Performance of underutilized forage legumes as an alternative to *Trifolium repens* under drought stress: yield, water utilization and nutritive value

Dissertation

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Ach Gott! die Kunst ist lang;
Und kurz ist unser Leben.
Mir wird, bei meinem kritischen Bestreben,
Doch oft um Kopf und Busen bang.
Wie schwer sind nicht die Mittel zu erwerben,
Durch die man zu den Quellen steigt!
[…]
Vom Eise befreit sind Strom und Bäche
Durch des Frühlings holden, belebenden Blick;
Im Tale grünet Hoffnungsglück;
[…]
Das also war des Pudels Kern!

(Goethe, Faust I)

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General Introduction

Permanent grassland covers more than 70% of the agriculturally utilized area worldwide and 35% in Europe (Panunzi, 2008; Smit et al., 2008) and thus forms an important agricultural resource (White et al., 2000; Isselstein et al., 2005). Grassland with its potentially high productivity and fodder quality is usually the basis for ruminant nutrition and livestock production (White et al., 2000; Hopkins & Wilkins, 2006). Due to their ability to fix atmospheric N and high protein contents, legumes are particularly important for grassland productivity and fodder quality, especially in swards with no or little input of nitrogen (N) from mineral fertiliser or manure. In spite of the high potential of forage legumes for grassland farming their proportion in European grasslands have decreased over the last decades (Peeters, 2009) mainly because of the ready availability of inorganic N-fertilizer (Rochon et al., 2004). With increasing prices of energy and N-fertiliser along with higher costs for concentrates, which are expected for the future, the use of grassland legumes becomes more attractive, not only for organic farming, but also for more intensive agricultural systems (Watson et al., 2002; Jensen & Hauggaard-Nielsen, 2003; Crews & Peoples, 2005; German Agricultural Research Alliance, 2012).

Trifolium repens L. is one of the most important forage legumes in European temperate grasslands (Frame et al., 1998; Gierus et al., 2012). Grass-*T. repens* mixtures are highly productive, as long as water is not limiting, and have a high nutritive value (Wilman & Williams, 1993; Wilkins et al., 1994; Topp & Doyle, 2004). As forecasted under conditions of climate change, water is likely to become more limiting in arid, semiarid and temperate climates as the probability of summer droughts increases (Alcamo et al., 2007; Schindler et al., 2007; Trenberth, 2011). *Trifolium repens* is sensitive to water shortages and responds with strongly decreasing yields (Marshall et al., 2001). The nutritive value is likely to be affected as well. Other legumes may be better adapted to water limited conditions and may therefore have an increasing potential in future forage production. However, knowledge about the agronomic potential of such alternative legume species under drought conditions is limited (Hopkins et al., 1996; Rochon et al., 2004; Hopkins & Wilkins, 2006; Sölter et al., 2007) and their cultivation and use insignificant.

In this study, we therefore tested the agronomic potential of a range of five forage legumes for temperate grassland as possible alternatives to *T. repens*. We chose *Lotus corniculatus* L., *L. uliginosus* Schkuhr, *Medicago lupulina* L., *M. falcata* L. and *Onobrychis viciifolia* Scop. and compared their performance with that of *T. repens* under control and drought conditions. We conducted a container experiment in a vegetation hall from 2009 (sowing year) to 2011. All legumes were sown in monoculture as well as in mixture with *Lolium perenne* L. as mixtures of grasses and legumes are common practise in grassland farming (Hopkins & Wilkins, 2006; Hopkins & Del Prado, 2007). The climate conditions followed normal seasonal pattern with frost in winter and higher temperatures in summer. Drought conditions were imposed during three periods in two years by temporary ceasing the watering of the containers. A moderate stress phase was set up in spring 2010 (April/May) followed by two periods of strong drought stress in summer 2010 (July/August) and spring 2011 (April/May).

The major aims of this study were:

1. To test the establishment, the early yield development and the competitive ability against the fast growing grass *L. perenne* under sufficient water supply. (Chapter 1)
2. To investigate yield and yield stability as well as water utilisation of alternative legumes and *T. repens* both in monoculture and mixture under temporary drought. (Chapter 2)
3. To examine the effects of drought stress on the nutritive value of legume monocultures and mixtures. (Chapter 3)

The investigation was part of the research co-operation “KLIFF Klimafolgenforschung in Niedersachsen” (Climate impact and adaptation research in Lower Saxony). Our study was part of the research area “Animal production”.

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Chapter I

Establishment and early yield development of five possible alternatives to *Trifolium repens* as a grassland legume

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Abstract

The performance of *Trifolium repens* as the main grassland legume in temperate climates may decrease under climate change due to more frequent water shortages. This calls for alternative legumes with agronomic potential. We examined germination rates, establishment, winter tolerance and yield potential of *Medicago lupulina*, *Medicago falcata*, *Lotus corniculatus*, *Lotus uliginosus* and *Onobrychis viciifolia* both in monoculture and in mixture with *Lolium perenne* in a two-year container experiment. Germination and establishment of all alternative legumes were comparable to *T. repens* except of *M. falcata* with a retarded initial development. *L. uliginosus* was the only species with an insufficient winter tolerance. In pure stands *M. lupulina* and *L. corniculatus* showed a yield potential almost as high as of *T. repens*. However, their performance in mixture with *L. perenne* was lower than *T. repens*. This has to be considered with the choice of less competitive grass partner species when designing seed mixtures.

Keywords: *Lotus corniculatus*, *Lotus uliginosus*, *Medicago lupulina*, *Medicago falcata*, *Onobrychis viciiifolia*, *Lolium perenne*, winter tolerance, early development

1. Introduction

Legumes are important for grassland productivity, especially in swards with low or no nitrogen (N) fertilisation due to their ability to fix atmospheric N. Nevertheless, in conventional agriculture in Europe the proportion of forage legumes in swards has decreased in the last decades (Peeters, 2009) mainly because of the ready availability of inorganic N-fertilizer (Rochon et al., 2004). However, with increasing energy and N-fertilizer prices along with higher costs for concentrates, the use of grassland legumes becomes more attractive (Jensen & Hauggaard-Nielsen, 2003; Crews & Peoples, 2005).

The main fodder legume in grasslands in Central Europe is *Trifolium repens* (Frame, Charlton, & Laidlaw, 1998). Under appropriate climatic conditions, *T. repens*/grass mixtures can produce high yields and a good fodder quality (Wilman & Williams, 1993; Wilkins, Gibb, Huckle, & Clements, 1994; Topp & Doyle, 2004). However, *T. repens* has been shown to need a good supply of water for growth (Foulds, 1978). This may become challenging in times of climate change, as summer rainfall is predicted to become sparse (Alcamo et al., 2007).

Other legumes may be better adapted to drier conditions or have special feeding values and may therefore have potential as alternatives for *T. repens*. Currently, the agronomic knowledge, including early stages of establishment in monoculture and in mixture with grasses, of other legumes of permanent grasslands is limited (Hopkins, Martyn, Johnson, Sheldrick, & Lavender, 1996; Rochon et al., 2004; Hopkins & Wilkins, 2006; Sölter, Hopkins, Sitzia, Goby, & Greef, 2007) and their cultivation and use insignificant. Early development determines to a great deal the successful establishment and yield contribution of legumes especially when sown in mixture with grasses (Petersen, 1967).

In this study, we therefore tested the agronomic potential in early development of five promising grassland legumes (Table 1) against *T. repens*. A container experiment was conducted in a vegetation hall from 2009 to 2010. All legumes were sown in monoculture and in mixture with *Lolium perenne*. The climatic conditions in the vegetation hall followed a normal seasonal pattern with frost in winter and higher temperatures in summer. We considered the germination rates, establishment, the response to a winter stress phase and the yield potential in the sowing and first main production year.

Table 1. Used plant species, cultivars, seed weight, ecological strategy types, tolerances (mowing, grazing, trampling) and nutritive values of all species

Plant species	Cultivar	Seed weight	Strategy type [1]	Tolerance			Nutritive value [2]
		[mg]		Mowing [3]	Grazing [2]	Trampling [2]	
Lotus corniculatus	Bull	1.45	csr	6	4	4	8
Lotus uliginosus	wild seeds	0.74	csr	4	4	4	7
Medicago lupulina	Ekola	1.69	csr	7	4	6	8
Medicago falcata	wild seeds	0.95	cs	5	2	2	7
Onobrychis viciifolia	Matra	21.90	c	6	2	2	8
Trifolium repens	Rivendel	0.62	csr	8	8	8	9
Lolium perenne	Signum	2.75	c	8	8	8	9

[1] according to Klotz, Kühn and Durka (2002); c: competitor. s: stress tolerator. r: ruderal

[2] according to Dierschke and Briemle (2002); values range from 1 (low) to 9 (high)

[3] according to Briemle and Ellenberg (1994); values range from 1 (low) to 9 (high)

2. Material and Methods

The experiment consisted of a germination test and a container experiment with six legumes and the grass *Lolium perenne*. Both, wild flower seeds and cultivars were used depending on the availability (Table 1). The trial was separated into three phases:

germination and establishment (establishment phase), effects of lower temperatures and short days (winter stress phase) and the first main production year (initial yield phase).

2.1 Germination Test

For the germination test, 30 seeds of each species were sown on Petri dishes (9 cm diameter, bottom covered with two pieces of moistened filter paper, Schleicher and Schuell Microscience). The lids were replaced and fastened with laboratory film (American National Can). Five replicate dishes for each species were arranged in a randomized block design in a greenhouse (night temperatures: 13 to 16°C, day temperatures 21 to 26°C, no extra lighting, germination between February 8th and March 4th 2009). Every two days, germinated seeds (with visible radicles) were counted and removed. The filter paper was kept moist (tap water).

2.2 Container Experiment

2.2.1 Experimental Setup

The container experiment was set up in 2009, sowing date was July 15th. For this experiment, 30 l containers (diameter 33 cm, height 42 cm) were filled with a homogeneous mixture of 20 kg air-dried sand (sieved to pass a mesh of 5 mm; August Oppermann Kiesgewinnung GmbH), 0.9 kg vermiculite (particle size 8–12 mm; Deutsche Vermiculite GmbH) and 5.5 kg compost (air dried; Bioenergiezentrum Göttingen GmbH) and covered with 1.5 kg compost as a seed bed. The six legumes and *L. perenne* were sown in monoculture (1000 germinable seeds per m^2 for legumes and 5000 for the grass) and the legumes also in mixture with *L. perenne* (half the amount of seeds of each species sown in monoculture). This resulted in 13 treatments, which were replicated four times, leading to 52 containers that were arranged in a randomized block design in a vegetation hall.

The minimum and maximum air temperatures were recorded daily at three locations distributed over the vegetation hall (Table 2) and temperatures adjusted by venting in summer and heating on frost days in winter (temperature should not fall below 0°C for more than 24 h). Nevertheless, *L. uliginosus* was strongly reduced in all containers during winter and had to be resown at full seed strength in March 2010. There was no extra lighting in the vegetation hall and lighting conditions followed seasonal patterns. No fertilisation took place, but all plots were treated with rhizobium solution (Radicin, Jost GmbH) three times in 2009 and twice in 2010 (per application, 0.015 ml Radicin mixed with 250 ml tap water per m^2). The Radicin solution mixture contained all rhizobia strains in same proportions for an effective infection of all legumes. Containers were kept moist during germination of the seeds. Starting two weeks after sowing, all containers were weighed regularly and irrigated when the water content was below 50% of field capacity.

Table 2. Temperatures [°C] in the vegetation hall from July 2009 until October 2010

Year	Month	Average minimum – maximum temperature
2009	July	14–31
	August	14–33
	September	13–32
	October	6–25
	November	6–14
	December	-1–8
2010	January	-1–5
	February	0–14
	March	5–25
	April	6–29
	May	9–26
	June	12–35
	July	16–36
	August	15–33
	September	11–26
	October	6–24

2.2.2 Sampling

The aboveground biomass was harvested two times in 2009 and five times in 2010. Harvesting took place 50 (establishment phase), 104, 272 (winter stress phase), 315, 356, 407, and 462 (yield phase) days after sowing. Shoots were cut 3–4 cm above the soil surface. Biomass of mixtures was sorted into species directly after harvesting. All samples were dried at 60°C for 72 h and weighed.

2.3 Statistical Analysis of Data

Statistical data analysis was carried out using the Genstat 6.1 software package. Analysis of variance (ANOVA) considered one factor. Residuals were used to check the validity of the models. Normality in data was achieved by applying logarithmic or square root transformations, if necessary. Where significant treatment effects ($\alpha<0.05$) were found by

ANOVA, least significant differences (Tukey Test) were used to compare mean values. Relationships between legume dry matter yield in monocultures and mixtures of the first harvest, in monocultures before and after winter and between the accumulated yield of the legume partner and the total mixture yield in the first main harvest year were examined with a linear regression model.

3. Results

3.1 Establishment Phase

The germination rate after 24 days on petri dishes ranged from 34% (*M. falcata*) and 100% (*M. lupulina*, Table 3). There were significant differences between *M. falcata*, the two *Lotus* species, which formed an intermediate group, and the other legumes, which had germination rates between 88 and 100% ($P<0.001$). The germination rate of the grass *L. perenne* was 93% and similar to that of the latter group of legumes.

The dry matter yield of the legumes in pure stands during the establishment phase (50 days after sowing) ranged from 5.0 g pot^{-1} to 16.0 g pot^{-1} for *M. falcata* and *O. viciifolia*, respectively (Table 3). Yields of the other legumes were intermediate, with *L. corniculatus*, *M. lupulina* and *T. repens* producing similar yields to *O. viciifolia*, and *L. uliginosus* being closer to the low yielding *M. falcata*. For comparison *L. perenne* produced in pure stands 27.8 g pot^{-1} in that first harvest. Mixtures of the single legumes and *L. perenne* did not differ in dry matter yield ($P=0.144$). Yield in mixtures was generally larger than in legume monocultures, but smaller than that of *L. perenne* in pure stand. The contribution of the legume partner to the total yield in mixture varied significantly among species ($P<0.001$), with yields increasing in the order *L. uliginosus*, *M. falcata*, *T. repens*, *M. lupulina*, *L. corniculatus*, and *O. viciifolia*.

Table 3. Germination rate after 24 days [%] on Petri dishes and dry matter yield of all species in pure and mixed stands (total yield and yield of the legume partner in the mixture) of the container experiment at the first harvest (Establishment phase).

Plant species	Germination after 24 days [%]	Dry matter yield [g pot^{-1}]		
		Pure stand	Mixed stand	
			Total	Legume
L. corniculatus	73 ± 4^{b}	14.3 ± 4.5^{ab}	20.8 ± 1.1	2.3 ± 0.3^{b}
L. uliginosus	71 ± 8^{b}	9.1 ± 1.4^{bc}	19.6 ± 1.9	$0.1\pm< 0.1^{f}$
M. lupulina	100 ± 0^{a}	11.8 ± 2.0^{ab}	25.8 ± 3.8	1.6 ± 0.2^{c}
M. falcata	34 ± 10^{c}	5.0 ± 1.2^{c}	22.5 ± 1.4	0.3 ± 0.1^{e}
O. viciifolia	89 ± 7^{a}	16.0 ± 2.1^{a}	22.1 ± 4.9	4.0 ± 0.5^{a}
T. repens	88 ± 5^{a}	11.4 ± 0.7^{ab}	21.5 ± 2.2	1.0 ± 0.3^{d}
L. perenne	93 ± 3	27.8 ± 2.4		
P-value	< 0.001	< 0.001	0.144	< 0.001

Shown are means and standard deviations. Different superscript letters indicate significant differences among species (ANOVA with Tukey Test (*a*<0.05) analysis; the last row gives the corresponding *P*-values). *L. perenne* was not included in the statistics.

