# Improved persistence of red clover (Trifolium pratense L.) increases the protein supplied by red clover/grass swards grown over four harvest years

Marshall, Athole; Collins, Rosemary; Vale, James; Lowe, Matthew

*Published in:*
European Journal of Agronomy

*DOI:*
10.1016/j.eja.2017.06.006

*Publication date:*
2017

*Citation for published version (APA):*
Marshall, A., Collins, R., Vale, J., & Lowe, M. (2017). Improved persistence of red clover (Trifolium pratense L.) increases the protein supplied by red clover/grass swards grown over four harvest years. *European Journal of Agronomy*, *89*, 38-45. https://doi.org/10.1016/j.eja.2017.06.006

# Improved persistence of red clover (*Trifolium pratense* L.) increases the protein supplied by red clover/grass swards grown over four harvest years

*Marshall, A. H., Collins, R.P., Vale, J. and Lowe, M.

Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Gogerddan, Aberystwyth, United Kingdom, SY233EB.

* Corresponding author
Email: thm@aber.ac.uk
Tel:01970823171

## Abstract

UK livestock agriculture can significantly reduce its protein imports by increasing the amount of forage based protein grown on-farm. Forage legumes such as red clover (*Trifolium pratense* L.) produce high dry matter yields of quality forage but currently available varieties lack persistence, particularly under grazing. To assess the impact of red clover persistence on protein yield, diploid red clover populations selected for improved persistence were compared with a range of commercially available varieties. All populations were grown over four harvest years in mixed swards with either perennial ryegrass (*Lolium perenne* L.) or perennial plus hybrid ryegrass (*L. boucheanum* Kunth). Red clover and total sward dry matter (DM) herbage yields were measured in Years 1-4, red clover plant survival in Years 3 and 4 and herbage protein (CP) yield and concentration in Years 2 and 4. In general, red clover DM yield in year 4 (3.4 t $ha^{-1}$) was lower than in year 1 (13.9 t $ha^{-1}$) but the red clover populations differed in the extent of this decline. Differences in the persistence of the red clover populations in terms of plant survival and yield were reflected in the contribution of red clover to the total sward yield in Year 4, which ranged from 61% for the highest yielding population, AberClaret, to 11% in the lowest yielding, Vivi.. Increased red clover DM yield was reflected in a greater CP yield (protein weight per unit area), which ranged from 1.6 t $ha^{-1}$ $year^{-1}$ to 2.9 t $ha^{-1}$ $year^{-1}$ in Year 2 and from 1.1 t $ha^{-1}year^{-1}$ to 1.9 t $ha^{-1}year^{-1}$ in Year 4. CP concentration (protein weight per unit herbage weight) of all of the red clover populations was within a range considered suitable for ruminant production. The implication of these results for the future use of red clover in sustainable grassland systems is discussed.


Keywords: *Trifolium pratense*, yield, persistence, protein yield, variety

## 1. **Introduction**

Increasing the amount of forage based protein that can be grown "on-farm" is one way of addressing the UK's protein deficit and increasing the efficiency of livestock production (Abberton and Marshall, 2005). Forage legumes such as white (*Trifolium repens* L.) and red clover (*T. pratense* L.) are important species for sustainable livestock production systems because in mixture with grasses they can fix, on average, 150 kg N ha $yr^{-1}$, some of which subsequently becomes available to the companion grass (Pirhofer-Walzl *et al*., 2012). They produce high yields of good quality forage with a crude protein (CP) content of 18-19% (Frame *et al*., 1997), which, in turn, leads to high voluntary intakes and improvements in livestock performance (Lüscher *et al*., 2014). Recent studies also show that red clover contains high levels of the enzyme polyphenol oxidase (PPO) in comparison with other forage legumes (Winters and Minchin, 2005). PPO has beneficial effects in improving nitrogen utilization in ruminants (Lee *et al*., 2006) and in protecting lipids from degradation, both 'in silo' as well as in the rumen, leading to a higher output of polyunsaturated fatty acids (PUFA) in ruminant products (meat and milk) (van Ranst *et al.,* 2011).

Despite these beneficial attributes of red clover, its use may be limited by its lack of persistence in swards. For example, under a typical UK management of 3 conservation cuts per year followed by a late autumn grazing, red clover tends to persist for only two to three years, after which dry matter (DM) yields decline (Frame, 2005). One challenge to improving the persistence of forage legumes is a lack of scientific consensus on how persistence is defined (Phelan *et al*., 2014). In this study we considered two aspects of persistence in red clover swards. The first of these was the number of individual red clover plants surviving in swards after three or more years (Choo, 1984). In contrast to the other major temperate forage legume, white clover, which has a stoloniferous growth habit and is therefore able to spread vegetatively in swards, red clover plants grow from apical meristems from the crown tissue, which means that poor plant survival has a significant impact on DM yield under managements that do not allow regeneration from seed *i.e.* red clover cannot replace plants that die. Secondly, we analysed the capacity of red clover based swards to produce 'functionally relevant' forage yield in the second and fourth growing seasons (Taylor and Quesenberry, 1996) *i.e.* an amount of CP sufficient to meet the nutritional requirements of animals ingesting the conserved forage. Efficient ruminant production requires forages with a CP concentration of 100 to 170g $kg^{-1}$ DM (Phelan et al., 2014) and the CP concentration of red clover silage has been measured as 171-181 g $kg^{-1}$ DM, depending upon growth stage (Dewhurst 2013). The maintenance of DM and CP yield is therefore crucial to red clover's agronomic value as a source of protein grown on-farm, and explains why persistence is a common breeding target of red clover breeding programmes in the UK (Marshall *et al*., 2012) and in other countries (Herrmann *et al*., 2008; Taylor, 2008; Riday 2010).

