Improvement of an empirical model for the quantification of symbiotic nitrogen fixation in grass-clover mixtures

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Table of Contents

List of Tables

List of Figures

Preface

This thesis report was written as one of the requirements for the completion of my Master's programme, Master of Science in Organic Agriculture. The study was conducted in the Farming Systems Ecology Group with Dr. Egbert A. Lantinga as my Thesis Supervisor. This report is presented in the format of a scientific paper format with the purpose of submitting the paper to a scientific journal for future publication where, Dr. Egbert A. Lantinga and Dina Volker will serve as co-authors.

The main focus of the study was to investigate the proportion of fixed N_2 that is invested in the non-harvested biomass (roots and stubbles) of white clover. In this case, the robustness and correctness of the Danish model for the quantification of symbiotic N_2 fixation and the N difference method for estimating symbiotic N_2 fixation in grass-clover mixtures was evaluated.

I would like to extend my gratitude to those people who generously helped me in the successful completion of this study. To Dr. Egbert Lantinga, my Thesis Supervisor for his supervision and guidance; Dine Volker and Oscar van de Vos, for their assistance in the data collection; Hennie Halm, for the laboratory analysis; and support staff of Unifarm-Agros particularly Andries Siepel for the preparation of the experimental field and provision of working space and equipment for data processing.

Likewise, to my family and friends who had been the source of my strength and encouragement. And above all, to the Almighty Father who made everything possible.

R. J. D. Flora

Abstract

The validity of the Danish model for the quantification of symbiotic dinitrogen (N_2) fixation and the experimental N difference method for estimating symbiotic N_2 fixation in perennial ryegrass-white clover mixtures was evaluated in a field experiment. Main focus was on the proportion of fixed N_2 that is invested in the non-harvested biomass (roots and stubbles) of white clover. The two approaches differ in their assumptions in terms of the net proportion of fixed N_2 allocated to the non-harvested biomass. On the one hand, the Danish model assumes that 25% of the total N_2 fixed ends up in the roots and stubbles (mainly stolons) of white clover. On the other hand, the N difference method assumes that there is no net investment of fixed N_2 in the roots and stubbles of white clover. To confirm the validity of these two approaches, we hypothesized that the net investment of fixed N_2 in the roots and stubbles is insignificant if both N yields and turnover rates of roots and stubbles of white clover and perennial ryegrass in monocultures as well as mixtures without N fertilisation are more or less similar. N yields of roots and stubbles of perennial ryegrass and white clover in monocultures and in mixtures were analysed using One-way ANOVA. Turnover rates were determined through desktop research and field assessment. Assessment of tagged perennial ryegrass tillers and white clover stolons was conducted for one experimental year to determine the turnover rates of these plant organs in the field.

There were variations in N yields of the roots and stubbles of both perennial ryegrass and white clover in monocultures and in mixtures when treated separately. N yields of the stubbles of white clover in monocultures and in mixtures were generally higher than that of perennial ryegrass in monoculture. However, in November 2011, perennial ryegrass in monoculture had similar N yield with that of both perennial ryegrass and white clover in mixtures. It also had similar yield with that of perennial ryegrass and white clover cultivar Riesling mixture in August 2012. In terms of root N yields, perennial ryegrass in monoculture had higher N yield than that of perennial ryegrass and white clover in mixtures and white clover in monocultures in August 2012. However, in November 2011 and October 2012, N yields of both plants in monocultures and in mixtures were not significant. Despite of the differences in N yields of the roots and stubbles of both plants, the average N yields of roots and stubbles together from the three sampling dates were not found to be significantly different. Average N yields of roots and stubbles of perennial ryegrass in monoculture, mixture grass-clover cultivar Riesling, mixture grass-clover cultivar Alice, white clover cultivar Riesling, and white clover cultivar Alice were 101, 105, 114, 97, 102 kg ha^{-1}, respectively.

Results of previous experiments conducted in England, Switzerland and UK, and of the present experiment showed variations in turnover rates of individual plant organs of perennial ryegrass (leaves, tillers and roots) and white clover plants (leaves, petioles, stolons and roots). Despite this,

differences in turnover rates of different plant organs of perennial ryegrass and white clover were found to be negligible. Although, leaves and petioles of white clover turned-over faster than perennial ryegrass leaves, the difference is counterbalanced by the slower turnover of white clover stolons as compared to perennial ryegrass tillers. The turnover rates of the roots of both plant species were comparable. One published mean value on turnover rate of white clover roots is 1.26 year^{-1} (0.59 – 13.52 year^{-1}) and that falls within the range of the turnover rates of perennial ryegrass roots (0.70 – 1.90 year^{-1} under defoliated tillers and 1.0 year^{-1} under undefoliated tillers) taken from previous studies. The present study confirmed that perennial ryegrass tillers turned-over faster than white clover stolons. The result of the assessment revealed that only 32% of the tagged tillers of perennial ryegrass in monoculture remained in the field after one experimental year while 60% and 53% of the tagged stolons remained in white clover cultivars Riesling and Alice in monoculture fields. In perennial ryegrass and white clover mixtures, only 25% and 30% of the tagged tillers remained alive in mixture grass-clover cultivar Riesling and mixture grass-clover cultivar Alice, respectively. However, 75% and 45% of the tagged stolons were still alive in mixture grass-clover cultivar Riesling and mixture grass-clover cultivar Alice, respectively.

The results of the present experiment demonstrated that throughout one experimental year, N yields of the roots and stubbles together of monocultures and mixtures of perennial ryegrass and white clover (two cultivars: Alice and Riesling) without N fertilisation were not significantly different. Likewise, the aggregated differences in turnover rate of stubbles and roots based on our field observations and previous studies were also found to be negligible. This means that N uptake and N turnover in the roots and stubbles of both perennial ryegrass and white clover are quite similar. In conclusion, the experiment revealed that there is no net investment of fixed N_2 in the roots and stubbles of white clover plants. It also confirms the robustness and correctness of the N difference method which implies the incorrectness of the assumption in the Danish model.

Keywords: N_2 fixation, nitrogen, white clover, perennial ryegrass, roots, stubbles, Danish model, N difference method

1. Introduction

Globally, symbiotic dinitrogen (N_2) fixation had been increasingly recognised because of its contribution to organic agricultural systems. It significantly supports sustainable crop production particularly in low-input grassland production systems (Carlsson and Huss-Danell, 2003; Jørgensen et al., 1999; Rasmussen et al., 2007). Symbiotic N_2 fixation is the most economical and sustainable source of nitrogen (N) in most grassland systems (Goodman, 1988; Kessel and Hartley, 2000; Paynel et al., 2001).

Symbiotic N_2 fixation is therefore an alternative for animal manure as source of organic fertilizer. It helps produce yield with less N fertiliser input (Nyfeler et al., 2006). In grass-clover mixtures, white clover *(Trifolium repens L.)* is predominantly used to symbiotically fix N_2 to increase the N supply to the associated grass. With the significant contribution of symbiotic N_2 fixation to organic farming, estimation models were designed and continuously studied to improve the quantification of the contribution of symbiotic N_2 fixation to the system and to construct nutrient budgets. Only when reliable estimations of fixed N_2 levels are available, N nutrient input from white clover will be included in agricultural nutrient budgets (Eurostat, 2011).

There exist several methods used to estimate N_2 fixation. Among these methods are acetylene reduction, ^{15}N Isotope dilution method, ^{15}N abundance method, N difference method (Carlsson and Huss-Danell, 2003) and the recently developed, Danish model. Acetylene reduction assay is a simple method, less expensive than others, to estimate N_2 fixation. With this method, plants are incubated in a closed container with 10% acetylene for a period of 0.5-2 hours. Acetylene is converted into ethylene with the use of nitrogenase enzyme. This method was used in 1970's, but was replaced by ^{15}N techniques (Ledgard and Steele, 1992) because of its inapplicability in field experiments (Danso et al., 1986; Goh and Edmeades, 1978).

^{15}N Isotope dilution is the most reliable method to estimate N_2 fixation (Danso et al., 1986; Giller, 2001). The method uses N-fixing and non N-fixing plants. Plants are applied with ^{15}N-labelled fertilizers. The technique assumes that both plants absorbed the same amount of ^{15}N from the soil (Danso et al., 1986; Ledgard et al., 1985; McNeill and Wood, 1990). Further, it assumes that N-fixing plants do not transfer fixed N_2 to the non-fixing plants during the growth period (McNeill and Wood, 1990). N_2 fixation is calculated based on the atom % ^{15}N excess in both plants.

The ^{15}N abundance method is relatively the same but less expensive than ^{15}N isotope dilution. The only difference is that ^{15}N abundance method uses natural enrichment of ^{15}N present in soils while Isotope dilution method used labelled ^{15}N application (Shearer and Kohl, 1988; Somado and Kuehne, 2006). Estimation of symbiotic N_2 fixation is also based on the excess atom% ^{15}N of legumes and reference plants. If well-calibrated, it can provide reliable measurement of symbiotic N_2 fixation

(Halliday and Pate, 1976; Ledgard and Steele, 1992).

The total N difference method, known to have comparable results with ^{15}N Isotope dilution method, is a cheap and simple way to quantify N_2 fixation (Carlsson and Huss-Danell, 2003; Martensson and Ljunggren, 1984). The method assumes that legumes (e.g., white clover) and the reference plant (e.g., perennial ryegrass) take up the same amount of mineral N from the soil. N_2 fixed is quantified based on the difference between the total amount of N found in legumes and the total amount of N in reference plants (Carlsson and Huss-Danel, 2003; Ledgard and Steele, 1992). This calculation is generally based on harvested biomass (> 4cm above the soil surface). Since calculation is only based on harvested shoot biomass, it further assumes that fixed N_2 is not invested in the roots and stubbles. The general equation for this method is:

$$N_2 \text{ fixation (kg ha}^{-1}\text{)} = \text{Total N in clover or grass-clover} - \text{Total N in grass monoculture}$$

The Danish model is an empirical model used to estimate N_2 fixation, developed by Høgh-Jensen et al. (2004). The model assumes that there is investment of fixed N_2 in the roots and stubbles of white clover. According to this model, the constant proportion of the fixed N_2 invested in the roots and stubbles of white clover is 25%. This is based on field measurements and assumptions. For grass-white clover swards in sandy soil, the equation and the description of components used in the model (Høgh-Jensen et al., 2004) is:

$$SNF = DM_{legume} \times N\% \times P_{fix} \times (1 + P_{root+stubble} + P_{trans\ soil})$$

DM_{legume}	=	accumulated amount of legume shoot dry matter above normal defoliation height;
N%	=	concentration of N in the dry matter of the legume (kg kg^{-1});
P_{fix}	=	fixed N_2 as proportion of total N in the shoot dry matter of the legume;
$P_{root+stubble}$	=	fixed N_2 in the root and stubble as proportion of the total fixed shoot N at the end of the growing period;
$P_{trans\ soil}$	=	below-ground transfer of fixed legume N_2 located in the grass in mixtures as proportion of the total fixed shoot N at the end of the growing period;

Of the different methods mentioned, N difference method and Danish model are the cheap and simple approaches to estimate N_2 fixation. Only when these methods are proven reliable and valid, however, can they be widely used for estimating symbiotic N_2 fixation.

Based on the assumptions of the Danish model that there is fixed N_2 allocated to the roots and stubbles, the validity of N difference method becomes questionable. The total N difference method disregards the contribution of roots and stubbles when estimating symbiotic N_2 fixation.

Quantification of symbiotic N_2 fixation might have been underestimated using this method. Hence, investigating the assumptions for both methods is necessary to improve the validity of the models.

Estimates of symbiotic N_2 fixation are generally important in organic grassland production. Among all legumes, white clover (*Trifolium repens* L.) is widely used in grassland swards mixed with perennial ryegrass (*Lolium perenne* L.). Of the white clover cultivars, not enough evidence on the performance of white clover cultivar Alice and white clover cultivar Riesling in terms of N_2 fixation is known (Starre, 2011). From the previous finding of Andela (2010), white clover cultivar Alice performed better in late summer and autumn while white clover cultivar Riesling performed better in spring and early summer in terms of N yield and apparent N_2 fixation. Aside from that, Starre (2011) noted that the amount of fixed N_2 in kg per Mg of white clover DM, regardless of cultivars, is inversely proportional to the percentage of clover in the total dry matter (DM) yield. These cultivars also need further evaluation to validate their performance, hence, they were used in the present study.

Quantification of white clover biomass in grass-clover mixtures is another important aspect to estimate N_2 fixation. There exist two methods to estimate clover biomass in the sward: direct and indirect methods. Direct method is destructive and demands more labour and time (Hermy, 1988). In this method, shoot biomass of grass and clover are harvested in the field. Shoot biomass is sorted out into clover and grass and oven-dried to determine the dry matter proportion of clover in the field. With the cited disadvantages of direct method, exploring indirect or non-destructive method is necessary. Of the different indirect methods to quantify clover biomass, visual estimation method for estimating visual ground cover of white clover is commonly used. In-depth understanding of the relationship of these methods is important to generate good estimates of the proportion of clover in the dry matter through visual estimation method. According to Gerard Oomen (personal communication) the ratio between clover DM proportion and clover visual ground cover is about 2/3.

With the goal of investigating the assumptions of the two approaches in estimating symbiotic N_2 fixation, the current study aims to investigate the proportion of fixed N_2 invested in the non-harvested biomass (roots and stubbles) of white clover. The study also aims to validate the seasonal performance of white clover cultivars (Alice and Riesling), evaluate N_2 fixation performance of white clover in grass-clover mixtures and evaluate the relationship of visual clover ground cover and dry matter proportion of white clover. In this study, we hypothesised that 1) there is no net proportion of fixed N_2 allocated to the roots and stubbles of white clover in monocultures and in mixtures; 2) the seasonal performance of white clover cultivar Alice and cultivar Riesling will still be the same as that of the previous result; 3) N_2 fixation per Mg clover DM decreases as the proportion of clover in the total dry matter in the grass-white clover sward increases; and 4) the assumption of G. Oomen about the ratio between white clover dry matter proportion and clover visual ground cover is correct.

