

Red clover varieties of Mattenklee type have higher production and persistence than Ackerklee types in grass-clover mixtures

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Abstract

Dairy farmers have increased interest in multiyear red clover–ryegrass ley pasture swards because of their high productivity and protein concentration but the adoption of such mixtures has been restricted because of poor persistency of many red clover cultivars currently used. We aimed (i) to assess dry-matter yield (DMY), persistence and nutritive value of four Mattenklee and four Ackerklee cultivars and (ii) to investigate the underlying plant morphological traits. We conducted a 4-year field experiment with red clover–ryegrass mixtures, in combination with a short-term pot experiment in which the red clover cultivars were grown both in monoculture and in mixture with *Lolium perenne* L. In the field, Mattenklee showed higher production (on average 42% higher) and clover content (on average 34% higher) in the third and fourth year of production compared to Ackerklees. In the fourth year, Mattenklee had slightly lower digestibility and lower protein concentration compared to Ackerklees cultivars; however, the digestible DMY and the protein yield were higher for Mattenklee. Both persistence and nutritive value of the different red clover cultivars were closely correlated with plant morphological traits. For stem length, there was close correlation ($r > .77$) between the results

from the pot experiment and the field experiment, suggesting that stem length may be an important trait for plant breeding.

Keywords: nutritive value, morphology, breeding, plant traits, *Trifolium pratense* L.

Introduction

In recent decades, the European forage sector has been confronted with new challenges. In order to reduce the adverse effects of agricultural practices on the environments, the European Union (EU) has implemented a series of guidelines to its member states (e.g., the EU Nitrates Directive Drinking Water Directive). These directives have resulted in a limitation in the amount of nutrients (particularly nitrogen) that can be applied to grassland. As a result, forage legumes with their ability for symbiotic N₂ fixation are playing an increasingly important role for grassland forage production in the dairy sector (Gierus et al., 2012; Lüscher et al., 2014; Mela, 2003). Red clover (*Trifolium pratense* L.) is the second-ranking forage legume worldwide after alfalfa (*Medicago sativa* L.) based on the amounts of seed produced, marketed and cultivar availability (Boller et al., 2010). Especially in the northwest of Europe, red clover is gradually gaining momentum as part of a rotation scheme with grass-clover and maize silage, although white clover (*Trifolium repens* L.) is the dominant forage legume species in this region (Gierus et al., 2012).

In The Netherlands, there is an increasingly high interest in red clover for ley pastures with dairy farmers, who see its value and potential in terms of symbiotic nitrogen fixation, protein concentration and production capacity (Iepema et al., 2006). One of the main constraints that restricts the adoption of such mixtures is the poor persistency of the red clover cultivars currently used by farmers (Iepema et al., 2006). These farmers are increasingly specialised in dairy production, have less arable crops to rotate the red clover with and would like to use these leys for more than three years. The latter would reduce costs for seeding but also positively influence soil organic matter build up, carbon sequestration and soil biodiversity (van Eekeren et al., 2008). Although a natural decline in plant populations is typical for clover swards (Frame et al., 1998), disease infestation, pest attacks, improper management, winter-killing and poor competitiveness have all been reported as important factors leading to poor persistence (Boller et al., 2010; Taylor, 2008; Taylor and Quesenberry, 1996). In the 20th century, considerable efforts of red clover breeding programs and research have aimed to improve red clover resistance to various fungal and virus diseases, pests and

nematodes (Christensen et al., 1994; Coulman and Lambert, 1995; Delclos et al., 1997; Marum et al., 1994; Page et al., 1997; Quesenberry et al., 1989; Rufelt, 1985). Although forage production and disease resistance of many red clover cultivars have been improved considerably during the last decades, most cultivars still suffer from poor persistence. In contrast, in Switzerland highly persistent and locally adapted red clover landraces, the so-called “Mattenklees”, show a high level of survival after three growing seasons in swards mixed with grass (as opposed to the traditional “Ackerklees”).

Red clover is mainly grown in mixture with grasses for a number of reasons, including nutritional composition of the forage, higher N fixation efficiency (Nyfeler et al., 2011), higher production, lower contamination with soil during the harvest and positive effects on soil quality (Mytton et al., 1993; Van Eekeren et al., 2009). The red clover proportion is an important feature for the forage quality as well as agronomic performance of binary mixtures with legumes and grasses (Kleen et al., 2011) and is determined by the competitive ability of red clover and the companion grass. Important plant traits in this respect are for example plant height and leaf area (competition for light), N-fixating capacity, plant survival and persistence.

There has been some effort to link easily measurable morphological characteristics to persistence. For example, Herrmann et al. (2008) showed that persistence is highly positively correlated with stem length. Also, flowering responses have been used as an indicator for persistence, with early flowering suggesting a higher persistence (Annicchiarico and Pagnotta, 2012; Ford and Barrett, 2011). In addition to differences in dry matter yield and production, red clover cultivars may differ in nutritional quality, e.g. protein concentration and digestibility (Frame et al., 1972).

The objective of this research was 1) to assess the DMY, persistence, nutritive value (CP-concentration, digestibility) and total N uptake of four Mattenlee and four Ackerlee cultivars grown in mixture with perennial ryegrass, and 2) to increase our insight in differences in persistency, competitive ability and nutritive value by relating them to plant morphological traits including stem length, leaf:stem ratio and plant density. To this end we conducted a four-year field experiment with red clover – grass mixtures in combination with a short pot-experiment in which red clover was grown both in monoculture and mixture with *Lolium perenne* L. We hypothesize that: 1) Mattenlee cultivars grown in mixture with perennial ryegrass show higher clover and total herbage yield compared to Ackerlee cultivars in the third and fourth production year, and 2) that this increased persistence can be correlated to plant morphological traits.

Materials and Methods

Field experiment

Experimental setup

The field experiment was established in August 2011 at an organic dairy farm located at Esbeek, The Netherlands (51°26'44"N, 5°7'13"E). The field had been previously used for arable production, with a crop rotation of green beans, potato and lettuce, operating in accordance with organic crop cultivation rules in the Netherlands (Skal). The field received 20 tons ha⁻¹ of farmyard manure in spring 2011 (104 kg N-total ha⁻¹, 78 kg P₂O₅ ha⁻¹, 268 kg K₂O ha⁻¹), in combination with 1 ton ha⁻¹ of ground limestone to restore soil pH. The soil texture was loamy sand with 2% clay, 18% silt and 76% sand. Soil fertility characteristics in the 0-10 cm soil layer were summarized as follows: soil pH-KCl, 5.4; soil organic matter, 3.9%; CEC, 50 mmol kg⁻¹; total nitrogen, 0.13%; C/N ratio, 17; available P, 0.9 mg P kg⁻¹; available K, 74 mg K kg⁻¹.

