Effect of white clover and perennial ryegrass genotype on yield and forage quality of grass-clover and grass-clover-forb mixtures

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Abbreviations, acronyms and symbols

asl	Above sea level
CaCl	Calcium chloride
cm	Centimeter
CV	Coefficient of variation
FAO	Food and Agriculture Organization
g	Gram
$IMPAC^3$	Novel genotypes for mixed cropping allow for improved sustainable land use across arable land, grassland and woodland
LSD	Least Significant Difference
mg kg^{-1}	Milligram per kilogram
m	Meter
m^2	Square meter
N	Nitrogen
ns	Not significant
t dm ha^{-1}	Ton dry matter per hectare
*	Significant at α=0.05
**	Significant at α=0.01
***	Significant at α=0.001
°C	Degrees Celsius
°N	Degrees north latitude
°E	Degrees east latitude

Chapter I: General introduction

1.1 Forage species mixture in grassland farming

Mixed cropping is an ancient practice involving two or more plant species or genotypes in proximity and coexisting for a time (Annicchiarico et al. 1994; Lüscher et al. 2014; Temperton et al. 2007). Before the 1940s, mixed cropping was commonly practiced in Europe and the United States (Machado 2009). It has declined drastically in developed countries due to the mechanization and the availability of synthetic fertilizer, which makes mono cropping an easy and efficient way to go (Horwith 1985; Machado 2009). In developing countries, where farmers have limited access to the mechanization and fertilizers mixed cropping is still widely used (Lithourgidis et al. 2011; Machado 2009).

According to the ecological literature, species richness increases both ecological services and ecological functioning (Loreau et al. 2001; Tilman 1999). In this context, mixed cropping is not an exception and it has shown many advantages over mono crops such as improving resource utilization, increasing the forage yield and yield stability, increasing forage nutritive value and decreasing pests and diseases (Annicchiarico et al. 1994; Brooker et al. 2015; Cardinale et al. 2007; Lüscher et al. 2014).

In the 21st century, grassland ecosystems are facing multiple challenges such as increasing world genotype and climate change (Rojas-Downing et al. 2017). A major challenge is to increase grassland productivity while reducing negative environmental impacts. Sustainable intensification is one way to tackle these problems (Tilman et al. 2011). The goal of sustainable intensification is to increase crop production from existing resources while minimizing the environmental impact of agriculture. In this context, legumes play an important role due to their ability to fix the atmospheric nitrogen (N) and increase the soil N pool, which may reduce the mixtures reliance on synthetic nitrogen fertilization (Nyfeler et al. 2011). Findings of Brophy et al. (2017) from a continental-scale experiment support the positive effect of including legumes in multi-species mixtures over the long-standing grass monoculture.

In temperate regions, binary mixtures of grass and clover species have shown to produce high dry matter yield and increased yield stability compared to grass monocultures (Annicchiarico et al. 1994; Ergon et al. 2016; Franco et al. 2015; Sleugh et al. 2000; Temperton et al. 2007). Complementarity and facilitation among component species in a mixture are the main mechanisms explaining the advantages of mixtures over monocultures (Duchene et al. 2017). Complementarity occurs when plant species utilize different resources, the same resources at different times or the same resources from different spaces (Hooper 1998; Duchene et al. 2017). Complementarity enables mixtures to exploit available resources more efficiently than corresponding monocultures (Duchene et al. 2017; Hoekstra et al, 2014; Hooper 1998). In ecology, if niches of two species are similar, the two species cannot coexist in the same community for a long time due to high interspecific competition for resources (Vandermeer 1992). If their niches are different, however, the species can coexist in a community because of complementary use of resources (Cardinale et al. 2011). Diverse plant communities containing species with a different shoot and root architecture can occupy a larger niche space and thus can acquire more unexploited soil resources compared to communities containing species with similar root distributions (Hooper 1998). Hoekstra et al. (2014) demonstrated that the inclusion of the deep-rooted species *Cichorium intybus* in grass-clover mixture increased the biomass production compared to grass-legume mixtures, especially under drought conditions. They showed that the inclusion of *C. intybus* in grass-legume mixtures improves vertical complementarity and increases the mixtures yield, since *C. intybus* has the potential for distinct vertical N capture compared to *Lolium perenne* with its shallow roots (Hoekstra et al. 2014). Species with different shoot architectures (tall-erect and short-prostrate) in a mixture may use light more efficiently than an individual species in a monoculture by partitioning the light among species (Husse et al. 2016; Hooper 1998). Phenological differences among species also improve the complementarity in mixtures, because they take up resources at different times (Hooper 1998). Where resource availability is limited, mixtures can be more productive because they utilize the available resources more efficiently (Hooper 1998). Facilitation; a positive interaction among component species which plants enhance the environment of their neighbors, is another reason for the over-yielding of mixtures (Lüscher et al. 2014; Newton et al. 2009). An outstanding example of facilitation regarding mixed cropping is the facilitative interaction between legumes and non-legumes where non-legumes benefit from the nitrogen fixed by legumes (Dhamala et al. 2017; Temperton et al. 2007).

Species belonging to different functional groups (e.g., legume and non-legume) are more likely to feature complementarity in mixtures (Hooper 1998). Grass-legume mixtures have been widely used in grassland farming and it has been shown that these more diverse mixtures increase productivity (Sanderson et al. 2005). Cong et al. (2017) examined the effect of including different forb e.g., chicory, caraway, and plantain in a red clover-ryegrass mixture. They found that including plantain in a grass-clover mixture significantly increased herbage yield while adding chicory and caraway maintained similar yields to the grass-clover mixture

Transgressive over-yielding, a phenomenon where mixtures' forage yield exceed the production in best-performing monoculture, has been attributed to the complementarity in resource use and minimal niche overlap between species (Nyfeler et al. 2009). Nyfeler et al. (2009) demonstrated that four-species mixtures yielded more than doubled the yield of the corresponding average monocultures and 57% of the mixtures were more productive than the most productive monoculture. Cardinale et al. (2007), in a meta-analysis summarizing 44 diversity experiments including non-agricultural systems found that the 79% of all mixtures were more productive than the average monoculture. However, in only 12% of the experiments, the mixtures were more productive than the most productive monocultures (transgressive overyielding). They also demonstrated that the probability of transgressive over-yielding of mixtures increased over time (Cardinale et al. 2007).

Many studies have emphasized the importance of plant species functional group on the productivity of mixtures (Craine et al. 2002; Finn et al. 2013). However, plant species genotype and environmental factors both singly and interactively may notably affect the productivity of mixtures via alteration in plant-plant interaction (competition and/or complementarity) (Collins et al. 1989; Sanderson et al. 2002). Amongst environmental factors, competition for nitrogen is of prime importance in determining the balance between the competitive outcomes of a mixtures component (Collins et al. 1996). Considering the ability of legume to fix the atmospheric nitrogen, the inclusion of legumes in a mixture increases the mixtures productivity (Dhamala et al. 2017; Lüscher et al. 2008).

In forage systems, it is desirable to achieve not only high yield, but also to obtain high forage nutritive value (Sturludotter et al. 2013). Legumes contain comparatively higher concentration of crude protein (CP) and lower concentration of fiber and water-soluble carbohydrate (WSC) than grasses (Brink et al. 2015; Lüscher et al. 2014). Therefore, including legumes in a mixture may improve the forage nutritive value, in particular increase the concentration of CP compared to the non-legume species monoculture (Brink et al. 2015; Lüscher et al. 2014; Temperton et al. 2007; Zemechik et al. 2002).

The maintenance of consistent forage yield and nutritive value under varying environmental conditions is as important as the overall yield of forage production (Sanderson 2010; Tracy et al. 2004). The varying responses of component species in a mixture to environmental fluctuation and disturbance can, over time, produce high yields and stable community dynamics (Brink et al. 2015). Higher stability of forage nutritive value in mixtures may partly be attributed to the forage reproductive development of component species in mixtures, which is distributed over a longer time span and balanced by the presence of species at a different stage of development (Ergon et al. 2016).

Overall, selecting the plant species for cultivation in mixtures needs to be strategically designed to include traits that maximize complementarity and minimize niche overlap to improve resource utilization and increase the yield of aboveground biomass (Brooker et al. 2015; Litrico et al. 2015).

1.2 The role of breeding new genotypes in mixture performance

In addition to plant functional group, the individual genotype of a plant species has the potential to affect mixture performance, as the competitive ability and persistence of genotypes may vary (Collins et al. 2003). Plant species genotypes may differ markedly in various morphological and physiological characters, which might reflect adaption in response to the different environmental condition, i.e., soil condition, water availability, temperature and neighboring other plant species (Annicchiarico et al. 2010; Rhodes 1970). To stabilize the production of mixture, genetic improvement of an individual species may enhance the compatibility of a plant species in a mixture (Annicchiarico et al. 2010).

Breeding programs seek to optimize key agronomic traits such as forage quality, biomass production and pest and disease resistance in monoculture without regard to the fact that forages are almost universally grown in mixtures (Collins et al. 1989; Helgadóttir et al.

2008; Litrico et al. 2015). The interactions between plants are considerably different in monoculture and mixture and the interspecific interaction in a mixture may affect the performance of a plant genotype (Maamouri et al. 2017). Thus, plant genotypes optimized for monocultures may not be those best suited for mixed-cropping systems (Litrico et al. 2015). Many studies show the importance of choosing varieties in a mixture for their competitive ability (Annachiarico et al. 2010; Collins et al. 2003; Collins et al. 1996; Rhodes 1970). Understanding the performance of plant genotypes in mixtures could help to select appropriate genotypes for balanced and high yielding mixtures.