2. Materials and methods

2.1 Experimental set-up

The experiment was conducted for one experimental year from July 2012 until June 2013 at the Droevendaal farm, an organic experimental and training farm owned by the Wageningen University, The Netherlands. It was carried out at the existing 4-year old experimental area planted with perennial ryegrass and white clover.

This experiment follows studies done by Andela (2010) and Van der Starre (2011), using the same experimental field. The current experiment was designed with 5 treatments replicated four times (4 blocks) in a randomised complete block design (RCBD). The treatments were: (A) Perennial ryegrass monoculture; (B) Perennial ryegrass-white clover cultivar Riesling mixture; (C) Perennial ryegrass-white clover cultivar Alice mixture; (D) White clover cultivar Riesling monoculture; and (E) White clover cultivar Alice monoculture. The experimental field is 540 m^2 with a dimension of 9 x 60 m. Each block of 9 x 15 m is divided into (five) plots. Each plot has a dimension of 9 x 3 m. The design and lay-out of the experiment is illustrated in Appendix I. White clover (*Trifolium repens* L.) with white clover cultivars, Alice and Riesling were used in the experiment as test legume. Perennial ryegrass (*Lolium perenne* L.) with cultivars in composition: 40% Premium, 18% Romark and 42% Asturion, were used as reference grass. The seeding rate at the start of the experiment in 2008 was 8 kg ha^{-1} for both white clover cultivars and 25 kg ha^{-1} for perennial ryegrass.

Two months before the start of the present experiment, the grass-clover sward was carefully mowed leaving 4-cm stubble above soil surface. This was to allow perennial ryegrass and white clover plants to regrow for two months prior to the first cut. Before the start of the actual field measurement, potassium (K) deficiency was observed in the field, and K fertiliser was applied at a rate of 300 kg ha^{-1}.

Measurements for the first cut in 2012 were carried on August. The second cut was conducted on October.

2.2 Estimation of the presence of white clover

Estimation of the presence of white clover in the field was conducted in two ways, visual estimation (VEM) and dry matter proportion method (DMPM). DMPM was used to check the reliability of VEM.

2.2.1 Visual estimation method (VEM)

Before the plots were harvested, white clover percentage ground cover was estimated using VEM. The researcher together with the two experts carried out the measurement. To estimate, three sample spots of 0.25 m^2 per plot were selected and staked with four plastic sticks at the four corners of the sampled areas. Plots were no longer uniform as it was during the first experiment. The plots were heterogeneous. Invasive grass and weed species existed and white clover also invaded other plots. Therefore, three representative sample spots were carefully selected in each plot based on the original treatments.

2.2.2 Dry matter proportion method (DMPM)

To check the validity of VEM, the dry matter proportion method (Andela, 2010; Van der Starre, 2011) was also carried out. The three representative sample spots selected during visual estimation were harvested after the estimation was completed. Each harvested shoot biomass sample (shoot biomass defoliated from 4 cm above the ground) was thoroughly mixed in the laboratory and divided into two sub-samples. One was for the determination of dry matter and N content of the shoot biomass and the other one was for determining the dry matter proportion of clover. Subsamples for DMPM were sorted out into white clover and perennial ryegrass. They were oven-dried for 48 hours at 70°C. Dry weight of perennial ryegrass and white clover were weighed to get the DM proportion of clover in the subsamples.

2.3 Harvesting of the above- and below-ground biomass

Harvesting of shoot and stubble (shoot material below defoliation height of 4 cm), and root (below-ground) biomass was done in late summer (August) and autumn (October). Harvesting the shoot biomass was done using metallic harvest frame with a dimension of 50 × 50 cm and a spinach knife (Lantinga et al., 2004) to cut at least 4 cm above ground. Three representative sample spots, with an area of 0.25 m^2 per plot were taken. Each sample was placed inside labelled bags.

After harvesting the shoot biomass from all treatment plots, stubble and root biomass samples were also collected within the sampled areas used in collecting shoot biomass. Stubble and root samples were taken carefully using sampling cylinder of 8 cm in diameter and 14 cm in height. To take the stubbles, the sampling cylinder was used to mark the area to be harvested. Then the stubbles were taken by cutting the stubbles at the surface of the soil using scissors. After that, root samples inside the marked area were collected using the sampling cylinder. Sub-samples of roots and stubbles were placed inside separate and labelled bags. After collecting all samples, the whole field was mowed. This was done every time measurements were completely taken to prepare the field for the next measurement period.

Root and stubble data for November 2011 were collected by Dine Volker, Researcher and Lecturer of Farming Systems Ecology, using similar methods as in 2012. However, the sampling cylinder for collecting root biomass was 2 cm larger and 6 cm deeper (20 cm depth compared with 14 cm in 2012). Sampling in 2012 somewhat underestimated the root biomass of the representative samples compared with November but the difference is not significant. Root biomass is known to decrease with increasing soil depth (Bolinder et al., 2002). According to Bolinder et al. (2002) the fraction of root biomass found within 0-15 cm soil depth ranged from 54-71%, while 21-37% was found within 15-30 cm. Similarly, Hebeisen (1997) as cited by Schneider et al. (2006) reported that 90-95% of root mass can be found at of 0-15 cm soil depth. Based on these figures, the use of 14 cm-depth soil cylinder in 2012 might have underestimated only 5-10% of the total root biomass compared with that using 20 cm depth cylinder in 2011.

2.4 Determination of dry matter and N content, and N yield of the above- and below-ground biomass

Samples were brought to Unifarm-Agros laboratory, where the shoot biomass of each sample was weighed to get the total fresh weight of the samples. Each sample was then mixed thoroughly and divided into two sub-samples. One was for the determination of dry matter yield and the other one was used to determine the N content. To determine the dry matter and N content, sub-samples were weighed to determine fresh weight and then oven-dried for 48 hours at 70°C. Dried sub-samples were weighed to get the DM content. For N analysis, sub-samples were ground and analysed based on Berthelot reaction (Houba et al., 1989).

For the root and stubble biomass, each sub-sample was washed thoroughly with running water to separate soil and other particles. Sub-samples were dried using paper towels, weighed to measure fresh weight, and then oven-dried for 48 hours at 70°C. The dried samples were mixed to make one sample for each plot. They were then ground to determine the N content based on Berthelot reaction (Houba et al., 1989).

2.5 Turnover rate (year^{-1}) of root and stubble biomass

To determine the turnover rates of stubbles, 10 representative sample tillers or stolons per plot in perennial ryegrass and white clover monocultures were tagged. Well-developed clover stolons (with 5-10 nodes) and newly-matured perennial ryegrass tillers (with 3-4 leaves) were selected as samples. In grass-clover mixtures, five representative perennial ryegrass tillers and five white clover stolons were tagged per plot. The selected white clover stolons were tagged with coloured wire (Jørgensen and Ledgard, 1997; Marriott and Smith, 1992; Sturite et al., 2007) and perennial ryegrass tillers with coloured thread. The tagging of perennial ryegrass tillers was based on the study by Hepp et al. (1996). Once the tagged perennial ryegrass tillers and white clover stolons were missing or the tissues became brown and soft, these were considered dead (Sturite et al., 2007). Tagging was done on July 25, 2012. The turnover of white clover stolons and perennial ryegrass tillers were examined and recorded every 7 to 14 days. Monitoring was continuously done starting at 1 week after tagging until November 2012 before onset of snow. Other assessments were done in February and June 2013 rounding the observation period to one experimental year. Observations on turnover rates of leaves and petioles of white clover and perennial ryegrass were disregarded in this experiment. Their inclusion requires a lot of effort and time which is difficult to manage with all other parameters considered in the experiment. The turnover rate equation is:

$$\text{Turnover rate (year}^{-1}\text{)} = \frac{\text{Number of days in a year}}{\text{Average lifespan of grass tillers or white clover stolons}}$$

Since the field is an established 4-year old sward, determination of the exact age of white clover stolons and perennial grass tillers when tagging is impossible. In this case, the age of clover stolon is estimated based on number of nodes at the start of tagging. The age of clover stolon is estimated based on node appearance rate of 0.86 nodes stolon^{-1} week^{-1} (Elgersma and Fengrui, 1997). Given the node appearance rate of 0.86 nodes stolon^{-1} week^{-1} and the number of nodes (5-10) of selected white clover stolons in the experiment, we estimated the age of stolons to be 50 – 70 days old. Since perennial ryegrass and white clover were sown at the same time in the field four years ago, we assumed that the age of white clover stolons derived from the figures was also the age of the tagged perennial ryegrass tillers. The age of the two plant organs might be underestimated using this method but this may not have significant impact to the study. Since only perennial ryegrass tillers and white clover stolons were monitored in this experiment, more information on turnover rates of plant organs of both grass species were sourced from other studies.

2.6 Analysis and calculations

One-way Analysis of Variance (ANOVA) for randomised complete block design was performed to determine significant differences using Genstat 15th edition. Results were further analysed to compare the significant differences of all treatment means using Fisher's Protected LSD test at 5% level of probability. Before executing the analyses, data were checked for normality and homogeneity of variance using Shapiro-Wilk test for Normality and Bartlett's test for homogeneity of variances.

Since it was found in the present study that there was no investment of fixed N_2 in the roots and stubbles, apparent N_2 fixation was calculated using the N difference method. The amount of N contained in each sample was converted on per hectare basis. The dry matter yield and N concentration in shoots were considered to calculate the amount of N in kg ha^{-1} using the formula:

$$\text{Total harvested shoot N yield (kg ha}^{-1}\text{)} = \text{\% N*harvested shoot dry matter yield}$$

Further, the amount of fixed N_2 was calculated using the formula of N difference method:

N_2 fixation (kg ha^{-1}) = Total N in clover or grass-clover – Total N in grass monoculture

Scatterplots were also used to evaluate relationships of variables and to generate equations.

3. Results and discussion

3.1 Weather conditions

Within the period of study, the average monthly minimum and maximum air temperatures reached 13°C and 24°C, respectively, in August 2012. They drastically went down to -1°C and 4°C, in January 2013, and remained low until March 2013. The highest cumulative monthly precipitation was observed in December 2012 (145 mm) during winter period and in July 2012 (105 mm) during summer. Least rainfall was observed in April 2013. Cumulative rainfall was zero for June 2013 because the observation period was only until June 8, 2013 (Fig. 1).

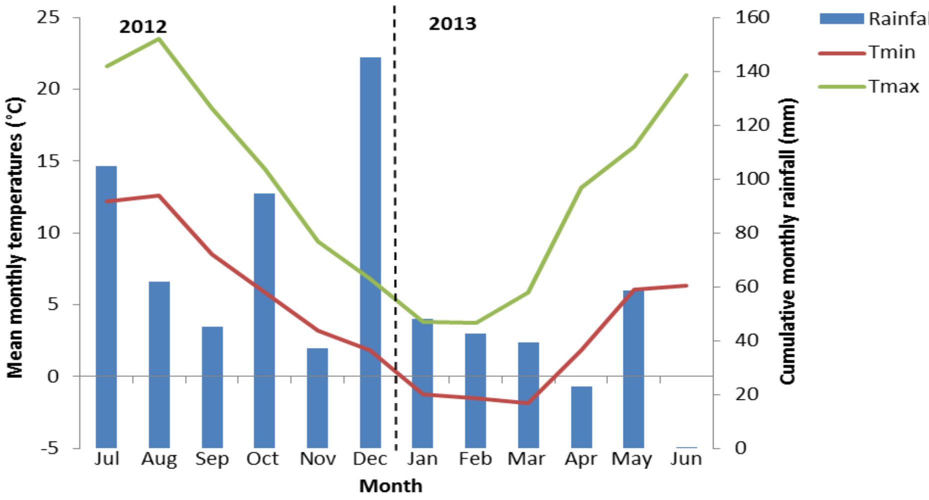

Fig. 1. Cumulative monthly rainfall, and mean Tmin and Tmax from July 2012-June 2013.

3.2 White clover roots and stubbles can be neglected in estimating N_2 fixation

The most important part of this study is to confirm if there is net proportion of fixed N_2 in the roots and stubbles of white clover. To answer this, we hypothesised that the net investment of fixed N_2 in the roots and stubbles is insignificant if both N yields and turnover rates of roots and stubbles of white clover and perennial ryegrass in monocultures as well as mixtures without N fertilization are more or less similar.

3.2.1 N yields of non-harvested biomass of white clover and perennial ryegrass are of the same order of magnitude

White clover in monocultures had significantly higher stubble N yield than perennial ryegrass in monoculture in all sampling dates. White clover and perennial ryegrass in mixtures had significantly higher N value than perennial ryegrass in monoculture in October 2012. Mixture grass-clover cultivar

Alice was also higher than perennial ryegrass in monoculture in August 2012 (Fig. 2A).

Differences in stubble N yield between white clover in monocultures and in mixtures, and perennial ryegrass in monoculture can be explained by the differences of both plant species in terms of N content and dry matter yield (data not shown) of stubbles. This is due to the remaining plant organs after defoliation. The defoliation of white clover at a stubble height of 4 cm resulted to the retention of newly formed leaf before the apex of white clover stolon and petioles. Harvesting of perennial ryegrass at a stubble height of 4 cm retained pseudo stem and portion of its leaf lamina. According to Nassiri and Elgersma (1998), when white clover is cut (>5 cm) 22% of total white clover DM is retained as stubbles. For perennial ryegrass, cutting retains 34% of its total DM. In contrast to this, we found out in the present experiment that white clover in monocultures and in mixtures generally had quite similar stubble DM yield than perennial ryegrass stubble. However, since white clover in monocultures and in mixtures generally had higher N concentration in total dry matter than perennial ryegrass, stubble N yields of white clover in monocultures and in mixtures were generally higher than perennial ryegrass in monoculture.

In terms of root N yields, perennial ryegrass in monoculture had higher N yield than that of perennial ryegrass and white clover in mixtures and white clover in monocultures in August 2012. However, in November 2011 and October 2012, N yields of both plants in monocultures and in mixtures were not significantly different (Fig. 2B).