The trial was arranged in a randomized block design with four replicates. Eight red clover (*Trifolium pratense* L.) cultivars including four Ackerklee landraces (cv. Avanti, Lemmon, Maro and Taifun) and four Mattenklee landraces (cv. Fregata, Larus, Milvus and Pavo) were selected, of which five were tetraploid (Avanti, Maro, Taifun, Fregata and Larus) and three diploid (Lemmon, Milvus and Pavo) cultivars. The red clover was sown in mixture with diploid perennial ryegrass (*Lolium perenne* L., 30 kg ha⁻¹) in August 2011 in plots of 4 m × 8 m. In order to achieve an equal red clover plant density for the eight red clover cultivars, seeding rates for the different cultivars were adjusted to 270 viable seeds m² based on 1000 seed weight and germination rate (Table A1).

The trial was managed with a four-cut regime (cut in May, July, August and October) under rainfed conditions during four years (2012-2015). Throughout the experiment, plots received mean annual fertilizer applications of 25 m³ ha⁻¹ cattle slurry and 300 kg ha⁻¹ potassium sulfate (K₂SO₄) (90 kg N ha⁻¹, 38 kg P₂O₅ ha⁻¹, 300 kg K₂O ha⁻¹)

Yield, persistence and chemical composition

Measurements were carried out during 2012, 2014 and 2015, the first, third and fourth year after sowing, and no measurements took place during 2013, as this year was deemed less relevant in terms of persistence. Total DMY at each harvest was determined by cutting a strip

of 0.84 m × 4 m within each plot with a front bar mower (EuroSystems, Italy), leaving a sward stubble height of approximately 6 cm. The samples were weighed and a subsample was taken for DM analysis. Samples were dried in a forced-draught oven at 70°C for 24 h and corrected for residual water content, by drying a subsample for 2 h at 105°C for DM determination. In 2015, for all harvests a subsample was analyzed for total nitrogen concentration (total-N) (BLGG AgroXpertus, The Netherlands), based on which the crude protein (CP) concentration and yield was calculated.

Additionally, for each harvest a fresh sub-sample of at least 0.2 kg was manually separated into red clover, white clover, perennial ryegrass and weeds and herbs for obtaining the botanical composition. The separated red clover sub-samples taken from the 3rd cut in 2012, 2nd cut in 2014 and 1st cut in 2015 were dried and analyzed for ash concentration, N-total and digestible organic matter in the dry matter (DOMD) (BLGG AgroXpertus, The Netherlands).

Clover morphology

Before the first harvest in 2015, plant density was determined by counting the number of red clover plants in a 1 m² quadrat in each plot. Additionally, morphological traits of the red clover cultivars were measured in three plants per plot, which were cut above root crown level. For each plant, ten normally developed medial leaflets (petiole not included) from the top 10-15 cm were cut for measuring the leaf area (cm²) with ImageJ (Schneider et al., 2012), following the protocol of standardized measurement of plant functional traits (Pérez-Harguindeguy et al., 2013). These leaves were dried at 40 °C for 72 h and corrected for residual water content, by drying a subsample for 2 h at 105 °C. The specific leaf area (SLA, cm² g DM⁻¹) was calculated as leaf area divided by leaf DM weight. For each plant, the number of stems was counted and the length of the longest stem was measured. The clover samples were divided into leaves and stems (petiole included) dried at 40°C for 72h to determine shoot weight (g plant⁻¹) and leaf:stem ratio (g g⁻¹).

Pot experiment

The four Mattenkee and four Ackerkee cultivars used in the field experiment and one perennial ryegrass (*Lolium perenne* L.) cultivar (cv. Mathilde) were sown in pots alone and in mixtures on the 20th August 2014 in a greenhouse at Wageningen University, The Netherlands (51°59'11"N, 5°40'5"E). The pot experiment was arranged in a randomized block design with four replicates and consisted of one grass monoculture, eight red clover monocultures and

eight red clover-perennial ryegrass 50:50 mixtures per block. All the pots included four plants per pot at a distance of 5 cm in a square, and the mixture contained two plants of each species per pot.

The soil was collected from Nergena experimental farm at Wageningen University (51°59'46"N, 5°39'29"E) and had a sandy texture. The soil was sieved through an aluminum mesh (50 mm) and mixed thoroughly. Each pot (1 L) was filled with the same amount of approximately 1 kg soil. The soil NH_4^+ -N and NO_3^- -N concentration were 2.0 mg kg^{-1} and 8.0 mg kg^{-1} , respectively. The temperature in the greenhouse was $20.9 / 16.5 \text{ }^\circ\text{C}$ (day / night), and all the pots were arranged under a natural long-day light cycle condition. The pots were watered every day to keep the water-holding capacity constant at 60%. The pots were hand-weeded and received no fertilizer. The few plants infested with powdery mildew were sprayed with an organic fungicide "Microsulfo spuitzwavel" (Bayer Garden, The Netherlands).

Eight weeks after sowing, all pots were harvested. Red clover and grass plant height were measured and clover leaf area (petiole included) per pot was measured by a leaf area scanner (LI-COR, USA). The clover plants were separated into leaves (petiole included) and stems and oven dried at $70 \text{ }^\circ\text{C}$ for 72 h for dry matter (DM) determination.

Statistical analyses

Data were subjected to analysis of variance (ANOVA) in Genstat (Genstat 18, VSB international). The different yield and morphological response variables in the field and pot experiment were tested for a cultivar effect ($n = 4$), for a clover type effect (Mattenklee vs Ackerklee, $n = 16$) and ploidy effect (diploid vs tetraploid, $n = 16$). Additionally, response variables from the pot experiment were tested for a mixture effect ($n = 16$). Data were checked for normality and log-transformed if required. Spearman correlation coefficients were conducted in Genstat to determine the association between different morphological traits, nutritive value and persistence indicators.