3.2 Winter Stress Phase

The winter phase lasted from beginning of November 2009 to early March 2010. Low temperatures (Table 2) associated with low radiation and short days limited plant growth similar to field conditions. Frost occurred but temperatures were prevented from falling below 0°C for more than 24 h.

To evaluate the effects of the winter phase the last harvest in 2009 (end of October) and the first harvest in 2010 (mid of April) were considered. For the harvest in October 2009, the legumes growing in pure stands showed two distinctive groups (*P*<0.001): a high-yielding group consisting of *L. uliginosus, T. repens* and *M. lupulina,* with yields between 27.1 and 34.4 g pot^{-1}, and a low-yielding group of *M. falcata*, *L. corniculatus,* and *O. viciifolia*, with yields between 7.8 and 15.2 g pot^{-1} (Table 4). At the harvest in April 2009, the dry matter yields of most legumes in pure stand (apart from *L. uliginosus*) were similar to or larger than before winter. While *L. uliginosus* produced only 0.4 g pot^{-1}, a significantly smaller yield than all other legumes (*P*<0.001; Table 4), *M. falcata, O. viciifolia* and *L. corniculatus* showed intermediate yields of 21.6- 29.9 g pot^{-1} and *M. lupulina* and *T. repens* were again the highest-yielding legumes (34.4 and 37.0 g pot^{-1}, respectively).

Total yields in mixed stands of legume and *L. perenne* before winter were largest in containers containing *M. lupulina,* the only legume besides *T. repens* that managed to produce appreciable amounts of biomass in this phase (Table 4). Containers with *M. falcata* or *O. viciifolia* as legume partner produced significantly less total biomass before winter (*P*=0.005), while yields of mixtures with *L. uliginosus* and *L. corniculatus* were intermediate. The yield contribution of the legume was smallest for *L. uliginosus* and also *M. falcata* and *L. corniculatus,* while only *M. lupulina* and *T. repens* produced considerable amounts of biomass. After winter, differences in biomass production of the mixtures were not significant (*P*=0.678). At that time only *T. repens* produced an appreciable yield of 3.7 g pot^{-1}, significantly larger than that of any other legume in mixture.

Table 4. Dry matter yield of all species in pure and mixed stands with *L. perenne* (total yield and yield of the legume partner in the mixture) at the last harvest before winter in 2009 and the first harvest after winter in spring 2010 (Winter stress phase).

Plant species	Dry matter yield [g pot^{-1}]					
	Pure stand		Mixed stand			
			Total		Legume	
	Before winter	After winter	Before winter	After winter	Before winter	After winter
L. corniculatus	15.2±2.8^{b}	25.6±4.1^{ab}	30.2±2.6^{ab}	33.3±5.6	0.4±0.1^{b}	0.5±0.4^{b}
L. uliginosus	27.1±6.6^{a}	0.4±0.4^{c}	32.5±1.1^{ab}	34.1±2.4	<0.1±0.1^{c}	<0.1±<0.1^{b}
M. lupulina	34.4±2.7^{a}	34.4±5.6^{a}	35.3±2.0^{a}	32.8±3.2	3.1±1.3^{a}	0.5±0.1^{b}
M. falcata	7.8±5.3^{b}	21.6±4.2^{b}	28.2±2.1^{b}	35.0±3.8	0.1±0.1^{c}	0.2±0.1^{b}
O. viciifolia	12.9±0.5^{b}	29.9±3.1^{ab}	27.9±4.0^{b}	33.9±2.5	0.8±0.2^{ab}	0.1±<0.1^{b}
T. repens	32.4±5.4^{a}	37.0±10.9^{a}	33.2±1.3^{ab}	36.8±3.5	3.5±2.2^{a}	3.7±2.4^{a}
L. perenne	26.1±3.7	35.9±2.4				
P-value	< 0.001	< 0.001	0.005	0.678	< 0.001	< 0.001

Shown are means and standard deviations. Different superscript letters indicate significant differences among species (ANOVA with Tukey Test (*a*<0.05) analysis; the last row gives the corresponding *P*-values). *L. perenne* was not included in the statistics.

3.3 Initial Yield Phase

The yield phase comprises of four harvests in the first main production year following the initial harvest after winter. The accumulated yield of the pure stands over the four main harvests 2010 ranged from 88.1 to 288.6 g pot^{-1}. It was smallest for *O. viciifolia* and *L. uliginosus,* significantly larger for *M. falcata* and *L. corniculatus* and largest for *T. repens* with *M. lupulina* being intermediate (*P*<0.001, Table 5). Accumulated yield of mixed stands was by far largest for mixtures with *T. repens,* followed by those with *M. lupulina* and *L. corniculatus*. In mixed stands *L. uliginosus* and *O. viciifolia* as the legume partner did not produce any biomass. Yield contribution of *T. repens* was largest with 0.59 (149.0

g pot^{-1}), while that of *M. lupulina, L. corniculatus* and *M. falcata* amounted to 0.26, 0.16 and 0.08, respectively. In pure stands the coefficient of variation (CV), as a measure of the yield variability among harvests, was largest for *M. falcata*, *L. corniculatus*, and *M. lupulina* and significantly smaller for *O. viciifolia* (P<0.001, Table 5). Generally, mixtures had a smaller CV than pure stands of legumes (Table 5). Greatest variability in yields between harvests (CV, P<0.001) was observed for the mixture with *T. repens* with a CV of 0.34 which was significantly larger than that of *L. uliginosus*, *M. lupulina*, *M. falcata* and *O. viciifolia* ranging from 0.18–0.21; *L. corniculatus* was intermediate with 0.24. Coefficients of variation considering the yields of the legume partner in mixtures were high and in a range from 0.71–0.95 but differences between legumes not significant (P=0.395).

Table 5. Accumulated dry matter yield over the four harvests 2010 (Initial yield phase) of all species in pure and mixed stands with *L. perenne* (total yield and yield of the legume partner in the mixture) and coefficients of variation over these harvests.

Plant species	Accumulated dry matter yield [g pot^{-1}]			Coefficient of Variation		
	Pure stand	Mixed stand		Pure stand	Mixed stand	
		Total	Legume		Total	Legume
L. corniculatus	213.0±15.3^{b}	137.4±16.4^{cb}	21.8±15.3^{b}	0.64^{a}	0.24^{ab}	0.95
L. uliginosus	125.4±29.0^{c}	118.9±9.2^{c}	n.p.$^{\#}$	0.56^{ab}	0.18^{b}	n.p.
M. lupulina	239.3±33.3^{ab}	165.5±7.6^{b}	42.4±5.7^{b}	0.62^{a}	0.21^{b}	0.76
M. falcata	192.4±18.2^{b}	121.0±9.0^{c}	9.9±2.2^{b}	0.69^{a}	0.20^{b}	0.85
O. viciifolia	88.1±21.0^{c}	119.7±5.3^{c}	n.p.	0.32^{c}	0.21^{b}	n.p.
T. repens	288.6±12.2^{a}	253.3±53.9^{a}	149.0±63.8^{a}	0.39^{bc}	0.34^{a}	0.71
L. perenne	115.4±10.1			0.19		
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.395

Shown are means and standard deviations. Different superscript letters indicate significant differences among species (ANOVA with Tukey Test (a<0.05) analysis; the last row gives the corresponding P-values). *L. perenne* was not included in the statistics.

$^{\#}$ n.p.: not present; in these mixtures. The legume partner did not produce any more biomass at these harvests.

4. Discussion

We compared five legumes as alternatives to *T. repens* in early development as pure stands and in mixture with *L. perenne*. To be agronomically competitive, it is important that legumes have a good establishment, show good winter tolerance, can perform in mixtures with partner species (here *L. perenne*) and thus provide good yields.

4.1 Establishment Phase

Generally, cultivated legumes had larger germination rates than wild seeds (*L. uliginosus* and *M. falcata*, Table 3). Especially *M. falcata*, from wild seeds, had very poor germination rates, with only 34% of seeds germinated after 24 days (Table 3). This was likely due to a higher percentage of hard seed. *Medicago* species may have up to 100% hard seed, depending on the habitat (Young, Evans, & Kay, 1970; Crawford, Lake, & Boyce, 1989). Hard seed coats help to survive unfavourable environmental conditions like long droughts (Kemp, 1989), but also influence both, water uptake and germination rate (Argel & Paton, 1999; Uzun & Aydin, 2004). Mechanical scarification may have increased germination of *M. falcata*. However, in the present experiment, we accounted for low germination rates by adapting the sowing density. The German Regulation for Seeds (Saatgutverordnung, 2006), requires a good and homogeneous germination for cultivars, consequently resulting in a smaller share of seeds with hard seed coats. This may have lead to the larger germination rates of the tested cultivars compared to the wild seeds. While germination is better in cultivars, the ability to survive periods of drought might be reduced and the timing of sowing and weather conditions during germination becomes more important. In the first harvest 50 days after sowing, cultivars also had larger dry matter yields than the wild type legumes both in monoculture and mixture (Table 3). Tauro, Nezomba, Mtambanengwe and Mapfumo (2009) observed similar results in their study. The size of seeds also has an effect on plant establishment; large-seeded species have often been found to have a better seedling establishment than small-seeded ones (Moles & Westoby, 2004), because of larger nutrient reserves in heavier seeds (Baker, 1972). This could only be confirmed in part in our experiment (Table 1 and Table 3): The species with the largest seeds, *O. viciifolia*, produced the largest biomass at the first harvest, both in pure stands and in mixtures. However, the large difference in seed weight between *O. viciifolia* and the next two legumes, *M. lupulina* and *L. corniculatus* (Table 1) was not reflected in large differences in yield (Table 3). Furthermore, *M. lupulina* and *T. repens* produced similar amounts of dry matter (Table 3) despite seed weights differing by a factor of 2.7 (Table 1). The absence of a strong relationship between seed weight and dry matter yield during the establishment might partly be explained by the use of wild seeds and cultivars for different legume species.

In mixtures, *L. perenne* was the main contributor to dry matter yields at the first harvest (Table 3). This was both due to the fast growth of *L. perenne* (Petersen, 1967) and the relatively high seed density of the grass compared to the legumes, deliberately chosen to test the competitive strength of the legumes. There was a positive linear correlation between the dry matter yield of legumes in monoculture and that in mixture (R^2=0.75; P=0.026). But, neither germination rate nor seed weight were good explanatory factors for the ability of species to compete in mixtures.

To sum up, *O. viciifolia* and *M. lupulina* did compete well with *T. repens* in germination rates. In terms of dry matter yield at the first harvest, *O. viciifolia* and *M. lupulina* again, but also *L. corniculatus*, were similar or even superior to *T. repens*. In mixtures with *L. perenne*, these three legumes produced significantly more biomass than *T. repens* at the first harvest. However, total yields in mixtures were only slightly larger and differences not significant (Table 3).

4.2 Winter Stress Phase

For legumes, the winter period is a crucial and sensitive phase, especially in newly established swards (Brandsæter, Smeby, Tronsmo, & Netland, 2000). All legumes in monoculture in this study, apart from *L. uliginosus,* survived the winter period well (Table 4). This is partly due to the conditions of a vegetation hall where moderate frost occurred, but long-term and sharp frost was prevented (Table 2). Results of only moderate winter tolerance for *L. uliginosus* have been reported before (Hedqvist, Murphy, & Nilsdotter-Linde, 2002). In line with that, the good winter tolerance found for *M. falcata*, *M. lupulina*, *T. repens*, *L. corniculatus* and *O. viciifolia* is in agreement with earlier findings (Frame et al., 1998; Brandsæter et al., 2000; Hedqvist et al., 2002). This was confirmed by a positive linear correlation between the dry matter yield of legumes in monocultures before and after winter (R^2=0.83; P=0.033, *L. uliginosus* not considered). Two legumes, *M. lupulina* and *T. repens*, had a fast establishment in the sowing year and a corresponding early development in the next spring, which was the basis for good yields in the first main production year. This is consistent with Petersen (1967).

As in the first harvest, *L. perenne* was the dominant plant in all mixtures in the harvests before and after winter (Table 4) - with the exception of *T. repens*, the yield of all legume partners in mixtures was well below one gram in the first harvest after winter (Table 4). This can be explained by a good and fast growth of *L. perenne* (Petersen, 1967) and temperatures that were closer to the optimum for grass than for the legumes (Wilson & Ford, 1971), (Table 2). For *T. repens* there is evidence that the presence of *L. perenne* is beneficial for the development of the legume in younger pastures (Turkington & Jolliffe, 1996).

In summary, only *M. lupulina* in monoculture produced a yield similar to *T. repens* at the harvests before and after winter and in mixture before winter. After winter, only *T. repens* produced a considerable yield in mixtures (Table 4).

4.3 Initial Yield Phase

A good establishment (Finch-Savage, 1995) of legumes associated with sufficient winter tolerance (Brouwer, Duke, & Osborn, 2000) is the basis for a good yield and sufficient yield contribution in a mixture in the first main production year. In our experiments, *T. repens* and *M. lupulina* but also *L. corniculatus* showed a good establishment and winter tolerance (Tables 3 and 4). These three legumes also produced the largest accumulated yields in monocultures (Table 5). Despite a good establishment and winter tolerance, *O. viciifolia* in monoculture had a small accumulated yield in the main production year (Table 5). This may be due to the low cutting height (3–4 cm) and the high cutting frequency (five times in the main harvest year) in this trial. *Onobrychis viciifolia* is generally known to be susceptible to a low cutting height and in particular a frequent defoliation (Slepetys, 2008), although some authors also consider this species to be moderately tolerant to cutting (Briemle & Ellenberg, 1994; see Table 1). Most likely, in our experiment a reduced cutting frequency of two to three cuttings per year would have increased the yield of *O. viciifolia*. The poor establishment and/or an inadequate or poor winter tolerance (Table 3 and 4) of *M. falcata* and *L. uliginosus* probably caused the only moderate accumulated yields in the first main harvest year (Table 5).

In mixtures, there was a positive linear correlation between the accumulated yield of the legume partner and that of the total mixture (grass and legume; R^2=0.99; P<0.001). *T. repens* was the most productive legume in mixtures with a yield proportion of nearly 60% of the total yield; this illustrates the strong competitive ability of *T. repens* (Petersen, 1967). A relative good competitive ability could be attributed to *M. lupulina* (Rehm & Espig, 1991) with a yield proportion in mixture with *L. perenne* of above 25%, while *L. corniculatus* had 16%. Where the yield of legumes in mixtures was small, as for *M. falcata*, a species with low competitive ability against fast-growing grasses (Petersen, 1967), the total mixture yield was also only slightly increased compared to the grass monoculture, and even smaller than that of the legume monoculture (Table 5). When the legume partner was no longer present, as was the case with *L. uliginosus* and *O. viciifolia*, mixtures produced a similar accumulated yield as *L. perenne* in monoculture (Table 5).

In general, legumes require higher temperatures for optimal growth than *L. perenne* (Wilson & Ford, 1971; Frame et al., 1998). Therefore, the growth of legumes in summer was faster compared to spring and autumn and yields differed between the harvests according to the individual temperature requirements of the respective legumes. This is well displayed in a higher coefficient of variation for the yields of all harvests for all six legumes in the main harvest year compared with the small CV for *L. perenne* (Table 5). In mixtures, the CV was depending on the legume partner and accordingly highest for the mixture with *T. repens*. In pure stands, *O. viciifolia* and *T. repens* showed the most stable yield over the first main production year.