Previous studies on red clover persistence have considered the potential of exploiting variation in morphological traits such as the development of adventitious roots (Montpetit and Coulmann, 1991) and stolons (Smith and Bishop, 1993). However, the most direct way to breed for persistence involves exposing plants to their target environment and selecting for survival (Taylor and Quesenberry, 1996). In the red clover breeding programme at IBERS, Aberystwyth the focus has been on identifying factors contributing to the poor persistence of red clover in swards and applying this information to the selection of improved red clover varieties that combine high forage yields with greater persistence. Two approaches to selection for persistence have been applied: (1) previous studies in spaced plants showed that crown diameter is the morphological characteristic most associated with plant mortality (Ortega and Rhodes, 1996), and a number of selections in the breeding programme were made on that basis (indirect selection); (2) survivor plants were collected at the end of long term variety evaluation experiments that were carried out for up to four years. These survivor plants were subsequently subject to a single round of polycrossing to produce a number of selection lines (direct selection).

This paper describes a field experiment undertaken to quantify the effects of differences in red clover persistence on sward DM production and CP yield over a four year period, using new red clover varieties with greater crown diameter (developed by indirect selection), survivor populations selected for improved persistence (developed by direct selection) and a range of current commercially available varieties. To avoid confusion, all red clover germplasm (selection lines and varieties) used in this study is referred to collectively as ‘populations’.

In this study the red clover populations were grown in binary mixtures with two companion grass treatments. In the UK, red clover is usually sown in mixtures with Italian (*Lolium multiflorum* Lam.) or hybrid ryegrass (*L. boucheanum* Kunth) (Abberton and Marshall, 2005), species that are high yielding but relatively short lived in comparison with perennial ryegrass (*L. perenne* L.). However, breeding red clover varieties that are more persistent will lead to greater longevity of mixed grass/red clover swards, beyond the current two or three years. This may necessitate the inclusion of the more persistent perennial ryegrass in such mixtures. No published information is currently available on the performance of red clover when sown with different companion grasses over more than three harvest years, so the effect of adding perennial ryegrass to hybrid ryegrass as a companion grass treatment was analysed here.

## 2.0 Materials and methods

### 2.1 Plant material and site

The experiment was established in July 2008 at IBERS, Aberystwyth, UK (52° 4’N, 4° 0’W) on soil of the Rheidol series (well drained fine loamy soil over gravel) with a pH value of 6.1.

Twelve red clover populations (Table 1), were sown in mixtures either with the tetraploid hybrid ryegrass cv. AberEcho on its own (‘companion grass treatment 1’) or with a mixture of the intermediate heading perennial ryegrass cv. AberDart and cv. AberEcho (‘companion grass treatment 2’). The red clover populations comprised eight commercial varieties in use in the UK at the time of sowing, including two (AberChianti and AberClaret) developed at IBERS and possessing increased crown diameter, a trait linked with improved persistence, plus four survivor populations selected from long term cutting and grazing trials carried out at IBERS experimental sites at Aberystwyth and Bronydd Mawr Upland Research centre at Brecon, Wales.

### 2.2 Experimental design

A randomized complete block (RCB) design was used. Each treatment combination (12 red clover populations with two companion grass treatments) was replicated three times, giving 72 experimental plots in total. Plots (5m x 1m) were sown by broadcasting seed. In both companion grass treatments, red clover was sown at a seed rate of 7.5 kg $ha^{-1}$. In 'companion grass treatment 1' hybrid ryegrass was sown at 35 kg $ha^{-1}$, and at 22.4 kg $ha^{-1}$ in 'companion grass treatment 2' together with perennial ryegrass at 12.6 kg $ha^{-1}$. Thus, the total seed rate for the grass component was the same in both companion grass treatment types. Plots were lightly topped in the establishment year and three harvests taken in each of the following four harvest years. In the autumn of each harvest year the plots were mob grazed with sheep to a sward height of 4cm.

The grass and red clover components of sward DM yield were quantified in three cuts per year in 2009 (Year 1: 20 May, 8 July, 24 August), 2010 (Year 2: 27 May, 19 July, 6 September), 2011 (Year 3: 31 May, 20 July, 12 September) and 2012 (Year 4: 31 May, 2 August, 10 October). Plots were cut with a Haldrup forage harvester to a height of 5cm. The harvested fresh weight was measured on each plot and grass and red clover content determined on a 300g subsample. DM yields were calculated after drying the subsample in a forced draught oven at 80°C for 18 h.

The density of red clover plants in each plot was recorded in spring of Years 3 and 4. Two 0.25$m^2$ quadrats were placed at random in each plot and the number of red clover plants in each quadrat was recorded.

The nitrogen (N) content of dried, milled samples of the total mixture (grass and red clover) from all plots harvested in Years 2 and 4 was determined by a rapid combustion method using a LECO FP- 428 analyses (Leco Corp., St. Joseph, MI, USA) and the CP content was calculated using the formula CP = N x 6.25.

### 2.3 Meteorological data

Weather conditions during the growing season (March-October) of the establishment year and the subsequent four harvest years are presented in Table 2, together with the 25-year average.

### 2.4 Statistical analysis

Data for annual red clover and total (red clover + grass) DM yields from each plot were analysed by ANOVA in each growing season according to the RCB design, and also over the four growing seasons as repeated measures. Red clover means were compared within growing seasons using the Student-Newman-Keuls method for multiple comparisons. The stability of annual red clover DM yield over the four growing seasons was examined using modified joint regression analysis (Digby, 1979) as implemented in procedure RJOINT of the statistical package GenStat (Payne et al, 2015). The stability (i.e. sensitivity) estimate for a population characterized the average linear relationship between the means for that population in each year and the overall site means. The stability estimates for all populations were standardized to have mean +1 (i.e. slope>1 = more sensitive (less stable) than average and slope<1 = less sensitive (more stable) than average).

Plant densities and CP content and yield compared between treatments across two growing seasons were analysed by repeated measures ANOVA. Bonferroni adjusted pairwise multiple comparisons were made between years within red clover populations. These analyses were carried out using GenStat (Payne et al., 2015).

## 3. Results

### 3.1 Meteorological conditions

Growing seasons in the establishment year (2008) and year 4 (2012) were wetter than average (100.6mm and 108.1mm respectively), and the growing season in Years 1 (718.0mm) and 3 (65.2mm) drier than average (81.5mm) (Table 2). Growing seasons in Years 1 and 3 were slightly warmer than average but in all other years conditions were comparable with the 25-year average for this location. The minimum temperature in harvest years 1 (9.1°C) and 3 (9.0°C) was higher than average (8.1°C) and Years 1 (16.1°C), 2 (15.8°C) and 3 (16.5°C) had a higher average maximum temperature than the 25 year mean (15.4°C).