Results

Field experiment

Red clover content

In 2012, the year of establishment, the red clover content (% of total herbage DMY) was similar for all cultivars with on average 58% of the herbage yield being red clover (Fig. 1). In 2014, the red clover cultivars differed in their proportional contribution to the herbage yield in

the grass-clover mixtures and clover content ranged from 59% for Avanti (AK) to 81% for cv Larus (MK) (significant cultivar effect at $P < 0.001$). There was also a significant difference between the Mattenkleee and Ackerkleee clover types ($P < 0.001$), with Ackerklees representing on average 61 % and Mattenklees 77 % of the herbage dry matter yield. In 2015, the clover content had decreased to 42 % and 60 % for Ackerkleee and Mattenkleee, respectively (significant clover type effect, $P < 0.001$). Clover content was highest for Larus (MK) (75%) and lowest for Lemmon (AK) (37%) (significant cultivar effect at $P < 0.001$). The decrease in red clover content from 2012 to 2015 was accompanied by an increase in the proportion of *Lolium perenne* from 31 % to 47 % and a decrease in the proportion of unsown species decreased from 11% to 2 % (data not shown).

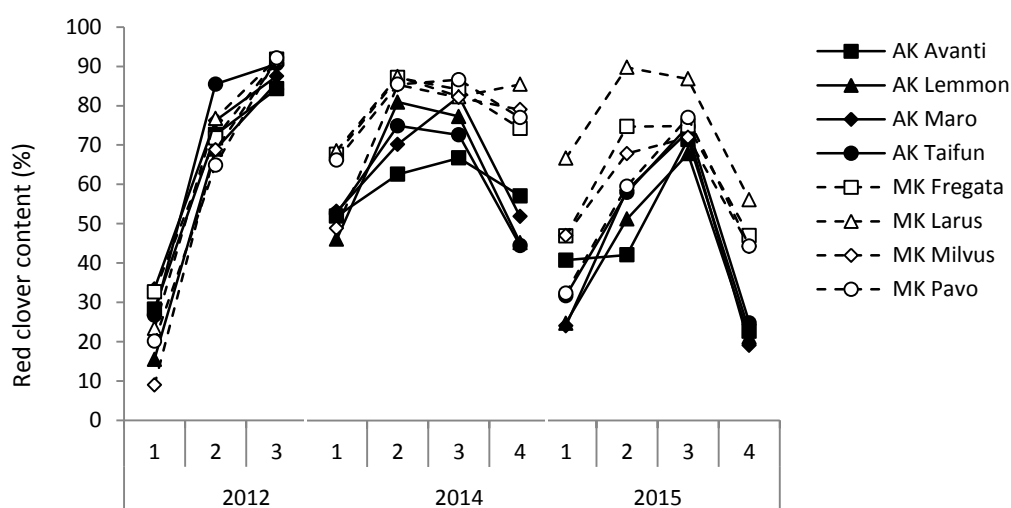


Figure 1. Red clover content (% of total herbage dry matter yield) of the four Ackerkleee cultivars (solid lines and symbols) and the four Mattenkleee cultivars (dashed lines and open symbols), during harvest 1-4 in 2012, 2014 and 2015.

Red clover dry matter yield

In 2012, there was no significant effect of clover type on red clover DMY (Table 1), which was on average 6.7 ton ha^{-1} . However in 2012, there was a significant effect of cultivar ($P < 0.01$) on red clover DMY, with the highest DMY for Maro (AK) (7.7 ton ha^{-1}) and the lowest DMY for Milvus (MK) (5.4 ton ha^{-1}). Moreover, the red clover DMY was significantly ($P < 0.01$) higher for the tetraploid compared to the diploid cultivars. In 2014 the red clover DMY was on average 9.4 ton ha^{-1} and was significantly different between clover types with DMY being 33% higher for the Mattenklees compared to the Ackerklees ($P < 0.001$). The clover DMY was also significantly different between red clover cultivars ($P < 0.001$) being highest for Larus and Pavo (MK) (11 ton ha^{-1}) and lowest for Avanti, Lemmon and Taifun (AK) (7.4 ,

7.9 and 7.9 ton ha⁻¹, respectively). The mean red clover DMY yield in 2015 was 5.5 ton ha⁻¹ and was significantly ($P < 0.001$) higher for the Mattenklees with 6.7 ton ha⁻¹ compared to the Ackerklees with 4.4 ton ha⁻¹. Clover DMY ranged from 3.7 ton ha⁻¹ for Lemmon (AK) to 8.4 ton ha⁻¹ for Larus (MK).

The cumulative red clover DMY across the three measurement years was significantly higher ($P < 0.001$) for the Mattenklees (23.9 ton DM ha⁻¹) compared to the Ackerklees (19.3 ton DM ha⁻¹, Table 1). Larus (MK) showed the highest and Lemmon (AK) the lowest cumulative red clover DMY.

The persistence of red clover quantified as the yield index (relative red clover performance in 2015 compared to 2012), was significantly ($P < 0.001$) higher for the Mattenklees compared to the Ackerklees, with 107 % for Mattenklees and 67 % for Ackerklees of the DMY production in 2012 (Table 1).

Table 1. Mean annual red clover dry matter yield (DMY) and total herbage dry matter yield of the four Ackerklees (AK) and four Mattenklees (MK) in 2012, 2014 and 2015 and the yield index (DMY 2015 / DMY 2012 x 100%). Cultivar data are means of four values. Values within columns followed by the same letters are not significantly different ($P > 0.05$)