4.4 Outlook and Need for Research

For the alternative legumes, an intensive breeding, like for *T. repens* in the last decades (Abberton & Marshall, 2005), might help to enhance not only the yield potential but also the competitive ability in mixtures with fast growing grasses. A good and lasting contribution of the legume in grass-clover mixtures is essential for a successful introduction of new species.

This experiment provides some worthwhile information on early development of some legumes as possible alternatives to *T. repens.* Nevertheless, further work is necessary to test these legumes under field conditions with different cutting regimes, soil conditions and fertilizer applications. Of particular interest would be the reactions of these alternative legumes in view of possible future climate change conditions like drought or higher temperatures.

5. Conclusion

General, the yield in the first main productive year relies on a good germination and establishment along with a sufficient winter tolerance. Furthermore, the yield of a mixture strongly depends on the yield contribution of the legume partner. Thus, a good competitive ability of legumes against fast-growing grasses like *L. perenne* (Petersen, 1967) is essential.

In conclusion, *M. lupulina* and - to a somewhat lesser extent - *L. corniculatus* in monoculture showed potential to produce similar yields as *T. repens* in the first main production year, however, yield stability for *M. lupulina* and *L. corniculatus* was not sufficient. In mixtures, *M. lupulina* and less so *L. corniculatus* showed some potential, but only *T. repens* showed a strong competitive ability against *L. perenne*. This has to be considered with the choice of less competitive grass partner species when designing seed mixtures.

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Chapter II

Water use efficiency and yield under drought stress of five grassland legumes as possible alternatives to *Trifolium repens*

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Abstract

Currently, *Trifolium repens* is the main fodder legume in temperate climates, but its comparatively large water requirements may become challenging under changing climatic conditions. This calls for alternative legumes. In a two-year container experiment, we examined *Medicago lupulina, Medicago falcata, Lotus corniculatus, Lotus uliginosus, Onobrychis viciifolia* and also *T. repens* in monoculture and in mixture with *Lolium perenne* concerning yield and agronomic water use efficiency under moderate and strong drought. Under moderate stress, the mean volumetric soil water content at the end of the drought period was 11 vol. % and under strong stress 6 vol. % (10 vol. % equalled -1.5 MPa). Changes in yield and agronomic water use efficiency under drought stress depended on the strength of the stress. Moderate drought stress had no or even slightly increasing effects on agronomic water use efficiency while strong stress usually decreased it. Yield decreased under drought stress. Alternative legumes, especially *M. lupulina*, but also *L. corniculatus* and *M. falcata,* often showed a higher tolerance to drought than *T. repens*. We found that changes in N fixation explained changes in yield and agronomic water use efficiency well. Intrinsic water use efficiency, measured as $\delta^{13}C$, increased under strong drought stress, while agronomic water use was usually decreased.

Key words: *Medicago* spp., *Lotus* spp., *Onobrychis viciifolia,* productivity, nitrogen fixation, $\delta^{13}C$

1. Introduction

The productivity of grassland swards is strongly dependent on nitrogen (N) availability. Increasing prices of energy and N-fertiliser along with higher costs for concentrates, which are expected for the future, will further stress the importance of grassland legumes and their N-fixation ability, not only for organic farming, but also for more intensive agricultural systems (Watson et al. 2002, Jensen and Hauggaard-Nielsen 2003, Crews and Peoples 2005, German Agricultural Research Alliance 2012).

Trifolium repens is currently the most important legume in European temperate grasslands (Frame et al. 1998; Gierus et al. 2012). Grass/*T. repens* mixtures are highly productive as long as water is not limiting and have a high nutritive value (Wilman and Williams 1993, Wilkins et al. 1994, Topp and Doyle 2004). While legumes would benefit from rising temperatures and elevated CO_2 (Soussana et al. 2010), as expected under conditions of climate change, water is likely to become limiting in temperate climates where the probability of summer droughts increases (Alcamo et al. 2007, Schindler et al. 2007). *Trifolium repens* is sensitive to water shortages and responds with strongly decreasing yields (Marshall et al. 2001). Other legumes may be better adapted to water limited conditions and may therefore have an increasing potential in future forage production. However, knowledge about the agronomic potential of such alternative legume species under drought is limited (Hopkins et al. 1996, Rochon et al. 2004, Hopkins and Wilkins 2006, Sölter et al. 2007) and their cultivation and use insignificant. Besides yield, agronomic water use efficiency, i.e. the yield per unit of water used, is an important factor for dealing with limited water resources (Gregory et al. 2000; Wallace 2000). The agronomic water use efficiency depends on several factors among which the intrinsic WUE, i.e. CO_2 assimilation divided by stomatal conductance, and the N availability are important. Nitrogen availability and intrinsic WUE are affected by drought stress and thus influence agronomic WUE (Condon et al. 2002; Farooq et al. 2009). Especially N fixation, as an important feature of legumes, is sensitive to drought stress (Frame et al. 1998).

In this study, we tested the hypothesis that under temporary drought some so far neglected grassland legumes use water more efficiently and provide biomass yields that are in a range of those found with *T. repens* and are of a higher stability when grown either as monocultures or mixtures.

We used a selection of five promising forage legumes for temperate grassland and compared their performance with that of *T. repens*. In a container experiment in a vegetation hall, drought conditions were imposed during three different periods over two years. Legumes were sown in monocultures and in mixtures with *Lolium perenne*.

We quantified yield and water use and calculated agronomic water use efficiency. Furthermore, we determined the stable carbon isotope composition ($\delta^{13}C$) for a strong drought stress period in summer 2010 as an indicator for intrinsic WUE. N-fixation (Ndfa in g N container^{-1}) as an important, but drought-stress sensitive feature of legumes, was determined as well.

2. Material and Methods

The experiment was set up in July 2009 (sowing date: 15 July) as a three-factorial container experiment. The three factors were (1) legume species (six legumes), (2) stand (legumes in monoculture or in mixture with *L. perenne*) and (3) drought stress (regular irrigation or water shortage). The legumes were *Lotus corniculatus* L. (var. Bull), *Lotus uliginosus* Schkuhr (wild seeds), *Medicago lupulina* L. (var. Ekola), *Medicago falcata* L. (wild seeds), *Onobrychis viciifolia* Scop. (var. Matra), and *Trifolium repens* L. (var. Rivendel); *Lolium perenne* L. (var. Signum) was used as a companion grass in mixtures and as a reference crop. The legumes were chosen according to their potential agronomic performance as an alternative to *T. repens* (Dierschke and Briemle 2002; Klotz et al. 2002).

2.1 Experimental Setup

The experimental containers (30 l, diameter 33 cm, height 42 cm) were filled with a homogeneous mixture of 20 kg air-dried sand (sieved to pass a mesh of 5 mm; August Oppermann Kiesgewinnung GmbH, Hann. Münden, Germany), 5.5 kg compost (air-dried; Bioenergiezentrum Göttingen GmbH, Göttingen, Germany) and 0.9 kg vermiculite (particle size 8-12 mm; Deutsche Vermiculite GmbH, Sprockhoevel, Germany) with a top layer of 1.5 kg compost as a seed bed. The pH of the soil (in $CaCl_2$ suspension), as well as the availability of P, K (extracted with calcium acetate lactate, continuous flow analyser [CFA]) and Mg ($CaCl_2$ extraction, CFA), were measured in summer 2011 (pH, 7.3; 292 mg P kg^{-1}; 430 mg K kg^{-1}; 364 mg Mg kg^{-1} oven-dry soil). The relation of volumetric soil water content and soil water tension was determined by a soil water retention curve carried out with a pressure plate extractor (Or and Wraith 2002).

The six legumes and *L. perenne* were sown in monoculture with 1000 germinable seeds per m^2 for legumes and 5000 for *L. perenne*. For the mixtures of each legume with *L. perenne* we used 500 germinable seeds per m^2 for legumes and 2500 for *L. perenne*. The experiment consisted of a total of 26 treatments, which were replicated four times, leading to 104 containers that were arranged in a randomized block design in a vegetation hall. We chose a vegetation hall as the conditions there followed a normal seasonal pattern with limited frost in winter and higher temperatures in summer, while the drought stress phases could be fully controlled and recorded.

Minimum and maximum air temperatures were recorded daily at three locations in the vegetation hall (Figure 1). Climatic conditions were controlled by forced venting in summer and by a heating system in winter that was switched on when temperatures fell below 0°C for longer than 24 h. Heating in winter was limited to a maximum of 5°C air temperature in the vegetation hall. No extra lighting was provided and no fertilisation was applied. In order to ensure nodulation of the legumes, all containers were treated with a rhizobium solution (Radicin, Jost GmbH, Iserlohn, Germany). The Radicin solution contained all rhizobia strains in the same proportions for an effective infection of all legume species. Starting two weeks after sowing, all containers were weighed regularly and were irrigated when the soil water content reached ~18 vol. % (-0.3 MPa). *L. uliginosus* did not survive the first winter and was therefore resown in March 2010.

The aboveground biomass was harvested twice in 2009 (calendar week 36 and 44), five times in 2010 (calendar week 15, 21, 27, 34 and 42) and two times in 2011 (calendar week 15 and 22). We here report data from the harvests (week 21 and 34 in 2010; week 22 in 2011) after three drought stress periods.

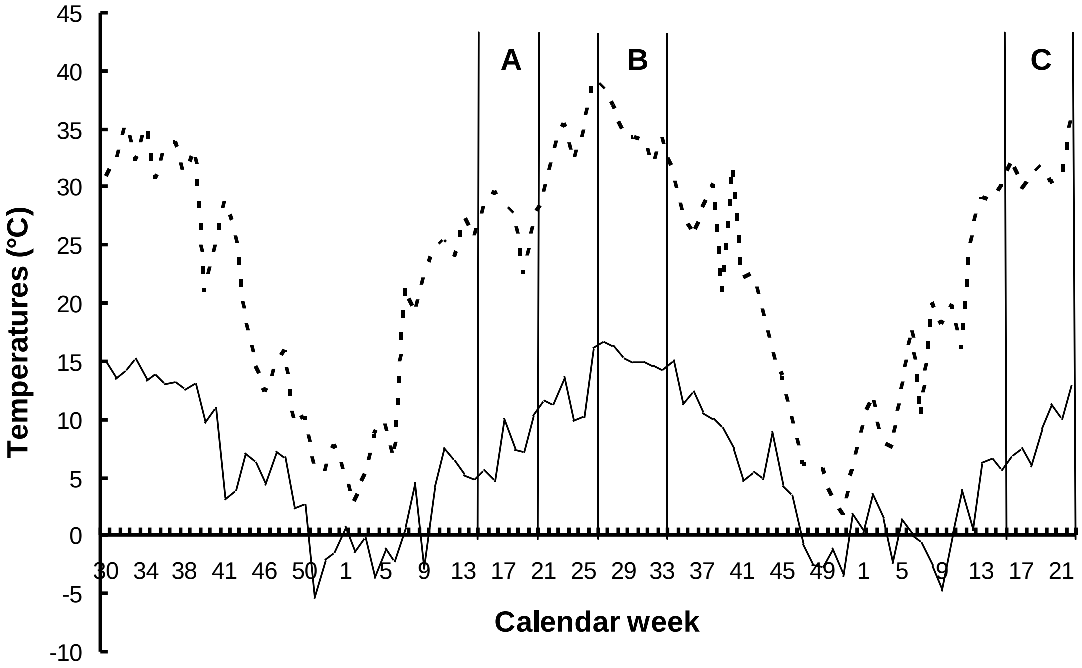

Figure 1. Weekly air temperature in the vegetation hall from July 2009 to May 2011. The solid and broken lines represent the mean minimal and maximal temperatures, respectively. Section A – moderate drought stress spring 2010; Section B – strong drought stress summer 2010; Section C – strong drought stress spring 2011.

2.2 Drought Stress Treatment

Drought stress was imposed during three periods with a varying severity, i.e. a moderate stress in spring 2010, and a severe stress in summer 2010 and spring 2011. Stress phases in spring were carried out after the first harvest of the year. There were intermittent periods with normal watering where plants were allowed to recover from drought. Drought stress was induced by temporarily ceasing the watering of the containers after an initial watering to 25% soil volumetric water content (-0.03 MPa). For the moderate drought stress, no water was given until three days after the first plants showed signs of drought (soil water tension -1.5 MPa; 10 vol. %). Containers were then watered again to 25 vol. % (-0.03 MPa) followed by a second drought cycle. In order to induce strong drought, the stress phase was extended to five days after first stress symptoms (e.g. wilting) had appeared and was repeated three times with two irrigations in between. The number of days until the soil water content reached 18 vol. % (-0.3 MPa) was counted (Table 1). Means of soil water content (vol. %) at the end of the respective drying cycles (Table 1) indicate the severity of drought for every plant species and mixture.

The control containers which did not receive any drought stress treatment were watered to approximately -0.03 MPa once their water content fell below -0.3 MPa. During the three investigated periods all containers were weighed regularly in order to determine the water use per container from accumulated weight losses.

Table 1. Number of days needed to reduce the soil water content from 25 to 18 vol.% (-0.3 MPa) under drought stress, and mean soil water content (in vol.%) at the end of each drying cycle for three periods with moderate and two strong drought stresses.

	Spring 2010 moderate				Summer 2010 strong				Spring 2011 strong			
Plant species	Monoculture		Mixture		Monoculture		Mixture		Monoculture		Mixture	
	Day	Vol. %	Day	Vol. %	Day	Vol. %	Day	Vol. %	Day	Vol. %	Day	Vol. %
Lotus corniculatus	16	13	12	10	10	4	10	6	13	4	14	5
Lotus uliginosus	16	24	10#	10#	13	10	10#	8#	18	8	15#	8#
Medicago lupulina	14	11	10	10	11	7	9	6	13	5	10	4
Medicago falcata	15	11	9	9	11	5	9	6	13	6	12	6
Onobrychis viciifolia	15	15	10#	9#	12	9	10#	6#	16	7	13#	7#
Trifolium repens	10	6	9	7	8	5	8	4	11	4	9	5
Lolium perenne	9	10			11	7			14	8		

The legume partner in this mixtures did not produce any more biomass at these periods.

2.3 Sampling and Measurement

Aboveground biomass was determined by cutting the plants at a height of 3–4 cm above the soil surface. The cut herbage was separated into species immediately after the harvest. Dry mass was measured after drying of the herbage sample at 60°C for 72 h.

Dry herbage was ground to pass a mesh of 1 mm size. The herbage crude protein (CP) content was obtained by near-infrared reflectance spectroscopy (NIRS). The spectra were analyzed using the large dataset of calibration samples from different kinds of grasslands by the Institute VDLUFA Qualitätssicherung NIRS GmbH, Kassel, Germany (Tillmann 2010).

A difference method was used to calculate nitrogen fixation (Gierus et al., 2012). *Lolium perenne* was used as a non-fixing reference crop. The nitrogen in the reference crop was used as a proxy of the nitrogen derived from soil. Nitrogen derived from atmosphere (NdfA in g N container^{-1}) was then assessed by nitrogen content (CP content divided to 6.25) in the legume minus N in the reference crop.

For determination of agronomic WUE, we divided yield per drought period by total water use (evaporation plus transpiration) in the same period (Gregory et al. 2000). As an indicator of intrinsic WUE we measured the stable carbon isotope composition ($\delta^{13}C$ signature) which is linearly correlated to intrinsic WUE (Farquhar et al. 1989) in the strong drought stress period in summer 2010. Plant samples (representative samples of the whole aboveground biomass) were ground to 0.2 mm. The isotopic analyses were carried out with an isotope ratio mass spectrometer Finnigan MAT 251 (IRMS; Finnigan, Bremen, Germany), linked with a Conflo II-Interface (Thermo-Finnigan, Bremen, Germany) to an elemental analyser NA1500 (Carlo Erba Instruments, Milano, Italy). The standard was V-PDB, with acetanilide as internal standard. The internal reproducibility of the ^{13}C measurements was better than ± 0.2‰.