While a large number of studies have shown that both, the number of species and the diversity of species functional group, would enhance productivity and stability of mixtures, the effect of genetic diversity and resource availability on mixtures is less well-understood (Collins et al. 1996; Prieto et al. 2015; Rhodes 1970). So far, little information is available regarding the effect of different genotype combinations within mixed cropping systems (Helgadóttir et al. 2008; Annicchiarico et al. 1997).

To investigate the effect of plant species genotype on mixtures performance, we established two different field experiments. We hypothesized that:

I. Non-legume species genotype affects the mixtures’ production.
II. Legume species genotype affects the mixtures’ production.
III. Legume species genotype affects the mixtures’ forage nutritive value.

1.3 References

Annicchiarico P, Piano E (1994) Interference effects in white clover genotypes grown as pure stands and binary. *Theor Appl Genet* 88:153-158.

Annicchiarico P, Proietti S (2010) White clover selected for enhanced competitive ability widens the compatibility with grasses and favours the optimization of legume content and forage yield in mown clover-grass mixtures. *Grass Forage Sci* 65: 318-324.

Brink GE, Sanderson MA, Casler MD (2015) Grass and legume effect on nutritive value of complex forage mixtures. *Crop Sci* 55: 1329-1337.

Brooker RW, Bennett AE, Cong W-F, Daniell TJ, George TS, Hallett PD, Hawes C, Iannetta PPM, Jones HG, Karley AJ, Li L, McKenzie BM, Pakeman RJ, Paterson E, Schöb C, Shen J, Squire G, Watson CA, Zhang C, Zhang F, Zhang J, White PJ (2015) Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytol* 206: 107-117.

Brophy C, Finn JA, Lüscher A, Suter M, Kirwan L, Sebastià MT, Helgadóttir Á, Baadshaug OH, Bélanger G, Black A., Collins RP, Čop J, Dalmannsdottir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Ghesquiere A, Golinska B, Golinski P, Grieu P, Gustavsson AM, Höglind M, Huguenin-Elie O, Jørgensen M, Kadziuliene Z, Kurki P, Llurba R, Lunnan T, Porqueddu C, Thumm U, Connolly J (2017) Major shifts in species' relative abundance in grassland mixtures alongside positive effects of species diversity in yield: a continental-scale experiment. *J. Ecol* 105(5): 1210-1222.

Cardinale BJ, Matulich KL, Hooper DU, Byrnes JE, Duffy E, Gamfeldt L, Balvanera P, O'Connor MI, Gonzalez A (2011) The functional role of producer diversity in ecosystems. *Am J Bot* 98: 572-592.

Cardinale BJ, Wright JP, Cadotte MW, Carroll IT, Hector A, Sirvastava DS, Loreau M, Weis JJ (2007) Impacts of plant diversity on biomass production increase through time because of species complementarity. Proceedings of the National Academy of Sciences of the USA 104: 18123 – 18128.

Collins RP, Fothergill M, Macduff JH, Puzio S (2003) Morphological compatibility of white clover and perennial ryegrass cultivars grown under two nitrate levels in flowering solution culture. *Ann. Bot* 92:247-258.

Collins RP, Fothergill M, Rhodes I (1996) Interactions between seedlings of perennial ryegrass and white clover cultivars in establishing swards. *Grass Forage Sci* 51: 163-169.

Collins RP, Rhodes I (1989) Yield of white clover populations in mixture with contrasting perennial ryegrasses. *Grass Forage Sci* 44:111. Doi: 10.1111/j.1365-2494.1989.tb01918.x

Cong WF, Jing J, Rasmussen J, Søegaard K, Eriksen J (2017) Forbs enhance productivity of unfertilised grass-clover leys and support low-carbon bioenergy. *Sci Rep* 7: 1422

Craine JM, Tilman D, Wedin D, Reich P, Tjoelker M, Knops, J (2002) Functional traits, productivity and effects on nitrogen cycling of 33 grassland species. *Funct Ecol* 16: 563-574.

Dhamala NR, Rasmussen J, Carlsson G, Søegaard K, Eriksen J (2017) N transfer in three species grass-clover mixtures with chicory, ribwort plantain or caraway. *Plant Soil* 413: 217.

Duchene O, Vian JF, Celette F (2017) Intercropping with legume for agroecological cropping systems: Complementarity and facilitation processes and the importance of soil microorganisms: a review. *Agric Ecosyst Environ* 240: 148-161.

Ergon A, Kirwan L, Fystro G, Bleken MA, Collins RP, Rognli OA (2016) Species interaction in a grassland mixture under low nitrogen fertilization and two cutting frequencies. II. Nutritional quality. *Grass Forage Sci* 72: 333-342.

Finn JA, Kirwan L, Connolly J, Sebastià MT, Helgadottir A, Baadshaug OH, Bélanger G, Black, A, Brophy C, Collins RP, Čop J, Dalmannsdóttir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Ghesquiere A, Golinska B, Golinski P, Grieu P, Gustavsson AM, Höglind M, Huguenin-Elie O, Jørgensen M, Kadziuliene Z, Kurki P, Llurba R, Lunnan T, Porqueddu C, Suter M, Thumm U, Lüscher A (2013) Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. *J Appl Ecol* 50: 365-375.

Franco JG, King SR, Masabni JG, Volder A (2015) Plant functional diversity improves short-term yields in a low-input intercropping system. *Agric Ecosyst Environ* 203:1-10,

Helgadóttir A, Connolly J, Collins R, Fothergill M, Kreuzer M, Lüscher A, Porqueddu C, Sebasti MT, Wachendorf M, Brophy C, Finn JA, Kirwan L, Nyfeler D (2008) The benefits of sward diversity for cultivated grasslands. *Grassland Science in Europe* 22: 39-51.

Hoekstra NJ, Finn JA, Hofer D, Lüscher A (2014) The effect of drought and interspecific interactions on depth of water uptake in deep-and shallow-rooting grassland species as determined by δ18O natural abundance. *Biogeosciences* 11: 4493-4506.

Hooper DU (1998) The Role of Complementarity and Competition in Ecosystem Responses to Variation in Plant Diversity. *J. Ecol* 79: 704-719

Horwith B (1985) A Role for Intercropping in Modern Agriculture, BioScience 35(5): 286–291.

Husse S, Huguenin-Elie O, Buchmann N, Lüscher A (2016) Larger yields of mixtures than monocultures of cultivated grassland species match with asynchrony in shoot growth among species but not with increased light interception. *Field Crops Res* 194: 1-11.

Lithourgidis AS, Dordas CA, Damalas CA, Vlachostergios DN (2011) Annual intercrops: an alternative pathway for sustainable agriculture. *AUST J CROP SCI* 5, 396-410.

Litrico I, Violle C (2015) Diversity in plant breeding: A new conceptual framework. Trends. *plant Sci* 20 (10): 604-613

Loreau M, Hector A (2001) Partitioning selection and complementarity in biodiversity experiments. *Nature* 412: 72-76.

Loreau M, Naeem S, Inchausti P, Bengtsson J, Grime J, Hector A, Hooper D, Huston M, Raffaelli D, Schmid B (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science* 294: 804-808.

Lüscher A, Finn JA, Connolly J, Sebastia MT, Collins R, Fothergill M, Porqueddu C, Brophy C, Huguenin-Elie O, Kirwan L, Nyfeler D, Helgadottir A (2008) Benefits of sward diversity for agricultural grasslands. *Biodversity* 9: 29-32.

Lüscher A, Mueller-Harvey I, Soussana JF, Rees RM, Peyraud JL (2014) Potential of legume-based grassland–livestock systems in Europe: a review. *Grass Forage Sci* 69(2): 206–228.

Maamouri A, Louarn G, Béguier V, Julier B (2017) Performance of lucerne genotypes for biomass production and nitrogen content differs in monoculture and in mixture with grasses and is partly predicted from traits recorded on isolated plants. *Crop Pasture Sci* 68: 942-951.

Machado S (2009). Does intercropping have a role in modern agriculture?. J. *Soil Water Conserv* 64: 55A-57A.

Newton AC, Begg GS, Swanston JS (2009) Deployment of diversity for enhanced crop function. *Ann Appl* Biol 154: 309-322

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

Nyfeler D, Huguenin-Elie, O, Suter M, Frossard E, Connolly J, Lüscher A (2009) Strong mixture effects among four species in fertilized agricultural grassland led to persistent and consistent transgressive overyielding. *J Appl Ecol* 46: 683–691.

Prieto I, Violle C, Barre P, Durand JL, Ghesquiere M, Litrico I (2015) Complementary effects of species and genetic diversity on productivity and stability of sown grasslands.*Nat Plants* 1(4): 15033.

Rhodes I (1970). The production of contrasting genotypes of perennial ryegrass (Lolium perenne L.) in monocultures and mixed cultures of varying complexity. *Grass Forage Sci* 25:285-288

Rojas-Downing MM, Nejadhashemi AP, Harrigan T, Woznicki SA (2017) Climate change and livestock: Impacts, adaptation, and mitigation. *Clim. Risk Manag* 16: 145-163.

Sanderson MA (2010) Stability of production and plant species diversity in managed grasslands: A retrospective study. *Basic Appl Ecol* 11(3): 216-224.

Sanderson MA, Elwinger GF (2002) Plant Density and Environment Effects on Orchardgrass–White Clover Mixtures. *Crop Sci* 42:2055–2063.

Sanderson MA, Soder KJ, Muller LD, Klement KD, Skinner RH, Goslee SC (2005) Forage mixture productivity and botanical composition in pastures grazed by cattle. *Agron J* 97: 1465–1471.