Lower N yield values of roots of white clover in monocultures and in mixtures than perennial ryegrass in August 2012 could be attributed to their differences in their growth response to season. An observed reduction in root growth of white clover and perennial ryegrass occurred during summer due to limited soil moisture (Caradus and Evans, 1977; Matthew, 1992). Low amount of moisture in the soil decreases nodal root formation of both species (Soper, 1958). This was confirmed in the study of Thomas (1984) under glasshouse condition where he reported that production of roots of perennial ryegrass and white clover significantly decline during drought period. Relatively, Evans (1978) demonstrated in his study that perennial ryegrass has higher root biomass than white clover in summer. Perennial ryegrass has denser root system. It can explore more moisture reserve in the soil than white clover during this season. Likewise, it produces vegetative tillers and roots before the onset of dry periods (Soper, 1958). This supports the result of the present experiment where perennial ryegrass had much higher root dry matter yield (data not shown) than white clover in summer (August 2012). Even though, it is generally known that N concentration of perennial ryegrass is lower than white clover, perennial ryegrass resulted to higher N value due to higher root DM yield than white clover.

Despite of some significant differences in N yields of stubbles and roots between white clover and perennial ryegrass in monocultures and in mixtures, the aggregated root and stubble mean N values of the different treatments averaged from the three sampling dates revealed no significant difference (P > 0.05) (Fig. 2C). The result revealed that white clover and perennial ryegrass took up relatively the same amount of mineral N from the soil.

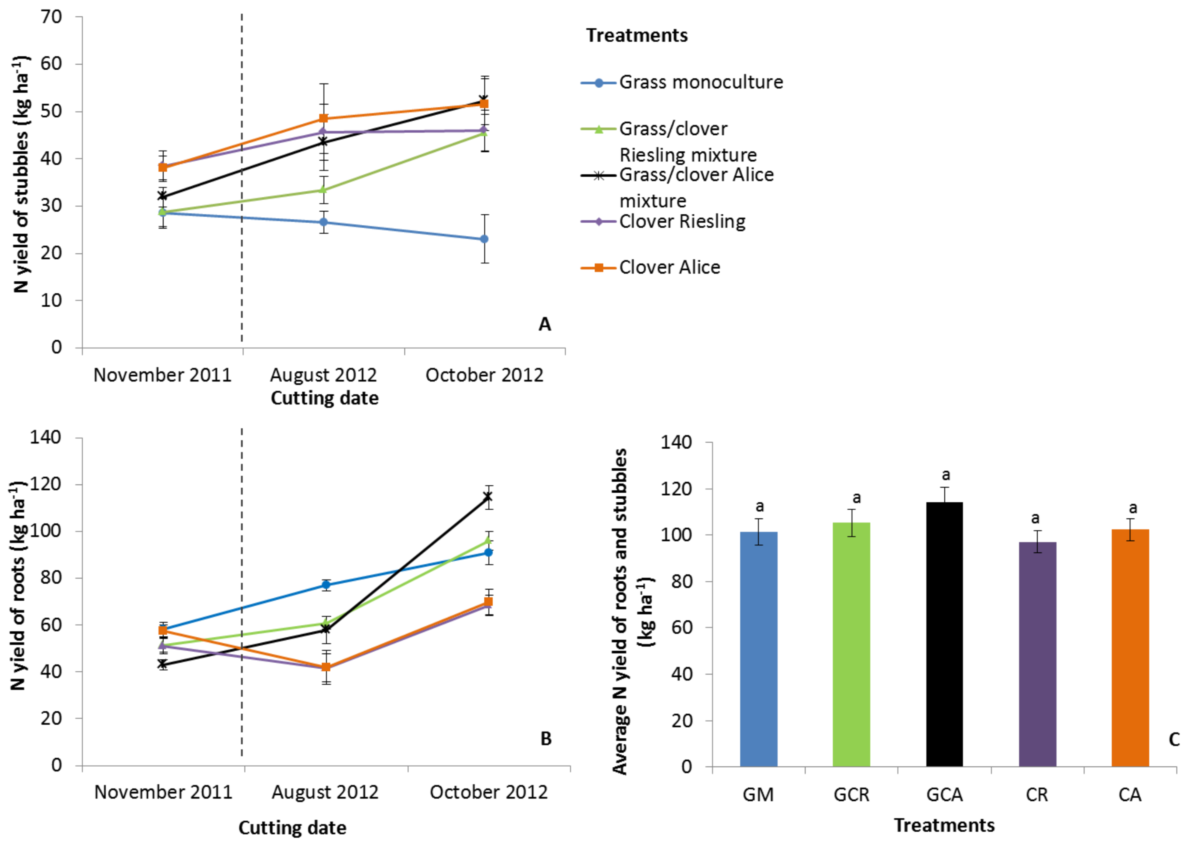

Fig. 2. N yields of stubbles (A) and roots (B) over time, and average N yield of roots and stubbles (C). The vertical bars represent standard error of the mean, (n = 4).

3.2.2 Turnover rates of non-harvested biomass of white clover and perennial ryegrass are about the same.

Turnover rates of white clover and perennial ryegrass stubbles and roots were obtained from previous studies. As reflected in Table 1, a Norwegian plot and root window experiment demonstrated that white clover leaves and petioles, and stolons have average turnover rates of 6.19 and 0.89 $year^{-1}$, respectively. Its roots turnover at an average rate of 1.26 $year^{-1}$. Perennial ryegrass leaves and tillers have average turnover rates of 2.40 and 1.80 $year^{-1}$, respectively. Its root turnover rate ranges from 0.70 - 1.90 $year^{-1}$ for defoliated tillers and 1.0 $year^{-1}$ for undefoliated ones based from the results of various studies. Figures on perennial ryegrass turnover rates were sourced from the results of grazing, field and pot experiments conducted in England, Switzerland and UK, respectively.

Table 1

Turnover rates of white clover leaves, petioles and roots; and perennial grass tillers, leaves and roots[1]

Plant species	Plant parts	Mean turnover rate ($year^{-1}$)	References
White clover	Stubble (0 – 4cm)		
	▪ *Leaves and petioles*	6.19 *(4.24 – 17.38)*	Sturite et al., 2007
	▪ *Stolons*	0.89 *(0.54 - 3.28)*	Sturite et al., 2007
	Roots	1.26 *(0.59 - 13.52)*	Sturite et al., 2007
Perennial ryegrass	Stubble (0 – 4 cm)		
	▪ *Leaves*	2.40 *(2.30 – 3.40)*	Schneider et al., 2006
	▪ *Tillers*	1.80	Bullock et al., 1994 as cited by Schneider et al., 2006
	Roots	*0.70 – 1.20 (defoliated tillers)*	Schneider et al., 2006
		1.00 $\pm$ 0.05 *(undefoliated tillers)*	Troughton et al., 1981
		1.91 $\pm$ 0.16 *(defoliated tillers)*	Troughton et al., 1981

[1]Data on turnover rates of plant organs were taken from experiments of temperate conditions.

Determination of the turnover rates of roots and stubbles of perennial ryegrass and white clover is one of the two important factors to confirm the net proportion of fixed N_2 allocated to the roots and stubbles of white clover. Hence, field observations on the turnover of white clover stolons and perennial ryegrass tillers were conducted in the existing experimental field for an experimental period of one year.

Fig. 3 reflects the remaining tagged stolons of white clover and tillers of perennial ryegrass in monocultures and in mixtures after one experimental year. The result of the field observations exhibited that within 7 months of assessment, none of the tagged stolons of white clover in monocultures died. Tagged stolons only drastically decreased from February until June 2013 resulting to 60% and 53% remaining stolons in white clover cultivar Riesling and white clover cultivar Alice monoculture plots, respectively. Perennial ryegrass tillers in monoculture markedly decreased from August 2012 until June 2013 resulting to 32% remaining tagged tillers in the field (Fig. 3A). In grass-clover mixtures, tagged white clover stolons in mixture grass-clover cultivar Riesling plots had no observed deaths until February 2013 but mixture grass-clover cultivar Alice had death of 5% of the tagged tillers. A marked decrease of tagged stolons occurred from February 2013 until June 2013 with remaining stolons of 75% in mixture grass-clover cultivar Riesling and 45% in mixture grass-clover cultivar Alice plots. Tagged perennial ryegrass tillers drastically decreased from August 2012 until June 2013, where 25% and 30% of the tagged tillers remained in grass-clover cultivar Riesling and grass-clover cultivar Alice mixtures within the observation period (Fig. 3B).

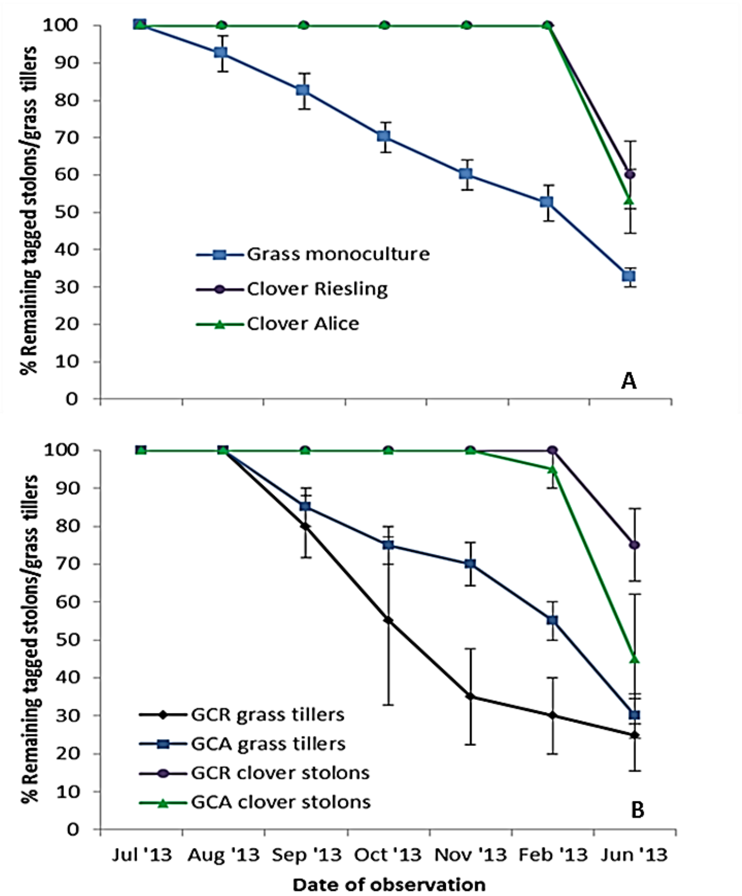

Fig. 3. Percentage remaining tagged grass tillers or clover stolons in monocultures (A) and in grass-clover mixtures (B) over time (July 2012 - June 2013). The vertical bar represents standard error of the mean (n=4).

Based on the assumed age of stolons and tillers which is 50-70 days at the time of tagging, and on the results of the field observations of the turnover of white clover stolons and perennial ryegrass tillers, we therefore conclude that less than an average of 43% and 78% of the total number of white clover stolons and perennial ryegrass tillers in monocultures turnover per year, respectively. In grass-clover mixtures, fewer than 40% and 75% of the total white clover stolons and perennial ryegrass tillers turned-over in one year, respectively. The field observations confirmed the results of the previous studies of Sturite et al. (2007) and Bullock et al. (1994) as cited by Schneider et al. (2006) exhibiting that white clover stolons turnover slowly compared to perennial ryegrass tillers.

Although there appeared to have variations in turnover rates between plant organs of white clover and perennial ryegrass, the difference was found to be relatively small. In terms of stubble turnover, the leaves and petioles of white clover turned-over faster than the leaves of perennial ryegrass. However, white clover stolons turned-over slower than perennial ryegrass tillers. Although the leaves and petioles of white clover turnover quite faster than perennial ryegrass, the difference was counterbalanced by the slower turnover rate of white clover stolons than perennial ryegrass tillers. As noticed in the previous results and present observations, root turnover rates of both species were comparable. If we look at the figures in Table 1, the average turnover rate of white clover roots which is 1.26 $year^{-1}$ (0.59 – 13.52 $year^{-1}$) falls within the range of the root turnover rates of perennial ryegrass (0.70 – 1.90 $year^{-1}$ under defoliated and 1.0 $year^{-1}$ undefoliated tillers) obtained from various experiments. Based on these results, we concluded that replacement rate of mineral N taken up by both plants from the soil through the recurrent turnover of different plant organs was relatively similar.

3.2.3 Symbiotically fixed N_2 is not invested in the roots and stubbles of white clover

Many researchers believed that fixed N_2 is also allocated to the roots and stubbles of white clover and that inclusion of the amount of fixed N_2 in these tissues is necessary to reliably estimate the total N_2 fixation of white clover. Jørgensen and Ledgard (1997) reported that the amount of fixed N_2 in the roots and stubbles of white clover is estimated to be 70% of the total amount of fixed N_2 in its shoots. They recommended a correction factor of 1.7 to estimate the total N_2 fixation. This assumed figure is impossible and is very far from the assumptions of Høgh-Jensen and Kristensen (1995) and Høgh-Jensen et al. (2004) who also believed that stubbles and roots have contribution to the total N_2 fixation of white clover. In the study of Høgh-Jensen and Kristensen (1995) they assumed that 19-25% of total fixed N_2 of white clover is underestimated if only the shoot biomass is considered in the estimation. The result was derived from a comparative study of N difference method and ^{15}N dilution method to estimate N_2 fixation. In the most recent study of Høgh-Jensen et al. (2004), the estimation was improved by developing a Danish model to estimate N_2 fixation in grass-clover mixtures. They assumed that a constant proportion of 25% of the total fixed N_2 is invested in the roots and stubbles of white clover. Although lots of studies claimed that there is fixed N_2 invested in these plant organs, there are still doubts on this aspect because they were all assumptions and were not yet proven in the field.

These assumptions were proven invalid based on the results of the present field experiment and previous studies. The two important conditions set to confirm the investment of fixed N_2 in the roots and stubbles of white clover were successfully met in the present study. Nitrogen yields of the roots and stubbles of perennial ryegrass and white clover was found similar. Likewise, there was a relatively small difference in the overall turnover rates of different plant organs of white clover and perennial ryegrass. Thus, white clover and perennial ryegrass took up the same amount of mineral N from the soil during their regrowth period and replaced mineral N taken up from the soil at relatively the same period of time. Regardless of root morphology and rooting depth of perennial ryegrass and white clover (Chalk, 1998), perennial ryegrass takes up the same amount of mineral N from the soil as that of white clover. Although, both plants differ in root depth and characteristics, their roots occupy almost the same surface area per unit dry weight in the soil (Evans, 1977). Concentration of plant roots are mainly found at 0-10 cm, where most of the mineral N is present (Ledgard et al., 1985). In this case, both species shared the same amount of mineral N from the soil. Thus, the difference in the uptake of mineral N of perennial ryegrass and white clover is negligible. The finding contradicts to the idea that roots and nodules of white clover may have relatively high amount of fixed N_2 (Carranca et al., 1999). Roots and nodules certainly have fixed N_2 but the amount is very small.