2.4 Statistical Analysis of Data

Statistical data analysis was carried out using the Genstat 6.1 software package. An analysis of variance (ANOVA) was calculated for every drought stress period and considered the effects of three factors (legume, stand and drought stress) on dry matter yield and agronomic WUE of all species in monoculture and in mixture with *L. perenne*. Two factors, legume species and drought stress, were considered for an ANOVA of yield contribution of the legume partner in mixture with *L. perenne*, nitrogen derived from atmosphere (Ndfa) of monocultures and $\delta^{13}C$ signatures of monocultures. Residuals were used to check the validity of the models. Normality in data was achieved by applying logarithmic or square root transformations, if necessary. In case of significant treatment effects (α <0.05), least significant differences (LSD values) were used to compare mean values. Relationships between selected variables were examined with a linear regression model.

3. Results

3.1 Dry Matter Yield

The main factors legume (L) and drought stress (DS) as well as the interaction L x DS had a significant effect on dry matter yield in all stress periods. The factor stand (S), i.e. whether the legume was grown in monoculture or in mixture with *L. perenne*, had no effect on dry matter production under moderate drought stress in spring 2010, but had so under strong stress in summer 2010 and in spring 2011 (Table 2). During these drought stress periods, dry matter yields of legumes were smaller in mixtures than in monoculture for both, control and stress treatments.

Under control conditions, when water was not limiting, *Trifolium repens* produced the largest dry matter yields of all legumes in all three investigated periods, in monoculture as well as in mixture. However, dry matter yields of *L. corniculatus*, *L. uliginosus* and *M. falcata* grown in monoculture in summer 2010 or of *L. uliginosus* and *M. lupulina* in spring 2011 were not significantly different from *T. repens*.

Generally, dry matter production was smaller under moderate and strong drought stress compared to the corresponding control treatments (Table 2). *Trifolium repens,* especially, showed significant and quite substantial reductions in yield even under moderate stress in monocultures and in mixtures. *Lotus corniculatus, M. falcata* and *M. lupulina* were less strongly affected by strong drought stress than *T. repens* and produced similar or even larger yields and showed a smaller yield decrease under these conditions.

We assessed Ndfa (g N container^{-1}) as an indicator for drought induced changes in physiology of the investigated legume monocultures (Table 3). Analysis of variance (ANOVA) showed significant effects of main factors L and DS on Ndfa in all drought periods, while the interaction of L x DS was significant only in both spring drought periods. In all drought periods *T. repens*, *M. lupulina*, *M. falcata* and *L. corniculatus* showed mostly larger Ndfa (g N container^{-1}) than *L. uliginosus* or *O. viciifolia*

Table 2. Dry matter yield of six legume species and *L. perenne* in monoculture and in mixture with *L. perenne* under different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4) and LSD.

Plant species	Dry matter yield [g container^{-1}]											
	Spring 2010 moderate				Summer 2010 strong				Spring 2011 strong			
	Monoculture		Mixture		Monoculture		Mixture		Monoculture		Mixture	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Lotus corniculatus	37.1	36.7	31.8	27.1	58.6	34.9	42.2	32.5	107.5	57.1	63.6	44.6
Lotus uliginosus	9.4	6.9	32.1[#]	30.1[#]	51.7	36.7	34.9[#]	28.9[#]	117.6	50.2	35.3[#]	28.8[#]
Medicago lupulina	47.8	41.7	33.6	32.2	48.1	45.9	48.8	39.5	120.4	57.3	68.0	56.0
Medicago falcata	39.7	37.8	29.2	29.7	53.6	38.9	37.8	30.0	86.6	50.3	49.4	40.3
Onobrychis viciifolia	19.1	19.0	32.8[#]	27.0[#]	31.8	22.5	35.8[#]	29.3[#]	82.1	41.5	40.4[#]	26.9[#]
Trifolium repens	70.5	44.9	49.6	40.6	60.9	39.1	62.7	35.3	128.9	57.0	117.1	54.9
Lolium perenne	29.1	27.3			36.1	32.5			38.2	31.4		
LSD values	7.65				10.95				14.29			
ANOVA Summary	*F*-ratio		*P*		*F*-ratio		*P*		*F*-ratio		*P*	
Legume (L)	67.78		< 0.001		12.16		< 0.001		35.30		< 0.001	
Stand (S)	1.28		0.263		11.74		< 0.001		178.21		< 0.001	
Drought Stress (DS)	19.74		< 0.001		65.27		< 0.001		331.88		< 0.001	
L x S	29.35		< 0.001		3.37		0.009		8.08		< 0.001	
L x DS	5.13		< 0.001		3.12		0.013		9.44		< 0.001	
S x DS	1.15		0.288		1.11		0.296		69.85		< 0.001	
L x S x DS	2.20		0.064		1.14		0.349		3.32		0.010	

Results from an Analysis of variance (ANOVA) considering the effects of legume, stand (monoculture or mixture) and drought stress. *L. perenne* was not included in the analysis.

[#] The legume partner in this mixtures did not produce any more biomass at these periods.

in control and stress treatments. Moderate drought stress led to significant decreases in Ndfa only for *T. repens*, whereas strong stress decreased Ndfa significantly for all legumes (Table 3).

Table 3. Nitrogen derived from atmosphere (Ndfa) in g N container^{-1} of six legume species in monocultures under different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4) and LSD.

Plant species	Ndfa [g N container^{-1}] Spring 2010 moderate		Summer 2010 strong		Spring 2011 strong	
	Control	Stress	Control	Stress	Control	Stress
Lotus corniculatus	1.17	1.13	1.46	0.58	3.38	1.70
Lotus uliginosus	-0.12	-0.15	0.82	0.28	4.19	1.12
Medicago lupulina	1.50	1.27	1.36	1.09	4.08	1.64
Medicago falcata	1.31	1.29	1.47	0.89	3.09	1.63
Onobrychis viciifolia	-0.02	0.01	0.29	0.13	1.60	0.65
Trifolium repens	2.64	1.50	1.68	0.80	4.70	1.73
LSD values	0.2449		0.6418		0.6424	
ANOVA Summary	*F*-ratio	*P*	*F*-ratio	*P*	*F*-ratio	*P*
Legume (L)	206.27	< 0.001	6.90	< 0.001	20.56	< 0.001
Drought Stress (DS)	24.04	< 0.001	19.33	< 0.001	265.60	< 0.001
L x DS	13.94	< 0.001	0.92	0.479	7.48	< 0.001

Results from an Analysis of variance (ANOVA) considering the effects of legume and drought stress.

3.2 Yield Contribution

The yield contribution of the legume partner in mixtures with *L. perenne* differed among the three periods. While the yield contribution to the respective mixtures differed among legumes species (L) during all drought periods, DS and the interaction L x DS led to significant differences in yield contribution only in spring 2011(Table 4).

The yield contribution of *T. repens* was generally larger than that of the other legumes. However, under conditions of drought stress, the yield contribution of *T. repens* was more strongly reduced in relation to the control than that of the other legumes. The yield contribution of *T. repens* (g container^{-1}) under stress was reduced by 47%–73%, while the average of the corresponding value for *L. corniculatus*, *M. lupulina* and *M. falcata* ranged from 25–31% over the three periods. Under conditions of strong drought stress in spring 2011, *M. lupulina* showed even a larger yield contribution than *T. repens*.

Table 4. Yield contribution of the legume partner in mixture with *L. perenne* with different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4) with LSD.

Plant species	Yield contribution [%] Spring 2010 moderate		Summer 2010 strong		Spring 2011 strong	
	Control	Stress	Control	Stress	Control	Stress
Lotus corniculatus	3.7 (0.37)	2.9 (0.35)	15.0 (1.11)	12.9 (1.04)	49.6 (1.68)	38.1 (1.57)
Medicago lupulina	12.8 (1.10)	10.8 (1.00)	18.1 (1.25)	16.8 (1.18)	46.2 (1.64)	57.1 (1.75)
Medicago falcata	2.8 (0.43)	2.2 (0.32)	9.6 (0.96)	8.2 (0.88)	37.5 (1.57)	30.0 (1.47)
Trifolium repens	40.8 (1.56)	28.2 (1.40)	49.9 (1.64)	25.2 (1.39)	70.8 (1.85)	49.7 (1.69)
LSD values	0.40		0.34		0.12	
ANOVA Summary	*F*-ratio	*P*	*F*-ratio	*P*	*F*-ratio	*P*
Legume (L)	31.99	< 0.001	9.62	< 0.001	12.51	< 0.001
Drought Stress (DS)	1.08	0.311	2.05	0.167	4.45	0.047
L x DS	0.10	0.962	0.30	0.825	3.91	0.023

Results from an Analysis of variance (ANOVA) considering the effects of legume and drought stress; analysis based on log-transformed data (ANOVA results, LSD values and means in brackets).

3.3 Water Use Efficiency

During the two strong drought stress periods, the main factors L, S and DS had highly significant effects on agronomic WUE, while in the moderate stress period only L was significant. There was a highly significant interaction of L x S in all periods and, additionally in spring 2011 the interaction of S x DS and the three-way interaction were highly significant as well (Table 5).

Under control conditions values for agronomic WUE ranged from 1.3 g l^{-1} for *L. uliginosus* in monoculture to 4.3 g l^{-1} for *M. lupulina* in monoculture (Table 5). *Trifolium repens* and *M. lupulina* in monoculture and in mixture had the highest agronomic WUE, values for *M. falcata* and *L. corniculatus* were slightly lower. Differences in WUE between legumes in monoculture and their respective mixtures with *L. perenne* were particularly pronounced in spring 2011. Moderate drought stress conditions in spring 2010 did not lead to lower WUE. In contrast, under strong drought stress in summer 2010 and especially in spring 2011, WUE was in most cases reduced compared to the control; this effect was stronger in monocultures than in mixtures. *Trifolium repens* particularly, reacted strongly to severe drought stress which led to a significantly reduced WUE in monoculture and in mixture. Other legumes like *M. lupulina* showed a higher WUE than *T. repens* under strong drought stress.

Table 5. Agronomic water use efficiency of six legume species (and *L. perenne*) in monoculture and in mixture with *L. perenne* with different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4) with LSD.

Plant species	Water use efficiency $[\mathrm{g\ l^{-1}}]$											
	Spring 2010 moderate				Summer 2010 strong				Spring 2011 strong			
	Monoculture		Mixture		Monoculture		Mixture		Monoculture		Mixture	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Lotus corniculatus	3.2	3.2	2.5	2.4	2.2	1.8	1.9	1.6	3.6	2.8	2.6	2.2
Lotus uliginosus	1.3	1.3	2.6[#]	2.8[#]	2.2	2.0	1.7[#]	1.6[#]	3.6	2.5	2.1[#]	1.8[#]
Medicago lupulina	3.6	3.7	2.4	2.9	2.7	2.8	2.1	2.0	4.3	2.9	2.8	2.9
Medicago falcata	3.4	3.3	2.4	2.7	2.6	2.1	1.9	1.6	3.9	2.6	2.4	2.2
Onobrychis viciifolia	2.0	2.1	2.5[#]	2.5[#]	1.5	1.3	1.8[#]	1.5[#]	3.3	2.5	2.0[#]	1.7[#]
Trifolium repens	3.5	3.5	3.1	3.3	2.5	2.1	2.3	1.7	3.6	2.8	3.4	2.6
Lolium perenne	2.4	2.6			1.8	1.6			2.1	1.8		
LSD values	0.67				0.30				0.36			
ANOVA Summary	*F*-ratio		*P*		*F*-ratio		*P*		*F*-ratio		*P*	
Legume (L)	19.66		< 0.001		30.21		< 0.001		27.52		< 0.001	
Stand (S)	2.25		0.138		68.78		< 0.001		227.69		< 0.001	
Drought Stress (DS)	1.27		0.264		42.80		< 0.001		180.49		< 0.001	
L x S	15.41		< 0.001		10.42		< 0.001		7.35		< 0.001	
L x DS	0.27		0.929		2.84		0.022		0.63		0.680	
S x DS	0.79		0.378		0.00		0.997		49.33		< 0.001	
L x S x DS	0.27		0.927		0.49		0.779		3.67		0.005	

Results from an Analysis of variance (ANOVA) considering the effects of legume, stand (monoculture or mixture) and drought stress. *L. perenne* was not included in the analysis.

[#] The legume partner in this mixtures did not produce any more biomass at these periods.

An analysis of variance (ANOVA) of $\delta^{13}C$ signatures showed significant effects of L (F=63.63; $P<0.001$), DS (F=75.38; $P<0.001$) and of the interaction L x DS (F=2.73; P=0.036). Drought stress in summer 2010 led to significant enrichments in $\delta^{13}C$ for *L. corniculatus*, *M. lupulina*, *M. falcata*, *O. viciifolia* and *T. repens* compared to control treatments; however, the extent of enrichment differed among legumes. Figure 2 shows $\delta^{13}C$ signatures of the six legumes (and *L. perenne*) in monoculture under strong drought stress in summer 2010 and the relationship between $\delta^{13}C$ and agronomic WUE. $\delta^{13}C$ signatures as an indicator of intrinsic WUE increased under drought stress, while agronomic WUE mostly decreased.

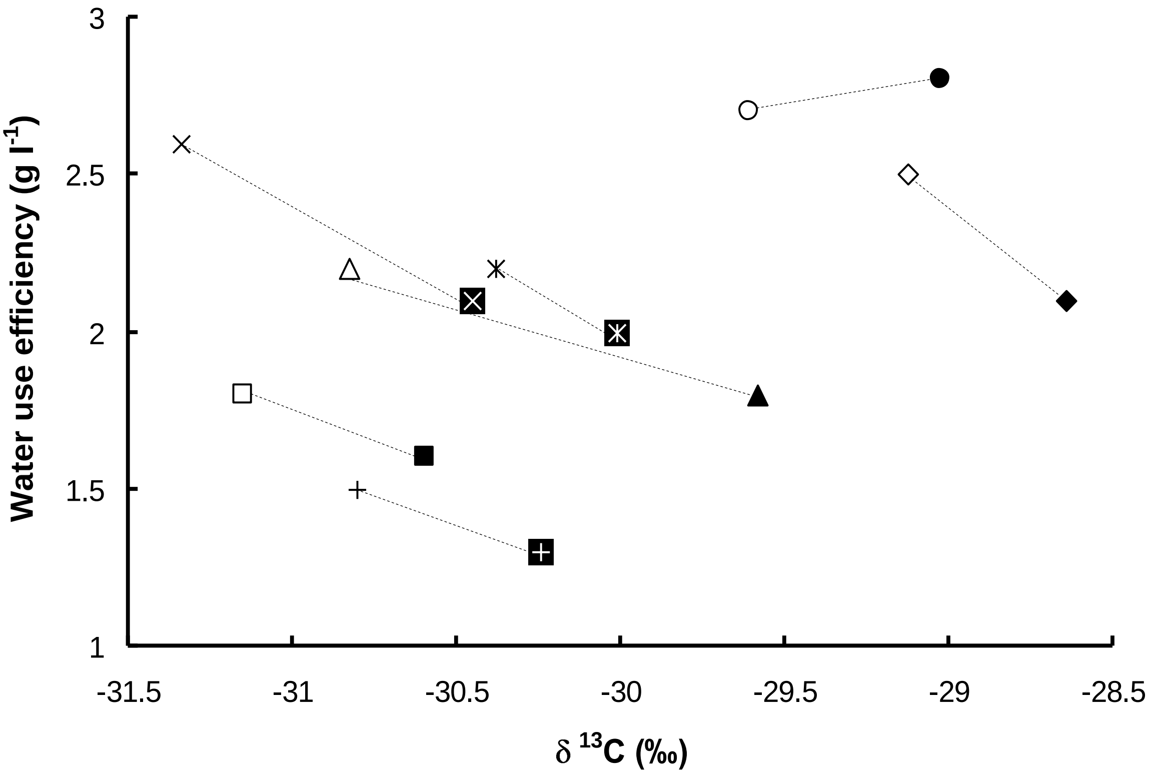

Figure 2. Agronomic water use efficiency and $\delta^{13}C$ signatures of six legume species and *L. perenne* in monoculture under strong drought stress (summer 2010).