Sleugh B, Moore KJ, George JR, Brummer EC (2000) Binary legume-grass mixtures improve forage yield, quality, and seasonal distribution. *Agron J* 92: 24–29.

Sturludóttir E, Brophy C, Bélanger G, Gustavsson AM, Jørgensen M, Lunnan T, Helgadóttir Á (2014) Benefits of mixing grasses and legumes for herbage yield and nutritive value in Northern Europe and Canada. *Grass Forage Sci* 69 (2): 229-240.

Temperton VM, Mwangi PM, Scherer-Lorenzen M, Schmid B, Buchmann N (2007) Postive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. Oecologia 151: 190-205.

Tilman D (1999) The ecological consequences of changes in biodiversity: A search for general principles. *Ecology* 80: 1455-1474.

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. Proceedings of the National Academy of Sciences 108 (50) 20260-20264.

Tracy BF, Sanderson MA (2004) Productivity and stability relationships in mowed pasture communities of varying species composition. *Crop Sci* 44: 2180–2186.

Vandermeer JH (1992) The ecology of intercropping, Cambridge university press.

Zemenchik RA, Albrecht KA, Shaver RD (2002) Improved Nutritive Value of Kura Clover– and Birdsfoot Trefoil–Grass Mixtures compared with Grass Monocultures. *Agron J* 94, 1131–1138.

Chapter II: Only small perennial ryegrass genotype effects on the performance of binary grass-clover and four-species mixtur

2.1 Abstract

Forage plants have frequently shown higher yields and higher yield stability when grown in mixtures compared to monocultures, with complementarity of species traits as an important determinant of mixture performance. Not only species, but also genotypes can be expected to have different complementarity to their mixture partners, so that relative performance of genotypes may differ between monocultures and mixtures. To investigate genotype effects on yield and yield stability of mixtures, we grew four genotypes of perennial ryegrass (*Lolium perenne*) differing in two traits (growth form: prostrate or upright, phenology: early or late heading) in monoculture, in binary mixture with white clover (*Trifolium repens*) and in four-species mixture with white clover and two forb species (ribwort plantain, *Plantago lanceolata* and dandelion, *Taraxacum officinale*). We expected (1) that the total dry matter yield and between- and within-year yield stability of perennial ryegrass genotypes would differ between cultivation as monoculture and as mixture, and that the early and upright genotype would show higher compatibility with white clover due to greater trait differences, (2) that the traits of the perennial ryegrass genotype would also affect yields of the mixture components and (3) that perennial ryegrass genotype effects on any of these variables would be weakest in the four-species mixture due to niche saturation. When grown in monocultures, the accumulated total dry matter yields over four years were higher for perennial ryegrass genotypes with an upright growth form compared to the prostrate growth form. This effect did not occur in binary or four-species mixtures. Accumulated total dry matter yields of the four-species mixtures exceeded those of the monocultures, even though only the latter received nitrogen fertilizer. Of the mixture component yields, that of white clover, ribwort plantain and of perennial ryegrass itself were affected by perennial ryegrass growth form. Between- and within-year yield stability was generally highest in the four-species mixtures and lowest in the monocultures, without consistent perennial ryegrass genotype effects. It is concluded that breeding perennial ryegrass for mixtures is likely to be less relevant the more complex the mixtures are and that breeding for yield stability rather than annual herbage yield is more promising.

Keywords: Grassland, White clover, Monoculture, Mixed stands, Forbs, Herbage yield, Yield stability

2.2 Introduction

Compared to grass monocultures, binary mixtures of one grass and one legume species have many advantages, most notably increased forage yield under reduced nitrogen fertilizer application (Annicchiarico and Piano 1994; Temperton et al. 2007; Sanderson et al. 2012; Lüscher et al. 2014; Brooker et al. 2015; Ergon et al. 2016). As a consequence, their use for forage production is relatively common. More recently it has been shown that adding more components to such binary mixtures may further increase yields (Assaf and Isselstein 2009; Finn et al. 2013; Sanderson et al. 2016; Jingying et al. 2017). While legumes increase the availability of nitrogen for the mixture through symbiotic nitrogen fixation, yield increases through addition of non-legume mixture partners can be attributed to a more efficient use of nutrients and other available resources (Hooper et al. 2005; Brooker et al. 2015). Compatibility in resource use has been shown to strongly depend on species differences in traits that are relevant for resource capture, such as rooting depth, growth form or phenology (Berendse 1982; Hill 1990; Frankow-Lindberg and Wrage-Mönnig 2015; Brooker et al. 2015; Husse et al. 2016; Ravenek et al. 2016). Besides increasing absolute yields, trait diversity between species may also lead to higher yield stability, as species responses to environmental fluctuations and disturbances vary (Loreau and de Mazancourt 2013).

In an agronomic context, not only the choice of crop species, but also that of genotypes can be expected to affect the yield and yield stability of mixed cropping systems. In binary mixtures, competition will be decreased and total resource use will be increased, if a genotype is chosen which traits are more complementary to its mixture partner (Figure 2.1). It has been shown that genotype mixtures could improve productivity and minimize the yield fluctuation (Lopez and Mundt 2000). Current breeding efforts, however, are generally based on monoculture performance, which is linked to traits that maximize resource acquisition without interspecific competition. As plant genotypes optimized for monocultures may not be those best suitable for mixed cropping, a new breeding framework with a focus on interaction traits would be essential (Litrico and Violle 2015). This is particularly true for binary mixtures. In more complex mixtures, niche saturation leads to a decreasing productivity gain with each added species (Hooper et al. 2005). This process may also make “fine-tuning” mixtures through the choice of genotypes with complementary traits less important.

To investigate genotype effects on yield and yield stability of mixtures we established a field experiment where different genotypes of perennial ryegrass were grown in monoculture, binary mixture with white clover or four-species mixture with white clover and two forb species over four years. The four perennial ryegrass genotypes were factorial combinations of different growth form (prostrate and upright) and phenology (early and late heading). We hypothesized (1) that relative performance of perennial ryegrass genotypes, in terms of total dry matter yield and yield stability, would differ between cultivation as monoculture and as mixture. Specifically, we expected the early and upright genotype to be most compatible with white clover due to the greatest trait differences, and therefore to cause the highest and most stable mixture yields. We also expected (2) that the yields of the mixture components would differ between mixtures containing different genotypes of perennial ryegrass, as interspecific competition should vary with compatibility. We finally hypothesized (3) that perennial ryegrass genotype effects on any of these variables would be weakest in the four-species mixture due to niche saturation.

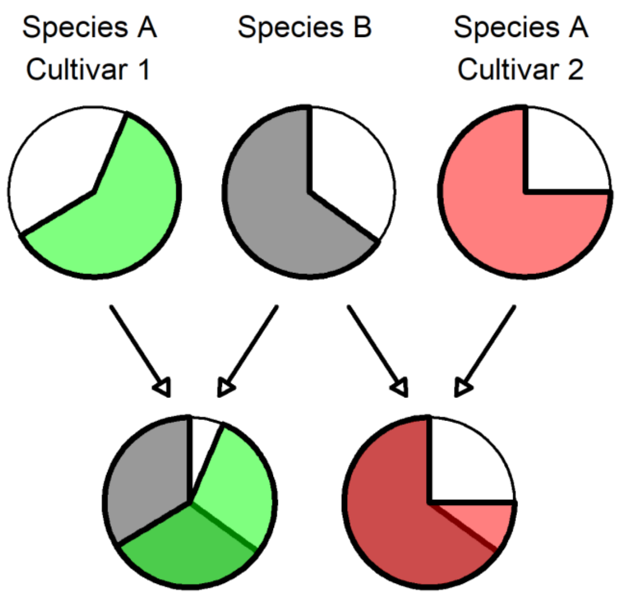

Figure 2.1: Conceptual graph of the hypothesized genotype effects on monoculture and mixture resource use. Circles represent total available resources, shaded areas represent plant resource use, which translates to herbage yield. In monocultures of species A, genotype 2 outperforms genotype 1, as it is able to use a greater share of the available resources. Binary mixtures of species A and B increase total resource use. However, the resource use pattern of genotype 1 is more complementary to species B than that of genotype 2, thus reversing the relative performance of the two genotypes in monoculture. Addition of further complementary species to the mixture is expected to further reduce the share of unused resources (white) and therefore decrease the effect of varying compatibility of single species' genotypes with the mixture partners.

2.3 Materials and methods

2.3.1 Study area

A field experiment was conducted at the research farm Reinshof (51.50° N, 9.93° E, 150 m asl) of the Georg-August-University Göttingen, Germany. The average annual rainfall and temperature during the four experimental years were 652 mm and 9.5 ˚C (Deutscher Wetterdienst, 51.50˚ N and 9.95° E). Weather conditions in the experimental years are shown in Figure 2.2. The seasonal rainfall (April to September) varied from 219 to 430 mm. The soil was classified as Haplic Luvisol according to the FAO classification system. In 0–30 cm depth, the soil contained 15% clay, 73% silt, and 12% sand, 0.1% total N and 1.0% total organic carbon content.