The results of the present study and previous findings exhibited that replacement of mineral N taken up from the soil by perennial ryegrass and white clover plants through tissue turnover is relatively the same. The turnover rates of plant organs of white clover and perennial ryegrass have quite small difference. This means that both plant species returned on average the same amount of organic N to the soil annually. Recurrent deaths of perennial ryegrass stubbles and roots have important contribution in sustaining the growth of perennial ryegrass as a whole (Eason and Newman, 1990). The result of the present study is contrary to the finding of Gylfadóttir et al. (2007) from a field experiment where they reported that white clover contributes a higher proportion of N in the soil than the associated grass. In their study, they used different grass species (Poa pratensis) and that the response might be different than perennial ryegrass. The method used in determining the amount of N is different considering that the proportion of N from clover is derived from the total soil sample and that contribution of N from root exudation is taken into account. Root exudation is the release of root-derived organic and inorganic substances into the soil (Gylfadóttir et. al., 2007). Apart from that the field was applied with NPK fertilisers. If we consider root exudation in the overall N turnover from white clover, N turnover of white clover might be a bit higher than perennial ryegrass. However, the difference may be quite small and is assumed to have no significant increase in the annual N turnover of white clover than perennial ryegrass.

In organic grasslands, atmospheric N deposition is another important N source apart from biological N_2 fixation. N input from atmospheric N deposition directly goes the pool of mineral N in the soil. It supplements the amount of mineral N in the soil that could not be supplied alone by decomposition and mineralisation of plant organs and from root exudation (Elgersma and Hassink, 1997). Recent estimates show that the total atmospheric N deposition in the Netherlands mainly through wet deposition by rainfall is not higher than 10-15 kg N ha^{-1} year (Rashid, 2013; Van Eeekeren et al., 2010).

In the present experiment, perennial ryegrass and white clover in monoculture plots is assumed to take up mineral N that comes from decomposed and mineralised plant organs and root exudations (Elgersma and Hassink, 1997; Laidlaw et al., 1996; Paynel at al., 2001; Rasmussen et al., 2008) and atmospheric N deposition (Elgersma and Hassink, 1997). In grass-clover mixtures, the sources are the same. However, below-ground N transfer between white clover and perennial ryegrass also plays an important role (Høgh-Jensen and Schjoerring, 2000). When N from decomposed plant organs of perennial ryegrass and white clover and root exudates (from white clover) are mineralised, these mineralised N contribute to the pool of N in the soil. During plant uptake, portions of N supplied by both species can possibly be taken up by perennial ryegrass and vice versa. The general assumption about N transfer is that N transfer takes place only from white clover to associated grass (Høgh-Jensen, 2006; Paynel, et al., 2008). However, it is recently recognised that N can also move from grass to legume plants (Høgh-Jensen, 2006). Høgh-Jensen and Schjoerring (2000) reported based on ^{15}N leaf feeding technique to estimate N transfer reported that 50% of the total above-ground N of white clover was transferred from white clover to perennial ryegrass and 8% of the total above-ground N of perennial ryegrass was transferred from perennial ryegrass to white clover. In another study conducted by Haystead and Marriott (2008) N transfer from white clover to perennial ryegrass in the field is only 6% of the total grass nitrogen. Rasmussen et al. (2008) found that N transfer from white clover contributed 40% of N in perennial ryegrass and N transfer from perennial grass contributed 5% of the total N in clover. Thus, it is assumed in this study that transfers of N occur between white clover and perennial ryegrass because these plant species in grass-clover mixture shared the same pool of mineral N in the soil. Likewise, they take up mineral N from different N sources combined including atmospheric N deposition.

From our observations and analyses, it is tentatively concluded that there existed relatively small difference in the annual investment of and turnover rates of N in the roots and stubbles of white clover and perennial ryegrass. Thus, the experiment confirms that there is no net investment of fixed

N_2 in the roots and stubbles of white clover plants. It also confirms the robustness and correctness of the N difference method and implies incorrectness of the assumption of the Danish model. The present claim can be further verified with the use of ^{15}N dilution method.

3.2.4 Synthesis

Since it is important to clearly understand the biological processes derived from the experiment, an illustration of N dynamics in grass-clover mixture is reflected in Fig. 4. In a cut grass-clover mixture without N fertilisation, external inputs of N mainly come from symbiotic N_2 fixation by white clover and atmospheric N deposition (Breckle et. al., 2008; Bouwman et al., 2005; Parton et al., 1988). Once N_2 is fixed by the microorganisms found in the root nodules of white clover, fixed N_2 is converted to ammonium and assimilated in the above-ground tissues of legumes (Postgate, 1998). N from atmospheric deposition contributes directly to the pool of mineral N in the soil (soluble N compounds) for plant N uptake. As per result of the present experiment, it was confirmed that fixed N_2 is mainly assimilated in the shoot biomass and this is exported from the field when shoot biomass is harvested. With this, only the tissue turnover of perennial ryegrass and white clover roots and stubbles contributes to the soil N pool as internal source of N. On the one hand, white clover as a legume recycles N taken up from the soil through two different pathways: (1) decomposition and mineralisation of plant organs (stubbles and roots) and (2) root exudation. On the other hand, perennial ryegrass contributes N in terms of decomposition and mineralisation of plant organs. Both plants turnover relatively the same amount of organic N to the soil based on the result of the present experiment. Soil N pool is the combination of N from atmospheric deposition, and from the two previously mentioned N pathways contributed by white clover and perennial ryegrass plants. Perennial ryegrass and white clover take up relatively the same amount of N from the soil N pool for regrowth. Since soil N pool is a combination of different N sources including N from atmospheric N deposition, there is a possibility that nitrogen contributed by white clover in the soil can be taken up by perennial ryegrass and vice versa. During harvesting, plant N and fixed N_2 from white clover and N of perennial ryegrass biomass are exported from the field by harvesting the shoot biomass as an output of the system. Hence, N from symbiotic N_2 fixation contributes less to the recycling of N in the mown field due to N export.

Understanding the contribution of roots and stubbles of white clover in the estimation of symbiotic N_2 fixation is geared towards an improved estimation of N_2 fixation in grass-clover mixtures. The confirmation of the correctness of N difference method in the present study is hoped to contribute in the construction of nutrient budgets for grass-clover sward by correctly estimating symbiotic N_2 fixation.

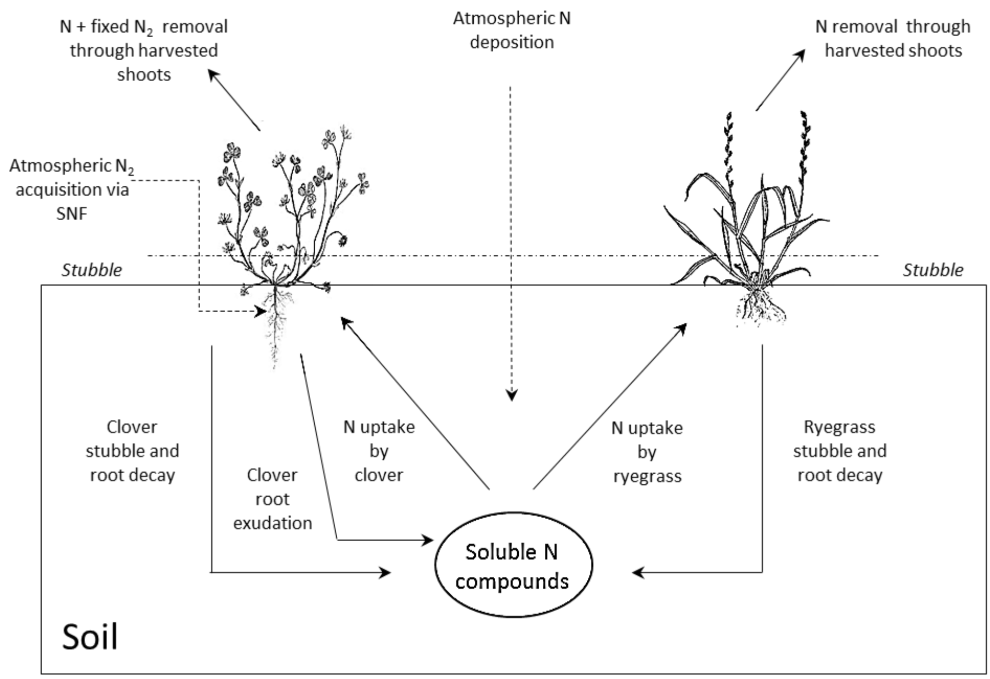

Fig. 4. Nitrogen dynamics in an organic grass-clover sward. Modified from: Paynel et al., (2008) and Stern (1993)

3.3 White clover cultivar Alice performed better than white clover cultivar Riesling

The seasonal performance of white clover cultivars Riesling and Alice was based on N yield and N_2 fixation of harvested shoots in monocultures and in mixtures. Harvested shoot dry matter yield performance was not considered in comparing the two cultivars. The experiment only selected representative plots for each treatment because of the heterogeneity of the field. Several weed species invaded each treatment fields. In this case, harvested shoot dry matter yield taken from small representative area is not that sufficient to be compared and subjected to statistical analysis. The total harvested shoot DM yield (> 4 cm) was only used as basis to compute N_2 fixation and N yield per ha (Table 2).

N yields of harvested shoots of white clover and perennial ryegrass in mixtures in August (late summer) and October (autumn), and white clover cultivars in monocultures in August were statistically similar. Harvested shoots of white clover cultivar Alice in monocultures had higher N values than white clover cultivar Riesling in October. In terms of apparent N_2 fixation, white clover cultivars in monocultures and in mixtures had statistically similar values for apparent N_2 fixation in August. However in October, white clover cultivar Alice in monocultures and in mixtures had statistically higher apparent N_2 fixation than white clover cultivar Riesling in monocultures and in mixtures (Table 3).

Table 2

Total harvested shoot DM yield (>4 cm) per cut (Mg ha^{-1})[1]

Treatments	August 2012	October 2012
Grass	2.17 (0.10)	1.31 (0.06)
Grass/clover Riesling	2.68 (0.14)	1.65 (0.05)
Grass/clover Alice	2.82 (0.16)	1.97 (0.10)
Clover Riesling	2.56 (0.10)	1.62 (0.09)
Clover Alice	2.58 (0.09)	1.76 (0.08)

[1]Data represent mean of four replicates ± s.e. in parenthesis.

Table 3

N yield and apparent N_2 fixation (kg ha^{-1}) from harvested shoot biomass (>4 cm)

Treatments	N yield (kg ha^{-1})		Apparent N_2 fixation (kg ha^{-1})	
	August	October	August	October
Grass	31 (1)a[1]	26 (2)a	-	-
Grass/clover Riesling	55 (4)b	47 (1)b	24 (4)a	20 (1)a
Grass/clover Alice	61 (5)bc	59 (3)b	30 (5)ab	32 (3)bc
Clover Riesling	67 (3)cd	52 (3)b	36 (3)bc	25 (3)ab
Clover Alice	72 (3)d	63 (3)c	41 (3)c	36 (3)c

[1]Different letters in the same column indicate significant differences (<0.05) between the treatments. Data represent mean of four replicates ± s.e. in parenthesis.

White clover cultivar Alice in monocultures and in mixtures generally had higher harvested shoot N yields and apparent N_2 fixation than white clover cultivar Riesling in August and October. The results of the present experiment confirms the findings of Andela (2010) where he also found that white clover cultivar Alice performed better than white clover cultivar Riesling in late summer and autumn in terms of N yield and apparent N_2 fixation. During the experimental period, white clover cultivar Alice both in monocultures and in mixtures was observed to have denser ground cover in the field than white clover cultivar Riesling in monocultures and in mixtures in both seasons.

3.4 N_2 fixation per Mg clover DM is inversely proportional to the proportion of white clover in the total DM

The average harvested shoot N yield of all treatments was plotted against white clover harvested shoot dry matter yield (Fig. 5A). Harvested shoot N yield linearly increased with the increase in white clover harvested shoot DM yield ($P < 0.05$). N yield per harvested shoot DM yield was relatively higher in August than October. Average value for harvested shoot N yield per Mg clover DM yield was 39 kg N (August) and 33 kg N (October). The difference in harvested shoot N yield between the two sampling dates slightly increased with the increase in white clover harvested shoot DM yield.

N_2 fixation linearly increased with the increase in white clover harvested shoot DM yield ($P < 0.05$). The average N_2 fixation was a bit higher in August than October. Average values of 38 and 34 kg fixed N_2 per Mg clover DM yield was found for August and October, respectively (Fig. 5B).

A quadratic relationship was found between clover proportion in the total dry matter and N_2 fixation (kg N ha^{-1}) (Fig. 5C). N_2 fixation seemed to reach its asymptotic value as the proportion of clover in the total dry matter in the sward reaches 100%.

Results of the present study agree with the findings of Van der Starre (2011) where she also found linear relationships of different variables that were also considered in the study. Average values ranging from 33 – 39 kg N per Mg clover DM was higher than the mean value of 22 kg N found by Van der Starre (2011) during spring and early summer. Likewise, mean values ranging from 34 – 38 kg N_2 fixated per Mg clover DM found in the present study was higher than values of N_2 fixation per Mg clover DM found by Van der Starre (2011) which is 22 kg N and by Andela (2010) which is 30 kg N. Comparing the results of the previous studies, the present findings showed increase in N yield per Mg clover DM by 11 to 17 kg N and N_2 fixation per Mg clover DM yield by 8 to 16 kg N.