▲/Δ *L. corniculatus* (stress/control) ⧆/* *L. uliginosus* (stress/control) ●/○ *M. lupulina* (stress/control) ⊠/× *M. falcata* (stress/control) ⊞/+ *O. viciifolia* (stress/control) ♦/◊ *T. repens* (stress/control) ■/□ *L. perenne* (stress/control)

4. Discussion

In the study presented here, we were looking for possible alternative legumes to *T. repens* with a potential to cope better with temporary drought. We determined biomass yield and agronomic WUE under drought and control conditions to evaluate the suitability of five legumes as possible agronomic alternatives to T. *repens*. Generally, yields and agronomic WUE decreased under strong stress; however, legumes differed in the extent of their reaction to drought.

In particular, *T. repens* was susceptible to drought. Under moderate drought stress yield of *T. repens* in monoculture was reduced by 36% and under strong stress yield reduction amounted up to 56%. While yield reductions for *M. lupulina*, *L. corniculatus* and *M. falcata* were relatively small under moderate drought stress (1 to 13%), decreases in yield for alternative legumes under strong stress were on average 36% which is considerable, but still less than for *T. repens*. When Foulds (1978) compared the reaction of legumes to soil moisture deficit, he also found that under drought, *M. lupulina* had smaller yield reductions than *T. repens* resulting in comparable yields of *M. lupulina* to *T. repens* under stress. Belaygue et al. (1996) explained a significant decrease in productivity for *T. repens* with a reduction in stolon number of up to one third under moderate stress and even greater reductions under stronger drought. In our mixtures, *T. repens* showed a strong competitive ability against the fast growing grass *L. perenne* when water was not limited (Table 4). This supports the findings of Petersen (1967). However, under strong drought stress, *T. repens* contributed less to the total yield of the mixture (up to 73% decrease). It seems that *T. repens* lost strongly in competitive ability under drought while the grass partner *L. perenne* was relatively unaffected. As a consequence, there was a considerable decrease in total yield of the mixture. The competitive ability of other legumes, especially of *M. lupulina,* but also of *L. corniculatus* and *M. falcata*, was less reduced under drought and decrease in yield contribution of the legume as well as yield of the whole mixture was smaller than with *T. repens*.

Legumes differed in their agronomic WUE and in the extent to which this parameter was affected by drought. Agronomic WUE of *T. repens* was drastically reduced under strong drought, while some of the alternative legumes were less affected. *Medicago lupulina* in monoculture showed similar or higher WUE under control or drought conditions and *M. lupulina*, *L. corniculatus* and *M. falcata* mixtures had a smaller decrease in agronomic WUE under strong drought than *T. repens*.

Biomass yield and agronomic WUE are linked and influenced by several factors (Ehlers and Goss, 2003), among which are: evaporation (Ehlers and Goss 2003), N supply (Ehlers and Goss 2003, Brueck, 2008), intrinsic WUE (Farquhar et al. 1989, Condon et al. 2002) and N fixation (Pimratch et al. 2013). These factors will thus determine the differences between legumes in their reaction and tolerance to water shortage as well (Kemp 1984).

Evaporation is a part of unproductive water loss (Ehlers and Goss, 2003) and amounted to max. 0.4 l day^{-1} for containers with no vegetation in the first two days after irrigation in our experiments. In the following days, evaporation continuously decreased because the thin top soil layer made of compost dried up and acted as an evaporations barrier. After two weeks evaporation ceased completely in containers with bare soil. It can be assumed that evaporation was considerably smaller in containers with vegetation. Despite the probably higher evaporation associated with higher unproductive water loss in control treatment, WUE was generally lower under strong drought stress. It seems that factors other than evaporation must contribute more to an explanation of decreased WUE under strong stress.

Nitrogen supply is known to increase agronomic WUE (Ehlers and Goss 2003, Brueck 2008). None of the containers in our experiment received N; N fixation, apart from N from soil sources, was the most important and only substantial external source for N. Consequently, we observed a positive linear correlation between Ndfa (g N container^{-1}) and agronomic WUE ($P<0.001$; $R^2=0.60$). The amount of Ndfa was affected by drought. Under strong drought stress the decrease in Ndfa was on average 15% larger than the decrease in yield (Table 2 and 3). This implies that N concentration in plant tissues in the stress treatment was lower than in control treatment (8% lower). It can be assumed that a reduction in Ndfa, and thus in N concentration in plant biomass, would negatively affect the efficiency of carbon metabolism of plant tissue or lead to an accelerated leaf senescence. This is associated with a reduced stomata control, which results in more unproductive water losses (Brueck 2008). In our experiments, particularly *T. repens* suffered under drought stress; even under moderate stress Ndfa was reduced by 43%. Alternative legumes seemed less affected and the reduction in Ndfa under moderate stress was only up to 16%. Strong drought stress led to decreases in Ndfa of up to 63% for *T. repens* and usually lower decreases for *M. lupulina*, *M. falcata* and *L. corniculatus* (on average 46%, Table 3). Legumes differed in their capacity to fix N from the atmosphere. *Trifolium repens*, *M. lupulina*, *M. falcata* and *L. corniculatus* had larger Ndfa than *L. uliginosus* and *O. viciifolia*. More Ndfa of total N in biomass was usually associated with a better agronomic WUE.

Another important factor to contribute to differences in agronomic WUE is the intrinsic WUE. Signatures of δ^{13}C are indicators for CO_2 uptake and water release (transpiration) by stomata and thus closely related to intrinsic WUE (Farquhar et al. 1989, Condon et al.

2002). In all legume monocultures we observed larger signatures of $\delta^{13}C$ in biomass from strong stress treatments compared to the control. This indicates that intrinsic WUE was higher under drought conditions. However, even if intrinsic WUE was higher under drought stress, this did not lead to a higher agronomic WUE (Figure 2). Measured $\delta^{13}C$, as an indicator for intrinsic WUE, can not be used to explain the whole agronomic WUE (Farquhar et al. 1989). Although $\delta^{13}C$ signatures, and therefore intrinsic WUE, can only partly explain agronomic WUE (Farquhar et al. 1989), differences in $\delta^{13}C$ signatures between control and stress might be taken as an indication for drought adaptation. A larger intrinsic WUE is realized by lower stomatal conductance and/or larger carbon assimilation (Farquhar et al. 1989, Köhler et al. 2010). We might conclude that legume species like *L. corniculatus, M. falcata* and *M. lupulina,* with an intrinsic WUE that increased more under drought conditions than that of *T. repens,* are probably better adapted to water shortage (Figure 2). Differences in water use among legumes may support this assumption. *Trifolium repens* in monoculture and in mixture with *L. perenne* consumed water very fast and reached -0.3 MPa within ten days. In contrast to that, *M. lupulina*, *L. corniculatus* or *M. falcata* used water slower and reached -0.3 MPa up to six days later than *T. repens*. Also, mean soil water content (vol. %) at the end of each drying cycle was usually lower for *T. repens* than for alternative legumes (Table 1). This means that water stress started earlier and was more severe for *T. repens*.

Apart from Ndfa and intrinsic WUE, there are other factors that influence the plant performance under drought. Farooq et al. (2009) mentioned that additional stress factors, like, for example, higher temperatures, could further enhance the disturbance of physiological and metabolic processes in growing plants under drought. In our experiments, both strong drought stress periods (summer 2010 and spring 2011) were accompanied by relatively high mean air temperatures. Average maximum temperature in spring 2010 (moderate stress) was ~27°C compared to ~35°C in summer 2010 and ~31°C in spring 2011 (Figure 1). We may assume that the combination of severe drought and higher temperatures led to a disturbance of the activity of various enzymes, to cell damages and even to a die-back of plant tissue and whole plants (McKersie and Leshem 1994, Lösch 2003, Farooq et al. 2009). In our experiments we observed increased wilting, tissue die-back and leave losses under strong stress.

Some plants have anatomical adjustments to drier conditions that will reduce stomatal and cuticular transpirations and result in a reduction of ineffective water losses (Schreiber and Riederer 1996, Lösch 2003). *Medicago lupulina* and *L. corniculatus* have waxy coatings on both sides of their leaves (Winstel and Rentschler 1975) and, additionally, *M lupulina* has hairs on the leaves (Klapp and Opitz von Boberfeld 2004). Leaves of *M. falcata* can also show some skleromorphic features (Klotz et al. 2002).

In conclusion, *M. lupulina* and, to a lesser extent, *L. corniculatus* and *M. falcata* showed potential as alternatives to *T. repens* under drought conditions. Reduction of yield and agronomic WUE under drought was mostly smaller for alternative legumes compared with *T. repens*.

We found that the amount of nitrogen derived from the atmosphere (N fixation) was a good indicator for the performance of the legumes under drought stress. High N fixation was related to larger yield and better agronomic water use efficiency in both, control and stress treatment. Intrinsic WUE was a poorer indicator for agronomic WUE: although intrinsic WUE increased under strong drought, agronomic WUE was mostly decreased. Still, the increase of intrinsic WUE implies some potential to drought adaptation. For future sustainable forage legume production it will be important to consider this range of main factors and important interactions and use this information to identify promising legume species for plant breeding and develop better-adapted varieties with.

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Chapter III

Influence of drought stress on nutritive value of perennial forage legumes

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Abstract

In the next decades, forage legumes are likely to become more important. However, predicted climate change may increase the risk of droughts and thus influence their agricultural performance. Decreases in yield due to water shortage are well documented, while influences on nutritive values are inconsistent. Therefore, we examined the effects of drought on crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and water-soluble carbohydrates (WSC) of six legumes, birdsfoot trefoil (*Lotus corniculatus* L.); marsh birdsfoot trefoil (*Lotus uliginosus* Schkuhr); black medic (*Medicago lupulina* L.); yellow alfalfa (*Medicago falcata* L.); sainfoin (*Onobrychis viciifolia* Scop.), and white clover (*Trifolium repens* L.) in monoculture and in mixture with perennial ryegrass (*Lolium perenne* L.) in a container experiment in a vegetation hall. Moderate and strong drought stress was applied during three periods in two years. Mean volumetric soil water content at the end of the moderate drought stress period was 11 vol. % and 6 vol. % under strong stress. The effect of drought on nutritive values was considerably less pronounced than on yield. While the impact of moderate stress on nutritive quality was negligible, we found decreases in CP, NDF, and ADF, and increases in WSC under strong stress. This may indicate that water scarcity could even increase fodder quality and digestibility. However, the choice of legume species and stand (monoculture or mixture) had stronger effects on nutritive values than drought. We conclude that the reaction of temporary drought on nutritive values seems to be less important for the selection of suitable forage legumes species than other agronomic properties under conditions of climate change.

Key words: Crude protein, NDF, ADF, Water-soluble carbohydrates

1. Introduction

Grassland with its potentially high productivity and good fodder quality forms the basis for ruminant nutrition. Legumes are important for grassland productivity and fodder quality, especially under conditions of limited input of nitrogen (N) from mineral fertilisers and/or manures, due to their ability to fix atmospheric N. Increasing prices for energy and N-fertiliser along with higher costs for concentrates, which are expected for the future, will further increase the importance of forage legumes (Watson et al., 2002; Jensen and

Hauggaard-Nielsen, 2003; Crews and Peoples, 2005; German Agricultural Research Alliance, 2012).

Forage production from grassland is dependent on adequate water supply (Hopkins and Del Prado, 2007). Under conditions of climate change, water is likely to become more limited in semiarid and in temperate climates as the probability of summer droughts increases (Alcamo et al., 2007; Schindler et al., 2007; Trenberth, 2011). Insufficient water supply can have strong effects on production of forage legumes. A decrease in yield, depending on the strength and duration of drought stress, is common (Foulds, 1978; Farooq et al., 2009; Jalleel et al., 2009). However, knowledge about the influence of drought on important characteristics of the nutritive value of legumes is inconsistent and limited. Under conditions of drought stress, Peterson et al. (1992) found reduced acid detergent fibre (ADF) and neutral detergent fibre (NDF) concentrations in a range of forage legumes, but inconsistent changes in crude protein (CP) concentrations. In contrast, Seguin et al. (2002) described an increased ADF concentration and a minor effect on CP and NDF concentration in cura clover (*Trifolium ambiguum* M.B.), red clover (*T. pratense* L.) and alfalfa (*Medicago sativa* L.). Nakayama et al. (2007) found an increased concentration of water-soluble carbohydrates (WSC) under water shortage in two cultivars of soybean (*Glycine max* L. Merrill). For clover species, Abberton et al. (2002) observed only a small effect of drought on WSC. More research is needed to gain knowledge about the influence of drought stress on the nutritive value of forage legumes.

In this study, we used six perennial forage legumes in monoculture and in mixture with perennial ryegrass (*Lolium perenne*) and examined the effects of drought stress and the interaction of legume species and drought on important parameters of nutritive value, like CP, WSC and the fibre components NDF and ADF. Due to N fixation, legumes are high in CP which is essential for ruminant nutrition. Water-soluble carbohydrates have a positive influence on fodder intake and are important for an efficient utilisation of dietary N. The NDF concentration gives an estimation of the structural part of the plant material (cellulose, hemicellulose and lignin) and is inversely related to the voluntary fodder intake. Acid detergent fibre includes lignin and cellulose and is negative correlated with cell wall digestibility (Sarwar et al., 1999; Hopkins and Wilkins, 2006; Moorby et al., 2006). It is well known that forage legumes differ in drought stress sensitivity (Dierschke and Briemle, 2002). White clover (*Trifolium repens*) is one of the most important legumes in agricultural production, but is also relatively drought-sensitive (Marshall et al., 2001). We selected five promising and better drought-adapted legumes as possible alternatives to white clover (*T.*

repens) for future forage production, namely birdsfoot trefoil (*Lotus corniculatus*), marsh birdsfoot trefoil (*L. uliginosus*), black medic (*Medicago lupulina*), yellow alfalfa (*M. falcata*) and sainfoin (*Onobrychis viciifolia*). A possibly better adaptation to drought of these legumes is supposed to stabilize yields and also the nutritive value under conditions of stress. Forage legumes are commonly grown in mixtures with grasses (Hopkins and Wilkins, 2006; Hopkins and Del Prado, 2007). All six legume species were therefore cultivated in monoculture and in mixture with perennial ryegrass (*Lolium perenne*) to examine if legume-typical reactions to drought were also apparent in the mixtures. In the present experiment, we investigated the following hypotheses: (1) the six legumes differ in nutritive value; (2) drought stress can change nutritive value; and (3) legume species differ in their reaction to drought stress, in monoculture as well as in mixtures with grass.