### 3.2 Dry matter yield

Repeated measures analysis over years showed that annual total yield and red clover yield were significantly influenced by red clover population ($P<0.001$), year ($P<0.001$) and a year x population interaction ($P<0.001$). Results for annual total and red clover DM yields are presented in Tables 3a and 3b respectively. There was a significant difference between grass treatments in Year 1, in which the addition of perennial ryegrass in grass treatment 2 resulted in significantly higher average total yield (17.2 vs. 18.0 t $ha^{-1}$ in grass treatments 1 and 2 respectively; P<0.008). However, this was the only year in which grass treatment had a significant effect on total yield. By contrast, the identity of the red clover population had a strong effect on total yield in all years. Mixtures containing AberClaret or Milvus were highest yielding in Year 1 (P<0.001); Milvus or Milvus (S) in Year 2 (P<0.001); and AberClaret or Milvus (S) in Year 3 (P<0.001). For total yield in Year 4, the means comparisons test at P<0.05 did not detect any differences between red clover populations even though the effect of red clover was highly significant (P=0.007). This may have been due to grouping of the means (Thomas, 1973). In order to identify differences between the populations the experiment-wise type I error rate was reduced. In absolute terms, the highest total yields in Year 4 were in mixtures containing Milvus (S), AberChianti and Pavo and the lowest yielding was Vivi (S). In all years, total yields in mixtures containing the population Vivi were the lowest yielding. There was no interaction between grass treatment and red clover population in terms of annual total yield in any year. Although mean total yields decreased over time, they were still agronomically acceptable in Year 4 (17.6, 15.9, 12.7 and 10.4 t $ha^{-1}$ in Years 1, 2, 3 and 4 respectively).

Average annual red clover yield was significantly affected by grass treatment in Year 1 only, when it was higher in mixtures containing perennial ryegrass (13.4 and 14.2 t $ha^{-1}$ in grass treatments 1 and 2 respectively; P<0.05). As observed for total sward yield, the yield of the red clover component differed significantly between populations in all years. In Year 1 the highest red clover yields were measured in Milvus and AberClaret (P<0.001); Milvus was again the highest yielding in Year 2 (P<0.001), while AberClaret and Milvus (S) were highest in Year 3 and Year 4

(P<0.001). Population Vivi was consistently low yielding in all years, although by Year 4 many of the other red clover populations were equally low yielding. The average red clover yield was very high in Years 1 and 2, but decreased substantially in Years 3 and 4 (13.8, 14.6, 8.6 and 3.4 t ha$^{-1}$, respectively). Milvus (S) Varieties developed by both indirect (AberClaret) and direct (Milvus (S)) selection strategies produced high red clover DM yields in Years 3 and 4 compared to the commercially available red clover varieties.

Yields of the grass component (averaged over grass treatments and red clover populations) were 3.8, 1.4, 4.1 and 7.1 t ha$^{-1}$ in Years 1, 2, 3 and 4 respectively. There was no effect of grass treatment on annual grass yield except in Year 3 when the yield of grass treatment 2 was higher than grass treatment 1 (P<0.05). Over time there was an inverse relationship between annual grass and red clover yield, so that as the yield of the latter decreased the contribution of the grass to total yield increased. The relationship between DM yields in Year 1 against subsequent yields in Years 2, 3 and 4 is shown in Figure 1. There was a significant positive relationship in all cases, indicating that performance in Year 1 was a good predictor of the performance in later years. The red clover and total yields of the 12 red clover populations were also broadly similar in Years 1, 2 and 3, as the slopes of the fitted lines in Year 1 compared with yield in Years 2 and 3 were similar. The comparison of yields in Years 1 and 4 however, showed a greater relative drop in performance in total yield in comparison with red clover yield, as the fitted lines diverged and the total yield exhibited a smaller slope than that of red clover. Figure 1 also shows the relative change in total and red clover yield over time. In a comparison of Year 1 vs Year 2, the slopes of both yields were not significantly different from 1, indicating there was no difference between red clover populations in performance between those years in either total or red clover yield. There was, however, a drop in performance in Year 3 as the slope of the fitted lines become significantly (P<0.05) less than 1, and a bigger drop again in Year 4 for both total and red clover yields, as the slope of each fitted line was significantly (P<0.001) less than 1.

### 3.3 Stability of red clover yield over growing seasons

The stability of red clover yield of each population over 4 years is shown in Figure 2. The closer the sensitivity estimates were to 1 the more similar the population stability was to the overall mean stability. Populations with sensitivities > 1 had less stable yields than the mean, and those with sensitivities < 1 were more stable than the mean. Milvus tended to be the least stable population. Yield in Vivi was more stable (P<0.05) than that in Vivi (S), Aa4577, Merviot, Amos, Pavo, Milvus and AberChianti.

### 3.4 Plant density

Analysis of plant density (count $m^{-2}$) in Years 3 and 4 (mean of the plant density data from May and September counts in each year) as repeated measures in each year showed significant effects of red clover population, year and the red clover population x year interaction. There was a significant effect (P<0.001) of population on the density of red clover plants in both years (Table 4). In Years 3 and 4 respectively, plant density was highest in mixtures containing Britta (S1) (85.4 and 66.3) and lowest in Vivi (24.5 and 16.7). However, only the populations Amos and Vivi (S) showed a significant decrease in plant density between Years 3 and 4

($P<0.05$), based on Bonferroni adjusted pairwise multiple comparisons between red clover populations. Effects of companion grass treatment, the interaction of grass treatment with red clover population and year, and the interaction between grass treatment, red clover population and year were not significant (P=0.725, 0.363, 0.129 and 0.088 respectively). The relationship between plant density and annual red clover DM yield in Years 3 and 4 was analysed using linear regression. The correlation coefficient for the regression in Year 3 was not significant, but became significant in Year 4 (r = 0.72; 10 df; $P<0.01$). The regression equation for this relationship was: Annual clover yield (t DM $ha^{-1}$) = 0.089 (±0.0275) x Plant density (count $m^{-2}$) - 0.47 (±1.298) ($R^2$=0.462, P=0.009, rsd=1.68).