Higher values of N and N_2 fixation per Mg clover DM compared to the previous studies may be due to the application of K fertiliser in the field. K is one of the most important macro-elements next to N and plays an important role in the many physiological functions of the plants. It is important in photosynthesis, protein synthesis (Sale and Campbell, 1986), activation of enzymes, stomatal closing and opening, etc. (Britto and Kronzucker, 2008). Blaser and Brady (1950) as cited by Parthipan and Kulasooriya (1989) noted that limited availability of K in the soil reduced the N_2 fixation. K application is positively related to the growth of shoots and roots, as well as N_2 fixation of legumes (Dietrich et al, 2001; Sangakkara et al., 1996). Relatively, a study by Parthipan and Kulasooriya (1989) showed that application of K fertiliser increased root growth, nodule number and nodule dry weight of winged bean (*Psophocarpus tetragonolobus).*

Variation on N yield and N_2 fixation per Mg clover DM in August and October can be explained by the response of white clover to the change in temperature. In October, the temperature is definitely lower than in August (Fig. 1). At low temperature, nodulation and N_2 fixation of white clover decreases (Bordeleau et al., 1994). White clover takes up most of the N from the soil (Nesheim and Boller, 1991) thus, fixes less N_2 from the atmosphere.

N_2 fixation per Mg harvested clover shoot DM linearly decreased with the increase in the proportion of clover in the total dry matter yield (P < 0.05) (Fig. 5D).

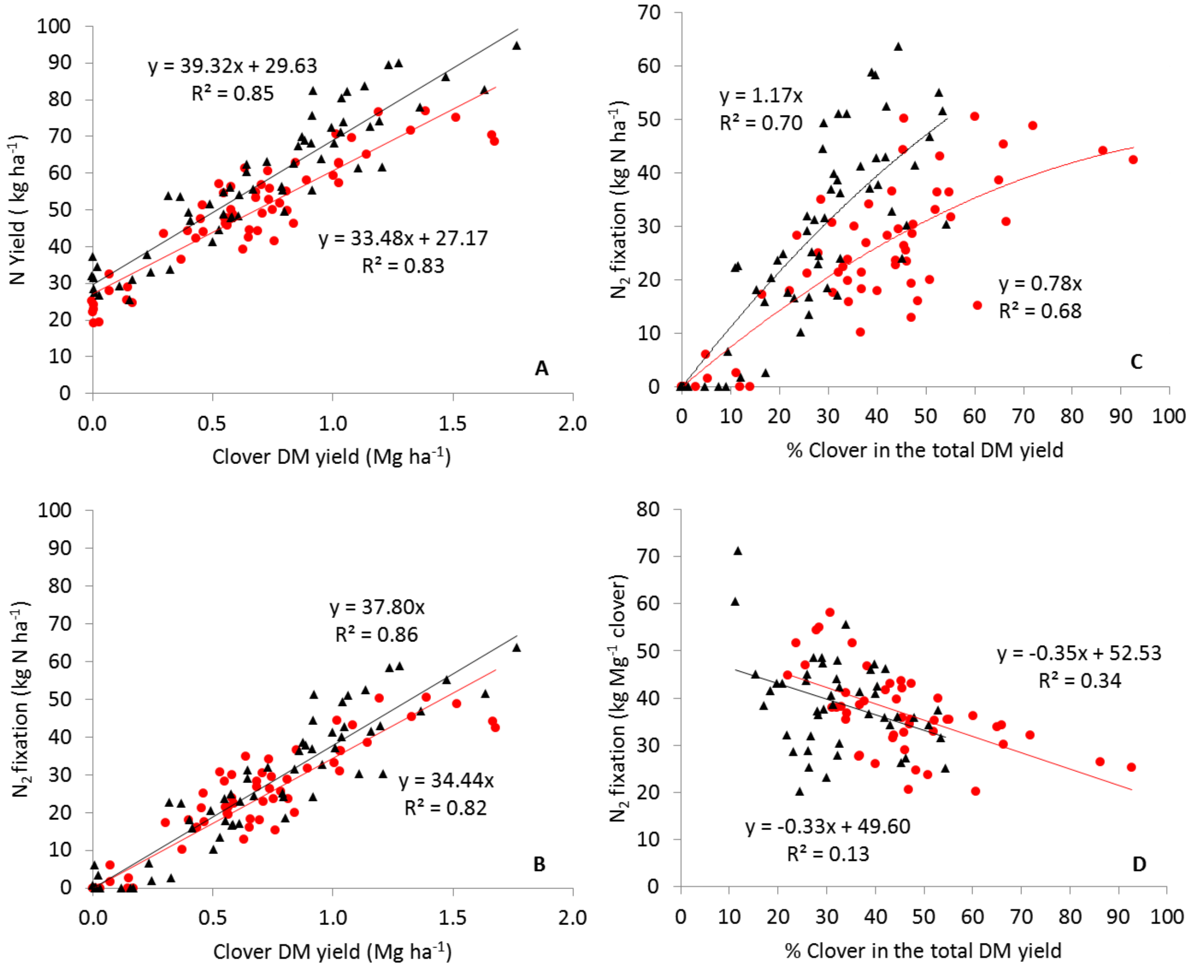

Fig. 5. Relations between harvested shoot total N yield and white clover harvested shoot DM (A); N_2 fixation and white clover harvested shoot DM (B); N_2 fixation and proportion of clover in harvested shoot total DM yield (C); and N_2 fixation and proportion of clover in harvested shoot total DM yield and harvested shoot N content (D) for August (▲) and October (●) cuts.

The decrease in N_2 fixation per Mg clover DM as the proportion of white clover in the total DM yield increases could be explained by the increasing amount of organic N turnover to the soil. When the proportion of white clover in the field increases the turnover of organic N to the soil also increases because the collective defoliation and turnover of leaves and petioles of white clover in the field becomes higher. Hence, soil with higher proportion of white clover are more fertile than with lower proportion of white clover. In this case, white clover relies more on mineral N from the soil rather than N_2 fixation at relatively more fertile soil.

Interestingly, the present finding agrees with the result of previous study by Van der Starre (2011). This implies that N_2 fixation per Mg clover DM increases as the proportion of clover in the grass-clover mixture decreases. In the present experiment, the average N_2 fixation per Mg clover DM is 34 – 38 kg at an average clover DM proportion of 53 to 75%. In the study of Van der Starre (2011) N_2 fixation per Mg clover DM is 22 kg at an average clover DM proportion of 70%.

It is interesting to note that at quite the same average proportion of clover with the previous study, the present experiment had higher value for average N_2 fixation. This suggests that K fertilizer application had significant effect in the increase of N_2 fixation per Mg clover DM.

3.5 The assumption of G. Oomen about the ratio between the white clover DM proportion and clover visual ground cover is correct.

The estimation of the presence of white clover was done by applying the two methods: visual estimation of clover visual ground cover (CVGC) and dry matter proportion method (DMPM). The result of the present study revealed that the ratio between the white clover DM proportion and the clover visual ground cover in August is 64% (R^2 = 0.79) and 78% in October (R^2 = 0.75) (Fig. 6). The result was on the average at the same level (2/3) as suggested by G. Oomen. Hence, G. Oomen's assumption is correct.

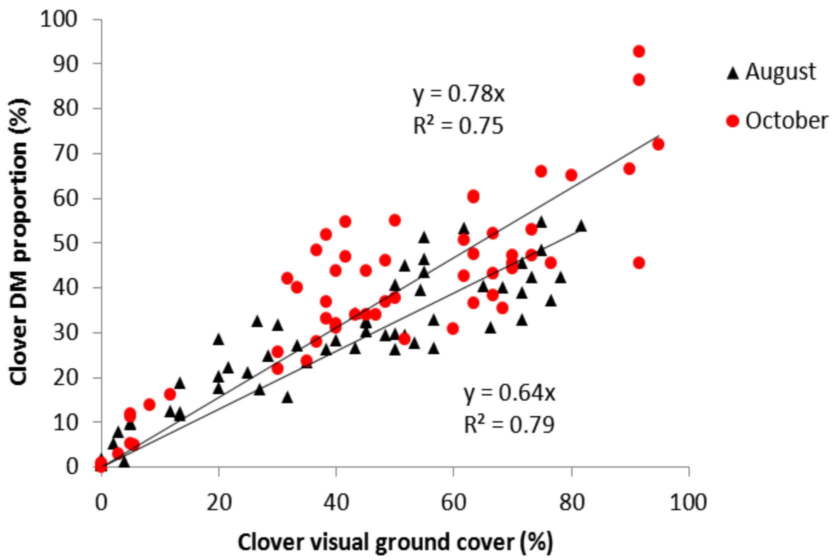

Fig. 6. Relation of clover visual ground cover and clover dry matter proportion during August (▲) and October (●) cuts.

Variation in the percentage of dry matter per visual clover ground cover in season can be explained by the height and density of white clover (Van der Starre, 2011). During summer, white clover performed well than autumn. White clover during this period had higher petioles and larger leaves. In this case, perennial ryegrass growing with it was hardly visible. The leaves of white clover cover most of the perennial ryegrass that grows beneath or along with it because perennial ryegrass normally has upright leaves, which makes estimation difficult. Thus, the dry matter proportion of white clover was overestimated. In autumn, the petioles of white clover were shorter and the leaves were smaller. Perennial ryegrass that grow in the mixture with white clover were more visible because small clover leaves have less ground covering capacity in autumn. White clover produces less biomass during this period. In this case, the visual ground cover estimation was better than in August because of nearly equal visibility of white clover and perennial ryegrass plants in the field.

4. Conclusions

Based on the result of the present study, N yields and turnover rates of the roots and stubbles of white clover and perennial ryegrass are quite similar. This means that uptake of mineral N from and turnover of organic N to the soil are about the same. We therefore, tentatively concluded that there existed relatively small difference in the annual investment of and turnover rates of N in the roots and stubbles of white clover and perennial ryegrass. Thus, the experiment confirms that there is no net investment of fixed N_2 in the roots and stubbles of white clover plants. It also confirms the robustness and correctness of the N difference method and implies the incorrectness of the assumption of the Danish model to estimate N_2 fixation. Hence, the inclusion of the assumed N_2 invested in the roots and stubbles in estimating N_2 fixation overestimates N_2 fixation as in the case of the Danish model.

White clover cultivar Alice in monocultures and in mixtures performed better than white clover Riesling in August (late summer) and October (autumn). The result is consistent with that of the previous finding of Andela (2010).

The relationship of N_2 fixation per Mg clover DM is linear. N_2 fixation per Mg clover DM decreases as the proportion of white clover in grass-clover mixture increases.

The assumption of G. Oomen about the ratio between white clover DM proportion and clover visual ground cover is correct.

To further validate the result of the study, it is recommended to conduct long-term and in-depth study on the investment of fixed N_2 and continue similar assessment of turnover rate of white clover and perennial ryegrass roots and stubbles including root exudation in the existing sward. It is also suggested to use ^{15}N dilution method to verify the investment of fixed N_2 allocated to the roots and stubbles of white clover. Explore doable methodology to collect empirical data on turnover rate of the roots applicable to the existing grass-clover field. Further investigate the effect of potassium fertiliser application in organic grass-clover mixture in terms of N_2 fixation per Mg clover DM.

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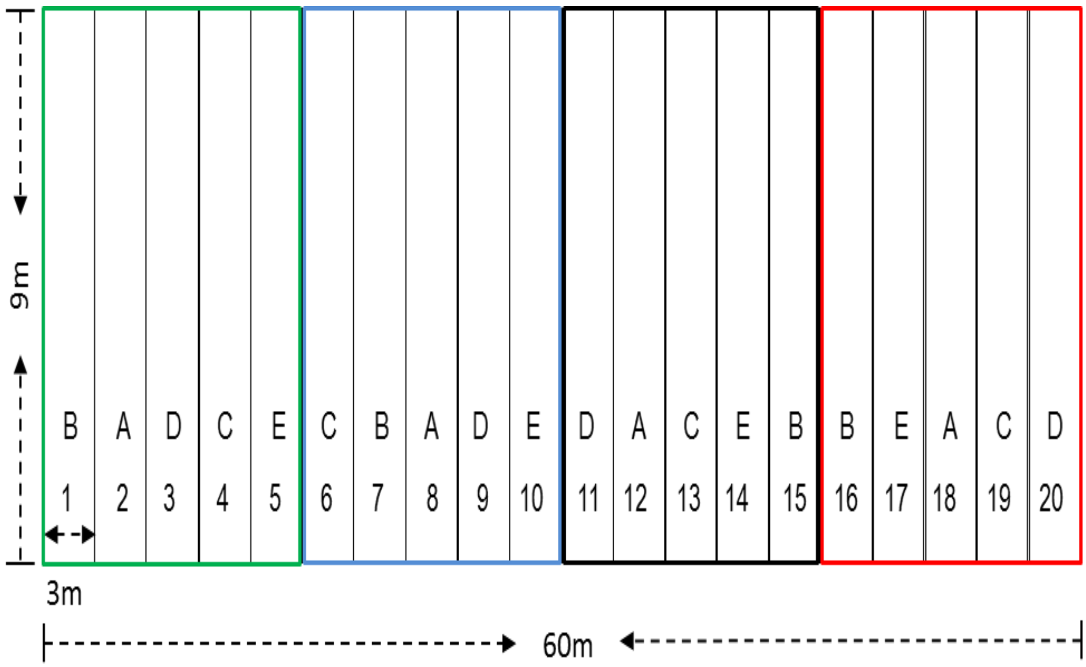
Appendices

Appendix I. Experimental design and lay-out



Legend:

A	Perennial ryegrass monoculture
B	Perennial ryegrass – white clover cultivar Riesling mixture
C	Perennial ryegrass – white clover cultivar Alice mixture
D	White clover cultivar Riesling monoculture
E	White clover cultivar Alice monoculture

Appendix II. Weather data

Daily weather data during the experimental period (July 2012 – June 2013)