2. Material and Methods

The study was conducted in 2009 (sowing date: 15th July) in a vegetation hall of the University of Göttingen, Germany, as a three-factorial experiment in a randomized complete blocks design with four replications. The factors were (1) legume species, (2) stand (legumes in monoculture or in mixture with *L. perenne*), and (3) drought stress (regular watering or water shortage). The legumes were birdsfoot trefoil (*Lotus corniculatus* L., var. Bull), marsh birdsfoot trefoil (*Lotus uliginosus* Schkuhr, wild seeds), black medic (*Medicago lupulina* L., var. Ekola), yellow alfalfa (*Medicago falcata* L., wild seeds), sainfoin (*Onobrychis viciifolia* Scop., var. Matra), and white clover (*Trifolium repens* L., var. Rivendel); perennial ryegrass (*Lolium perenne* L., var. Signum) was used as a companion grass in mixtures. Drought stress was imposed during three periods with varying severity, i.e. a moderate stress in spring 2010 and a strong stress in summer 2010 and spring 2011.

2.1 Experimental Setup

The growing substrate was a homogeneous mixture composed of 20 kg sand (air-dried, sieved to pass a mesh of 5 mm; August Oppermann Kiesgewinnung GmbH, Hann. Münden, Germany), 0.9 kg vermiculite (particle size 8-12 mm; Deutsche Vermiculite GmbH, Sprockhoevel, Germany), and 5.5 kg compost (air-dried; Bioenergiezentrum Göttingen GmbH, Göttingen, Germany) per container (round plastic containers, diameter 33 cm, height 42 cm, volume 30 l), covered with 1.5 kg compost as seed bed. The pH of the soil (in $CaCl_2$ suspension) as well as the availability of P, K (extracted with calcium acetate lactate, continuous flow analyser [CFA]) and Mg ($CaCl_2$ extraction, CFA) measured in summer 2011 were 7.3; 292 mg P kg^{-1}; 430 mg K kg^{-1}; 364 mg Mg kg^{-1} (oven-dry soil), respectively.

The six legumes and perennial ryegrass were sown in monoculture with 1000 germinating seeds per m^2 for legumes and 5000 for grass. For the mixtures of each legume with perennial ryegrass, we used 500 germinating seeds per m^2 for legumes and 2500 for grass. This considerably high sowing density was used in order to establish a dense sward immediately after seedling emergence.

We chose a vegetation hall as the conditions there followed a normal seasonal pattern with mild frost in winter and higher temperatures in summer, while drought stress could be fully controlled and recorded. Temperatures were recorded daily at three locations in the vegetation hall. The average temperatures ranged from 8°C (min.) to 27°C (max.) in spring regrowth 2010, from 15°C to 35°C in summer regrowth 2010 and from 9°C to 31°C in spring 2011. Climatic conditions were controlled by ventilation in summer and by a heating system in winter that was switched on when temperatures were below 0°C for longer than 24 hour. Heating in winter was limited to a maximum of 5°C air temperature in the vegetation hall. No extra lighting was provided and no fertilisation applied. In order to ensure nodulation of the legumes, all containers were treated with a rhizobium solution (Radicin, Jost GmbH, Iserlohn, Germany). The Radicin solution contained all rhizobia strains in same proportions for an effective infection of all legumes. Marsh birdsfoot trefoil did not survive the first winter and was therefore re-sown in March 2010. The aboveground biomass was harvested two times in 2009 (2th September and 25th October), five times in 2010 (12th April, 25th May, 5th July, 24th August and 18th October) as well as two times in 2011 (11th April and 30th May).

2.2 Drought Stress Treatment

A moderate stress was induced in spring 2010 (April/May) followed by two periods with strong drought stress in summer 2010 (July/August) and spring 2011 (April/May). In spring, drought stress periods were carried out after the first harvest of the year. After each drought stress period, plants were allowed to recover with regular irrigation and harvests of all containers at the end of the recovery periods. Drought stress was induced by temporarily ceasing the watering of the containers after an initial watering up to a volumetric soil water content of 25 vol. % (-0.03 MPa). For the moderate drought stress, no water was given until three days after the first plants showed signs of drought (e.g. wilt of leaves; ~10 vol. %, -1.5 MPa). Containers were then watered again (~25 vol. %) followed by a second cycle of drying up. In order to induce strong drought, the stress phase was extended to five days after first stress symptoms had appeared and was carried out three times with two waterings in between. The average water content of the containers (except marsh birdsfoot trefoil monoculture) ranged from 15 vol. % to 6 vol. % at the end of the moderate drought stress and from 10 vol. % to 4 vol. % under strong drought stress. All containers were weighed at intervals of one to three days during the stress periods. The control containers (no drought stress) were watered to ~25 vol. % if their water content was below ~18 vol. % (-0.3 MPa).

2.3 Sampling and Measurement

Aboveground biomass was determined by cutting the plants at a height of 3-4 cm above the soil surface. The cut herbage was separated into species immediately after harvest. Dry weight was determined after drying of the herbage samples at 60°C for 72 hours in a drying oven (ULM 800, Memmert GmbH und Co KG, Schwabach, Germany).

For analysis of CP, NDF, ADF and WSC, dried samples were ground to 1 mm and analysed by near-infrared reflectance spectroscopy (NIRS). The spectra were analysed using the large dataset of calibration samples from different kinds of grasslands by the Institute VDLUFA Qualitätssicherung NIRS GmbH, Kassel, Germany (Tillmann, 2010). Mixtures were separated into grasses and legumes for yield assessment, but the nutritive value was only analysed on the bulk sample.

2.4 Statistical Analysis of Data

Statistical data analysis was carried out using the Genstat 6.1 (VSN International, Hemel Hempstead, UK) software package. We did a three-factorial analysis of variance (ANOVA) for CP, NDF, ADF and WSC concentrations of all species in monoculture and in mixture with perennial ryegrass of the harvest following each stress period (Payne, 2002). The three factors were legume species (L), stand (S) and drought stress (DS). In case of significant treatment effects ($\alpha < 0.05$), least significant differences (LSD values) were used to compare mean values. Relationships between selected variables were examined with a linear regression model.

3. Results

The effect of the main factors legume species (L) and stand (S) as well as that of the interaction L X S on all parameters of nutritive value was in most cases significant ($P < 0.05$) in all three drought periods. Drought stress (DS) led to significant effects in spring 2011. Interactions between L X DS and S X DS, as well as the three-way interaction, were not significant, with the exception of some cases after strong drought stress; the pattern, however, was inconsistent. Generally, the effects of drought stress on the nutritive value were considerably smaller than on yield. It was only during the last strong drought stress period in spring 2011 that effects became apparent and statistically significant.

Table 1. Yield reduction (%) of legume species (plus *L. perenne*) in monoculture (Mono) and in mixture (Mix) with *L. perenne* under different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4).

	Yield reduction [%]					
Forage plant species	Spring 2010 moderate		Summer 2010 strong		Spring 2011 strong	
	Mono	Mix	Mono	Mix	Mono	Mix
Birdsfoot trefoil[1]	1	15	40	23	47	30
Marsh birdsfoot trefoil[2]	27	6[#]	29	17[#]	57	18[#]
Black medic[3]	13	4	5	19	52	18
Yellow alfalfa[4]	5	-2	28	21	42	19
Sainfoin[5]	1	18[#]	29	18[#]	49	33[#]
White clover[6]	36	18	36	44	56	53
Perennial ryegrass[7]	6		10		18	

Scientific names: [1] *Lotus corniculatus*; [2] *L. uliginosus*; [3] *Medicago lupulina*; [4] *M. falcata*; [5] *Onobrychis viciifolia*; [6] *Trifolium repens*; [7] *Lolium perenne*

[#] The legume partner did not produce any biomass in these periods.

3.1 Crude Protein Concentration

Crude protein concentrations in legume monocultures and mixtures were hardly affected by drought stress, but there was a tendency for reduced concentrations in monoculture under strong stress (Table 2). Among the legume species, particularly yellow alfalfa, but also white clover, black medic and birdsfoot trefoil had high CP concentrations in monocultures in all stress periods. For these legumes, CP values ranged from 225 g kg^{-1} DM for birdsfoot trefoil to 274 g kg^{-1} DM for yellow alfalfa with no drought stress, and from 212 g kg^{-1} DM for birdsfoot trefoil to 278 g kg^{-1} DM for yellow alfalfa under water shortage. In contrast, CP values for sainfoin and marsh birdsfoot trefoil in monoculture were usually rather small. Generally, the grass-legume mixtures had a smaller CP concentration than corresponding monocultures. Mixtures with white clover had highest CP concentrations, followed in most cases by the mixture containing black medic. When the drought stress treatment started in spring 2010, marsh birdsfoot trefoil and sainfoin had already been outcompeted by perennial ryegrass in the mixed sowings and did not produce any biomass.

3.2 Neutral Detergent Fibre Concentration

Neutral detergent fibre concentrations varied with the severity of drought stress. Moderate drought stress had no or a relatively small effect on NDF, while strong stress, particularly in spring 2011, decreased NDF concentrations (Table 3). When legumes were grown in monoculture, highest NDF values were found in yellow alfalfa in both control and stress treatment (439 g kg^{-1} DM; 425 g kg^{-1} DM), while for white clover NDF concentrations were always the lowest in both treatments (315 g kg^{-1} DM; 293 g kg^{-1} DM). Mixtures had considerably higher NDF concentrations than monocultures in both, control and stress treatments. Concentrations of NDF in perennial ryegrass were comparatively high. Grass-legume mixtures with yellow alfalfa, especially, had high NDF concentrations of up to 599 g kg^{-1} DM, while white clover mixtures always showed lowest concentrations ranging from 405 to 554 g kg^{-1} DM.

3.3 Acid Detergent Fibre Concentration

The ADF concentrations differed relatively little between control and drought treatments in monocultures and in mixtures, but decreased under strong stress, especially in spring 2011 (Table 4). Values for ADF concentrations in legume monocultures ranged from 242 g kg^{-1} DM for marsh birdsfoot trefoil to 328 g kg^{-1} DM for sainfoin with sufficient water supply (control), and from 236 g kg^{-1} DM for marsh birdsfoot trefoil to 304 g kg^{-1} DM for yellow alfalfa under drought stress. Grass-legume mixtures usually showed slightly higher ADF concentrations than the corresponding monoculture in both, control and stress treatments.

3.4 Water-soluble Carbohydrates Concentration

The influence of drought stress on WSC concentrations was generally small, but there was a trend to higher concentrations under strong drought (Table 5). Sainfoin and marsh birdsfoot trefoil monocultures had high concentrations of WSC of up to 129 g kg^{-1} DM, while the WSC concentrations of yellow alfalfa and birdsfoot trefoil were comparatively low, ranging from 15 g kg^{-1} DM to 59 g kg^{-1} DM. WSC concentrations were in most cases higher in mixtures than in the corresponding legume monoculture.

Table 2. Crude protein (CP) values of legume species (plus *L. perenne*) in monoculture and in mixture with *L. perenne* under different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4).

Forage plant species	CP [g kg^{-1} DM]											
	Spring 2010 moderate				Summer 2010 strong				Spring 2011 strong			
	Monoculture		Mixture		Monoculture		Mixture		Monoculture		Mixture	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Birdsfoot trefoil[1]	270	259	95	100	226	212	144	146	227	229	162	149
Marsh birdsfoot trefoil[2]	205	220	92[#]	91[#]	179	149	113[#]	115[#]	251	188	77[#]	81[#]
Black medic[3]	253	249	114	112	261	230	144	149	239	222	152	167
Yellow alfalfa[4]	274	278	96	93	248	239	122	128	261	252	156	141
Sainfoin[5]	135	132	93[#]	97[#]	186	203	113[#]	119[#]	162	158	81[#]	86[#]
White clover[6]	272	264	175	150	240	224	188	150	254	234	205	169
Perennial ryegrass[7]	93	90			113	115			87	79		
LSD value	20.4				28.9				17.6			
ANOVA Summary	*F*-ratio		*P*		*F*-ratio		*P*		*F*-ratio		*P*	
Legume (L)	91.79		< 0.001		22.24		< 0.001		133.09		< 0.001	
Stand (S)	1807.98		< 0.001		371.02		< 0.001		1190.42		< 0.001	
Drought Stress (DS)	0.62		0.432		3.92		0.052		24.55		< 0.001	
L x S	49.48		< 0.001		5.87		< 0.001		24.65		< 0.001	
L x DS	1.25		0.296		1.55		0.187		4.45		0.001	
S x DS	0.18		0.676		1.70		0.196		5.32		0.024	
L x S x DS	0.82		0.537		1.26		0.291		6.72		< 0.001	

Results from an Analysis of variance (ANOVA) considering the effects of legume, stand (monoculture or mixture) and drought stress. *L. perenne* was not included in the analysis.

Scientific names: [1] *Lotus corniculatus*; [2] *L. uliginosus*; [3] *Medicago lupulina*; [4] *M. falcata*; [5] *Onobrychis viciifolia*; [6] *Trifolium repens*; [7] *Lolium perenne*

[#] The legume partner did not produce any biomass in these periods.

Table 3. Neutral detergent fibre (NDF) values of legume species (plus *L. perenne*) in monoculture and in mixture with *L. perenne* under different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4).

	NDF [g kg^{-1} DM]											
Forage plant species	Spring 2010 moderate				Summer 2010 strong				Spring 2011 strong			
	Monoculture		Mixture		Monoculture		Mixture		Monoculture		Mixture	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Birdsfoot trefoil[1]	345	346	513	523	390	383	580	583	384	320	475	452
Marsh birdsfoot trefoil[2]	337	324	560#	530#	373	354	618#	606#	364	352	504#	504#
Black medic[3]	398	376	528	531	383	389	576	578	391	334	480	443
Yellow alfalfa[4]	378	379	535	530	439	425	598	599	395	362	477	483
Sainfoin[5]	358	361	522#	530#	354	333	631#	599#	414	351	510#	509#
White clover [6]	340	324	469	487	366	366	490	554	315	293	411	405
Perennial ryegrass[7]	520	527			574	590			478	468		
LSD value	31.6				35.0				26.0			
ANOVA Summary	*F*-ratio		*P*		*F*-ratio		*P*		*F*-ratio		*P*	
Legume (L)	12.01		< 0.001		13.46		< 0.001		46.47		< 0.001	
Stand (S)	1317.22		< 0.001		1634.38		< 0.001		927.73		< 0.001	
Drought Stress (DS)	0.58		0.449		0.24		0.627		47.69		< 0.001	
L x S	5.55		< 0.001		13.77		< 0.001		3.87		0.004	
L x DS	0.87		0.503		2.61		0.032		3.46		0.008	
S x DS	0.84		0.364		1.71		0.195		17.52		< 0.001	
L x S x DS	0.67		0.650		1.16		0.337		1.03		0.409	

Results from an Analysis of variance (ANOVA) considering the effects of legume, stand (monoculture or mixture) and drought stress. *L. perenne* was not included in the analysis.

Scientific names: [1] *Lotus corniculatus*; [2] *L. uliginosus*; [3] *Medicago lupulina*; [4] *M. falcata*; [5] *Onobrychis viciifolia*; [6] *Trifolium repens*; [7] *Lolium perenne*

$^{\#}$ The legume partner did not produce any biomass in these periods.

Table 4. Acid detergent fibre (ADF) values of legume species (plus *L. perenne*) in monoculture and in mixture with *L. perenne* under different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4).