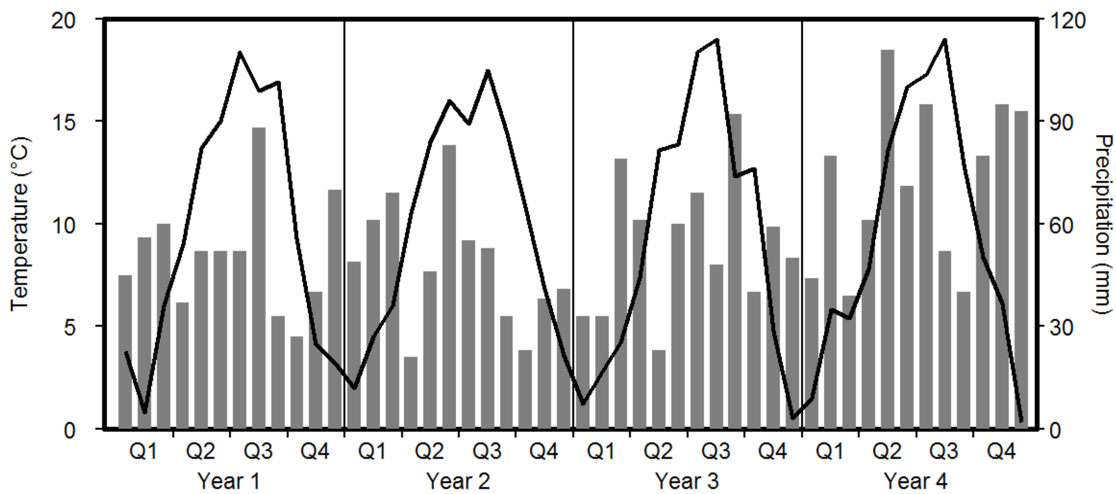

Figure 2.2: Monthly sum of precipitation (grey bars) and average temperature (black line) during the four experimental years.Q shows the quarter of the year.

2.3.2 Experimental design

The experiment was set up in August as a randomized block design with four replications. Four perennial ryegrass (*Lolium perenne*) genotypes were each sown in different crop stands: monocultures (G), binary mixtures with white clover (*Trifolium repens*) (0.75:0.25) (G/C) and four-species mixtures with white clover, ribwort plantain (*Plantago lanceolata*) and dandelion (*Taraxacum officinale*) (0.4:0.2:0.2:0.2, proportions based on number of germinable seeds per m²) (G/C/F). Sowing density in all crop stands was 2000 germinable seeds per m². The perennial ryegrass genotypes were chosen to provide factorial combinations of traits, namely phenology (early and late heading) and growth habit (prostrate, upright). For these traits the genotypes represent almost the full range of variability of the whole ryegrass assortment while for other important traits such as persistence, sward density, and susceptibility to diseases or yield potential they are similar among each other and close to the average of the ryegrass assortment. For details see Appendix Table A.1. Establishment of both perennial ryegrass genotypes and other species in all crop stands were good. The perennial ryegrass monoculture was fertilized with 200 kg N ha^{-1} per year, while mixtures did not receive nitrogen fertilizer. This design was chosen in order to assess ryegrass genotype performance under agronomically relevant conditions both in monocultures and in mixtures. While grass-legume mixtures are of greatest relevance in low-input and organic farming systems where they remain unfertilized, perennial ryegrass monocultures invariably receive nitrogen fertilization, as otherwise no satisfactory crop stand can be achieved. No phosphorus or potassium fertilizer was applied. Extractable (calcium acetate lactate) soil nutrient concentrations at the end of the experiment were 90 mg kg^{-1} phosphorus and 130 mg kg^{-1} potassium, with a pH of 5.9 (CaCl). The experimental plots were harvested by a forage combine harvester at a cutting height of 5 cm. There were four harvests per year over a period of four years (Table 2.1). Subsamples of fresh herbage were hand-separated into perennial ryegrass, white clover, ribwort plantain, dandelion, and non-sown species, and dried (60 °C, 48h) to determine the component yields. No such separation was done in the fourth cutting of year three due to very low dry matter yields of on average 0.36 t dm ha^{-1}.

Table 2.1: Harvest dates in the four experimental years

Harvest	Year 1	Year 2	Year 3	Year 4
1	May 18	May 10	May 14	May 14
2	Jun 22	Jun 15	Jun 25	Jun 17
3	Aug 4	Aug 1	Aug 13	Jul 15
4	Oct 11	Sep 27	Sep 20	Sep 16

2.3.3 Statistical analyses

Statistical analyses were performed with R 3.0.2 (R Core Team 2015). Target variables were annual total dry matter yields, the sum of total dry matter yields over four years, the sum of mixture component yields over four years, and within-year and between year variability of total dry matter yield. Within-year variability was calculated for each year as the coefficient of variation (CV) of total dry matter yields of the four single harvests. The CV of the total dry matter yield of each of the four experimental years was taken as a measure of between-year variability.

The effects of perennial ryegrass growth form (prostrate/upright), perennial ryegrass phenology (early/late) and crop stand (monoculture, binary and four-species mixture) on the target variables were analyzed by linear mixed-effects models using the software package “nlme” (Pinheiro et al. 2017). At first, full models including all possible interactions between the fixed effects (growth form, phenology, crop stand, and in the case of within-year variability, year) were fit. Experimental block and plot nested in block for the analysis of within-year variability was included as random effects. All models were visually checked for homogeneity of variance and normal distribution of the residuals. To fulfill model assumptions, grass and total dry matter yield over four years were log-transformed. Appropriate variance structures were fit for the analysis of within and between-year variability using the function “varIdent”. After validation of the full model, model reduction was performed using the second-order Akaike Information Criterion (AICc) as a selection criterion. For each target variable, the model with the lowest AICc was chosen as the final model. For significant effects in the final model, means were compared using post-hoc pairwise contrasts and tested for significance with the LSD test as implemented in the package “lsmeans” (Lenth 2016). A significance level of $\alpha = 0.05$ was chosen throughout.

2.4 Results

2.4.1 Herbage production

The total dry matter yield accumulated over four full harvest years was significantly affected by the perennial ryegrass growth form, the crop stand and their interaction (Table 2.2). The upright growth form of perennial ryegrass was higher yielding than the prostrate one. However, this growth form effect diminished from monoculture to four-species mixtures (Table 2.3). For the prostrate genotypes there was a significant yield increase from monoculture to the four-species stand while this was not the case for the upright genotypes (Table 2.3). The phenology of the ryegrass had no significant effect on the annual herbage yield. Figure 2.3 shows the total dry matter yield in the single years. In monocultures the growth form effect remained stable over years while in the mixtures the yield was hardly affected by either growth form or phenology of perennial ryegrass. The significant effect of the crop stand on the accumulated total dry matter yield could clearly be attributed to the four-species mixture, which on average over all treatments produced higher yields than the binary mixtures and the fertilized monocultures (Table 2.3). The effect of the crop stand varied among years. In the first year, the monocultures produced higher yields compared to the mixtures while in the other years the mixtures caught up and the four-species mixture showed the highest total dry matter yield (Figure 2.3).

A ryegrass growth form effect was also found for the component yield of perennial ryegrass, white clover and ribwort plantain (Table 2.2). There was no interaction effect growth form x crop stand on these target variables. Dandelion and weed dry matter were not significantly affected by any of the factors or interactions (Table 2.2).

2.4.2 Variability of herbage production between years

The coefficient of variation (CV) of the annual total dry matter yield among the four years was calculated as a measure for the yield stability between years for the different treatments. The results show that the growth form of the perennial ryegrass genotype, the crop stand as well as their interaction significantly affected the CV values (Table 2.4). They decreased from the monoculture over the binary to the four-species mixture. When grown in monocultures the upright genotypes showed higher yield stability than the prostrate ones; in binary and four-species mixtures no difference among the growth forms was found (Figure 2.4a).

Table 2.2: Results of linear mixed effects models for analyzing annual total dry matter yields (TDM) and accumulated dry matter yields over four years for TDM, component dry matter yield of perennial ryegrass (GDM), white clover (CDM) and ribwort plantain (PDM). Four perennial ryegrass genotypes differing in growth form (Form) and phenology (Phen) were grown in monoculture, binary mixture with white clover or four-species mixture with white clover, ribwort plantain and dandelion (crop stand). F and p values are only given for factors and interactions that remained in the final model; models for accumulated dry matter yield of dandelion and weeds only retained the intercept.

Factor		TDM				TDM	GDM	CDM	PDM
		Year 1	Year 2	Year 3	Year 4	Accumulated			
Stand	*F*	27.40	5.75	35.67	10.36	5.50	255.69	1.75	-
	p	<.0001***	0.0067**	<.0001***	0.0002***	0.0076**	<.0001***	0.1979^{ns}	-
Form	*F*	5.10	5.76	18.12	10.23	9.42	28.66	4.70	5.53
	p	0.0300*	0.0218	<.0001***	0.0027**	0.0039**	<.0001***	0.0403^{ns}	0.0384*
Phen	*F*	-	-	-	-	-	-	1.93	-
	p	-	-	-	-	-	-	0.1780^{ns}	-
Stand x Form	*F*	-	-	11.23	4.56	3.48	-	-	-
	p	-	-	<.0001***	0.0166*	0.0405*	-	-	-