Year	2012																	
Month	July			August			September			October			November			December		
Day	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall
	Min	Max	sum	Min	Max	sum	Min	Max	sum	Min	Max	sum	Min	Max	sum	Min	Max	sum
	°C	°C	mm	°C	°C	mm	°C	°C	mm	°C	°C	mm	°C	°C	mm	°C	°C	mm
1	9.0	19.4	0.8	12.0	27.2	6.8	7.1	19.0	0.0	6.6	18.4	0.4	5.4	10.3	2.8	1.2	4.7	4.3
2	8.3	21.0	0.0	13.0	21.5	0.0	9.1	19.6	0.0	12.4	16.4	0.0	4.1	10.1	1.0	-2.5	6.0	1.2
3	11.7	24.6	0.0	12.6	22.3	0.2	9.9	22.3	0.0	10.1	15.3	18.6	3.6	8.7	3.1	-0.9	6.2	5.2
4	15.9	26.6	0.0	13.6	23.6	0.0	8.9	23.9	0.0	9.4	14.6	12.6	0.3	9.2	3.1	-0.3	6.4	6.7
5	16.9	27.4	0.2	12.9	24.1	1.2	11.2	20.0	0.0	9.0	16.7	11.0	2.3	9.9	2.1	-1.3	3.5	0.6
6	13.9	22.9	0.0	14.6	21.0	2.6	9.8	18.5	0.0	2.4	14.9	9.6	-0.6	10.1	4.9	-3.2	2.5	1.3
7	12.6	24.0	0.0	13.7	19.6	2.2	10.4	22.6	0.0	1.1	14.6	0.0	9.2	10.6	1.3	-3.2	1.4	10.0
8	15.2	20.9	13.8	12.5	21.1	0.0	10.5	25.2	0.0	0.5	13.9	0.0	8.8	10.9	0.3	-13.1	1.4	0.4
9	13.8	19.9	0.2	9.5	21.4	0.0	7.7	27.8	0.0	0.6	13.8	0.0	7.1	10.4	1.7	1.2	7.4	11.7
10	11.9	18.7	0.0	7.0	21.4	0.0	13.0	23.2	0.0	1.1	14.3	0.0	7.6	11.6	1.7	-0.8	5.4	0.0
11	12.0	18.3	4.6	7.4	20.9	0.0	10.4	17.7	1.6	0.4	14.6	0.2	5.9	11.3	0.0	-3.5	2.3	0.0
12	11.7	18.2	3.8	13.6	24.0	0.0	8.8	16.7	1.8	6.4	14.5	13.2	4.6	8.1	0.0	-1.9	2.8	4.3
13	11.8	19.5	13.6	14.7	22.9	6.0	9.0	17.4	0.0	6.9	11.8	8.8	6.5	9.3	1.0	-2.5	0.2	0.0
14	12.2	17.3	8.6	14.0	26.2	3.6	10.3	16.7	1.2	5.2	12.2	0.8	0.0	11.9	0.0	-0.2	9.6	8.2
15	12.2	18.5	0.0	13.0	28.7	0.8	7.3	18.5	0.0	6.8	13.3	0.2	-0.2	5.8	0.0	6.7	9.5	0.0
16	12.6	17.5	23.6	13.6	23.4	0.0	7.5	19.7	0.0	8.3	14.0	1.0	0.6	4.9	0.0	3.8	8.2	2.9
17	15.2	20.0	1.6	13.1	26.9	0.0	13.1	19.8	0.6	5.9	15.5	4.6	0.5	8.0	0.4	2.8	7.4	5.8
18	14.9	20.6	0.8	15.5	31.9	0.0	7.7	17.6	1.6	13.5	19.5	0.0	-1.4	9.2	5.3	3.6	6.4	3.0
19	11.3	18.4	10.4	19.2	32.8	0.0	5.1	14.7	4.2	13.7	21.3	0.0	-1.6	9.9	0.1	3.7	6.0	0.0
20	9.2	18.9	0.0	15.6	26.9	0.0	4.9	15.3	0.0	13.3	17.0	0.0	4.3	11.7	0.0	1.9	4.2	5.2
21	6.5	17.7	0.0	15.6	26.0	0.0	7.3	15.9	0.0	10.5	16.1	0.0	3.4	10.2	0.2	2.2	7.1	2.6
22	5.6	20.8	0.0	12.8	21.7	0.0	2.2	15.4	0.0	9.4	22.4	0.0	4.4	11.1	0.0	6.3	8.3	13.8

23	10.3	24.9	0.0	11.9	22.2	0.0	0.7	13.8	9.4	10.1	19.4	0.0	3.2	8.2	3.1	8.3	13.2	20.0
24	10.3	28.0	0.0	13.9	23.3	1.0	9.7	21.5	11.0	9.4	12.9	0.0	3.6	8.9	3.6	7.3	12.4	2.0
25	12.6	29.2	0.0	14.3	21.9	0.2	11.1	16.5	2.8	7.6	13.0	0.0	6.6	13.3	0.4	7.4	10.3	17.2
26	13.7	27.8	0.0	10.7	19.2	25.6	9.2	15.7	1.2	-0.6	8.6	0.0	3.8	9.7	0.0	5.8	9.3	5.2
27	16.2	28.6	0.4	9.7	21.8	0.0	9.2	15.1	9.0	-2.6	8.2	0.0	5.1	8.9	1.0	4.8	9.7	1.4
28	15.0	22.8	19.2	11.3	22.6	0.6	9.2	16.9	0.0	-4.1	9.9	0.0	2.9	8.3	0.0	0.7	9.8	3.8
29	13.0	20.3	12.4	9.9	24.3	0.0	8.5	16.0	1.4	4.5	8.6	10.6	-0.3	6.4	0.0	8.7	11.4	1.0
30	11.2	19.2	10.8	11.6	21.1	6.2	6.0	16.5	0.0	2.6	9.1	3.0	-4.8	5.4	0.3	5.4	9.1	2.6
31	10.7	17.8	10.4	8.3	16.5	5.0	-	-	-	1.6	12.7	0.0	-	-	-	8.1	10.1	4.6

Daily weather data during the experimental period (July 2012 – June 2013) *(continued)*

Year	2013																	
Month	January			February			March			April			May			June		
Day	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall	Temperature		Rainfall
	Min	Max	sum	Min	Max	sum	Min	Max	sum	Min	Max	sum	Min	Max	sum	Min	Max	sum
	°C	°C	mm	°C	°C	mm	°C	°C	mm	°C	°C	mm	°C	°C	mm	°C	°C	mm
1	3.2	8.5	13.2	1.2	7.1	5.8	2.0	4.8	0.3	-5.2	7.3	0.0	2.0	17.8	0.0	6.7	15.3	0.0
2	3.2	7.9	0.2	0.2	5.6	3.5	1.4	4.1	0.0	-3.4	8.5	0.0	5.1	16.9	0.0	3.5	17.1	0.0
3	7.0	10.7	1.0	1.3	6.9	4.1	-1.7	6.1	0.5	-2.2	7.9	0.0	3.3	19.2	0.0	3.0	15.8	0.0
4	8.2	9.9	0.0	6.2	10.0	2.0	-4.3	10.8	0.0	0.7	4.8	0.0	3.3	17.9	0.0	4.3	22.2	0.0
5	7.1	8.6	0.0	0.7	6.5	3.8	-0.7	15.9	0.0	1.6	7.0	0.0	5.5	19.8	0.0	6.6	23.5	0.0
6	6.6	9.6	0.0	0.2	4.3	9.4	3.5	16.9	0.0	-2.2	9.4	0.0	4.0	24.6	0.0	8.5	25.9	0.0
7	6.1	8.3	0.0	-0.5	4.0	2.7	5.2	11.0	0.4	-6.0	10.2	0.0	6.9	23.2	1.3	8.9	25.8	0.0
8	5.7	7.9	0.0	-2.7	3.4	0.8	6.1	13.3	2.8	0.1	10.8	0.0	13.4	22.0	11.9	9.5	22.4	0.0
9	5.3	7.1	4.8	-4.5	0.8	1.6	2.5	6.3	27.0	-1.5	10.7	1.0	6.0	16.8	0.1	-	-	-
10	-1.0	7.5	1.4	-2.4	0.9	1.3	-2.1	2.8	3.6	0.1	11.7	0.1	7.5	14.6	0.0	-	-	-
11	-3.7	3.6	0.0	-3.9	0.7	0.0	-3.0	-1.4	0.0	4.9	11.4	5.1	6.2	13.7	6.9	-	-	-
12	-4.0	2.3	0.0	-5.8	0.0	0.0	-7.1	0.4	0.0	4.5	14.6	3.2	6.6	13.9	3.7	-	-	-

13	-5.7	0.4	0.0	-5.4	2.3	0.2	-8.3	4.2	0.0	6.0	14.5	0.3	7.7	14.1	3.3	-	-	-
14	-6.8	-1.3	0.0	-2.8	0.8	4.4	-6.3	3.5	0.3	11.1	20.7	0.0	6.9	12.8	3.8	-	-	-
15	-5.9	-2.2	0.0	0.7	6.2	0.2	-5.6	2.7	1.6	4.7	16.0	1.8	6.9	17.2	0.3	-	-	-
16	-12.5	-4.8	0.0	1.4	7.1	0.6	1.9	7.5	0.4	3.4	17.5	0.0	6.7	12.8	9.5	-	-	-
17	-7.1	-3.0	1.4	-2.0	6.0	0.0	1.6	9.0	2.0	8.4	20.7	0.0	8.5	10.7	3.6	-	-	-
18	-3.3	-0.4	0.2	-1.7	7.0	0.0	1.5	11.6	0.2	8.1	17.1	0.0	1.9	12.2	0.0	-	-	-
19	-5.4	-2.4	0.0	-1.3	5.3	0.1	0.8	6.1	0.2	2.1	12.3	1.7	0.8	16.4	0.0	-	-	-
20	-5.9	-2.4	0.0	-4.1	2.3	0.0	-0.7	2.3	0.2	-1.8	12.1	0.0	8.6	13.2	0.2	-	-	-
21	-3.0	-1.2	1.1	-5.0	1.3	0.0	-3.4	6.0	0.0	-1.2	14.6	0.0	8.0	12.8	4.9	-	-	-
22	-11.9	-1.4	0.0	-5.7	0.8	0.0	-3.9	3.8	0.0	-2.1	14.7	0.0	3.7	12.3	0.2	-	-	-
23	-12.2	-3.0	0.0	-5.8	0.7	0.0	-2.8	1.8	0.0	4.5	14.7	1.5	2.3	9.4	3.8	-	-	-
24	-7.7	-0.7	0.0	-1.3	0.9	1.7	-3.9	2.8	0.0	4.3	20.4	0.0	2.0	13.4	0.0	-	-	-
25	-12.3	-2.2	0.0	0.7	2.5	0.2	-2.9	4.5	0.0	9.4	22.9	0.0	1.4	14.0	4.5	-	-	-
26	-4.5	1.3	0.3	0.8	5.7	0.0	-3.8	5.0	0.0	4.0	11.9	7.1	6.4	13.2	0.7	-	-	-
27	1.2	5.4	5.2	0.3	1.9	0.0	-4.2	5.8	0.0	0.3	11.2	0.0	6.3	19.6	0.0	-	-	-
28	1.4	7.2	3.2	-1.0	4.3	0.0	-4.3	3.3	0.0	-3.0	12.3	0.0	7.7	21.5	0.0	-	-	-
29	7.2	12.9	7.0	-	-	-	-5.0	2.2	0.0	2.8	12.8	1.2	10.5	12.4	0.1	-	-	-
30	7.1	13.6	7.6	-	-	-	-3.9	4.1	0.0	2.6	14.3	0.0	9.8	16.4	0.0	-	-	-
31	6.4	9.5	1.4	-	-	-	-5.9	3.8	0.0	0.0	0.0	0.0	11.2	21.0	0.0	-	-	-

Appendix III. Experimental Data

Data on stubble and root biomass (before defoliation height of 4 cm)

November 2011

Block	Plot	Code	Stubble biomass			Root biomass			Total Root & Stubble N yield (kg ha^{-1})
			DM yield (Mg ha^{-1})	N content (g 100^{-1} g)	N yield (kg ha^{-1})	DM yield (Mg ha^{-1})	N content (g 100^{-1} g)	N yield (kg ha^{-1})	
1	1	a	1.47	2.32	34.20	9.50	1.29	122.52	156.72
1	1	b	1.03	2.32	23.92	3.50	1.29	45.21	69.13
1	1	c	0.75	2.32	17.33	3.51	1.29	45.24	62.57
1	2	a	1.73	2.03	35.12	4.44	1.00	44.43	79.55
1	2	b	0.48	2.03	9.67	10.42	1.00	104.21	113.88
1	2	c	1.49	2.03	30.18	7.45	1.00	74.47	104.65
1	3	a	1.02	3.52	35.86	1.28	2.43	31.07	66.93
1	3	b	0.92	3.52	32.26	2.21	2.43	53.75	86.01
1	3	c	1.10	3.52	38.63	2.45	2.43	59.59	98.22
1	4	a	1.31	2.48	32.41	2.81	1.53	43.06	75.47
1	4	b	1.12	2.48	27.88	1.72	1.53	26.26	54.14
1	4	c	1.38	2.48	34.16	3.24	1.53	49.64	83.80
1	5	a	1.51	3.80	57.21	2.87	2.40	68.94	126.15
1	5	b	0.95	3.80	35.93	3.12	2.40	74.96	110.89
1	5	c	1.11	3.80	42.27	1.87	2.40	44.98	87.25
2	6	a	1.12	3.06	34.27	4.60	1.59	73.17	107.44
2	6	b	1.09	3.06	33.47	1.76	1.59	27.95	61.42
2	6	c	0.77	3.06	23.62	1.11	1.59	17.59	41.21
2	7	a	0.66	2.32	15.41	0.85	1.65	13.96	29.37
2	7	b	0.64	2.32	14.75	1.44	1.65	23.72	38.47
2	7	c	1.98	2.32	45.85	4.66	1.65	76.97	122.82
2	8	a	1.08	1.87	20.17	3.92	1.00	39.17	59.34
2	8	b	2.06	1.87	38.52	4.65	1.00	46.45	84.97
2	8	c	1.73	1.87	32.41	3.73	1.00	37.34	69.75
2	9	a	1.38	3.98	55.11	2.34	2.55	59.62	114.73
2	9	b	0.62	3.98	24.56	2.07	2.55	52.69	77.25
2	9	c	1.59	3.98	63.34	2.28	2.55	58.07	121.41
2	10	a	0.69	3.98	27.33	1.68	2.30	38.58	65.91
2	10	b	1.02	3.98	40.55	2.32	2.30	53.37	93.92
2	10	c	1.16	3.98	46.17	2.57	2.30	59.03	105.20
3	11	a	0.75	3.76	28.18	2.35	2.34	55.09	83.27
3	11	b	0.93	3.76	34.88	1.68	2.34	39.36	74.24
3	11	c	0.79	3.76	29.85	2.06	2.34	48.20	78.05
3	12	a	2.07	1.96	40.49	5.53	1.13	62.54	103.03
3	12	b	0.69	1.96	13.57	4.98	1.13	56.32	69.89