Forage plant species	ADF [g kg^{-1} DM]											
	Spring 2010 moderate				Summer 2010 strong				Spring 2011 strong			
	Monoculture		Mixture		Monoculture		Mixture		Monoculture		Mixture	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Birdsfoot trefoil[1]	248	254	284	289	270	279	339	330	289	258	292	280
Marsh birdsfoot trefoil[2]	242	236	314[#]	285[#]	286	280	355[#]	337[#]	278	290	276[#]	269[#]
Black medic[3]	277	267	301	301	254	265	335	334	280	262	298	284
Yellow alfalfa[4]	261	264	298	293	302	304	343	340	286	272	288	291
Sainfoin[5]	294	300	285[#]	293[#]	296	279	362[#]	334[#]	328	292	282[#]	277[#]
White clover[6]	257	252	287	284	274	292	320	337	263	256	291	266
Perennial ryegrass[7]	288	286			328	331			263	251		
LSD value	18.9				21.2				18.8			
ANOVA Summary	*F*-ratio		*P*		*F*-ratio		*P*		*F*-ratio		*P*	
Legume (L)	9.36		< 0.001		6.31		< 0.001		6.46		< 0.001	
Stand (S)	120.93		< 0.001		348.43		< 0.001		1.63		0.206	
Drought Stress (DS)	0.98		0.326		0.35		0.554		22.90		< 0.001	
L x S	10.78		< 0.001		3.04		0.015		9.16		< 0.001	
L x DS	1.83		0.118		3.43		0.008		1.98		0.093	
S x DS	0.24		0.625		2.49		0.119		1.24		0.270	
L x S x DS	0.70		0.623		0.15		0.980		2.32		0.052	

Results from an Analysis of variance (ANOVA) considering the effects of legume, stand (monoculture or mixture) and drought stress. *L. perenne* was not included in the analysis.

Scientific names: [1] *Lotus corniculatus*; [2] *L. uliginosus*; [3] *Medicago lupulina*; [4] *M. falcata*; [5] *Onobrychis viciifolia*; [6] *Trifolium repens*; [7] *Lolium perenne*

[#] The legume partner did not produce any biomass in these periods.

Table 5. Water-soluble carbohydrates (WSC) values of legume species (plus *L. perenne*) in monoculture and in mixture with *L. perenne* under different levels of drought stress in spring 2010 (moderate stress), summer 2010 (strong stress) and spring 2011 (strong stress); means (n = 4).

Forage plant species	WSC [g kg^{-1} DM]											
	Spring 2010 moderate				Summer 2010 strong				Spring 2011 strong			
	Monoculture		Mixture		Monoculture		Mixture		Monoculture		Mixture	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Birdsfoot trefoil[1]	30	40	222	207	47	59	66	71	25	51	110	129
Marsh birdsfoot trefoil[2]	113	109	198[#]	227[#]	103	129	87[#]	100[#]	53	95	240[#]	242[#]
Black medic[3]	56	68	178	179	63	78	85	75	55	78	133	126
Yellow alfalfa[4]	37	33	195	211	20	26	90	85	15	25	135	147
Sainfoin[5]	129	121	224[#]	210[#]	86	92	78[#]	97[#]	74	101	230[#]	229[#]
White clover [6]	64	74	132	162	71	72	69	72	67	83	87	142
Perennial ryegrass[7]	218	217			109	123			235	260		
LSD value	22.9				24.2				27.2			
ANOVA Summary	*F*-ratio		*P*		*F*-ratio		*P*		*F*-ratio		*P*	
Legume (L)	40.29		< 0.001		18.01		< 0.001		58.55		< 0.001	
Stand (S)	1365.26		< 0.001		9.51		0.003		677.44		< 0.001	
Drought Stress (DS)	2.52		0.117		4.56		0.036		22.35		< 0.001	
L x S	26.58		< 0.001		11.74		< 0.001		25.75		< 0.001	
L x DS	1.90		0.106		0.78		0.567		1.08		0.377	
S x DS	0.62		0.434		0.95		0.332		1.96		0.166	
L x S x DS	1.99		0.091		0.55		0.734		2.31		0.053	

Results from an Analysis of variance (ANOVA) considering the effects of legume, stand (monoculture or mixture) and drought stress. *L. perenne* was not included in the analysis.

Scientific names: [1] *Lotus corniculatus*; [2] *L. uliginosus*; [3] *Medicago lupulina*; [4] *M. falcata*; [5] *Onobrychis viciifolia*; [6] *Trifolium repens*; [7] *Lolium perenne*

[#] The legume partner did not produce any biomass in these periods.

4. Discussion

Temporary drought influenced biomass yield depending on strength and duration of stress. Moderate and strong drought stress reduced yields up to 36 % and 57 %, respectively (Table 1). Moderate stress had no effect on nutritive value, while strong stress had a significant effect (spring 2011). Our hypothesis (2) that drought stress can change the nutritive value of legumes could thus be rejected for moderate stress while for strong stress it could not. The six forage legumes in our study differed in their nutritive value under conditions of sufficient water supply, which confirmed our first hypothesis. Also Peterson et al. (1992) and Fulkerson et al. (2007) found differences in nutritive value among legume species.

Interactions between legume species (L) and drought stress (DS) as well as between stand (S) (monoculture or mixture) and DS were not significant under moderate stress. There were significant interactions L X DS, and S X DS under strong drought stress, but they were usually weak and inconsistent among the different parameters. Therefore, our hypothesis (3) that legume species react differently to drought needs to be rejected for moderate stress, and can only partially be confirmed for strong stress.

Irrespective of the water supply treatment, the legumes showed a nutritive value comparable to values found in the literature (Peterson et al., 1992; Fulkerson et al., 2007); they would be ranked as having a moderate to high quality (Buxton, 1996; Schwarz, 2008).

4.1 Crude Protein Concentration

CP concentrations differed among the legume species. Strong drought stress showed a tendency to decrease CP concentrations in monoculture. The CP concentration of legumes is generally depending on the amount of available N. For legumes, especially under N limited conditions as was the case in our experiment, N fixation is very important for N nutrition (Zahran, 1999; Watson et al., 2002), and it differs among legume species. We used the difference method (Gierus et al., 2012) to investigate the N fixation performance (g N per container). According to the results, legumes could be divided in two groups. The group containing yellow alfalfa, white clover, black medic and birdsfoot trefoil had 10 to 30% higher Ndfa (mean N derived from atmosphere in %) than marsh birdsfoot trefoil and sainfoin, also under drought stress. This resulted in an at least 40% higher N fixation performance (g N per container) in the high fixing group and led to differences in CP concentration among the legumes. Marked differences in CP concentration among a variety of temperate clover species were also found by Ates (2011).

Nitrogen fixation determines the availability of N, but the N concentration in the plant is also depending on the amount of biomass production. A specific CP concentration is then the result of N uptake and the development of biomass production in time which is greatly determined by water availability. Nakayama et al. (2007) found an impaired N uptake in soybean under drought and Pimratch et al. (2013) measured a decreased N fixation under drought stress in peanuts (*Arachis hypogaea* L.). In our study, strong drought stress resulted in a reduction of N fixation performance that was on average 15% larger than the decrease in yield. This explains why the CP concentration in the stress treatments was smaller than the corresponding concentrations in the control treatment.

As CP concentrations were considerably higher in legumes than in perennial ryegrass, CP concentrations of mixtures strongly depended on the yield contribution of the legume component of mixture (R^2 up to 0.95; $P < 0.001$). When the legume partner was no longer present, as was the case with marsh birdsfoot trefoil and sainfoin, mixtures produced similar CP concentrations as perennial ryegrass in monoculture (Table 2). It seemed that under strong drought stress, the competitive ability of the legume against the grass partner decreased. This effect was enhanced by the fact that perennial ryegrass was much less affected by drought. Particularly white clover suffered from drought stress (decrease in yield contribution in mixture of up to 73%) and lost strongly in competitive ability against perennial ryegrass. Therefore, total mixture yield decreased and CP concentration in the mixture was reduced as well.

4.2 Neutral Detergent Fibre and Acid Detergent Fibre Concentrations

We observed a tendency for lower NDF and, to a lesser extent, ADF concentrations under strong drought stress. Legume species generally differed in fibre concentration.

Fibre concentration is generally influenced by many interacting factors among which are the stage of plant development, leaf-stem ratio, environmental conditions (drought, temperature, photoperiod etc.) or availability of nutrients (Peterson et al., 1992; Buxton, 1996; Fulkerson et al., 2007).

The reduction of NDF and ADF concentration under strong stress supports the findings of Peterson et al. (1992) and Buxton (1996) that a delayed maturity under drought is associated with lower NDF and ADF concentrations.

Drought effects on NDF (including cellulose, hemicellulose and lignin) were stronger than for ADF (including cellulose and lignin). This might be explained by the fact that the hemicellulose concentration, as a part of NDF, is more affected by drought than cellulose and lignin. However, results on the effects of drought on hemicellulose concentrations are inconsistent in the literature: some authors have reported decreased hemicellulose concentrations under drought (Jiang et al., 2012), while other reported increases (Al-Hakimi, 2006).

The cell walls of monocots and dicots differ in their composition. The lignification of cell walls in dicots is stronger, but the concentration of hemicellulose is smaller (Buxton and Mertens, 1995; Ebringerová et al., 2005) resulting in higher NDF of grasses and grass-legume mixtures than that of legumes (Buxton, 1996; Table 4). Additionally, the ADF concentration in most legumes is approximately 100 g kg^{-1} lower than that of NDF, while this difference is usually about 200 g kg^{-1} for most grasses (Buxton, 1996). Similar results were found in our experiment (Tables 3 and 4). A lower fibre concentration may lead to a higher herbage intake and to an increase in digestibility of forage (Buxton, 1996).

4.3 Water-soluble Carbohydrates Concentration

Under strong drought stress in summer 2010 and spring 2011 WSC concentrations mostly increased. Positive effects of drought stress on WSC have been reported elsewhere (DaCosta and Huang, 2006; Nakayama et al., 2007). An increase in the WSC concentration in plants will change the osmotic potential, which maintains the uptake of soil water under drought stress conditions (Morgan, 1984; Nakayama et al., 2007). This osmotic adjustment is a physiological mechanism in response to drought (DaCosta and Huang, 2006). Apart from stress, the WSC concentrations varied among legume species and differed between legume monoculture and legume-grass mixture (Table 5). WSC concentrations were highest in marsh birdsfoot trefoil and sainfoin. It seems that in legume monocultures higher WSC concentrations are related to lower CP concentrations (Sanada et al., 2007). Water-soluble carbohydrate concentrations in perennial ryegrass are usually higher than in legumes (Ulyatt et al., 1988; Dewhurst et al., 2003; Marshall et al., 2004). There were significant ($P < 0.05$) negative linear correlations between legume contents in the mixtures and WSC concentrations of the mixture in all three periods and in both, control and stress treatments.

Digestibility of legumes may even increase under strong drought stress due to a tendency to higher WSC and lower fibre concentrations. Moreover, higher WSC concentrations, associated with a lower ratio of CP to WSC, which is an indicator for N utilisation, could enhance N use and reduce N excretion in urine of ruminants (Moorby et al., 2006).

5. Conclusion

The effect of drought stress on the nutritive value of six different legume species was considerably less pronounced than the influence on yield. The impact of drought was more visible under strong drought stress than under moderate stress. Strong drought led to increased WSC concentrations and decreased fibre concentrations which may increase the digestibility of the herbage. Also the ratio of CP to WSC, an indicator for N utilisation, was smaller under drought and which could thus enhance the ruminal N retention and decrease the N surplus in ruminates. However, in most cases legume species and stand (monoculture or mixture) influenced quality parameters stronger than drought stress. We conclude that the effect of temporary drought on the nutritive value of legumes seems to be less important than other properties for the selection of suitable forage legumes for agronomic productions under conditions of predicted climate change.

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General Discussion

Under prognosticated climate change, water is likely to become more limiting during the vegetation period in the next decades, (Alcamo et al., 2007; Schindler et al., 2007; Trenberth, 2011). *Trifolium repens* as one of the most important forage legumes in temperate grassland (Frame et al., 1998; Gierus et al., 2012) is sensitive to water shortage and responds with strongly decreasing yields (Marshall et al., 2001) and may also loose nutritive value. In this context, the study was conducted to provide information about the agronomic potential of a range of five promising and maybe better drought adapted forage legumes for temperate grassland as possible alternatives to *T. repens*. The results of this study should give answers to the following questions: Do possible alternative legumes have similar establishment, early yield development and competitive ability against fast growing grass (*Lolium perenne*) compared to *T. repens* under sufficient water supply? Do possible alternative legumes and their mixtures with *L. perenne* have higher and more stable yields as well as better water utilisation under temporary drought than *T. repens*? Does temporary drought have an effect on the nutritive value of the investigated legume monocultures and mixtures?

The development and yield of the investigated legumes in the first main production year generally relies on a good establishment including germination along with a sufficient winter tolerance. These support the findings of Finch-Savage (1995) and Brouwer et al. (2000). Germination and establishment of alternative legumes were comparable to *T. repens* except for *M. falcata* with a very poor germination rate associated with a retarded initial development. All legumes in monoculture, apart from *L. uliginosus*, survived the winter period well. *Trifolium repens* and *M. lupulina* but also *L. corniculatus* showed a good establishment as well as winter tolerance and produced the largest accumulated yields in monocultures in the first main production year. However, the yield stability for *M. lupulina* and *L. corniculatus* was not sufficient. Despite a good establishment and winter tolerance, *O. viciifolia* had a small accumulated yield in monoculture, maybe due to the low cutting height (3–4 cm) and the high cutting frequency (five times in the main harvest year) (Slepetys, 2008). The yield of mixtures strongly depended on the yield contribution of the legume partner. Thus, a good competitive ability of legumes against fast-growing grasses like *L. perenne* (Petersen, 1967) is essential. *Trifolium repens* was the most productive legume in mixture under unlimited water supply with a yield proportion of nearly 60% of the total yield; this illustrates the strong competitive ability of *T. repens* (Petersen, 1967). *Medicago lupulina* and less so *L. corniculatus* showed some potential in mixture, with a yield proportion of 25% and 16%. Mixtures with small (*M. falcata*) or no (*L. uliginosus* and *O. viciifolia*) legume yield contribution produced lowest accumulated yields.

Drought stress can have strong effects on the agricultural performance of forage legumes. The impact is strongly depending on the strength and duration of drought (Farooq et al., 2009). In this study, moderate drought reduced the yield of *T. repens* by 36% while strong stress decreased yield by up to 56%. Yield reductions for *M. lupulina*, *L. corniculatus* and *M. falcata* were mostly smaller particularly under moderate but also under strong stress. Smaller yield reductions under drought associated with sometimes slightly higher yields for alternative legumes like *M. lupulina* compared with *T. repens* were also found by Foulds (1978). Agronomic water use efficiency (relation of yield and water use) as an important factor for dealing with limited water resource (Gregory et al., 2000; Wallace, 2000) was quite stable under moderate stress but mostly lower under strong stress. Compared with *T. repens*, especially *M. lupulina* displayed similar or even higher agronomic WUE in monocultures under not water limited conditions and drought. Furthermore, for *M. lupulina*, *L. corniculatus* and *M. falcata* mixtures we observed smaller decreases in agronomic WUE under strong drought and thus more stable agronomic WUE. Differences between legumes in their reaction and tolerance to water shortage are based on several factors (Kemp 1984). We found that changes in N fixation explained changes in yield and agronomic water use efficiency well. A good supply with N, in our case N fixation, mostly increased yield and agronomic WUE with and without drought stress. These support the findings of Ehlers and Goss (2003) and Brueck (2008). Nitrogen fixation of the investigated legumes particularly decreased under strong stress but mostly to a larger extent for *T. repens* compared to alternative legumes. Intrinsic WUE (ratio of assimilated CO2 and stomatal conductance), measured as $\delta^{13}C$, was a poorer indicator for agronomic WUE: although intrinsic WUE increased under strong drought, agronomic WUE was mostly decreased. Nevertheless, a larger increase of intrinsic WUE in combination with decreased water use rapidity under drought could be a hint for drought adaptation. In this study, *L. corniculatus*, *M. falcata* and *M. lupulina* increased intrinsic WUE more and used water more slowly than less drought adapted *T. repens* under drought stress. In addition, there are some anatomical adjustments (e.g. waxy coatings on both sides of leaves, hairs on the leaves) to drier conditions that will reduce stomatal and cuticular transpirations of *M. lupulina*, *L. corniculatus* and *M. falcate* (Winstel & Rentschler 1975; Schreiber & Riederer, 1996; Lösch, 2003; Klapp & Opitz von Boberfeld, 2004).