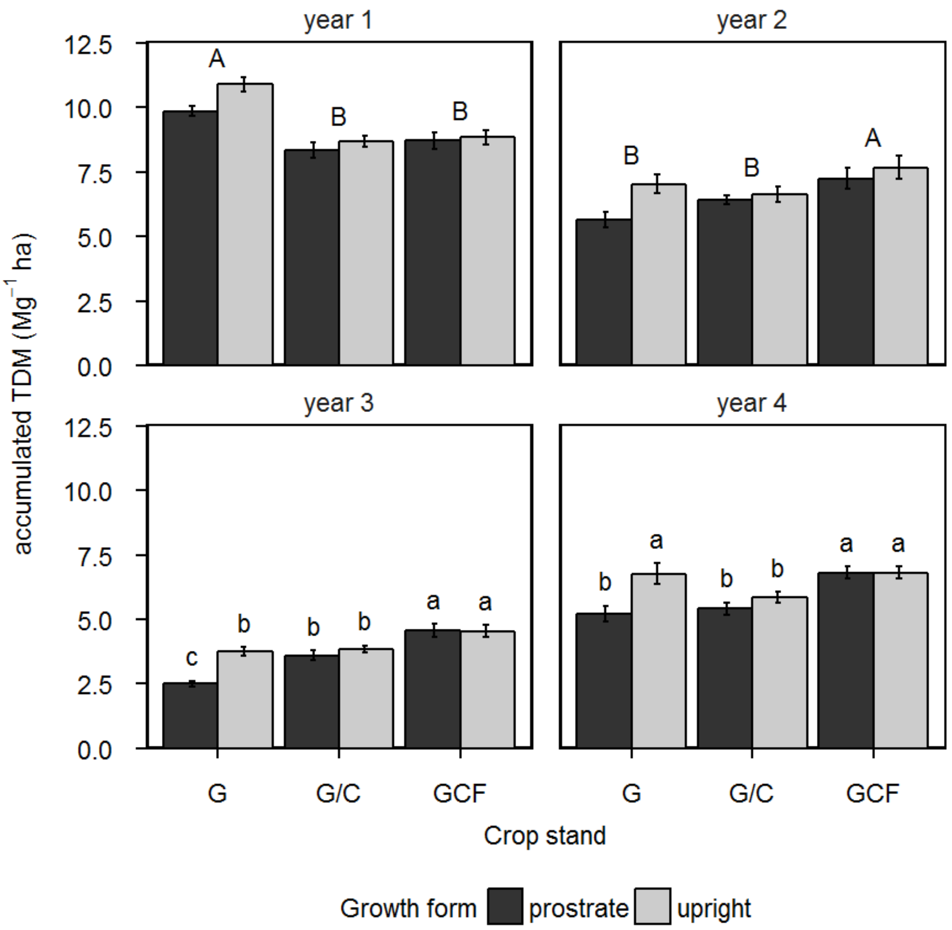

Figure 2.3: Annual total dry matter yield (TDM) of perennial ryegrass genotypes differing in growth form (prostrate/upright), grown in monoculture (G), binary mixture with white clover (G/C) and four-species mixture with white clover and two forb species (G/C/F). Lower case letters (year 3, year 4) indicate significant differences between growth form x crop stand means, averaged over phenology; upper case letters (year 1, year 2) indicate significant differences between crop stands, averaged over phenology and growth form ($P < 0.05$); error bars: standard error of the mean.

Table 2.3: Herbage yields accumulated over four years for total dry matter (TDM) and component dry matter yields of perennial ryegrass (GDM), white clover (CDM), ribwort plantain (PDM), dandelion (DDM) and weeds (WDM). Perennial ryegrass genotypes differing in growth form (form; P: prostrate, U: upright) and phenology (Phen) were grown in three crop stands (Stand): monoculture, binary mixture with white clover and four-species mixture with white clover, ribwort plantain and dandelion. All values are means over the two levels of phenology (early/late heading). Letters indicate significant differences between values in the same column within each level of comparison ($p < 0.05$).

Stand	Form	TDM	GDM	CDM	PDM	DDM	WDM
G	P	23.2c	22.2	-	-	-	0.7
	U	28.5a	27.2	-	-	-	0.6
G/C	P	23.7c	10.9	11.9	-	-	0.7
	U	25.0bc	13.2	10.9	-	-	0.6
G/C/F	P	27.3ab	4.5	12.3	8.2	1.4	0.7
	U	27.9ab	7.1	11.5	7.2	1.3	0.5
G	-	25.8	24.7a	-	-	-	0.6
G/C	-	24.4	12.0b	11.4	-	-	0.7
G/C/F	-	27.6	5.8c	11.9	7.2	1.4	0.6
-	P	24.8	12.5b	12.1	8.2a	1.4	0.7
-	U	27.1	15.8a	11.2	7.2b	1.3	0.6

Table 2.4: Results of linear mixed effects models analyzing the coefficient of variation of total dry matter yield between years and within years. Perennial ryegrass genotypes differing in growth forms (Form) and phenology (Phen) were grown in three crop stands (Stand): monoculture, binary mixture with white clover and four-species mixture with white clover, ribwort plantain and dandelion (Stand). F and p values are only given for factors and interactions that remained in the final model.

Factor	F value	p value
Between years		
Stand	121.84	<.0001***
Form	21.48	0.0011**
Stand x Form	10.69	0.0002***
Within years		
Stand	82.53	<.0001***
Form	1.61	0.2067^{ns}
Phen	0.11	0.7435^{ns}
Year	542.25	<.0001***
Stand x Form	9.69	0.0001***
Stand x Phen	12.18	<.0001***
Stand x Year	12.78	<.0001***
Form x Phen	8.32	0.0045**
Form x Year	4.51	0.0046**
Phen x Year	143.86	<.0001***
Stand x Phen x Year	13.40	<.0001***
Form x Phen x Year	4.26	0.0064**

2.4.3 Variability of herbage production within years

Similar to the stability of the herbage production between years the coefficient of variation was also used to assess the yield stability within years. Neither growth form nor phenology of perennial ryegrass genotype showed a significant main effect on the CV. However, there were significant interactions (Table 2.4). Compared to monocultures of ryegrass, the CV values were significantly lower in mixed stands, particularly so in the four-species mixtures (Figure 2.4b). In contrast to the herbage yield (Table 2.2), genotype effects were also significant in the mixtures, with pronounced differences between years: while early heading genotypes had a higher CV than late heading genotypes in the first two years, the opposite was true in years three and four. The prostrate growth form generally showed more within-year variability than the upright, but depending on year this was only true for either the early or the late heading genotypes (Figure 2.4b).

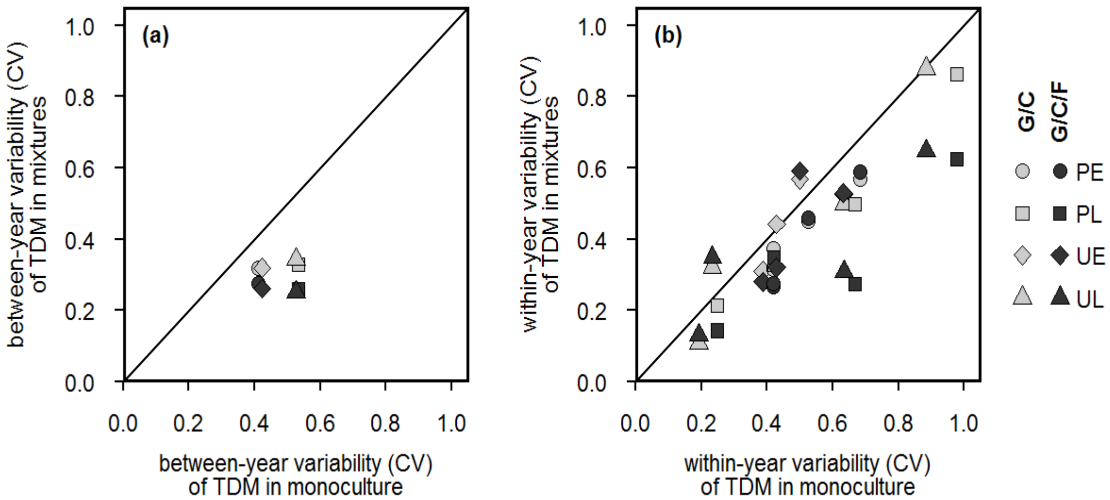

Figure 2.4: Relationship between the coefficient of variation (CV) of total dry matter yield (TDM) of mixtures and monocultures of four perennial ryegrass genotypes (a) between years, (b) within years. Perennial ryegrass genotypes differed in growth form (P: prostrate, U: upright) and phenology (E: early heading, L: late heading). Mixtures were either binary mixtures with white clover (G/C) or four-species mixtures with white clover and two forb species (G/C/F).

2.5 Discussion

Binary grass-clover and multi-species mixtures have received considerable attention in forage research in recent years. There is reasonable evidence that in contrast to highly fertilized pure grass sowings such systems have a considerable potential for the sustainable intensification of grassland management (Huyghe et al. 2012; Kirwan et al. 2007; Lüscher et al. 2014; Nyfeler et al. 2009; Weigelt et al. 2009). Mixture benefits have been shown to occur as overyielding compared to the respective monocultures but also as increasing yield stability (Ergon et al. 2016; Frankow-Lindberg et al. 2009). These benefits could be related to differences in plant traits for resource use and growth of the different species in mixtures ensuring a higher compatibility rather than competition among the partner species (Finn et al. 2013; Husse et al. 2016). Important modes of complementarity are differences in the nitrogen acquisition (legume vs non-legume species), temporal development (early vs late reproductive growth), shoot characteristics (small vs tall growing), or root growth pattern (shallow vs deep rooting). Based on the knowledge about complementarity it has been suggested to strategically utilize trait variability among forage species to design the optimal composition of mixtures (Huyghe et al. 2012; Finn et al. 2013). Although the genetic improvement of forage crops is a core activity of forage research and plant traits affecting agricultural performance as well as environmental services have successfully been altered (Barth 2012; Helgadottir et al. 2016) there is remarkably little consideration of traits and their complementarity in mixtures in plant breeding. In addition, the development and testing of new forage germplasm is usually done in pure stands and does not account for potential mixture effects.