Cultivar	Red clover DMY (t ha ⁻¹)				Total herbage DMY (t ha ⁻¹)				
	2012	2014	2015	Yield index	2012	2014	2015	Yield index	
Avanti (AK, 4n)	6.2 ^{cde}	7.4 ^c	4.8 ^{def}	80 ^{ab}	11.0 ^{bc}	12.4 ^d	10.6 ^{bcd}	98 ^{bc}	
Lemmon (AK, 2n)	5.9 ^{de}	7.9 ^c	3.7 ^f	68 ^a	10.9 ^{bc}	12.7 ^{cd}	10 ^{cd}	92 ^{ab}	
Maro (AK, 4n)	7.7 ^a	9.1 ^{bc}	4 ^{ef}	53 ^a	12.6 ^a	14.3 ^a	9.8 ^d	79 ^a	
Taifun (AK, 4n)	7.5 ^{ab}	7.9 ^c	5 ^{de}	68 ^a	11.7 ^{ab}	12.9 ^{bcd}	10.8 ^{abc}	94 ^{ab}	
Fregata (MK, 4n)	6.7 ^{abcd}	10.7 ^{ab}	6.8 ^b	105 ^{bc}	11.4 ^{bc}	13.7 ^{abc}	11.6 ^a	104 ^{bc}	
Larus (MK, 4n)	7.4 ^{abc}	11.1 ^a	8.4 ^a	116 ^c	12.0 ^{ab}	13.8 ^{abc}	11.2 ^{ab}	94 ^{ab}	
Milvus (MK, 2n)	5.4 ^e	9.9 ^{ab}	6.3 ^{bc}	125 ^c	10.2 ^c	13.8 ^{abc}	11.2 ^{ab}	112 ^c	
Pavo (MK, 2n)	6.4 ^{bcd}	11 ^a	5.2 ^{cd}	82 ^{ab}	11.6 ^{ab}	14.1 ^{ab}	10.5 ^{bcd}	92 ^{ab}	
SED	0.58	0.86	0.53	14.9	0.59	0.61	0.45	7.3	
P-value	**	***	***	***	*	*	**	*	
Mean Type									
	AK	6.8	8.1	4.4	67	11.6	13.1	10.3	91
	MK	6.5	10.7	6.7	107	11.3	13.8	11.1	100
SED		0.39	0.43	0.41	8.3	0.36	0.34	0.26	4.3
P-value		ns	***	***	***	ns	*	**	**

Total herbage dry matter yield

Total dry matter yield followed a trend similar to the red clover dry matter yield, as described above, however, the differences between the clover types and cultivars were less pronounced (Table 1). For example, in 2015 total herbage DMY of the Mattenklees was only 8 % higher

than Ackerklees (compared to 54 % for red clover DMY). Mean total herbage DMY was 11.4, 13.5 and 10.7 ton ha⁻¹ during 2012, 2014 and 2015, respectively.

The total herbage DMY cumulative across the three measurement years was significantly ($P < 0.05$) but only slightly higher for the Mattenklees (36.3 ton DM ha⁻¹) compared to the Ackerklees (35.0 ton DM ha⁻¹, Table 1). For the individual cultivars, Avanti and Lemmon (both AK) had a significantly lower total herbage DMY compared to Maro (AK), Fregata (MK) and Larus (MK).

The yield index for total herbage DMY was significantly ($P < 0.001$) higher for the Mattenklees compared to the Ackerklees, both in 2014 and 2015 (Table 1), and in 2015 was on average 100 % for Mattenklees and 91% for Ackerklee.

Table 2. Red clover crude protein (CP) concentration (g kg⁻¹ DM) of the four Ackerklees (AK) and four Mattenklees (MK) during the third harvest (H3) in 2012, the second harvest (H2) in 2014 and the first harvest (H1) in 2015 and total average herbage CP concentration and cumulative herbage CP yield during 2015. Cultivar data are means of four values. Values within columns followed by the same letters are not significantly different ($P > 0.05$)

Cultivar	Red clover CP concentration (g kg ⁻¹ DM)			Herbage CP concentration (g kg ⁻¹ DM)	Herbage CP yield (kg ha ⁻¹)	
	2012-H3	2014-H2	2015-H1	2015	2015	
Avanti (AK, 4n)	209	194	215 ^b	169 ^{bcd}	1777 ^{bc}	
Lemmon (AK, 2n)	211	202	215 ^b	151 ^e	1510 ^d	
Maro (AK, 4n)	210	209	221 ^b	163 ^{cd}	1602 ^{cd}	
Taifun (AK, 4n)	196	200	242 ^a	162 ^{de}	1760 ^{bc}	
Fregata (MK, 4n)	193	199	182 ^c	176 ^{ab}	2041 ^a	
Larus (MK, 4n)	208	198	190 ^c	182 ^a	2034 ^a	
Milvus (MK, 2n)	201	203	189 ^c	173 ^{abc}	1945 ^{ab}	
Pavo (MK, 2n)	207	196	190 ^c	171 ^{bcd}	1800 ^{bc}	
SED	8.9	6.2	55.4	4.8	103.8	
P-value	ns	ns	***	***	***	
Type	AK	206	201	223	161	1654
	MK	202	199	188	175	1955
SED		4.5	3.1	40.4	3.0	61.4
P-value		ns	ns	***	***	***

Crude protein concentration and yield

In 2012-H3 and 2014-H2, there were no significant differences in red clover crude protein (CP) concentration between the cultivars (Table 2). In 2015-H1, there was a significant red clover cultivar ($P < 0.001$) and type ($P < 0.001$) effect on the red clover CP concentration, which was on average 223 for the Ackerklees and 188 g kg⁻¹ DM for the Mattenklees (Table 2). For all years there was a significant ($P < 0.05$) cultivar effect on the red clover CP yield (Table A2), however, the order of the cultivars differed between years. For example, Maro

(AK, 4N) had the highest CP yield in 2012-H3, but the lowest CP yield in 2015-H1. During 2012-H3 there was no significant clover type effect on clover CP yield, whereas in 2014-H2 and 2015-H1 clover CP-yield was significantly ($P < 0.05$) higher for the Mattenklees compared to Ackerklees (27 % higher during 2014 and 39 % higher during 2015).

For the total herbage CP concentration and yield (measured in 2015 only), there was a significant effect of cultivar and clover type ($P < 0.001$) (Table 2). Herbage CP concentration was 9 % higher and herbage CP yield was 18 % higher, for the Mattenlee cultivars (in particular Fregata and Larus) compared to the Ackerlee cultivars.

Red clover digestible organic matter concentration

Red clover digestible organic matter concentration (DOMD) was on average 72 % in 2012-H3, 73 % in 2014-H2 and 77% in 2015-H1 (Table 3). During 2015, there was a significant cultivar ($P < 0.001$) and type ($P < 0.001$) effect on the red clover DOMD, which was on average 78.8 % for the Ackerklees and 76.6 % for the Mattenklees. Clover digestible organic matter yield ranged from 1006 kg ha⁻¹ (Lemmon, 2015-H1) to 2369 kg ha⁻¹ (Maro, 2012-H1) (Table A2). Clover DOM yield was higher for Mattenklees compared to Ackerklees, but only significantly so during 2014-H2 ($P < 0.05$).