The influence of drought stress on fodder quality was generally considerably lower than the effects on yield. The impact of moderate stress on quality was relatively small. Nevertheless, stronger stress (summer 2010 and spring 2011) increased the impact on fodder quality with more visible effects. By trend, we mostly found decreases in crude protein, neutral detergent fibre and to less extend acid detergent fibre and increases for water-soluble carbohydrates under strong drought. This is in line with Peterson et al., (1992) and Nakayama et al., (2007). Due to the tendency towards raised water-soluble carbohydrate concentration and the deceased fibre components under strong drought stress, water scarcity may even increase quality and digestibility (Peterson et al, 1992; Miller et al. 2001). Also the proportion of crude protein to water-soluble carbohydrates, an indicator for N utilisation, lowered under drought and could, thus, enhance N use and decrease N surplus in ruminates. Interactions between drought stress and legume as well as drought stress and mixture were comparatively weakly pronounced. In general, there were no substantial differentiations between legume species regarding changes in fodder quality under drought stress. However, legume species and stand (monoculture or mixture) influenced nutritive values more strongly than drought. Therefore, the reaction of temporary drought on nutritive value is less important compared to other agronomic properties for selection of suitable forage legumes.

In conclusion, particularly *M. lupulina* and, to less extent, *L. corniculatus* and *M. falcata* showed potential as alternative to *T. repens* also under drought stress. Given some time for establishment, *M. lupulina,* but also *L. corniculatus* and *M. falcata,* developed more stable and produced even larger yields than *T. repens* under drought stress. Also regarding fodder quality, the above named alternative legumes were comparable to *T. repens*. Nevertheless, an intensive breeding for alternative legumes, as has been the case for *T. repens* during the last decades (Abberton and Marshall 2005), might help to enhance not only the yield potential in general but also the competitive ability of these legumes in mixtures with fast growing and N sensitive grasses. Our experiment provides some valuable information on drought stress tolerance and agronomic features of some legumes as possible alternatives to *T. repens*.

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Summary

Grassland with a high productivity and fodder quality forms the basis for ruminant nutrition. In grassland swards with no or little input of nitrogen (N) from mineral fertiliser or manure, legumes are essential for productivity and fodder quality. This is mainly due to their ability to fix atmospheric N. *Trifolium repens* L. is currently the most important legume in European temperate grasslands. However, *T. repens* has been shown to need a good supply of water for growth. This may become challenging in times of climate change, as summer rainfall is predicted to become sparse. Other fodder legumes may be better adapted to drier conditions and may, therefore, have an increasing potential in future fodder production. However, knowledge on the agronomic potential of such alternative legume species especially under drought is limited. In this study, we investigate a range of five promising and maybe better drought adapted forage legumes for temperate grassland as possible alternative to *T. repens*. We chose *Lotus corniculatus* L., *L. uliginosus* Schkuhr, *Medicago lupulina* L., *M. falcate* L. and *Onobrychis viciifolia* Scop.. First, we examined the agronomic potential in establishment and early yield development under sufficient water supply. Furthermore, we studied yield and yield stability as well as water utilisation of alternative legumes under temporary drought and compared their performance with that of *T. repens*. Besides this, we examined the effects of drought stress on important nutritive values (crude protein, neutral detergent fibre, acid detergent fibre and water-soluble carbohydrates) of all investigate legumes.

A container experiment was conducted in a vegetation hall from 2009 (sowing year) to 2011. All legumes were sown in monoculture as well as in mixture with *Lolium perenne* L., which is used more frequently in common practice. The climate conditions followed a normal seasonal pattern with frost in winter and higher temperatures in summer. Drought conditions were imposed on three periods during two years by temporary ceasing the watering of the containers. A moderate stress phase was set up in spring 2010 (April/May) followed by two periods of strong drought stress in summer 2010 (July/August) and spring 2011 (April/May).

In our experiment, germination and establishment of all alternative legumes are comparable to *T. repens* except of *M. falcata* with a retarded initial development. In monoculture, *M. lupulina* and *L. corniculatus* show a yield potential almost as high as of *T. repens*. However, their performance in mixture with *L. perenne* showed some potential, but was smaller than that of *T. repens*.

Our data show that drought stress decreased yield and influenced agronomic water use efficiency (relation of yield and water use). Changes in yield and agronomic water use efficiency under drought stress depended on the strength and duration of the stress. Strong and even moderate drought stress led to a substantial decrease in yield up to 56% for *T. repens*. Alternative legumes like *M. lupulina* but also *L. corniculatus* and *M. falcata* displayed only little reductions under moderate drought and mostly lower decrease than *T. repens* under strong drought. Agronomic water use efficiency was quite stable under moderate stress but mostly lower under strong stress. *M. lupulina* in particular displayed a similar or even higher agronomic water use efficiency than *T. repens* in monocultures under control and stress conditions. Furthermore, we observed smaller decreases in agronomic water use efficiency for *M. lupulina, L. corniculatus* and *M. falcata* mixtures under strong drought. This confirms the drought sensitivity of *T. repens* and makes other legumes obviously better suited to drought stress. We found that changes in N fixation explained changes in yield and agronomic water use efficiency well. High N fixation performance mostly led to larger yield and water use efficiencies. Intrinsic water use efficiency (ratio of assimilated CO2 and stomatal conductance), measured as $\delta^{13}C$, was a poorer indicator for agronomic water use efficiency: although intrinsic water use efficiency increased under strong drought, agronomic water use efficiency mostly decreased. Still, the increase of intrinsic water use efficiency implies some potential to drought adaptation.

In our study, the influence of drought stress on fodder quality was considerably lower than effects on yield. Particularly moderate drought showed relatively low effects on nutritive value, while stronger stress increased the impact on fodder quality with more visible effects. Under strong stress, we mostly found decreases in crude protein, neutral detergent fibre and acid detergent fibre and increases for water-soluble carbohydrates. This may indicate that water scarcity could even increase fodder quality and digestibility. However, legume species and stand (monoculture or mixture) mostly influenced nutritive values stronger than drought. Therefore, the reaction of temporary drought on nutritive value is less important compared to other agronomic properties for the selection of suitable forage legumes.

In conclusion, especially *M. lupulina* and, to less extent, *L. corniculatus* and *M. falcata* showed potential as alternatives to *T. repens* also under drought stress. Given some time for establishment, *M. lupulina*, but also *L. corniculatus* and *M. falcata,* developed more stable and showed even larger yields than *T. repens* under drought stress. Also regarding fodder quality, the above named alternative legumes were comparable to *T. repens*.

Zusammenfassung

Grünland mit hoher Produktivität und Futterqualität bildet die Grundlage der Wiederkäuerernährung. In Grünlandbeständen mit ausbleibender oder geringer Stickstoffdüngung sind Leguminosen unerlässlich für Produktivität und Futterqualität, was auf die Fähigkeit von Leguminosen Luftstickstoff zu binden zurückzuführen ist. Gegenwärtig ist *Trifolium repens* L. eine der wichtigsten Futterleguminosen im Grünland der gemäßigten Zonen Europas. Es ist allerdings bekannt, dass *T. repens* eine gute Wasserversorgung benötigt, um einen hohen Ertrag zu erzielen. Verringerte Niederschlagsmengen in der Vegetationsperiode, die unter Klimawandelbedingungen vorausgesagt werden, könnten somit die Ertragsleistung von *T. repens* verringern. In Zukunft steigt dadurch möglicherweise auch die Bedeutung anderer Futterleguminosen, die besser an trockenere Bedingungen angepasst sind und somit als Alternative für *T. repens* dienen könnten. Da die Kenntnisse über das agronomische Potenzial solcher möglichen alternativen Leguminosen begrenzt sind, haben wir in dieser Studie fünf vielversprechende und wahrscheinlich besser an Trockenheit angepasste Leguminosen untersucht. Für unsere Versuche haben wir *Lotus corniculatus* L., *L. uliginosus* Schkuhr, *Medicago lupulina* L., *M. falcate* L. und *Onobrychis viciifolia* Scop. ausgewählt. In einem ersten Schritt wurde das agronomische Potenzial der Leguminosen im Hinblick auf Etablierung und frühe Ertragsentwicklung mit nicht limitierter Wasserversorgung getestet. Weiterhin wurden der Ertrag und die Ertragsstabilität sowie die Wassernutzung der alternativen Leguminosen bei temporärer Trockenheit untersucht und mit der von *T. repens* verglichen. Der Einfluss von Trockenstress auf wichtige Futterwert bestimmende Inhaltsstoffe der Leguminosen (Rohprotein, neutrale Detergenzienfasern, saure Detergenzienfasen und wasserlösliche Kohlenhydrate) war überdies Gegenstand der Betrachtungen.

Um die oben genannten Parameter zu untersuchen, wurde von 2009 (Einsaatjahr) bis 2011 ein Experiment in Großgefäßen in einer Vegetationshalle durchgeführt. In diesem Versuch wurden alle Leguminosen sowohl in Monokultur als auch in einer praxisüblichen Mischung mit *Lolium perenne* L. angesät. Im Versuchszeitraum folgten die klimatischen Bedingungen in der Vegetationshalle einem normalen jahreszeitlichen Verlauf, der Frost im Winter und höhere Temperaturen im Sommer umfasste. Der für den Versuch notwendige Trockenstress wurde in drei Aufwüchsen innerhalb von zwei Jahren durch temporären Bewässerungsstopp erzeugt. Dabei wurde im Frühjahr 2010 (April/Mai) ein moderater und im Sommer 2010 (Juli/August) sowie im Frühjahr 2011 (April/Mai) je ein starker Trockenstress induziert.

Mit Ausnahme von *M. falcata*, welches eine verzögerte Anfangsentwicklung zeigte, waren die Keimung und die Etablierung von allen alternativen Leguminosen vergleichbar mit der von *T. repens*. Die Ertragsleistung von *M. lupulina* und *L. corniculatus* in Monokultur war ähnlich hoch wie die von *T. repens*. In Mischung zeigten beide alternativen Leguminosen zwar Potenzial, aber die Ertragsleistungen waren dennoch geringer als die der *T. repens*/*L. perenne* Mischung.

In unserem Versuch führte Trockenstress zu verringertem Ertrag und er beeinflusste auch die agronomische Wassernutzungseffizienz (Verhältnis von Ertrag zu Wasserverbrauch). Ausschlaggebend waren dabei die Stärke und die Dauer des Trockenstresses. Besonders starker, aber auch bereits moderater Trockenstress führten bei *T. repens* zu erheblichen Ertragsverlusten von bis zu 56%. Demgegenüber zeigten vor allem *M. lupulina*, aber auch *L. corniculatus* und *M. falcata* lediglich marginale Ertragsverluste bei moderatem Trockenstress und meist geringere Ertragsverluste als *T. repens* bei starkem Stress. Die agronomische Wassernutzungseffizienz war bei moderatem Stress verhältnismäßig stabil, wohingegen starker Stress im Vergleich zur Kontrolle meist zu einer geringeren agronomischen Wassernutzungseffizienz führte. Sowohl unter Kontroll- als auch unter Stressbedingungen zeigte speziell *M. lupulina* in Monokultur eine ähnliche oder sogar eine höhere agronomische Wassernutzugseffizienz als *T. repens*. Zudem war die agronomische Wassernutzungseffizienz der Mischungen mit *M. lupulina*, *L. corniculatus* und *M. falcata* weniger negativ von starkem Trockenstress betroffen als die Mischung mit *T. repens*. Dies bestätigte zum einen die Trockenheitsempfindlichkeit von *T. repens* und zum anderen die bessere Anpassung der alternativen Leguminosen an trockenere Bedingungen. Wir beobachteten, dass eine Änderung in der Stickstofffixierungsleistung der Leguminosen eine gute Erklärungsgröße für Änderungen des Ertrags und der agronomischen Wassernutzungseffizienz darstellt. Hohe Stickstofffixierungsleistung geht dabei meist mit höherem Ertrag und höherer agronomischer Wassernutzungseffizienz einher. Die intrinsische Wassernutzungseffizienz (Verhältnis von assimiliertem CO_2 und stomatärer Leitfähigkeit), gemessen als $\delta^{13}C$, war ein schlechterer Indikator für die agronomische Wassernutzungseffizienz: Obwohl die intrinsische Wassernutzungseffizienz unter starkem Trockenstress zunahm, sank die agronomische Wassernutzungseffizienz meist ab. Dennoch besitzt die Erhöhung der intrinsischen Wassernutzungseffizienz ein gewisses Potenzial als Anpassung an trockenere Bedingungen

Der Trockenstresseffekt auf die Futterqualität war in unserer Studie generell deutlich geringer als der Effekt auf den Ertrag. Besonders moderater Stress hatte wenig Einfluss auf die Futterqualität, während sich die Effekte bei starkem Stress verstärkten. Starker Trockenstress führte meist zu einer Verringerung des Rohprotein- und Fasergehalts (neutrale und saure Detergenzienfasern), wohingegen sich der Gehalt an wasserlöslichen Kohlenhydraten erhöhte. Dies könnte ein Hinweis darauf sein, dass sich die Futterqualität bei Trockenstress sogar verbessert. Nichtsdestotrotz hatten in unserem Versuch die Leguminosenart und die Einsaat als Monokultur oder Mischung einen größeren Einfluss auf die Futterqualität als der Trockenstress. Der Einfluss von Trockenstress auf die Futterqualität ist deshalb bei der Wahl einer geeigneten Futterleguminose weniger von Bedeutung als andere agronomische Eigenschaften.

Zusammenfassend ist zu sagen, dass besonders *M. lupulina* und in geringerem Maße auch *L. corniculatus* und *M. falcata* Potenzial als Alternative für *T. repens* bei Trockenstress zeigen. Nach ausreichender Etablierungszeit entwickeln sich besonders *M. lupulina* aber auch *L. corniculatus* und *M. falcata* stabiler und können sogar höhere Erträge als *T. repens* bei Trockenstress produzieren. Bezüglich der Futterqualität sind oben genannte alternative Leguminosen ebenfalls vergleichbar mit *T. repens*.

Curriculum Vitae

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1997	A-Levels (Abitur) at Johann-Gottfried Seume Gymnasium Vacha

Teaching Experience

Advised Thesis:	4 B.Sc.-Thesis and 3 M.Sc.-Thesis
2006-2008	Teacher at Theo-Koch comprehensive school Grünberg

Country Experience

09/2012	Field trip trough Kyrgyzstan; Course in pasture and water

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08/2011	Field trip trough Siberia, Altai region; Course in Economic and Social Geography of Altai State University
04/2008-06/2008	Research project for Arpolith GmbH at Kingdom of Saudi-Arabia, Riyadh, Ministry of Agriculture, Department of Research and Development, National Agriculture and Animal Resources Research Centre
07/2006-08/2006	Field trip trough European Russia; Course in Soil Science, Geography and Agriculture University of Bayreuth

Language Skills

German	Native speaker
English	Fluent spoken and written
Russian	Basic knowledge

Further Qualifications

Relevant Courses:	Treatment of archaeological objects (in German); Dendrochronology (in German); Measurements of biodiversity (in German); Statistics and STATISTICA; Scientific writing
Hobbies:	Rennsteig cross run (13 participations); Fulda 84km March (3 participations); travelling; history and culture

Publications

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