**3.5 Forage quality**

Annual CP yield (t CP $ha^{-1}$ $year^{-1}$) and the CP concentration of the harvested herbage (g CP $kg^{-1}$ DM) of the mixtures in Years 2 and 4 were analysed as repeated measures with Bonferroni adjusted multiple comparisons between years and within red clover population. Values are shown in Figure 3 (a) and (b).

CP yield of the harvested herbage was higher in Year 2 than Year 4 (2.55 vs. 1.39 t $ha^{-1}$, s.e.d. 0.048, $P<0.001$) with no effect of grass treatment (1.96 and 1.98 t $ha^{-1}$ for treatments 1 and 2 respectively, s.e.d. 0.038, P=0.520), but a significant red clover population x year interaction (Figure 3a) (s.e.d. 0.165 between years within populations otherwise 0.149; $P<0.001$). All red clover populations showed a decrease ($P<0.05$) in CP yield between Years 2 and 4 except Vivi which had its lowest CP yield in Year 2.

CP concentration of the harvested herbage was higher in Year 2 than in Year 4 (161.1 vs 129.7 g kg$^{-1}$ DM, s.e.d. 1.37, P<0.001) and, averaged over years, was higher in grass treatment 1 than treatment 2 (147.5 vs. 143.3 g kg$^{-1}$ DM, s.e.d. 1.15, P<0.001) (data not shown), but there was no interaction between grass treatment and either red clover population or year. There was a significant interaction between red clover population and year (P<0.001) due to a significant decrease in CP concentration between Years 2 and 4 in all populations except Milvus (S), Milvus, AberChianti, AberClaret and Pavo, *i.e.* those populations that maintained a higher percentage contribution of red clover to total annual yield between these years. CP concentration of the red clover populations ranged from 150.3 to 174.3g kg$^{-1}$ DM in Year 2, and from 103.4 to 152.3g kg$^{-1}$ DM in Year 4 (Figure 3 b). The strong effect of the percentage red clover contribution to total mixture DM on the CP concentration of the herbage is illustrated in Figure 4.

## 4. Discussion

### 4.1 Variation in dry matter yield

Typically in the UK, red clover performs well for two years in mixed swards, but its subsequent decrease in DM yield has a negative impact on the amount of protein supplied by the sward. Analysis of the impact of red clover persistence on forage DM yield and CP yield is therefore important for evaluating the dynamics of forage quality in red clover-based swards, and also for establishing targets for genetic improvement programmes. To investigate this we analysed the performance of red clover germplasm selected with an *a priori* expectation of improved persistence, namely, four populations (Britta (S1), Milvus (S), Britta (S2) and Vivi (S)) developed using direct selection for DM yield persistence, plus two commercial populations (AberChianti and AberClaret) derived from crosses carried out at IBERS, Aberystwyth using germplasm selected for the larger crowns associated with red clover plant persistence (indirect selection). The primary objective of this experiment was to compare the persistence (using the criteria outlined in the Introduction) of these populations with that of five red clover populations that are currently commercially available in the UK.

Red clover yields of all the populations declined in the third and fourth harvest year compared with Year 1. The decline in the total sward yield over time was less evident, as mixtures with a lower red clover yield showed a concomitant increase in grass yield in compensation. As shown in other studies (e.g. Ford and Barrett, 2011), there were clear differences here in the persistence of the red clover populations in terms of both plant survival and maintenance of DM yield. These differences were reflected in the contribution of red clover yield to the total sward yield in Year 4 which ranged from 61% for the highest yielding population, AberClaret, to 11% in the lowest yielding, Vivi. Despite the differences in red clover yield, however, all of the populations demonstrated the capacity to produce swards containing functional levels of CP in Year 4 i.e. herbage CP concentration never fell below the level required for efficient ruminant production (Phelan *et al*., 2014), although in some mixtures CP concentration had declined to a level that was approaching the lower end of the acceptable range for ruminant production.

**4.2 Stability of red clover yield over harvest years**

Regression values (slope) >1 describe populations with less yield stability over years, whilst those with slope values <1 show above- average stability compared to the overall mean stability. This approach has been used in previous studies to quantify the adaptation of varieties to environment in plant breeding programmes (Finlay and Wilkinson, 1963) and to analyse the stability of seed yield of accessions of forage species when multiplied in different environments (Hinton Jones et al., 2007). In the current study, this approach has been combined with yield data to provide some indication of the yield stability of different populations over years. The results confirmed that, although not significantly different, AberClaret and Milvus (S) were the most stable varieties in terms of producing high red clover yields over the four years, while Milvus and Merviot were the least stable of the higher-yielding varieties.

**4.3 Variation in plant survival**

The overall decline in red clover yield over the duration of the experiment was a consequence of poor plant survival, and this deficiency was greater in some populations than others. Plant density in Year 4 showed a good correlation with red clover yield, and there were significant differences between populations in plant survival. The highest red clover yields in Year 4 were produced by populations Milvus (S) and AberClaret. Milvus (S) was produced through direct selection from survivors of long term field trials that included Milvus, whilst AberClaret was derived from crosses specifically aimed at increasing crown diameter (indirect selection). Evidence from the current experiment suggests that both strategies could be used to improve persistence of red clover. Recent studies have shown that persistence in red clover is also positively correlated with length of the longest stem of each plant, which may therefore be useful as another candidate trait for indirect selection (Herrmann *et al*., 2008).

**4.4 Contribution of red clover to total dry matter yield and protein yield**

Differences in plant persistence between the populations had an impact on the red clover content of the total DM yield and on the CP yield of the mixtures. There was a strong temporal effect on the contribution of red clover to the total annual yield such that it was 86% for the best red clover population in Year 1 and nearly 60% in Year 4. In the lowest yielding red clover populations this contribution was closer to 11% in Year 4. However, despite this six-fold difference in red clover content, in Year 4 the total DM yield of all mixtures only ranged from 9.3 to 11.4 t $ha^{-1}$. Thus, mixtures with low red clover DM yields had a proportionally higher grass DM yield. It is possible that in these mixtures increased mortality over time in red clover resulted in more physical spaces being available to fill for the grass component. However, previous results have shown a decrease in ryegrass plant density due to the vigorous growth of red clover (Eriksen *et al*., 2012). Similar reductions in tiller formation of ryegrass when grown in mixtures with tall legumes were found by Roscher *et al*., (2008). In the latter study the depression of ryegrass yield occurred despite a very low content of red clover in the seed mixture (1kg $ha^{-1}$) which was considerably lower than the 7.5 kg $ha^{-1}$ used in the present study.