Table 3. Red clover digestible organic matter concentration in the dry matter (DOMD, g kg⁻¹ DM) of the four Ackerklees (AK) and four Mattenklees (MK) during the third harvest (H3) in 2012, the second harvest (H2) in 2014 and the first harvest (H1) in in 2015. Cultivar data are means of four values. Values within columns followed by the same letters are not significantly different ($P > 0.05$)

Cultivar	Red clover DOMD (g kg ⁻¹ DM)			
	2012-H3	2014-H2	2015-H1	
Avanti (AK, 4n)	721	732	787 ^{ab}	
Lemmon (AK, 2n)	726	737	785 ^{ab}	
Maro (AK, 4n)	723	743	791 ^a	
Taifun (AK, 4n)	714	724	791 ^a	
Fregata (MK, 4n)	704	727	753 ^d	
Larus (MK, 4n)	714	724	773 ^{bc}	
Milvus (MK, 2n)	704	737	763 ^{cd}	
Pavo (MK, 2n)	724	736	773 ^{bc}	
SED	9.6	10.4	8.3	
P-value	ns	ns	***	
Type	AK	721	733	788 ^a
	MK	712	731	766 ^b
SED		5.0	5.1	4.4
P-value		0.07	ns	***

Red clover morphology

There was a significant ($P < 0.001$) effect of cultivar on plant density, which was highest for Larus (40 plants m^{-2}) and lowest for Lemmon (12 plant m^{-2}) (Table 4). Plant density was significantly ($P < 0.001$) higher for the Mattenklees compared to the Ackerklee type, with 32 and 21 plants m^{-2} , respectively. Shoot weight was also significantly ($P < 0.05$) higher for the Mattenklees (12.7 g $plant^{-1}$) compared to the Ackerklees (9.5 g $plant^{-1}$). The specific leaf area was on average 231 $cm^2 g DM^{-1}$) and there was no significant effect of cultivar, type or ploidy. Mean stem length (cm) ranged from 34 cm for Lemmon (AK) to 49 cm for Larus (MK). The Mattenklees had a significantly ($P < 0.001$) longer stem than the Ackerklees. The leaf:stem ratio ranged from 0.45 $g g^{-1}$ for Milvus (MK) to 0.62 $g g^{-1}$ for Taifun (AK) (significant cultivar effect at $P < 0.01$). The leaf:stem ratio tended to be higher for the Ackerklees, and was significantly ($P < 0.01$) higher for tetraploid compared to diploid cultivars.

Table 4. Morphological traits of the four Ackerklee (AK) and four Mattenlee (MK) red clover cultivars measured in the first cut during 2015. Cultivar data are means of four values. Values within columns followed by the same letters are not significantly different ($P > 0.05$)

Cultivar	Plant density (# m^{-2})	Shoot weight (g $plant^{-1}$)	SLA ($cm^2 g DM^{-1}$)	Stem length (cm)	Leaf:stem ratio ($g g^{-1}$)	
Avanti (AK, 4n)	26.7 ^{cd}	10.0	231	38.9 ^{cd}	0.60 ^{ab}	
Lemmon (AK, 2n)	12.3 ^f	9.0	248	33.8 ^d	0.49 ^{cd}	
Maro (AK, 4n)	21.8 ^e	8.6	239	41.7 ^{bc}	0.55 ^{abc}	
Taifun (AK, 4n)	23.4 ^{de}	10.3	222	39.8 ^c	0.62 ^a	
Fregata (MK, 4n)	31.6 ^b	10.6	221	47.9 ^a	0.55 ^{abc}	
Larus (MK, 4n)	40.3 ^a	13.4	222	48.9 ^a	0.50 ^{cd}	
Milvus (MK, 2n)	32.5 ^b	13.7	237	47.5 ^{ab}	0.45 ^d	
Pavo (MK, 2n)	25.5 ^{cd}	13.2	233	45.9 ^{ab}	0.52 ^{bcd}	
SED	1.67	2.71	16.4	2.88	0.037	
P-value	***	ns	ns	***	**	
Type	AK	21.1	9.5	235	38.6	0.56
	MK	32.5	12.7	228	47.6	0.51
SED		2.17	1.26	7.9	1.52	0.023
P-value		***	*	ns	***	0.06

Pot experiment

Red clover and grass yield

There was a significant cultivar effect ($P < 0.001$) and clover type effect ($P < 0.001$) on the red clover yield in the pot experiment, and monoculture yields ranged from 4.6 g pot^{-1} for

Avanti (AK) to 6.0 g pot^{-1} for Fregata (MK) (Fig. 2). Red clover yield was significantly ($P < 0.001$) lower in mixtures (3.9 g pot^{-1}) compared to monocultures (5.4 g pot^{-1}). However, the relative yield ($\text{RY} = \text{mix} / \text{mono}$) which takes into account planted red clover density in the mixture relative to the monoculture was on average 0.7 (which was higher than 0.5 as expected based on sown proportion). This indicates that the yield per red clover plant was significantly higher in mixtures compared to monocultures (Fig. 3a). There was no significant interaction between cultivar (or clover type) and mixture, indicating that all cultivars were equally affected by growing in a mixture with *Lolium perenne*.

The grass yield was on average 0.5 and 0.2 g pot^{-1} for monocultures and mixtures, respectively. The grass RY was on average 0.5 (indicating that the yield per grass plant was not affected by growing in mixture with red clover) and was not significantly affected by red clover cultivar or type.

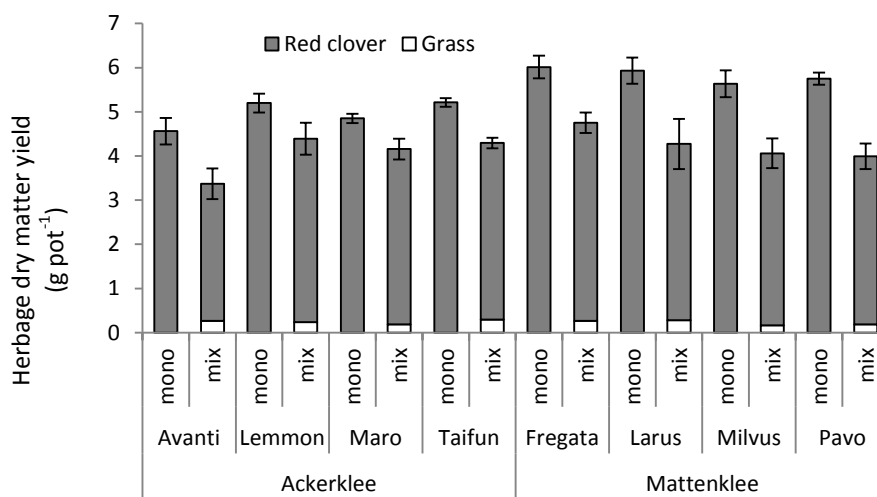


Figure 2. Grass and red clover dry matter yield of the four Ackerlee and the four Mattenlee cultivars grown in monoculture or mixture in the pot experiment.