In the present study trait variability among perennial ryegrass genotypes was used to investigate whether and to what extent the performance of mixtures of forage species can be varied by choosing genotypes which have a potentially higher compatibility with the partner species. More specifically, we expected that the growth form and the phenology of perennial ryegrass are traits that strongly determine the temporal and spatial overlap of resource use of the grass and its partner species and thereby significantly affect the total mixture yield and yield stability.

When grown in monocultures we found marked differences in the herbage production between the ryegrass genotypes, with the upright genotypes clearly better performing than the prostrate ones. It is well established that growth characteristics of ryegrass germplasm strongly determine herbage production (Wilkins and Lovatt 2011) and variation in the respective traits provide the basis for breeding.

When grown in mixtures, this growth form effect was reduced but still visible (albeit not significant) in the binary mixture and almost completely disappeared in the four-species mixture. Thus, the performance of the mixtures was much more determined by the species composition (either binary or four-species mixtures) than by the ryegrass genotype. This was true for both herbage yield and yield stability. This is in line with work being done on grass-clover mixtures with either inconsistent (Elgersma and Schlepers 1997) or no significant (Nassiri and Elgersma 2002) ryegrass genotype effect. The phenology of the ryegrass genotypes did not significantly affect the annual herbage yield. In general, grass-clover mixtures are known to show a characteristic pattern of within season growth. The grass has lower temperature requirements to achieve high growth rates compared to white clover; that is why maximum growth rates of ryegrass usually occur in spring while clover grows best in summer. Accordingly, Evans et al. (1985) found a higher compatibility of ryegrass and clover in mixtures when the seasonal pattern of growth was more differentiated. It was therefore expected that the early heading ryegrass genotypes should have a higher compatibility compared to the late heading ones. Presumably, this was not the case because of temporal limitations in water availability which – among other reasons such as nitrogen limitation - might have restricted growth even if temperatures were favorable.

In contrast to the herbage yield, the yield stability was significantly affected by interactions of ryegrass phenology with the other factors. When grown in monocultures the late heading ryegrass genotypes showed a lower within-season variation compared to the early heading ones. This effect decreased in the binary and even more so in the four-species mixtures. Obviously, the companion species in the mixtures compensated for phenology-related patterns of resource use of the grass, thus, at sward level, a more even growth was possible. As expected, the binary and four-species mixtures had significantly higher between-years yield stability than the ryegrass monocultures. The low dry matter yields of year three are interesting in this respect as the yield drop compared to the other years was clearly lower in the mixtures than in the monocultures. Although we cannot provide a definite reason for the low yield we assume that the grass component was particularly weak in that year.

The second hypothesis of the present experiment was that the yields of the mixture components would differ between mixtures containing different genotypes of perennial ryegrass, as interspecific competition should vary with compatibility. This hypothesis could partly be confirmed. The ryegrass, clover and ribwort plantain component yields were significantly different between the upright and prostrate ryegrass genotypes while neither the the dandelion nor the weed components showed this response. In general, the yield of white clover was rather stable among the different treatments. This is interesting as the competitiveness of white clover was either little affected by the companion species, or the different companion species exerted a similar competitive effect on the clover. This is confirmed by the inclusion of forbs in the four-species mixtures which markedly reduced the component yield of the grass but not of clover. As the mixtures did not receive any nitrogen fertilizer, nitrogen limitation was likely to be a key factor determining herbage growth. Given this situation, forbs were a strong competitor against the grass but not the clover.

The results of the present experiment quite clearly confirmed that unfertilized forage mixtures that include white clover have a similar yield potential as fertilized grass monocultures. In addition, adding forbs to binary grass-clover mixtures further increased and stabilized herbage yields. This latter finding is probably due to increasing niche saturation with an increasing complexity of forage mixtures (Assaf and Isselstein 2009; Jingying et al. 2017; Sanderson et al. 2016). In the third hypothesis of the present experiment we stated that perennial ryegrass genotype effects would be weakest in the four-species mixture due to niche saturation. This assumption was clearly supported by the sward level herbage yield. Neither phenology nor growth form of ryegrass showed any effect on the herbage yield of the four-species mixture. Apart from the niche saturation effect this finding could also be attributed to the relatively low yield share of ryegrass in that mixture. In contrast to the herbage yield, the yield stability not only of the monoculture but also of the mixtures responded to both the ryegrass growth form and phenology.

2.6 Conclusion and practical implications

Perennial ryegrass covers a considerable range of growth traits which are markedly expressed and visible when the grass is grown in monocultures. This trait variation was expected to affect the compatibility with companion species and the performance of mixed stands. This expectation could only partly be confirmed. Compared to the overall strong yield effect of including clover and forbs in forage mixtures, the additional yield variation due the use of different grass genotypes was small. However, yield stability responded more strongly to the genotype traits than herbage yield. This is noteworthy as a more even distribution of herbage growth within and among years is of importance for forage-dependent livestock husbandry, in particular grazing systems where fresh herbage is consumed.

While the agronomic advantage of binary and in particular multi-species mixtures are quite obvious, the consequences of this research for ryegrass breeding are less so. We assume that an attempt to design ryegrass germplasm through breeding in order to maximize the herbage potential will be decreasingly successful the more complex the mixtures are at the species level. Yet, we have to concede that in the present study only four contrasting ryegrass genotypes were employed which only cover a part of the totally available variation among the perennial ryegrass assortment.

2.7 Acknowledgments

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2.8 References

Annicchiarico P, Piano E (1994) Interference effects in white clover genotypes grown as pure stands and binary. *Theor Appl Genet* 88,153-158.

Assaf T, Isselstein J (2009) Evaluation of dandelion as a potential forage species in mixed-species swards. *Crop Sci* 49: 714-721.

Barth S (2012) Breeding strategies for forage and grass improvement. *Ann Bot* 110: 1261-1262.

Berendse F (1982) Competition between plant-populations with different rooting depths. III. Field experiments. *Oecol* 53: 50-55.

Brooker RW, Bennett AE, Cong W-F, Daniell TJ, George TS, Hallett PD, Hawes C, Iannetta PPM, Jones HG, Karley AJ, Li L, McKenzie BM, Pakeman RJ, Paterson E, Schöb C, Shen J, Squire G, Watson CA, Zhang C, Zhang F, Zhang J, White PJ (2015) Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytol* 206: 107-117.

Elgersma A, Schlepers H (1997) Performance of white clover/perennial ryegrass mixtures under cutting. *Grass Forage Sci* 52: 134-146.

Ergon Å, Kirwan L, Bleken MA, Skjelvåg AO, Collins RP, Rognli OA (2016) Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: 1. Dry-matter yield and dynamics of species composition. *Grass Forage Sci* 71: 667–682.

Evans DR, Hill J, Williams TA, Rhodes I (1985) Effects of coexistence on the performance of white clover-perennial ryegrass mixtures. *Oecol* 66: 536-539.

Finn JA, Kirwan L, Connolly J, Sebastià MT, Helgadottir A, Baadshaug OH, Bélanger G, Black A, Brophy C, Collins RP, Čop J, Dalmannsdóttir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Ghesquiere A, Golinska B, Golinski P, Grieu P, Gustavsson AM, Höglind M, Huguenin-Elie O, Jørgensen M, Kadziuliene Z, Kurki P, Llurba R, Lunnan T, Porqueddu C, Suter M, Thumm U, Lüscher A (2013) Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. *J. Appl. Ecol* 50: 365-375.

Frankow-Lindberg BE, Halling M, Höglind M, Forkman, J (2009) Yield and stability of yield of single- and multi-clover grass-clover swards in two contrasting temperate environments. *Grass Forage Sci* 64: 236-245.

Frankow-Lindberg BE, Wrage-Mönnig N (2015) Light availability is improved for legume species grown in moderately N-fertilized mixtures with non-legumes species. *Basic Appl*. Ecol 16: 403-412.

Helgadóttir A, Connolly J, Collins R, Fothergill M, Kreuzer M, Lüscher A, Porqueddu C, Sebasti MT, Wachendorf M, Brophy C, Finn JA, Kirwan L, Nyfeler D (2008) The benefits of sward diversity for cultivated grasslands. *Grassland Science in Europe* 22: 39-51.

Hill J (1990) The three C's- competition, coexistence and coevolution- and their impact on the breeding of forage crop mixtures. *Theor. Appl. Genet* 79: 168-176.

Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Setälä H, Symstad AJ, Vandermeer J, Wardle DA (2005) Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecol. Monogr* 75: 3-35.

Husse S, Huguenin-Elie O, Buchmann N, Lüscher A (2016) Larger yields of mixtures than monocultures of cultivated grassland species match with asynchrony in shoot growth among species but not with increased light interception. *Field Crops Res* 194: 1-11.

Huyghe C, Litrico I, Surault F (2012) Agronomic value and provisioning services of multi-species swards. *Grassland Sci. Eur* 17: 35-46.

Jingying J, Søegaard K, Cong WF, Eriksen J (2017) Species diversity effects on productivity, persistence and quality of multi-species swards in a four-year experiment. *PLoS ONE* 12, e0169208.

Kirwan L, Lüscher A, Sebastià MT, Finn JA, Collins RP, Porqueddu C, Helgadottir A, Baadshaug OH, Brophy C, Coran C, Dalmannsdottir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Golinski P, Grieu P, Gustavsson AM, Hoglind M, Huguenin-Elie O, Iliadis C, Jorgensen M, Kadziuliene Z, Karyotis T, Lunnan T, Malengier M, Maltoni S, Meyer V, Nyfeler D, Nykanen-Kurki P, Parente J, Smit HJ, Thumm U, Connolly J (2007) Evenness drives consistent diversity effects in intensive grassland systems across 28 European sites. *J. Ecol* 95: 530-539.