A key objective of this study was to ascertain the effect of persistence of the red clover populations on the CP yield and CP concentration of the herbage mixtures. The CP yield of the 12 red clover populations ranged from 1.6 t $ha^{-1}$ $year^{-1}$ to 2.9 t $ha^{-1}$ $year^{-1}$ in Year 2 and from 1.1 t $ha^{-1}year^{-1}$ to 1.9 t $ha^{-1}year^{-1}$ in Year 4, reflecting differences in the proportion of red clover in the mixtures and highlighting the importance of red clover persistence to the feed value of the sward. Such differences in CP yield will have a significant impact on the carrying capacity of the sward in terms of the number of livestock that can be supported and therefore on the economic value of the sward to the livestock farmer. Ruminant production requires forages with a CP concentration of 100 to 170g kg$^{-1}$ DM (Phelan et al., 2014) and the CP concentration of red clover silage has been measured as 171-181 g kg$^{-1}$ DM, depending upon growth stage (Dewhurst 2013). In the present study, the herbage CP concentration in Year 2 of the 12 red clover populations, averaged over the three cuts, ranged from 150.3 to 174.3 g kg$^{-1}$ DM and from 103.4 and 152.3 g kg$^{-1}$ DM in Year 4. Although the CP concentration declined between Years 2 and 4 as the proportion of red clover in the mixtures decreased, in Year 4, it was still in the acceptable range for ruminant production. However for some of the red clover populations CP concentration had declined to a level that was approaching the lower end of the acceptable range for ruminant production.

### 4.5 The effect of companion grass

An additional objective here was to consider the role of the companion grass species in the mixtures. Persistent red clover varieties may produce high DM yields for up to 4 or 5 years, significantly extending the life of the red clover based leys, and this may require the use of more persistent perennial ryegrass varieties than the high yielding, short lived grass species currently used for 2 year leys. The present study compared 2 grass treatments; tetraploid hybrid ryegrass cv. AberEcho on its own (‘companion grass treatment 1’) or with a mixture of the intermediate heading perennial ryegrass cv. AberDart and cv. AberEcho (‘companion grass treatment 2’).

AberDart, the perennial ryegrass variety used in the study, has a high WSC content, which is known to also improve silage quality (Conaghan *et al*., 2008). In the current study, the grass DM yield was not generally influenced by grass treatment, though over time there was an inverse relationship between annual grass and red clover yield, so that as the yield of the latter decreased the contribution of the grass to total yield increased. Further work is needed to consider the interaction between red clover variety, ley duration and type of management e.g. persistence under grazing, as this may have a greater impact on the choice of companion grass species than the management system included in this study. This is particularly important if the proposed management system will involve some longer and more severe grazing, and if it includes an element of sheep grazing in each growing season, as in this study, or cattle grazing, as in other studies (Ford and Barrett, 2011).

The results presented here also have implications for official variety testing systems. The current UK red clover variety testing system evaluates variety performance over two harvest years. Our data show that although the major differences between populations in persistence and yield were not apparent until the third and fourth harvest year, performance of the populations in year 1 was a good indicator of their performance in year 4. . This suggests that testing beyond two years may not be necessary  to identify appropriate varieties that are suitable for longer leys.

## 5. Conclusion

Increasing the longevity of red clover based swards is increasingly attractive to livestock farmers, but lack of persistence of red clover has a significant impact on the yield and quality of mixed red clover/grass swards. Evidence from this field experiment carried out over four harvest years shows that inadequate plant persistence can significantly reduce red clover DM yields and CP yield. New germplasm, selected for improved persistence, can maintain a higher red clover yield into the fourth harvest year, and the yield decline between harvest Years 1 and 4 is considerably less than in other commercially available varieties. Selection for improved persistence was reflected in improved plant survival, and consequently higher DM yield in the third and fourth harvest years, which also resulted in a higher CP yield per hectare in comparison with unselected material.

## Acknowledgements

The authors acknowledge financial support from the UK Department for the Environment, Food and Rural Affairs (Defra) and Ruth Sanderson for additional statistical analysis.

## References

ABBERTON, M.T. and MARSHALL, A.H. (2005). Progress in breeding perennial clovers for temperate agriculture. *Journal of Agricultural Science*, **143**, 117-135.

CHOO, T.M. 1984. Association between growth habit and persistence in red clover. *Euphytica*, **33**, 177-185.

CONAGHAN, P., O'KIELY, P., HOWARD, H., O'MARA, F.P. and HALLING, M.A. (2008). Evaluation of *Lolium perenne* L. cv. AberDart and AberDove for silage production.*Irish Journal of Agricultural and Food Research* **47**, 119-134.

DEWHURST, R. (2013). Milk production from silage: comparison of grass, legume and maize silages and their mixtures. *Agric. Food Sci*. (22 (1): 57-69.

DIGBY, P.G.N.  (1979). Modified joint regression analysis for incomplete variety x environment data. *Journal of Agricultural Science Cambridge*, **93**, 81-86.

ERIKSEN, J., ASKEGAARD, M. and SOEGAARD, K. (2014). Complementary effects of red clover inclusion in ryegrass-white clover swards for grazing and cutting. *Grass and Forage Science* **69**, 241-250.

FINLAY, K.W. and WILKINSON, G.N. 1963. The analysis of adaptation in a plant breeding programme. *Australian Journal of Agriculture Research*, **14**, 742-754.

FORD, J.L. and BARRETT, B.A. 2011. Improved red clover persistence under grazing. *Proceedings of the New Zealand Grassland Association,* **73**, 119-124.

FRAME, J. (2005). Forage legumes for temperate grasslands. Plymouth, UK: Science Publishers, Inc.