3	12	c	1.39	1.96	27.16	4.91	1.13	55.49	82.65
3	13	a	1.92	2.31	44.34	4.04	1.36	54.93	99.27
3	13	b	0.86	2.31	19.83	2.10	1.36	28.58	48.41
3	13	c	1.12	2.31	25.81	2.69	1.36	36.65	62.46
3	14	a	0.86	3.93	33.76	1.77	2.34	41.35	75.11
3	14	b	1.05	3.93	41.20	2.65	2.34	61.98	103.18
3	14	c	0.78	3.93	30.49	2.47	2.34	57.73	88.22
3	15	a	0.95	2.67	25.37	1.27	1.43	18.11	43.48
3	15	b	1.60	2.67	42.72	4.82	1.43	68.94	111.66
3	15	c	1.38	2.67	36.91	5.07	1.43	72.46	109.37
4	16	a	0.89	2.59	23.08	2.51	1.50	37.67	60.75
4	16	b	1.28	2.59	33.06	3.53	1.50	52.91	85.97
4	16	c	1.25	2.59	32.39	2.66	1.50	39.90	72.29
4	17	a	0.63	4.44	28.12	2.60	2.27	59.00	87.12
4	17	b	0.92	4.44	40.95	3.38	2.27	76.66	117.61
4	17	c	0.73	4.44	32.49	2.35	2.27	53.31	85.80
4	18	a	0.99	2.01	19.99	4.27	1.04	44.38	64.37
4	18	b	1.55	2.01	31.09	6.76	1.04	70.35	101.44
4	18	c	2.15	2.01	43.30	6.04	1.04	62.87	106.17
4	19	a	1.62	2.69	43.48	4.77	1.56	74.46	117.94
4	19	b	1.25	2.69	33.72	2.77	1.56	43.26	76.98
4	19	c	1.12	2.69	30.20	2.53	1.56	39.42	69.62
4	20	a	0.83	4.17	34.77	1.58	2.57	40.52	75.29
4	20	b	0.93	4.17	38.87	2.69	2.57	69.19	108.06
4	20	c	1.06	4.17	44.10	1.74	2.57	44.75	88.85

Data on stubble and root biomass (below defoliation height of 4 cm)

August 2012

Block	**Plot**	**Code**	**Stubble biomass**			**Root biomass**			**Total Root & Stubble N yield (kg ha^{-1})**
			DM yield (Mg ha^{-1})	**N content (g 100^{-1} g)**	**N yield (kg ha^{-1})**	**DM yield (Mg ha^{-1})**	**N content (g 100^{-1} g)**	**N yield (kg ha^{-1})**	
1	1	a	0.85	1.70	14.52	3.62	1.01	36.48	51.00
1	1	b	1.45	1.70	24.65	6.14	1.01	61.94	86.59
1	1	c	1.83	1.70	31.07	7.28	1.01	73.36	104.43
1	2	a	2.17	1.08	23.31	10.40	0.76	78.74	102.05
1	2	b	2.25	1.08	24.17	10.24	0.76	77.54	101.71
1	2	c	1.31	1.08	14.11	6.40	0.76	48.48	62.59
1	3	a	1.09	3.01	32.89	1.37	2.01	27.50	60.39
1	3	b	1.51	3.01	45.45	1.97	2.01	39.46	84.91
1	3	c	1.69	3.01	50.84	1.69	2.01	33.88	84.72
1	4	a	2.58	1.69	43.78	5.84	1.04	60.83	104.61
1	4	b	3.02	1.69	51.19	7.53	1.04	78.42	129.61
1	4	c	2.54	1.69	43.10	4.71	1.04	49.04	92.14
1	5	a	1.33	2.93	39.01	1.57	2.00	31.39	70.40
1	5	b	1.55	2.93	45.42	2.54	2.00	50.86	96.28

1	5	c	2.66	2.93	78.03	3.56	2.00	71.13	149.16
2	6	a	1.59	2.46	39.20	2.78	1.06	29.41	68.61
2	6	b	3.62	2.46	89.18	6.72	1.06	71.00	160.18
2	6	c	1.47	2.46	36.26	4.47	1.06	47.26	83.52
2	7	a	1.81	2.05	37.03	3.98	0.97	38.72	75.75
2	7	b	1.49	2.05	30.52	4.97	0.97	48.40	78.92
2	7	c	2.11	2.05	43.14	9.56	0.97	93.12	136.26
2	8	a	1.99	1.50	29.86	9.15	0.78	70.88	100.74
2	8	b	2.03	1.50	30.46	9.84	0.78	76.27	106.73
2	8	c	2.13	1.50	31.95	9.76	0.78	75.66	107.61
2	9	a	1.61	2.94	47.28	1.93	2.06	39.70	86.98
2	9	b	1.85	2.94	54.28	1.97	2.06	40.51	94.79
2	9	c	2.43	2.94	71.21	2.66	2.06	54.84	126.05
2	10	a	1.83	3.09	56.45	1.41	1.80	25.40	81.85
2	10	b	2.43	3.09	74.86	3.08	1.80	55.45	130.31
2	10	c	1.59	3.09	49.09	1.97	1.80	35.41	84.50
3	11	a	0.85	2.53	21.59	3.10	1.84	57.03	78.62
3	11	b	3.72	2.53	93.88	2.72	1.84	50.08	143.96
3	11	c	1.15	2.53	29.12	1.53	1.84	28.15	57.27
3	12	a	1.77	1.49	26.40	10.42	0.73	76.01	102.41
3	12	b	1.09	1.49	16.32	7.22	0.73	52.66	68.98
3	12	c	1.79	1.49	26.70	10.64	0.73	77.61	104.31
3	13	a	4.41	1.55	68.48	6.36	1.00	63.31	131.79
3	13	b	1.13	1.55	17.58	6.30	1.00	62.72	80.30
3	13	c	0.91	1.55	14.19	4.27	1.00	42.54	56.73
3	14	a	1.27	1.61	20.53	2.23	1.77	39.33	59.86
3	14	b	5.96	1.61	96.24	2.52	1.77	44.59	140.83
3	14	c	0.91	1.61	14.76	2.39	1.77	42.13	56.89
3	15	a	1.27	2.50	31.82	9.15	0.99	90.27	122.09
3	15	b	0.89	2.50	22.37	3.14	0.99	31.01	53.38
3	15	c	1.67	2.50	41.76	9.34	0.99	92.24	134.00
4	16	a	1.81	2.51	45.47	4.51	1.04	47.08	92.55
4	16	b	1.31	2.51	32.98	7.10	1.04	74.05	107.03
4	16	c	1.81	2.51	45.47	4.23	1.04	44.18	89.65
4	17	a	0.56	2.63	14.62	2.09	1.69	35.30	49.92
4	17	b	1.59	2.63	41.78	1.75	1.69	29.59	71.37
4	17	c	1.93	2.63	50.66	2.47	1.69	41.69	92.35
4	18	a	1.89	1.41	26.62	11.73	0.73	85.84	112.46
4	18	b	1.61	1.41	22.70	11.81	0.73	86.42	109.12
4	18	c	3.24	1.41	45.67	16.18	0.73	118.42	164.09
4	19	a	2.60	1.86	48.39	6.50	1.02	66.05	114.44
4	19	b	1.69	1.86	31.40	7.12	1.02	72.31	103.71
4	19	c	2.07	1.86	38.42	5.07	1.02	51.51	89.93
4	20	a	1.05	2.84	29.89	3.28	1.63	53.35	83.24
4	20	b	1.39	2.84	39.47	2.62	1.63	42.68	82.15
4	20	c	1.09	2.84	31.01	2.03	1.63	32.98	63.99

Data on stubble and root biomass (below defoliation height of 4 cm)

October 2012 (including the average root and stubble N from the three sampling dates)

Block	Plot	Code	Stubble biomass			Root biomass			Total Root & Stubble N yield (kg ha^{-1})	Average Root & Stubble N Yield from 3 cuts (kg ha^{-1})
			DM yield (Mg ha^{-1})	N content (g 100^{-1} g)	N yield (kg ha^{-1})	DM yield (Mg ha^{-1})	N content (g 100^{-1} g)	N yield (kg ha^{-1})		
1	1	a	0.60	2.62	15.60	8.65	1.05	90.70	106.30	104.68
1	1	b	1.75	2.62	45.77	5.59	1.05	58.59	104.36	86.69
1	1	c	1.75	2.62	45.77	12.98	1.05	136.16	181.93	116.31
1	2	a	2.64	1.47	38.74	10.76	0.81	86.63	125.37	102.32
1	2	b	0.64	1.47	9.32	7.46	0.81	60.05	69.37	94.98
1	2	c	0.42	1.47	6.12	9.34	0.81	75.26	81.38	82.87
1	3	a	0.97	3.49	34.00	2.33	2.26	52.57	86.57	71.30
1	3	b	1.47	3.49	51.35	2.39	2.26	53.92	105.27	92.07
1	3	c	1.79	3.49	62.45	3.34	2.26	75.49	137.94	106.96
1	4	a	2.45	2.61	63.78	7.69	1.18	90.78	154.56	111.54
1	4	b	1.67	2.61	43.55	5.29	1.18	62.39	105.94	96.56
1	4	c	1.97	2.61	51.33	5.63	1.18	66.38	117.71	97.88
1	5	a	1.45	3.51	50.89	1.79	2.48	44.42	95.31	97.29
1	5	b	1.69	3.51	59.25	2.29	2.48	56.76	116.01	107.73
1	5	c	1.67	3.51	58.56	3.70	2.48	91.81	150.37	128.92
2	6	a	1.35	3.15	42.63	6.68	2.44	163.24	205.87	127.31
2	6	b	1.07	3.15	33.85	6.76	2.44	165.19	199.04	140.21
2	6	c	2.03	3.15	63.94	8.59	2.44	209.88	273.82	132.86
2	7	a	1.61	2.74	44.08	6.46	1.24	80.40	124.48	76.54
2	7	b	1.93	2.74	52.79	8.17	1.24	101.68	154.47	90.62
2	7	c	1.35	2.74	37.01	9.82	1.24	122.21	159.22	139.43
2	8	a	0.85	1.98	16.96	10.20	0.72	73.48	90.44	83.51
2	8	b	0.68	1.98	13.41	12.64	0.72	91.10	104.51	98.74

2	8	c	0.85	1.98	16.96	16.90	0.72	121.76	138.72	105.36
2	9	a	1.93	3.64	70.10	1.83	2.62	47.87	117.97	106.56
2	9	b	1.31	3.64	47.70	2.29	2.62	59.84	107.54	93.20
2	9	c	1.57	3.64	57.09	2.50	2.62	65.57	122.66	123.37
2	10	a	1.09	3.50	38.23	3.80	2.16	81.84	120.07	89.28
2	10	b	1.53	3.50	53.53	2.92	2.16	62.99	116.52	113.58
2	10	c	1.45	3.50	50.75	2.13	2.16	45.85	96.60	95.44
3	11	a	1.99	3.15	62.68	3.24	2.24	72.46	135.14	99.01
3	11	b	1.19	3.15	37.61	3.42	2.24	76.46	114.07	110.76
3	11	c	0.78	3.15	24.45	3.40	2.24	76.02	100.47	78.59
3	12	a	0.74	1.76	12.95	11.17	0.73	81.31	94.26	99.90
3	12	b	1.35	1.76	23.79	9.50	0.73	69.16	92.95	77.27
3	12	c	1.13	1.76	19.94	10.50	0.73	76.39	96.33	94.43
3	13	a	1.53	2.51	38.38	5.27	1.24	65.07	103.45	111.50
3	13	b	2.68	2.51	67.29	6.48	1.24	80.05	147.34	92.02
3	13	c	1.67	2.51	41.87	8.45	1.24	104.36	146.23	88.47
3	14	a	1.01	3.33	33.79	2.13	2.23	47.39	81.18	72.05
3	14	b	1.41	3.33	47.04	1.87	2.23	41.63	88.67	110.90
3	14	c	2.47	3.33	82.15	5.37	2.23	119.57	201.72	115.61
3	15	a	1.59	2.63	41.76	6.60	1.38	91.28	133.04	99.54
3	15	b	1.21	2.63	31.84	4.53	1.38	62.69	94.53	86.52
3	15	c	2.60	2.63	68.38	8.01	1.38	110.80	179.18	140.85
4	16	a	2.09	2.65	55.40	9.07	0.95	86.15	141.55	98.29
4	16	b	1.77	2.65	46.96	11.09	0.95	105.42	152.38	115.13
4	16	c	2.27	2.65	60.15	11.13	0.95	105.80	165.95	109.30
4	17	a	1.39	2.99	41.64	4.04	1.95	78.82	120.46	85.84
4	17	b	0.58	2.99	17.25	3.64	1.95	71.05	88.30	92.43
4	17	c	2.84	2.99	85.07	4.85	1.95	94.74	179.81	119.32
4	18	a	1.27	2.09	26.65	11.59	0.89	103.07	129.72	102.18

4	18	b	3.42	2.09	71.63	15.63	0.89	138.96	210.59	140.38
4	18	c	0.95	2.09	19.99	12.90	0.89	114.74	134.73	135.00
4	19	a	1.33	2.54	33.82	3.68	1.25	45.80	79.62	104.00
4	19	b	2.05	2.54	52.00	7.77	1.25	96.80	148.80	109.83
4	19	c	3.78	2.54	95.92	17.99	1.25	224.04	319.96	159.84
4	20	a	1.05	3.39	35.70	2.74	2.05	56.18	91.88	83.47
4	20	b	1.23	3.39	41.77	5.88	2.05	120.51	162.28	117.49
4	20	c	0.78	3.39	26.27	3.18	2.05	65.14	91.41	81.42