Lenth RV (2016) lsmeans: Least-Squares Means. R package version 2.21-1. url: http://CRAN.R-project.org/package=lsmeans.

Litrico I, Violle C (2015) Diversity in plant breeding: a new conceptual framework. *Trends Plant Sci* 20: 604-613.

Lopez CG, Mundt CC (2000) Using mixing ability analysis from two-way cultivar mixtures to predict the performance of cultivars in complex mixtures. *Field Crops Res* 68: 121-132.

Loreau M, de Mazancourt C (2013) Biodiversity and ecosystem stability: a synthesis of underlying mechanisms. *Ecol. Lett* 16: 106-115.

Lüscher A, Mueller-Harvey I, Soussana JF, Rees RM, Peyraud JL (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass Forage Sci* 69: 206-228.

Nassiri M, Elgersma A (2002) Effects of nitrogen on leaves, dry matter allocation and regrowth dynamics in *Trifolium repens* L. and *Lolium perenne* L. in pure and mixed swards. *Plant Soil* 246: 107-121.

Nyfeler D, Huguenin-Elie O, Suter M, Frossard E, Connolly J, Lüscher A (2009) Strong mixture effects among four species in fertilized agricultural grassland led to persistent and consistent transgressive overyielding. *J. Appl. Ecol* 46: 683-691.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2017) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-128, url: http://CRAN.R-project.org/package=nlme.

Ravenek JM, Mommer L, Visser EJW, Ruijven J, van der Paauw JW, Smit-Tiekstra A, de Caluwe H, de Kroon H (2016) Linking root traits and competitive success in grassland species. *Plant Soil* 407: 39-53.

R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.

Sanderson MA, Brink G, Ruth L, Stout R (2012) Grass-legume mixtures suppress weeds during establishment better than monocultures. *Agron. J* 104: 36-42.

Sanderson MA, Stout R, Brink G (2016) Productivity, botanical composition, and nutritive value of commercial pasture mixtures. *Agron. J* 108: 93-100.

Temperton VM, Mwangi PN, Scherer-Lorenzen M, Schmid B, Buchmann N (2007) Positive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. *Oecol* 151: 190- 205.

Weigelt A, Weisser WW, Buchmann N, Scherer-Lorenzen M (2009) Biodiversity for multifunctional grasslands: equal productivity in high-diversity low input and low-diversity high input systems. *Biogeosci* 6: 1695-1706.

Wilkins PW, Lovatt JA (2011) Gains in dry matter yield and herbage quality from breeding perennial ryegrass. *IJAFR* 50: 23-30.

Chapter III: White clover genotype effects on the productivity and yield stability of mixtures with perennial ryegrass and chicory

3.1 Abstract

Due to complementary resource use of the components, grass-clover mixtures are usually higher yielding compared to grass pure stands. The number and identity of species in a mixture are important factors for the mixture's productivity. Little is known about the influence of plant species genotype on the productivity of mixtures. We established an experiment to investigate how and to what extent different genotypes of white clover (*Trifolium repens* L.) affect the mixtures' performance. Eight novel genotypes of white clover and one variety of perennial ryegrass (*Lolium perenne* L.) and chicory (*Cichorium intybus* L.) each were grown as monocultures and in two- and three-species mixtures at two sites differing in soil fertility. Aboveground herbage was cut twice in the establishing year and four times in each of the three following years. The accumulated dry matter yield was calculated as the sum of the four annual dry matter yields. There was no significant interaction between white clover genotype x crop stand (i.e., monoculture or mixture) on dry matter yield accumulated over four years; the white clover genotype that performed well in monoculture also did so in mixtures. On both sites, the binary mixtures of white clover and chicory produced significantly higher dry matter yields than the white clover monocultures and other mixtures. Inclusion of chicory in mixtures significantly increased the stability of yield production. Site condition strongly affect the performance of forage species mixtures. We found that the benefit of mixtures over the monocultures more related to the identity of species in mixture than the white clover genotype.

Keywords: White clover, Monoculture, Mixed stands, Forbs, Herbage yield, Yield stability

3.2 Introduction

In temperate regions, cultivating grasses and legumes in mixtures often provides higher yields compared to the individual species grown in monoculture (Finn et al., 2013; Lüscher et al., 2014; Nyfeler et al., 2009). A yield advantage of mixtures is termed overyielding when the performance of the mixture is better than the performance of the average monoculture (Nyfeler et al., 2009). Transgressive overyielding happens when the yield advantage exceeds the best performing monoculture (Cardinal et al., 2007; Ergon et al., 2016; Nyfeler et al., 2009). In a meta-analysis summarizing 44 diversity experiments including non-agricultural systems, Cardinale et al. (2007) found that overyielding is a common phenomenon while transgressive overyielding is rather rare; 79% of the mixtures showed higher yields than the average of the referring monocultures while only 12% were more productive than the best performing monoculture.

The highest possible yield, however, may not always have the highest priority from a farming point of view. Yield stability between years is also very important (Frankow-Lindberg et al., 2009). Mixtures often exhibit a more even seasonal growth than mono-crops, as different plant species may respond to environmental fluctuation and disturbance differently (Brink et al., 2015; Litrico and Violle, 2015; Sanderson et al., 200).

Selecting species or genotypes with complementary functional traits for mixtures has recently been considered as a promising strategy to enhance productivity and yield stability (Hooper, 1998; Lüscher et al., 2014). Mixture communities containing species with different root and shoot architecture can occupy a larger niche space and thus can acquire more unexploited resources compared to communities containing species with similar root and shoot distributions (Hooper, 1998). An example of complementary root architecture has been given by Hoekstra et al. (2014) who showed that the inclusion of deep-rooting forbs such as *Cichorium intybus* in grass-legume mixtures increase the biomass production. However, research by Cong et al. (2017) did not confirm this beneficial effect of *C. intybus*, probably because water was less than in the experiment of Hoekstra et al. (2014). However, Cong et al. (2017) found that, in contrast to *C. intybus*, *Plantago lanceolata* did increase the performance of a grass-clover mixture, suggesting other mechanism of complementarity than rooting depth.

In general, the role of functional group and species diversity of mixtures for improved resource utilization is well known (Finn et al., 2013; Lüscher et al., 2014). However, the effect of plant species genotype on the performance of mixtures is not that obvious. Plant species genotypes may differ markedly in major morphological and physiological characters. These variations might reflect adaption in response to different environmental conditions, e.g., soil properties, water availability, temperature or neighboring species (Annicchiarico and Proiretti, 2010; Collins and Rhodes, 1989; Rhodes, 1970; Turkington et al., 1979). A possible avenue to enhance the general compatibility of plant species in mixture may be through genetic improvement (Annicchiarico, 2010). So far, the breeding effort has concentrated on optimizing key agronomic traits, such as biomass production, forage quality, and pest and disease resistance (Litrico and Violle, 2015). Commonly, grassland plant species have been bred separately and tested in monocultures with little consideration of the fact that they are usually grown in mixtures with other species (Collins et al., 1989). When the breeding target is a crop mixture, in addition to all agronomic traits that have been optimized, other traits must also be incorporated and optimized, including the ability to live and perform with others (compatibility).

Earlier studies highlighted the general effect of the botanical composition of mixtures and the plant species genotype on the productivity of mixtures (Collins and Rhodes, 1989; Finn et al., 2013; Hill and Michaelson-Yeates, 1987; Lüscher et al., 2014). There is, however, a knowledge gap on how the combination of plant species genotype and botanical composition would affect the mixtures' performance under different environmental conditions. The objective of this study is to assess the effect of white clover genotype on yield and yield stability of different binary and multi-species mixtures. We hypothesized that white clover genotypes perform differently in white clover monocultures and mixtures of white clover with non-legume species. Differences between monocultures' and mixtures' performances are expected to depend on the partner species and the resource availability. Accordingly, a field experiment was established on two contrasting sites differing in climatic and soil conditions. Eight novel genotypes of white clover were sown as monocultures and in mixtures either with perennial ryegrass or chicory or a combination of the two.

3.3 Material and methods

3.3.1 Study area

This study was part of the IMPAC³ experiment and was carried out during 2014-2017 at two experimental stations of the Georg-August-University Goettingen, Germany. The two sites were noticeably different in terms of soil depth, soil texture, and precipitation. The fertile site (51.29 N, 9.55 E) has a Gleyic Fluvisol (FAO classification system) with 21% of clay, 11% sand and 68% silt in the Ah horizon, making up the top 30 cm soil layer. The marginal site (51.34 N, 9.58 E) with a Calcaric Leptosol (FAO classification system) has a shallower Ah horizon of 25 cm depth. The clay content is higher than on the fertile site (34% clay, 2% sand and 55% silt). The marginal site is located at a higher elevation (342 m versus 157 m above sea level of the fertile site). During the years 2014-2017, it had a lower mean annual precipitation (591.60 mm versus 636.83 mm) and a lower average temperature (8.96 °C versus 10.05 °C) than the fertile site. The monthly average temperature and rainfall per experimental year are given in Appendix (Fig. A1).