FRAME, J., CHARLTON, J. F. L. and LAIDLAW, A.S. (1997) Red clover. In: Frame J., Charlton, J.F.L and Laidlaw, A.S. (eds) *Temperate forage legumes*. CAB International, Wallingford, UK, pp.181-224.

HERRMANN, D., BOLLER, B., STUDER, B., WIDMER, F. AND KOLLIKER, R. 2008. Improving persistence in red clover: insights from QTL analysis and comparative phenotypic evaluation. *Crop Science*, **48**, 269-277.

HINTON-JONES, M., MARSHALL, A.H., THOMAS, I.D., HUMPHREYS, M.O., MARUM, P., ŠEVČÍKOVÁ, M. ŠRÁMEK, P. DE SOUSA, M.M.T., NIELSON, N. CHR. and DHANOA, M.S. 2007. Environmental effects on seed yield and costs of temperate forages during regeneration. *European Journal of Agronomy*, **26**, 235-248.

LEE, M.R.F., OLMOS COLMENERO, J.J., WINTERS, A.L., SCOLLAN, N.D. and MINCHIN, F. (2006). Polyphenol oxidase activity in grass and its effect on plant-mediated lipolysis and proteolysis of *Dactylis glomerata* (cocksfoot) in a simulated rumen environment. *Journal of the Science of Food and Agriculture*, **86**, 1503-1511.

LÜSCHER, A., MUELLER-HARVEY, I., SOUSSANA, J.F., REES, R.M. AND PEYRAUD, J.L. (2014). Potential of legume-based grassland livestock systems in Europe: a review. *Grass and Forage Science* **69**, 206-228.

MARSHALL, A.H. LOWE, M., and VALE, J. (2012). Improved persistence of red clover (*Trifolium pratense* L.) in mixed swards. In: *Grassland – a European Resource. Grassland Science in Europe,* **17**, 73-75.

MONTPETIT, J.M. and COULMANN, B.E. (1991). Response to divergent selection for adventitious rot growth in red clover (*Trifolium pratense* L.). *Euphytica,* **58**, 119-127.

ORTEGA F.M. and RHODES, I. (1996). Characteristics associated with mortality in red clover (*Trifolium pratense* L.). In: Younie D. (eds) *Legumes in Sustainable Grassland Systems.* Occasional Symposium No. 30 British Grassland Society pp. 92-93.

PAYNE R.W., MURRAY, D.A., HARDING S.A., BAIRD, D.B. and SOUTAR, D.M. (2015) Introduction to GenStat® for Window™ (18$^{th}$ Edition). VSN International, Hemel Hempstead, UK.

PHELAN P., MOLONEY, A.P., MCGEOUGH, E.J., HUMPHREYS, J., BERTILSSON, J., O'RIORDAN, E.G. AND O'KIELY, P. (2014). Forage legumes for grazing and conserving in ruminant production systems. *Critical Reviews in Plant Sciences* **34,** 281-326.

PIRHOFER-WALZL, K., RASMUSEN, J., HØGH-JENSEN, H., ERIKSEN J., SØEGAARD, K AND RASMUSSEN, J. (2012). Nitrogen transfer from forage legumes to nine neighbouring plants in a multi-species grassland. *Plant and Soil*, **350**, 71-84.

RIDAY 2010 Progress made in improving red clover (*Trifolium repens* L.) through breeding. *International Journal of Plant Breeding* 4 (1) 22-29.

VAN RANST G., LEEM. R. F., FIEVEZ V. (2011). Red clover polyphenol oxidase and lipid metabolism. *Animal* 5 512–521

ROSCHER, C., SCHUMACHER, J., WEISSER, W.W. AND SCHULZE E.-D. (2008). Genetic identity affects performance of species in grasslands of different plant diversity: an experiment with *Lolium perenne* cultivars. *Annals of Botany*, **102**, 113-125.

SMITH, R.S. and BISHOP, D.J. (1993). Astred, a stoloniferous red clover. 421-423.In: *Proceedings of the XVII International Grassand Congress, Palmerston*, North, New Zealand.

TAYLOR, N.L. (2008) A century of clover breeding development in the United States. *Crop Science*, **48**, 1-13.

TAYLOR, N.L. and QUESENBERRY, K.H. 1996. Red clover science. Kluwer Academic Publishers, Dordrecht, The Netherlands.

THOMAS, D.A.H. (1973). Multiple comparisons among means - a review. The Statistician, **22,** 16-42.

WINTERS, A.L., and MINCHIN, F.R. (2005). Modifications of the Lowry assay to measure proteins and phenols in covalently bonded complexes. *Analytical Biochemistry*, **346**, 43-48.

**Figure legends**

Figure 1. The relationship between total (grass + clover) yield and red clover DM yield (t $ha^{-1}$) in Year 1 with yields in Years 2, 3 and 4 of 12 red clover varieties grown in a field experiment at IBERS, Aberystwyth. Data are averaged over two companion grass treatments.

Figure 2. Sensitivity estimates for annual red clover yield (t DM $ha^{-1}$) for 12 red clover varieties over 4 years (vertical bars indicate 95 per cent simultaneous confidence intervals).The Y-axis is stability (sensitivity) coefficient for annual red clover yield from modified joint regression analysis (Digby, 1979) and X-axis is mean annual red clover yield over 4 years. The dotted line represents overall mean sensitivity.

Figure 3. a) Crude protein yield (t $ha^{-1}year^{-1}$) and b) crude protein concentration (g $kg^{-1}$ DM) in Years 2 (black columns) and 4 (shaded columns) of 12 red clover varieties grown in mixed swards with a grass companion. Yields are from 3 cuts in each harvest year. Measurements were made on total (grass + clover) forage samples, and data were averaged over two companion grass treatments. Crude protein yield: Year x Variety $P<0.001$ s.e.d. 0.149 (83 df) and within variety s.e.d. 0.165 (44 df); Crude protein content: Year x Variety $P<0.001$ s.e.d. 0.149 (83 df) and within variety s.e.d. 0.165 (44 df) Grass $P<0.001$ – grass treatment 1 147.5 v grass treatment 2 143.3, s.e.d. 1.15.

