller BC (1991) Nitrogen-fixation by white clover when competing with grasses at moderately low-temperatures. Plant Soil 133:47–56. doi:10.1007/BF00011898

Nyfeler D, Huguenin-Elie O, Suter M, Frossard E, Lüscher A (2011) Grass-legume mixture can yield more nitrogen than legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-symbiotic source. Agric Ecosyst Environ 140:155–163. doi:10.1016/j.agee.2010.11.022

Oke V, Long SR (1999) Bacteroid formation in the *Rhizobium*-legume symbiosis. Curr Opts Microbiol 2:641–646. doi:10.1016/S1369-5274(99)00035-1

Parsons AJ, Chapman DF (2000) The principles of pasture growth and utilization. In: Hopkins A (ed) Grass, its production & utilization, 3rd edn. British Grassland Society by Blackwell Science, Oxford, pp 31–89

Paynel F, Murray PJ, Cliquet JB (2001) Root exudates: a pathway for short-term N transfer from clover and ryegrass. Plant Soil 229(2):235–243. doi:10.1023/A:1004877214831

Peeters A (2009) Importance, evolution, environmental impact and future challenges of grasslands and grassland-based systems in Europe. Grassland Sci 55:113–125. doi:10.1111/j.1744-697X.2009.00154.x

Penning PD, Newman JA, Parsons AJ, Harvey A, Orr RJ (1997) Diet preferences of adult sheep and goats grazing ryegrass and white clover. Small Rumin Res 24:175–184. doi:10.1016/S0921-4488(96)00930-3

Pérez-de-Luque A, Rubiales D, Cubero JI, Press MC, Scholes J, Yoneyama K, Takeuchi Y, Plakhine D, Joel DM (2005) Interaction between *Orobanche crenata* and its host legumes: unsuccessful haustorial penetration and necrosis of the developing parasite. Ann Bot 95:935–942. doi:10.1093/aob/mci105

Rassmusen J, Eriksen J, Jensen ES, Esbensen KH, Høgh-Jensen H (2007) In situ carbon and nitrogen dynamics in ryegrass-clover mixture: transfer, deposition and leaching. Soil Biol Biochem 39:804–815. doi:10.1016/j.soilbio.2006.10.004

Rassmusen J, Søegaard K, Pirhofer-Walzi K, Eriksen J (2012) N_2-fixation and residual N effect of four legume species and four companion grass species. Eur J Agron 36:66–74. doi:10.1016/j.eja.2011.09.003

Romão-Dumaresq AS, de Araújo WL, Talbot NJ, Talbot NJ (2012) RNA interference of endochitinases in the sugarcane endophyte *Trichoderma virens* 223 reduces its fitness as biocontrol agent of pineapple disease. PLoS ONE 7: e47888. doi:10.1371/journal.pone.0047888

Rook AJ, Harvey A, Parsons AJ, Penning PD, Orr RJ (2002) Effect of long-term changes in relative resource availability on dietary preference of grazing sheep for perennial ryegrass and white clover. Grass Forage Sci 57:54–60. doi:10.1046/j.1365-2494.2002.00301.x

Ruzin SE (1999) Plant microtechniques and microscopy. Oxford University Press, New York, USA

Ryder LS, Harris BD, Soanes DM, Kershaw MJ, Talbot NJ, Thornton CR (2012) Saprotrophic competitiveness and biocontrol fitness of a genetically modified strain of the plant-growth-promoting fungus *Trichoderma hamatum* GD12. Microbiology 158:84–97. doi:10.1099/mic.0.051854-0

Shaban WI, El-Bramawy MA (2011) Impact of dual inoculation with *Rhizobium* and *Trichoderma* on damping off, root rot diseases and plant growth parameters of some legumes field crop under greenhouse conditions. International Res. J Agric Sci Soil Sci 1(3):98–108

Shoresh M, Harman GE, Mastouri F (2010) Induced systemic resistance and plant responses to fungal biocontrol agents. Ann Rev Phytopathol 48:21–43

Sreenivasaprasad S, Sharada K, Brown A, Mills P (1996) PCR-based detection of *Colletotrichum acutatum* on strawberry. Plant Pathol 45:650–655

Studholme D, Harris B, Le Cocq K, Winsbury R, Perera V, Ryder L, Ward JL, Beale MH, Thornton CR, Grant M (2013) Investigating the beneficial traits of *Trichoderma hamatum* GD12 for sustainable agriculture – insights from genomics. Front Plant Sci 4:1–13. doi:10.3389/fpls.2013.00258

Talbot NJ, Salch YP, Ma M, Hamer JE (1993) Karyotypic variation within clonal lineages of the rice blast fungus, Magnaporthe grisea. Appl Environ Microbiol 59:585–593

Vance CP, Johnson LEB, Hardarson G (1980) Histological comparisons of plant and *Rhizobium* induced ineffective nodules in alfalfa. Physiol Plant Pathol 17:167–173. doi:10.1016/0048-4059(80)90049-1

Vargas WA, Mandawe JC, Kenerley CM (2009) Plant-derived sucrose is a key element in the symbiotic association between *Trichoderma virens* and maize plants. Plant Physiol 151: 792–808. doi:10.1104/pp. 109.141291

Vasse J, De Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bacteriol 8:4295–4306

Viterbo A, Horwitz BA (2010) Mycoparasitism. In: Borkovich KA, Ebbole DJ (eds) Cellular and biology of filamentous fungi. American Society for Microbiology, Washington, pp 676–693

Walker JA, King JR (2010) Does relative time of emergence affect stand composition and yield in a grass-legume mixture? Kura

clover (*Trifolium ambiguum*)-meadow bromegrass (*Bromus biebersteinii*) and Kura clover-orchardgrass (*Dactylis glomerata*) mixtures. Grass Forage Sci 65:237–347. doi:10.1111/j.1365-2494.2010.00742.x

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: Guide Methods Appl 18:315–322

Woledge J, Davidson K, Dennis WD (1992) Growth and photosynthesis of tall and short cultivars of white clover with tall and short grasses. Grass Forage Sci 47:230–238. doi:10.1111/j.1365-2494.1992.tb02267.x

Yedidia I, Srivastva AK, Kapulnik Y, Chet I (2001) Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. Plant Soil 235:235–242. doi:10.1023/A:1011990013955