Red clover morphology

Red clover plant height, SLA and leaf:stem-ratio were all significantly ($P < 0.001$) affected by red clover cultivar and type (Fig. 3b-d). Red clover plant height ranged from 20.4 cm for Lemmon (AK) to 27.2 cm for Fregata (MK), the SLA ranged from $168 \text{ cm}^2 \text{ g}^{-1} \text{ DW}$ for Fregata (MK) to $208 \text{ cm}^2 \text{ g}^{-1} \text{ DW}$ for Avanti (AK) and the leaf:stem-ratio was lowest for Fregata and Milvus (MK, 1.0 g g^{-1}) and highest for the Ackerlees (1.2 g g^{-1}). For red clovers grown in mixture compared to monoculture the plant height was significantly ($P < 0.001$) higher, whereas the leaf:stem-ratio was significantly ($P < 0.001$) lower (Fig. 3).

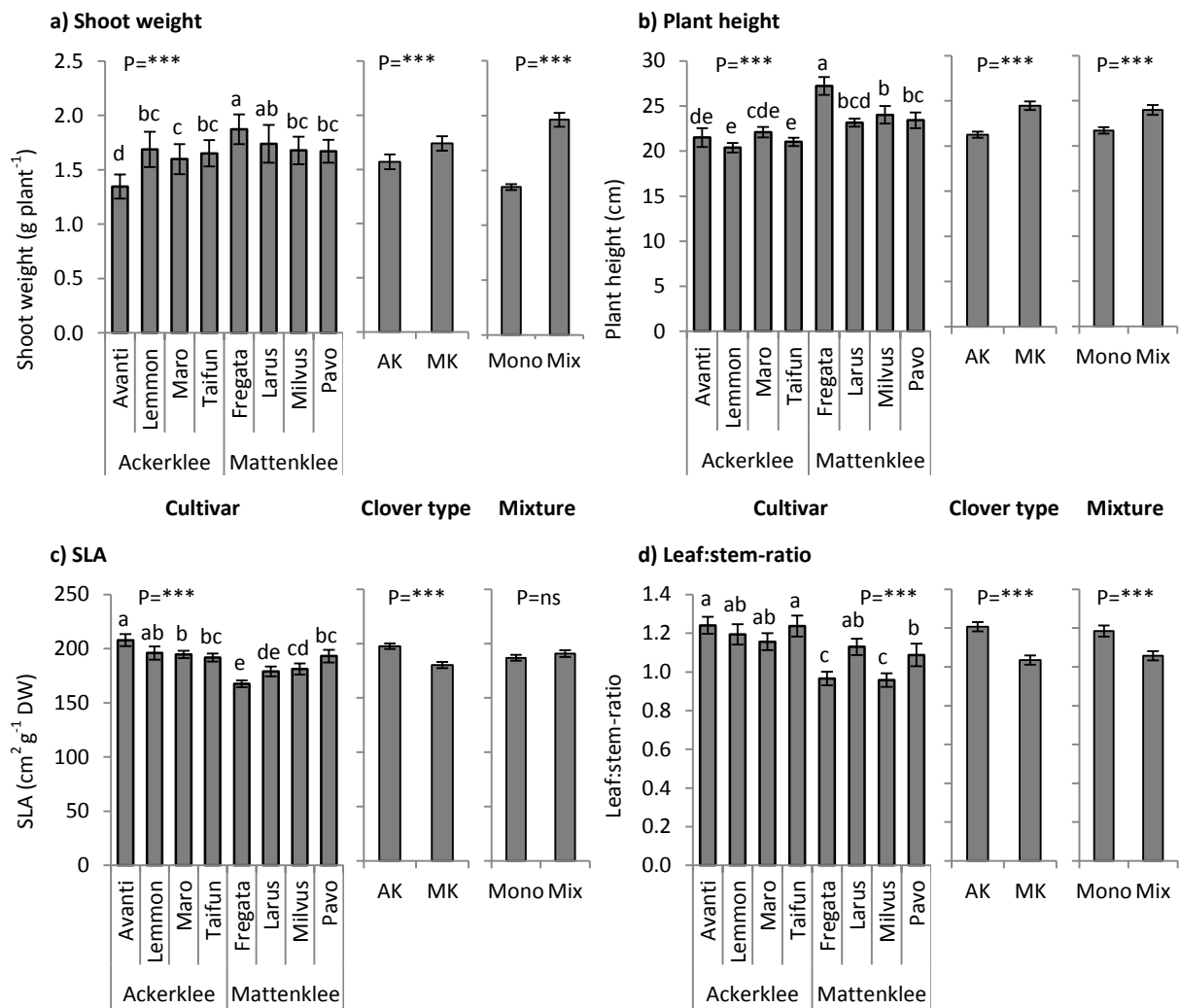


Figure 3. The effect of red clover cultivar, red clover type (Ackerklee vs Mattenklee) and mixture (monoculture vs mixture) on a) the shoot weight, b) plant height, c) SLA and d) leaf:stem-ratio of the eight red clover cultivars grown in the **pot experiment**. Error bars represent the standard error (n=4 for cultivars, n=16 for clover type and mixture means). Bars with the same letters are not significantly different (P>0.05)

Correlations

Correlation between pot and field results

There was a good correlation ($r = 0.77$; $P < 0.05$) between the plant height (or stem length) measurements in the pot (both mono and mixture) and the field experiment (Table 5). All the other traits (shoot weight, SLA and leaf:stem ratio) showed a positive (but non-significant) correlation between pot and field measurements (with the exception of mixture shoot biomass). In general, the pot monoculture measurements showed stronger correlations with the field experiment compared to the mixture measurements.