Figure 4. The effect of % red clover contribution to annual total DM on CP concentration (g $kg^{-1}$ DM) in the herbage in Year 2 (●) and Year 4 (○). $Y=0.5322(0.0253)x +112.85 (1.75)$ rsd=9.80, r=0.873, ($P<0.001$)

Table 1 Red clover (*Trifolium pratense* L.) populations included in the field experiment.

| Population | Country of origin | Description |
|---|---|---|
| Britta (S1) | UK | Survivor ex. Britta |
| Britta (S2) | UK | Survivor ex. Britta |
| Milvus (S) | UK | Survivor ex. Milvus |
| Vivi (S) | UK | Survivor ex Vivi |
| AberChianti | UK | Variety selected for strong crowns |
| AberClaret | UK | Variety selected for strong crowns |
| Amos (tetraploid) | Czech Republic | Variety |
| Britta | Sweden | Variety |
| Merviot | Belgium | Variety |
| Milvus | Switzerland | Variety |
| Pavo | Switzerland | Variety |
| Vivi | Sweden | Variety |

Table 2 Meteorological data (rainfall (mm), average minimum and maximum temperature (°C), at IBERS, Aberystwyth in 2008, 2009, 2010, 2011 and 2012, with 25 year (1988-2012) mean for comparison.

| | Rainfall (mm) | | | | | | Average minimum temperature (°C) | | | | | | Average maximum temperature (°C) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2008 | 2009 | 2010 | 2011 | 2012 | *25 year mean* | 2008 | 2009 | 2010 | 2011 | 2012 | *25 year mean* | 2008 | 2009 | 2010 | 2011 | *2012* | *25 year mean* |
| March | 107.8 | 40.4 | 78.6 | 24.2 | 22.6 | *76.0* | 3.6 | 3.5 | 1.9 | 2.4 | 4.7 | *3.1* | 9.5 | 10.4 | 10.1 | 11.5 | 12.8 | *9.5* |
| April | 62.8 | 48.6 | 34.0 | 20.2 | 93.2 | *60.8* | 5.1 | 5.4 | 4.0 | 6.8 | 3.9 | *4.2* | 12.0 | 13.7 | 13.6 | 16.5 | 10.9 | *12.0* |
| May | 22.0 | 99.6 | 59.6 | 62.4 | 73.8 | *60.4* | 9.5 | 8.2 | 5.5 | 9.2 | 7.4 | *6.9* | 15.1 | 15.3 | 14.9 | 15.6 | 15.8 | *15.1* |
| June | 65.4 | 31.0 | 40.4 | 96.4 | 184.0 | *74.5* | 9.9 | 10.8 | 10.1 | 9.3 | 10.5 | *9.7* | 17.5 | 19.1 | 19.3 | 17.4 | 16.6 | *17.5* |
| July | 98.8 | 152.0 | 149.8 | 65.2 | 89.4 | *79.1* | 13.0 | 12.9 | 13.6 | 10.6 | 11.7 | *11.9* | 19.0 | 18.7 | 18.8 | 18.7 | 17.6 | *19.0* |
| August | 117.6 | 51.2 | 54.4 | 45.4 | 108.0 | *90.1* | 13.6 | 13.1 | 11.9 | 11.6 | 13.4 | *11.7* | 19.0 | 19.1 | 17.8 | 18.2 | 19.5 | *19.0* |
| September | 102.0 | 50.2 | 118.2 | 118.2 | 138.4 | *97.1* | 9.3 | 9.9 | 10.7 | 12.0 | 9.5 | *9.8* | 17.1 | 17.2 | 17.5 | 18.0 | 15.9 | *17.1* |
| October | 228.6 | 104.4 | 96.6 | 89.2 | 155.6 | *114.2* | 7.8 | 8.8 | 7.1 | 10.2 | 6.9 | *7.4* | 14.2 | 15.4 | 14.7 | 15.8 | 13.5 | *14.2* |
| *AVERAGE* | *100.6* | *71.8* | *78.9* | *65.2* | *108.1* | *81.5* | *8.9* | *9.1* | *8.1* | *9.0* | *8.5* | *8.1* | *15.4* | *16.1* | *15.8* | *16.5* | *15.3* | *15.4* |

Table 3 a) Annual total (grass + clover) yield (t DM ha$^{-1}$) of 12 red clover populations grown in plots with hybrid ryegrass (Treatment 1) or a hybrid/perennial ryegrass mixture (Treatment 2) over 4 harvest years. Yield is based on 3 cuts in each harvest year.

| Clover Population | Year 1 | | | Year 2 | Year 3 | Year 4 |
|---|---|---|---|---|---|---|
| | Treatment 1 | Treatment 2 | Mean | Mean | Mean | Mean |
| Britta (S1) | 15.0 | 17.9 | 16.5$^{e}$ | 16.2$^{bcd}$ | 12.8$^{cde}$ | 10.2$^{ab}$ |
| Britta (S2) 60 | 15.2 | 15.4 | 15.3$^{e}$ | 12.5$^{e}$ | 10.7$^{ef}$ | 10.8$^{ab}$ |
| Milvus (S) | 19.4 | 20.5 | 19.9$^{bc}$ | 19.0$^{b}$ | 16.5$^{a}$ | 11.8$^{a}$ |
| Vivi (S) | 15.6 | 16.1 | 15.9$^{e}$ | 15.4$^{de}$ | 11.2$^{def}$ | 9.3$^{b}$ |
| AberChianti | 20.2 | 18.6 | 19.4$^{bcd}$ | 18.2$^{bc}$ | 14.2$^{bcd}$ | 11.4$^{ab}$ |
| AberClaret | 21.0 | 23.0 | 22.0$^{a}$ | 17.7$^{bc}$ | 16.5$^{a}$ | 11.1$^{ab}$ |
| Amos | 18.8 | 18.3 | 18.6$^{de}$ | 16.1$^{bcd}$ | 12.2$^{cdef}$ | 9.7$^{ab}$ |
| Britta | 14.5 | 15.1 | 14.8$^{e}$ | 13.9$^{de}$ | 10.4$^{ef}$ | 9.9$^{ab}$ |
| Merviot | 17.5 | 18.3 | 17.9$^{d}$ | 16.0$^{bcd}$ | 11.2$^{def}$ | 9.5$^{b}$ |
| Milvus | 20.8 | 21.3 | 21.1$^{ab}$ | 19.3$^{b}$ | 13.1$^{cde}$ | 10.4$^{ab}$ |
| Pavo | 19.2 | 20.1 | 19.6$^{bcd}$ | 18.1$^{bc}$ | 14.6$^{bc}$ | 11.4$^{ab}$ |
| Vivi | 9.6 | 11.3 | 10.4$^{f}$ | 9.1$^{f}$ | 9.1$^{f}$ | 9.4$^{b}$ |
| *Mean* | 17.2 | 18.0 | | 15.9 | 12.7 | 10.4 |
| Prob Grass | | 0.008 | | 0.063 | 0.584 | 0.483 |
| Clover | | <0.001 | | <0.001 | <0.001 | 0.007 |
| Grass.Clover | | 0.148 | | 0.556 | 0.242 | 0.446 |
| s.e.m. Grass | | 0.19 | | 0.31 | 0.31 | 0.22 |
| Clover | | 0.47 | | 0.75 | 0.75 | 0.53 |
| Grass.Clover | | 0.67 | | 1.06 | 1.06 | 0.75 |