Data of harvested shoot biomass

August 2012

Block	**Plot**	**Code**	**FM yield (Mg ha^{-1})**	**DM (%)**	**DM yield (Mg ha^{-1})**	**Clover in DM (%)**	**Clover visual ground cover (%)**	**Clover DM yield (Mg ha^{-1})**	**N in DM (g 100^{-1} g)**	**N yield (kg ha^{-1})**	**N_2 fixation (kg ha^{-1})**	**N_2 fixation (kg Mg^{-1} clover)**
1	1	a	13.03	17.11	2.23	26.20	56.67	0.58	2.15	47.92	16.78	28.74
1	1	b	10.04	20.47	2.05	24.46	28.33	0.50	2.01	41.29	10.15	20.20
1	1	c	9.62	19.61	1.89	17.26	20.00	0.33	1.79	33.78	2.64	8.11
1	2	a	7.94	23.27	1.85	0.58	0.00	0.01	1.48	27.36	0.00	-
1	2	b	8.68	25.68	2.23	0.41	0.00	0.01	1.28	28.52	0.00	-
1	2	c	7.15	23.83	1.70	9.22	5.00	0.16	1.49	25.40	0.00	-
1	3	a	17.20	15.18	2.61	38.65	71.67	1.01	2.61	68.15	37.01	36.68
1	3	b	15.18	14.67	2.23	54.40	75.00	1.21	2.76	61.45	30.31	25.03
1	3	c	16.73	16.18	2.71	36.85	76.67	1.00	2.67	72.30	41.16	41.25
1	4	a	13.45	18.07	2.43	32.63	56.67	0.79	2.27	55.17	24.03	30.30
1	4	b	10.84	18.58	2.01	26.33	43.33	0.53	2.21	44.53	13.39	25.24
1	4	c	12.92	19.34	2.50	23.17	35.00	0.58	1.91	47.72	16.58	28.64
1	5	a	21.47	14.22	3.05	53.58	81.67	1.64	2.71	82.74	51.60	31.54

1	5	b	15.88	15.19	2.41	48.02	75.00	1.16	3.01	72.60	41.46	35.80
1	5	c	15.67	16.81	2.63	32.66	71.67	0.86	2.56	67.44	36.30	42.19
2	6	a	25.18	12.99	3.27	39.14	54.33	1.28	2.75	89.96	58.82	45.94
2	6	b	27.09	14.64	3.97	44.59	51.67	1.77	2.39	94.80	63.66	35.99
2	6	c	15.84	17.83	2.82	25.88	38.33	0.73	2.23	62.98	31.84	43.57
2	7	a	21.28	15.53	3.31	31.34	30.00	1.04	2.15	71.07	39.93	38.54
2	7	b	13.36	19.89	2.66	18.48	13.33	0.49	1.94	51.54	20.40	41.55
2	7	c	13.98	19.78	2.77	19.83	20.00	0.55	1.98	54.76	23.62	43.07
2	8	a	9.33	23.09	2.15	0.00	0.00	0.00	1.48	31.89	0.00	-
2	8	b	8.83	27.35	2.42	4.84	2.00	0.12	1.21	29.24	0.00	-
2	8	c	8.28	26.84	2.22	7.62	3.00	0.17	1.39	30.91	0.00	-
2	9	a	21.26	12.60	2.68	51.00	55.00	1.37	2.91	77.96	46.82	34.27
2	9	b	15.87	13.97	2.22	43.16	55.00	0.96	2.88	63.86	32.72	34.19
2	9	c	21.57	12.89	2.78	52.97	61.67	1.47	3.10	86.21	55.07	37.39
2	10	a	18.37	14.74	2.71	34.00	43.33	0.92	3.04	82.28	51.14	55.58
2	10	b	19.83	13.60	2.70	42.20	78.33	1.14	3.10	83.60	52.46	46.09
2	10	c	14.63	15.04	2.20	40.35	50.00	0.89	3.13	68.88	37.74	42.50
3	11	a	19.65	16.10	3.16	29.02	51.67	0.92	2.39	75.64	44.50	48.45
3	11	b	16.21	16.15	2.62	39.96	65.00	1.05	2.82	73.86	42.72	40.82
3	11	c	17.72	15.43	2.73	32.04	45.00	0.88	2.55	69.72	38.58	44.04
3	12	a	9.79	23.22	2.27	0.00	4.00	0.00	1.52	34.56	3.42	-
3	12	b	10.97	26.88	2.95	0.00	0.00	0.00	1.26	37.14	6.00	-
3	12	c	8.34	23.51	1.96	0.00	0.00	0.00	1.60	31.36	0.00	-
3	13	a	16.79	19.66	3.30	32.28	45.00	1.07	2.49	82.16	51.02	47.90
3	13	b	12.22	21.45	2.62	15.39	31.67	0.40	1.88	49.26	18.12	44.94
3	13	c	10.67	22.55	2.41	17.13	27.00	0.41	1.95	46.94	15.80	38.32
3	14	a	16.21	17.59	2.85	41.99	73.33	1.20	2.60	74.14	43.00	35.91
3	14	b	17.58	17.66	3.11	39.88	68.33	1.24	2.88	89.43	58.29	47.07
3	14	c	13.42	18.55	2.49	25.94	50.00	0.65	2.42	60.26	29.12	45.08

3	15	a	12.04	20.89	2.52	21.90	21.67	0.55	1.94	48.79	17.65	32.05
3	15	b	19.51	18.24	3.56	29.26	48.33	1.04	2.26	80.44	49.30	47.34
3	15	c	12.42	21.72	2.70	11.75	13.33	0.32	1.99	53.71	22.57	71.16
4	16	a	15.38	17.34	2.67	30.05	45.00	0.80	1.86	49.59	18.45	23.03
4	16	b	21.35	13.86	2.96	30.86	66.33	0.91	2.30	68.07	36.93	40.44
4	16	c	19.10	14.98	2.86	29.39	50.00	0.84	2.19	62.68	31.54	37.50
4	17	a	15.58	13.02	2.03	45.28	71.67	0.92	2.72	55.18	24.04	26.17
4	17	b	16.22	14.47	2.35	27.44	53.33	0.64	2.66	62.42	31.28	48.58
4	17	c	15.22	15.79	2.40	46.23	55.00	1.11	2.55	61.31	30.17	27.14
4	18	a	10.18	19.61	2.00	12.21	11.67	0.24	1.65	32.94	1.80	-
4	18	b	8.21	23.22	1.91	0.00	0.00	0.00	1.40	26.68	0.00	-
4	18	c	11.48	21.05	2.42	9.58	5.00	0.23	1.56	37.70	6.56	-
4	19	a	14.58	15.06	2.20	28.03	40.00	0.62	2.46	54.02	22.88	37.16
4	19	b	16.96	17.38	2.95	26.83	33.33	0.79	1.91	56.31	25.17	31.82
4	19	c	16.08	20.53	3.30	11.19	13.33	0.37	1.62	53.48	22.34	60.46
4	20	a	12.71	14.92	1.90	32.18	26.67	0.61	2.54	48.15	17.01	27.89
4	20	b	14.24	16.60	2.37	28.35	20.00	0.67	2.35	55.58	24.44	36.45
4	20	c	14.76	18.67	2.76	20.90	25.00	0.58	2.03	55.94	24.80	43.06

Data of harvested shoot biomass

October 2012

Block	Plot	Code	FM yield (Mg ha^{-1})	DM (%)	DM yield (Mg ha^{-1})	Clover in DM (%)	Clover visual ground cover (%)	Clover DM yield (Mg ha^{-1})	N in DM (g 100^{-1} g)	N yield (kg ha^{-1})	N_2 fixation (kg ha^{-1})	N_2 fixation (kg Mg^{-1} clover)
1	1	a	9.46	17.55	1.66	50.76	61.67	0.84	2.79	46.31	20.00	23.75
1	1	b	7.68	17.45	1.34	46.94	41.67	0.63	2.93	39.26	12.95	20.59
1	1	c	8.91	19.44	1.73	40.00	33.33	0.69	2.56	44.30	17.99	25.96

1	2	a	4.76	27.41	1.30	0.35	0.00	0.00	1.69	22.05	0.00	-
1	2	b	4.57	27.13	1.24	11.80	5.00	0.15	2.05	25.48	0.00	-
1	2	c	4.65	26.26	1.22	13.86	8.33	0.17	2.02	24.62	0.00	-
1	3	a	6.83	19.80	1.35	48.35	36.67	0.65	3.14	42.43	16.12	24.66
1	3	b	8.42	19.24	1.62	45.47	76.67	0.74	3.26	52.74	26.43	35.89
1	3	c	10.68	18.49	1.97	52.24	66.67	1.03	3.17	62.64	36.33	35.24
1	4	a	8.77	20.11	1.76	46.05	61.67	0.81	2.83	49.85	23.54	28.98
1	4	b	8.69	19.69	1.71	47.28	73.33	0.81	3.21	54.98	28.67	35.44
1	4	c	7.77	21.11	1.64	34.02	46.67	0.56	2.81	46.14	19.83	35.52
1	5	a	11.47	15.80	1.81	92.66	91.67	1.68	3.79	68.70	42.39	25.24
1	5	b	12.54	16.83	2.11	71.95	95.00	1.52	3.56	75.13	48.82	32.15
1	5	c	11.86	16.98	2.01	65.95	75.00	1.33	3.56	71.69	45.38	34.17
2	6	a	13.04	17.75	2.31	60.14	63.33	1.39	3.32	76.83	50.52	36.30
2	6	b	11.69	17.49	2.04	52.87	73.33	1.08	3.40	69.52	43.21	39.97
2	6	c	7.87	20.54	1.62	43.84	45.00	0.71	3.04	49.12	22.81	32.20
2	7	a	7.98	21.52	1.72	43.74	40.00	0.75	2.91	49.98	23.67	31.51
2	7	b	7.12	24.01	1.71	33.90	43.33	0.58	2.93	50.08	23.77	41.02
2	7	c	7.26	24.97	1.81	22.05	30.00	0.40	2.44	44.22	17.91	44.83
2	8	a	3.33	31.09	1.04	2.81	3.00	0.03	1.88	19.48	0.00	-
2	8	b	4.91	27.35	1.34	11.15	5.00	0.15	2.16	28.97	2.66	-
2	8	c	5.25	25.75	1.35	5.34	5.00	0.07	2.06	27.84	1.53	-
2	9	a	11.41	17.00	1.94	51.91	38.33	1.01	3.06	59.37	33.06	32.83
2	9	b	9.91	16.33	1.62	42.14	31.67	0.68	3.38	54.67	28.36	41.61
2	9	c	11.24	16.70	1.88	54.82	41.67	1.03	3.34	62.71	36.40	35.37
2	10	a	12.73	15.17	1.93	86.34	91.67	1.67	3.65	70.47	44.16	26.49
2	10	b	9.29	17.49	1.62	55.15	50.00	0.90	3.57	58.00	31.69	35.37
2	10	c	8.65	17.21	1.49	47.45	63.33	0.71	3.81	56.74	30.43	43.06
3	11	a	11.34	16.84	1.91	38.38	66.67	0.73	3.17	60.55	34.24	46.71

3	11	b	8.09	15.48	1.25	60.62	63.33	0.76	3.32	41.58	15.27	20.11
3	11	c	6.43	15.75	1.01	36.67	63.33	0.37	3.61	36.58	10.27	27.63
3	12	a	4.52	25.39	1.15	0.80	0.00	0.01	2.00	22.94	0.00	-
3	12	b	5.39	26.61	1.43	0.00	0.00	0.00	1.76	25.22	0.00	-
3	12	c	4.92	26.14	1.29	0.46	0.00	0.01	1.88	24.20	0.00	-
3	13	a	16.12	16.25	2.62	45.58	70.00	1.19	2.92	76.52	50.21	42.03
3	13	b	10.04	16.69	1.68	44.33	70.00	0.74	3.33	55.79	29.48	39.70
3	13	c	8.52	20.94	1.78	36.79	48.33	0.66	2.50	44.59	18.28	27.85
3	14	a	10.37	14.93	1.55	66.48	90.00	1.03	3.70	57.27	30.96	30.09
3	14	b	12.52	17.93	2.24	45.41	91.67	1.02	3.15	70.69	44.38	43.55
3	14	c	10.60	16.61	1.76	64.97	80.00	1.14	3.69	64.95	38.64	33.79
3	15	a	8.96	19.55	1.75	32.09	40.00	0.56	2.72	47.67	21.36	37.98
3	15	b	9.05	19.46	1.76	25.63	30.00	0.45	2.70	47.54	21.23	47.05
3	15	c	9.18	19.78	1.82	37.70	50.00	0.68	2.93	53.23	26.92	39.31
4	16	a	9.56	18.60	1.78	33.07	38.33	0.59	2.74	48.72	22.41	38.11
4	16	b	7.50	16.93	1.27	34.15	45.00	0.43	3.33	42.26	15.95	36.81
4	16	c	9.25	16.27	1.50	36.80	38.33	0.55	3.17	47.70	21.39	38.63
4	17	a	11.32	14.50	1.64	35.39	68.33	0.58	3.43	56.31	30.00	51.64
4	17	b	7.54	15.87	1.20	47.12	70.00	0.56	3.82	45.70	19.39	34.40
4	17	c	11.41	15.12	1.73	30.73	60.00	0.53	3.31	57.10	30.79	58.09
4	18	a	4.33	24.87	1.08	0.67	0.00	0.01	1.77	19.07	0.00	-
4	18	b	5.81	24.87	1.45	4.92	5.67	0.07	2.24	32.40	6.09	-
4	18	c	8.63	21.35	1.84	16.31	11.67	0.30	2.36	43.50	17.19	-
4	19	a	10.94	17.94	1.96	43.14	66.67	0.85	3.20	62.81	36.50	43.11
4	19	b	11.75	18.97	2.23	28.51	51.67	0.64	2.75	61.30	34.99	55.05
4	19	c	10.50	22.05	2.32	23.67	35.00	0.55	2.36	54.65	28.34	51.71
4	20	a	9.15	18.57	1.70	45.98	48.33	0.78	3.05	51.84	25.53	32.67
4	20	b	9.21	16.22	1.49	31.05	40.00	0.46	2.94	43.93	17.62	37.98
4	20	c	10.39	15.89	1.65	27.85	36.67	0.46	3.11	51.35	25.04	54.45