Table 5. Coefficients of correlation of plant morphological traits measured in the pot experiment (in monocultures and mixtures) and in the field experiment. Significant correlations are indicated by * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

	Pot: Mono vs mix		Pot-mono vs field		Pot-mix vs field	
	r	P	r	P	r	P
Shoot weight	0.65	0.08	0.68	0.06	-0.04	ns
SLA	0.78	*	0.41	ns	0.46	ns
Plant height	0.87	**	0.77	*	0.78	*
Leaf:stem-ratio	0.86	**	0.68	0.07	0.44	ns

Competition

In the field experiment, there was a strong negative correlation ($r = -0.84$, $P < 0.001$) between the red clover dry matter yield and the grass dry matter yield during all three years. In contrast, the total herbage dry matter yield was positively correlated to the red clover dry matter yield ($r = 0.93$, $P < 0.001$). In the pot experiment there was no correlation between clover and grass performance.

Persistence, nutritive value and morphology

There was a strong and highly significant ($P < 0.001$) correlation in the field experiment between the three red clover persistence indicators in 2015: the red clover DMY during the first harvest in 2015, the cumulative red clover DMY over 2015 and the red clover yield index in 2015 (Table 6).

Table 6. Coefficients of correlation of plant morphology traits measured during H1 in 2015 (Plant density, shoot weight, leaf area, leaf:stem-ratio, # stems per plant, SLA and stem length) with red clover performance (red clover dry matter yield during H1 and total 2015 and clover yield index) and with clover nutritive value (crude protein (CP) concentration and digestible organic matter (DOMD) concentration). Significant correlations are indicated in bold: * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

	Clover performance			Clover nutritive value	
	Clover DMY (H1)	Clover DMY (T)	Clover Yield index	CP concentration	DOMD concentration
Clover DMY (H1)	-	0.95 ***	0.88 **	-0.60	-0.58
Clover Yield index	0.88 **	0.87 **	-	-0.77 *	-0.81 *
Plant density	0.93 ***	0.93 ***	0.81 *	-0.60	-0.58
Shoot weight	0.68 .	0.72 *	0.81 *	-0.65 .	-0.57
Leaf area	0.65 .	0.56	0.35	-0.01	-0.01
Leaf:stem ratio	-0.26	-0.28	-0.56	0.63 .	0.49
SLA	-0.60	-0.68 .	-0.36	0.12	0.29
Stem length	0.71 *	0.85 **	0.76 *	-0.75 *	-0.75 *
# of stems	0.63 .	0.63 .	0.83 **	-0.54	-0.62

All three persistence indicators were significantly and positively correlated to the red clover plant density, stem length and red clover shoot weight (except red clover DMY_H1)

(Table 6). Also, the number of stems per plant was positively correlated to the red clover yield index. In 2015-H1 red clover persistence (red clover DMY-H1 and red clover yield index) was negatively correlated to red clover CP and DOMD concentration (Table 6). However, during 2012-H3 and 2014-H2 there were no significant correlations between clover DMY and clover CP or DOMD concentration (data not shown).

The difference in nutritive value in 2015-H1 could be linked to clover morphology as there was a significant negative correlation between the stem length and clover CP and DOMD, whereas the leaf:stem ratio was positively correlated to the CP and DOMD.

Even though the clover CP concentration in 2015-H1 showed a (non-significant) negative correlation with clover DMY (Fig. 4a), the red clover DMY was positively correlated with the red clover CP-yield ($r = 0.97^{***}$) (Fig. 4b). Additionally, the total herbage CP concentration was positively correlated with the proportion of red clover in the herbage biomass ($r=0.87^{**}$, Fig. 4c).

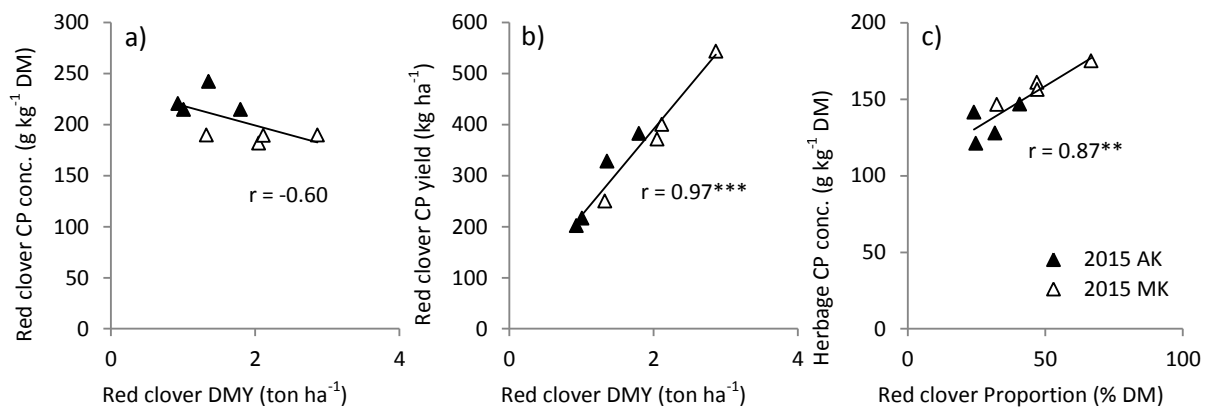


Figure 4. Correlation of red clover dry matter yield (DMY) with **a)** red clover crude protein (CP) concentration and **b)** red clover CP-yield, and **c)** the correlation of total herbage CP content with the proportion of red clover in total herbage biomass for the four Ackerlee (closed symbols) and Mattenlee (open symbols) cultivars during the first cut in 2015.

Discussion

Higher persistence of Ackerlee red clover cultivars

There was no significant difference in red clover DMY between the Ackerlees and the Mattenlees during the first year after sowing (2012). However, the red clover DMY of the Mattenlees in comparison to the Ackerlees was on average 33 % higher in the third year (2014) and 54 % higher in the fourth year (2015). This was in line with our expectations and indicates that the breeding effort into more persistent red clover cultivars is effective

(Lehmann and Briner, 1998), also under the local conditions in The Netherlands. Cumulatively across the three measurement years (2012, 2014, 2015), Larus and Fregata (both MK) performed best with the highest red clover DMY, whereas Avanti and Lemmon (both AK) had the lowest clover DMY.

The higher red clover DMY of the Mattenklees was the result of a higher number of remaining plants m^{-2} combined with a higher shoot DMY (Table 4 and 6). So after four years both the survival rate of the plants and the production per plant were higher for the Mattenklees compared to the Ackerklees. Persistence is often measured as the number of surviving plants (Choo, 1984), however, persistence may also be defined as the plants' ability to produce satisfying forage yield in the third or fourth growing season (Taylor and Quesenberry, 1996). In other words, good persistence should be a combination of plant survival and plant vigor and in practice these traits are often correlated. In the current experiment, Larus (MK) had both the highest number of surviving plants and the highest shoot weight, whereas Lemmon (AK) had the lowest plant density and the second lowest shoot weight (Table 4).