$^{a-e}$; Means with differing superscripts differ (P<0.05); df for within years = 46

df for within years = 46

Table 3 b) Annual red clover yield (t DM ha$^{-1}$) of 12 red clover populations grown in plots with hybrid ryegrass (Treatment 1) or a hybrid/perennial ryegrass mixture (Treatment 2) over 4 harvest years. Yield is based on 3 cuts in each harvest year.

| Clover Population | Year 1 | | | Year 2 | Year 3 | Year 4 |
|---|---|---|---|---|---|---|
| | Treatment 1 | Treatment 2 | Mean | Mean | Mean | Mean |
| Britta (S1) | 12.4 | 13.1 | 12.8$^{de}$ | 14.7$^{abc}$ | 8.3$^{dc}$ | 2.1$^{c}$ |
| Britta (S2) | 11.4 | 11.4 | 11.4$^{e}$ | 10.6$^{d}$ | 5.0$^{e}$ | 2.0$^{c}$ |
| Milvus (S) | 14.2 | 17.0 | 15.6$^{abc}$ | 17.5$^{ab}$ | 12.7$^{a}$ | 6.9$^{a}$ |
| Vivi (S) | 12.6 | 12.2 | 12.4$^{de}$ | 14.1$^{bc}$ | 7.4$^{cde}$ | 1.7$^{b}$ |
| AberChianti | 16.4 | 15.2 | 15.8$^{abc}$ | 17.2$^{ab}$ | 11.2$^{ab}$ | 5.2$^{b}$ |
| AberClaret | 17.3 | 17.8 | 17.6$^{a}$ | 16.5$^{ab}$ | 12.8$^{a}$ | 6.8$^{a}$ |
| Amos | 13.4 | 14.8 | 14.1$^{cd}$ | 14.8$^{abc}$ | 8.5$^{cd}$ | 1.9$^{c}$ |
| Britta | 11.0 | 11.6 | 11.3$^{e}$ | 12.2$^{dc}$ | 5.7$^{de}$ | 1.9$^{c}$ |
| Merviot | 13.9 | 15.4 | 14.6$^{bcd}$ | 14.6$^{abc}$ | 6.9$^{cde}$ | 1.3$^{c}$ |
| Milvus | 17.4 | 18.7 | 18.1$^{a}$ | 18.1$^{a}$ | 9.4$^{bc}$ | 4.3$^{b}$ |
| Pavo | 16.0 | 18.0 | 17.0$^{ab}$ | 17.0$^{ab}$ | 11.1$^{ab}$ | 5.0$^{b}$ |
| Vivi | 5.3 | 5.9 | 5.6$^{ff}$ | 7.5$^{e}$ | 5.0$^{e}$ | 1.0$^{c}$ |
| Mean | 13.4 | 14.2 | | .. 14.6 | 8.66 | 3.4 |
| Prob Grass | 0.050 | | | 0.245 | 0.439 | 0.979 |
| Clover | <0.001 | | | <0.001 | <0.001 | <0.001 |
| Grass.Clover | 0.774 | | | 0.406 | 0.278 | 0.470 |
| s.e.m. Grass | 0.28 | | | 0.34 | 0.29 | 0.18 |
| Clover | 0.68 | | | 0.82 | 0.70 | 0.45 |
| Grass.Clover | 0.96 | | | 1.16 | 0.99 | 0.64 |

$^{a-f}$; Means with differing superscripts differ ($P<0.05$); df for within years = 46

Table 4. Number of red clover plants m$^{-2}$ in Years 3 and 4, averaged over two companion grass treatments. Data are derived from the mean of duplicate 0.25m$^2$ quadrats and observations in May and September.

| Clover Variety/Selection line | Year 3 | Year 4 | *Mean* |
|---|---|---|---|
| Britta (S1) | 85.4 | 66.3 | *75.9* |
| Britta (S2) Aa 4560 | 57.3 | 42.5 | *49.9* |
| Milvus (S) | 77.0 | 66.0 | *71.5* |
| Vivi (S) | 66.0[a] | 28.8[b] | *47.4* |
| AberChianti | 61.8 | 64.8 | *63.3* |
| AberClaret | 62.7 | 57.7 | *60.2* |
| Amos | 47.3[a] | 20.5[b] | *33.9* |
| Britta | 55.8 | 46.2 | *51.0* |
| Merviot | 34.5 | 20.5 | *27.5* |
| Milvus | 45.0 | 49.0 | *47.0* |
| Pavo | 55.8 | 47.3 | *51.6* |
| Vivi | 24.5 | 16.7 | *20.6* |
| *Mean* | *56.1* | *43.9* | |

| | | |
|---|---|---|
| Probability | Clover | <0.001 |
| | Year | <0.001 |
| | Year x Clover | 0.020 |
| s.e.m. | Year x Clover | 6.09 (84 df) |
| | within Clover | 5.32 (46 df) |

[a, b]; indicates a significant change in plant density between years based on Bonferroni adjusted pairwise multiple comparisons within red clover population