The red clover yield showed a marked decline in 2015 compared to 2014 for both clover types, but the decline was less pronounced for Mattenklees compared to Ackerklees (37% and 46%, respectively). A progressive decline in annual DM production with age is typical of clover swards, even in the absence of pests and diseases, due to natural decline in plant populations (Frame et al., 1998), however we cannot exclude a role for stochastic year to year variation.

Red clover persistence and competitiveness related to plant morphological traits

The differences in red clover DMY were broadly reflected in the total DMY, however the differences in total herbage DMY between cultivars and clover types were much less pronounced (only 6% and 8% respectively). This was related to the increasing proportion of red clover in the total DMY of the Mattenklees. Due to the high competitiveness of these cultivars some of the increase in red clover yield was off-set by a reduction in the yield of the accompanying perennial ryegrass. Mixtures of red clover and perennial ryegrass usually show higher DMY compared to the average of both monoculture yields, because of facilitation and complementarity effects (Nyfeler et al., 2011). Red clover acquires a large proportion of its N through symbiotic N_2 fixation. Perennial ryegrass can benefit from this in two ways: 1) directly through recycling of symbiotically fixed N and 2) indirectly through increased N availability in the soil as a result of the lower soil N uptake of clover plants compared to

perennial ryegrass plants (Nyfeler et al., 2011). In the pot experiment we did not yet see a benefit for perennial ryegrass of growing in mixture with red clover. This is likely due to the short time-span of the pot experiment which did not yet enable the increase of soil N by the red clover via N-fixation and root turnover.

The differences in competitiveness of the clover cultivars compared to the grass may be related to the higher plant height (stem length) of Mattenkee compared to Ackerklee (Table 4), which results in advantage for light competition (Den Hollander et al., 2007). Also, Herrmann et al. (2008) reported that persistence in the field was positively correlated to stem length. However, in a greenhouse study with 85 accessions of red clover from the NPGS-USDA core collection Dias et al. (2008) found that persistence was negatively correlated with plant height. Plant height or stem length may be a more appropriate indicator of persistence when plants are grown in mixture rather than monoculture, i.e. it will give a competitive advantage in interspecific competition, but not in intra-specific competition. This highlights the need of cultivar assessments in both monocultures and agronomic mixtures in greenhouse and long-term field experiments and test of their consistency (Lehmann and Briner, 1998).

Interestingly, plant heights measured in both monocultures and mixtures in the short pot experiment (Fig. 3) showed a strong correlation with the stem length measured in the field experiment. The apparent robustness of this measurement strengthens the potential of this plant trait for use in plant selection and breeding.

Next to competition, persistence can be related to diseases. The difference in red clover plant density in 2015 between the cultivars indicates that there was a difference in the number of plants that didn't survive or difference in the establishment of new seedlings. This may be the result of a difference in the susceptibility to disease.

Potential trade-off between persistence and nutritive value in red clover biomass but not in total herbage biomass

During 2012-H3 and 2014-H2 there were no significant differences in CP concentration and DOMD between the different cultivars. However, in 2015-H1, CP concentration and DOMD were significantly lower for the MK compared to the AK cultivars (Table 2 and 3), indicating a trade-off between DM yield and nutritive quality, as highlighted by the trend of a negative correlation between CP and DOMD and red clover DMY (Table 6). These lower CP and DOMD concentrations were negatively correlated with the stem length and positively with leaf:stem ratio (Table 6). This is in line with literature indicating that red clover leaves have a

higher digestibility and CP concentration than the stems (Kleen et al., 2011; Rinne and Nykanen, 2000; Tucak and Popovic, 2013; Wilman and Altimimi, 1984).

However, the decrease in CP concentration of the red clover material, had no negative impact on the CP concentration and yield of the total herbage. Firstly, the decrease in clover CP concentration did not result in a lower CP yield, in fact, the opposite was true. As a result there was a positive correlation between red clover DMY and red clover CP yield (Fig. 4b). Secondly, in the mixtures, a higher red clover DMY was achieved partly at the cost of the grass yield, resulting in an increased proportion of red clover in the total herbage dry matter. Because the CP concentration of red clover is higher than that of perennial ryegrass (King et al., 2012), the CP concentration of the total herbage increased. Therefore, the clover CP concentration was not correlated (or even negatively) to the total herbage CP concentration. Instead, the proportion of red clover in the total herbage biomass was a much better predictor of the total herbage CP concentration.

Even though there was some evidence of slightly lower DOMD values for the Mattenklees compared to the Ackerklees during 2015-H1 (766 and 788 g kg⁻¹ respectively, Table 3), the digestible organic matter yield was actually higher for Mattenlee compared to Ackerlee. The difference between the cultivar with the highest and the lowest DOMD was 5%. We have no measurements of the total herbage DOMD. Literature shows that the digestibility of red clover may be slightly lower (Frame et al., 1972), or comparable (King et al., 2012; Laidlaw and McBratney, 1980; Moharrery et al., 2009) to perennial ryegrass, depending on season, management and cultivar.

We only measured a significant effect of clover type on nutritive value in 2015. This may have been related to the diverging development of the two clover types in relation to their differences in persistence. Additionally, in 2015, red clover nutritive value was determined in the first cut, whereas in 2012 and 2014 it was determined in the third and fourth cut, respectively, which may also have had an effect on the differentiation between cultivars. However, the latter is unlikely as research by Frame et al. (1972) shows that differences in digestibility were actually smaller (and non-significant) in the first harvest in comparison to the following harvests.

In line with our hypothesis, the Mattenklees showed a higher production and a higher clover content in the third and fourth year of production compared to the Ackerklees, indicating that these cultivars are suitable for inclusion in a multiple (> 4 yr) grass-clover ley. Even though in the first harvest of the fourth measurement year the Mattenlee cultivars had a slightly lower

digestibility and lower protein content in the red clover material, the protein content, digestible dry matter yield and protein yield in the total herbage biomass were higher for Mattenkleef cultivars.

Both persistence and nutritive value of the different red clover cultivars were closely correlated to plant morphological traits, particularly stem length and leaf:stem-ratio. For plant height (or stem length) there was close correlation between the results from the pot experiment and the field experiment, indicating that this may be an important parameter for breeding.

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