3.3.2 Experimental design

In July 2014, eight novel genotypes of white clover from the Deutsche Saatveredelung (DSV, Asendorf, Germany), one variety of perennial ryegrass (*Lolium perenne* L., ELP 060687) and one variety of chicory (*Cichorium intybus* L., Puna II) were sown as monocultures, in binary mixtures of white clover with either ryegrass or chicory, in binary mixture of ryegrass with chicory and in three-species mixtures of white clover with ryegrass and chicory. The white clover genotypes differed in phenology, leaf size and yield potential. On a scale of one to nine for phenology, leaf size and yield potential for white clover varieties included on the German National List of Varieties (Bundessortenamt, 2016), the phenology of white clover in the present study was rated between three and nine, and both leaf size and yield potential between three and seven. The seed rate of each species in mixtures was calculated based on a monoculture seed rate of 1000 seeds per m^2. Sowing ratios were 0.4 : 0.6 (white clover : ryegrass, white clover : chicory), 0.5 : 0.5 (ryegrass : chicory) and 0.4 : 0.3 : 0.3 (white clover : ryegrass : chicory). Crop stands with white clover did not receive any fertilizer. Two levels of fertilization, 0 and 240 kg total N $ha^{-1}yr^{-1}$ (NH_4NO_3+$CaCO_3$), were applied to ryegrass and chicory monocultures and also to the mixture of the two species. Fertilizer applications were split into four doses (80, 60, 60 and 40 kg N ha^{-1}), applied at the start of the vegetation period and after the first, second and third cut each year.

The experiment followed a split-plot design with four replications (block) at both sites. In each block, experimental treatments were arranged with five plots each nested within eight main plots corresponding to the white clover genotypes. This resulted in 160 plots (5 m × 2.8 m) at each site. Buffer areas of 0.2 m width between adjacent plots were regularly treated with glyphosate to prevent the incursion of white clover stolons into adjoining plots. All plots were cut twice during the establishment year (2014), in mid-August and mid-October. In the following years, four cuts per year were carried out in six-week intervals from mid-May to mid-October. The whole plots were cut to a height of 5 cm with a combine forage harvester (Wintersteiger hd 1500, Wintersteiger AG, Ried im Innkreis, Austria). At each harvest, the fresh biomass yield from a core area of 1.4 m × 5 m from each plot was determined. A subsample of approximately 250 g was dried at 60°C for 48 hours to estimate the dry-matter concentration of the harvested biomass, which was used to calculate the dry matter yield. The results presented in this paper focus on white clover monoculture and mixtures including white clover.

3.3.3 Statistical analysis

The accumulated dry matter yield was calculated as the sum of the four annual dry matter yields.The stability of dry matter yields was quantified using the coefficient of variation (CV) of annual dry matter yields from the three years following the establishment (2015–2017). Transgressive overyielding (TO) of legume/non-legume mixtures over the most productive unfertilized and fertilized monocultures was determined as:

$$\mathrm{TO} = \frac{\mathrm{DM}_{mix} - \mathrm{DM}_{mono}}{\mathrm{DM}_{mono}}$$

DM_{mix} = accumulated dry matter yield of mixture, DM_{mono}= accumulated dry matter yield of the mixture component species with the highest yield when grown as monoculture in the same block. The binary mixture of the non-legume species ryegrass and chicory was considered as a monoculture when evaluating transgressive overyielding of the three-species mixture of white clover with ryegrass and chicory.

The effect of white clover genotype, crop stand, site and their interactions on accumulated dry matter yield, transgressive overyielding and the stability of total dry matter yield between years was analyzed in the R 3.4.2 statistical software environment (R Core Team, 2017), using linear mixed effects models implemented in the software package “nlme” (Pinheiro et al, 2017). Block and main plot were included as nested random effects.

All models were visually checked for homogeneity of variance and normal distribution of residuals, and variance was allowed to vary between factor levels where necessary to account for heteroscedasticity. The full model containing all main effects and interactions was simplified based on the second-order Akaike Information Criterion (AICc). Means were then compared using post-hoc pairwise contrasts and tested for significance with the LSD test, as implemented in the “emmeans” package (Lenth, 2017). A significance level of $\alpha < 0.05$ was chosen throughout.

3.4 Results

No significant interaction was seen between white clover genotype × crop stand for accumulated dry matter yield (Table 3.1). The white clover genotype itself affected accumulated dry matter yield, but this effect was site-dependent (Table 3.1). The differences between white clover genotypes were larger at the fertile site compared to the marginal site (Fig. 3.1B).

Table 3.1: Results of linear mixed effects models analyzing accumulated dry matter yields (DM) over four years, yield stability between years, and transgressive overyielding over the unfertilized (TO-N0) monoculture or the fertilized monoculture (TO-N1). Eight white clover genotypes were grown in four crop stands (Stand): monoculture, binary mixture with either perennial ryegrass or chicory and three species mixture with perennial ryegrass and chicory at two sites, either fertile or marginal. *F* and *p* values are only given for factors and interactions that remained in the final model.

Factor	Accumulated DM		Yield stability		Transgressive overyielding TO-N0		Transgressive overyielding TO-N1	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Genotype	7.3	<0.0001	-	-	-	-	4.1	<0.0001
Stand	158.9	<0.0001	94.1	<0.0001	16.7	<0.0001	183.6	<0.0001
Site	12.4	0.0124	5.5	0.0561	0.2	0.6572	-	-
Genotype × site	2.4	0.0308	-	-	-	-	-	-
Stand × site	49.2	<0.0001	-	-	28.4	<0.0001	-	-

The advantage of mixtures over white clover monocultures differed between sites (Table 3.1). While accumulated dry matter yields of white clover monocultures did not differ between the two sites, the yields of all mixtures were higher at the fertile than at the marginal site (Fig. 3.1A, 3.2). As a consequence, all mixtures showed higher yields than the white clover monocultures on the fertile site, while on the marginal site, this was only true for the two mixtures including chicory (Fig. 3.1A, 3.2). At both sites, all mixtures yielded higher than the average of the unfertilized partner species monoculture but not all mixtures yielded higher than the fertilized monocultures. (Fig. 3.2)

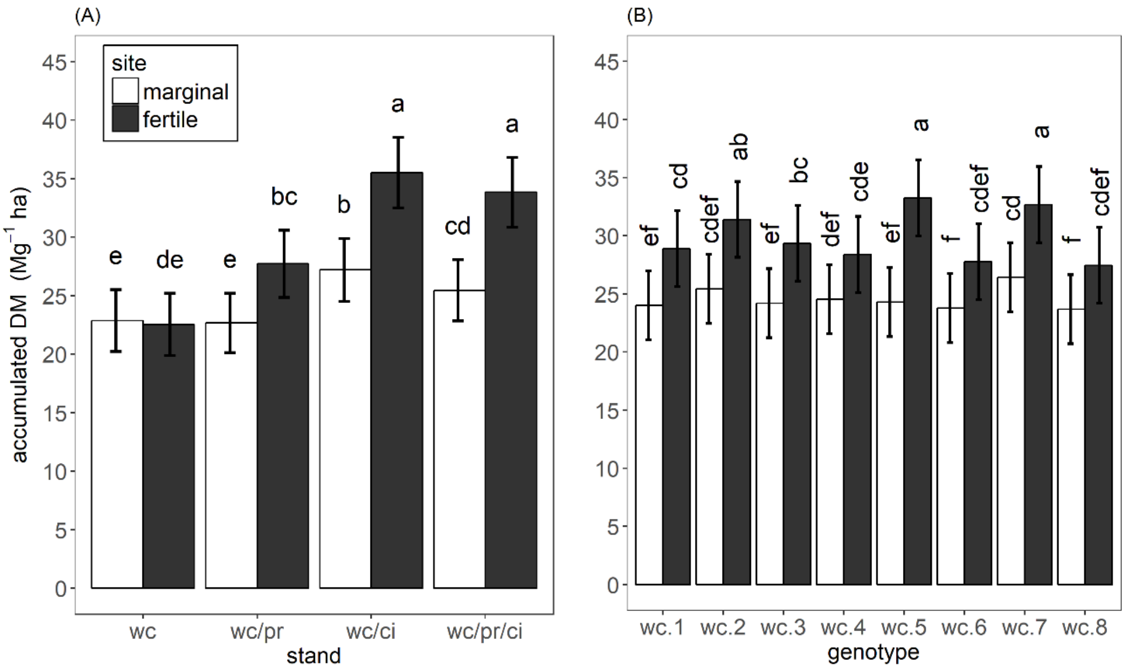

Figure 3.1: Dry matter yield of white clover monocultures and mixtures at two sites accumulated over four years (A): effect of crop stand (stand) averaged over white clover genotypes for white clover monoculture (wc), binary mixture of white clover with perennial ryegrass (wc/pr), binary mixture of white clover with chicory (wc/ci), and three-species mixture of white clover, perennial ryegrass and chicory (wc/pr/ci). (B): effect of white clover genotypes averaged across crop stands. Letters indicate significant ($\alpha < 0.05$) differences between crop stand × site (A) and white clover genotype × site model estimates (B), respectively; error bars: confidence interval.

At the fertile site, the binary mixures of white clover either with rygrass (TO = 0.13; confidence interval 0.05–0.18) or chicory (TO = 0.095; confidence interval -0002-0.192) showed significant transgressive overyielding compared to the correspondig best performing unfertilized monocultures while the three-species mixture (TO = -0.030; confidence interval -0.117-0.056) did not. At the marginal site, the binary mixture of white clover and chicory (TO = 0.139; confidence interval 0.042-0.236) as well as binary mixture of white clover and ryegrass (TO = 0.001; confidence interval -0.097-0.099) transgressively overyielded the corresponding best performing unfertilized monocultures. In contrast to the fertile site, the three species mixture of white clover-ryegrass-chicory at the marginal site was superior to the best performing unfertilized ryegrass-chicory mixture.

Among the mixtures only the binary mixtures of white clover and ryegrass yielded higher than the corresponding best performing fertilized ryegrass monoculture. The mixtures including chicory did not yield higher than the best fertilized respective monocultures.

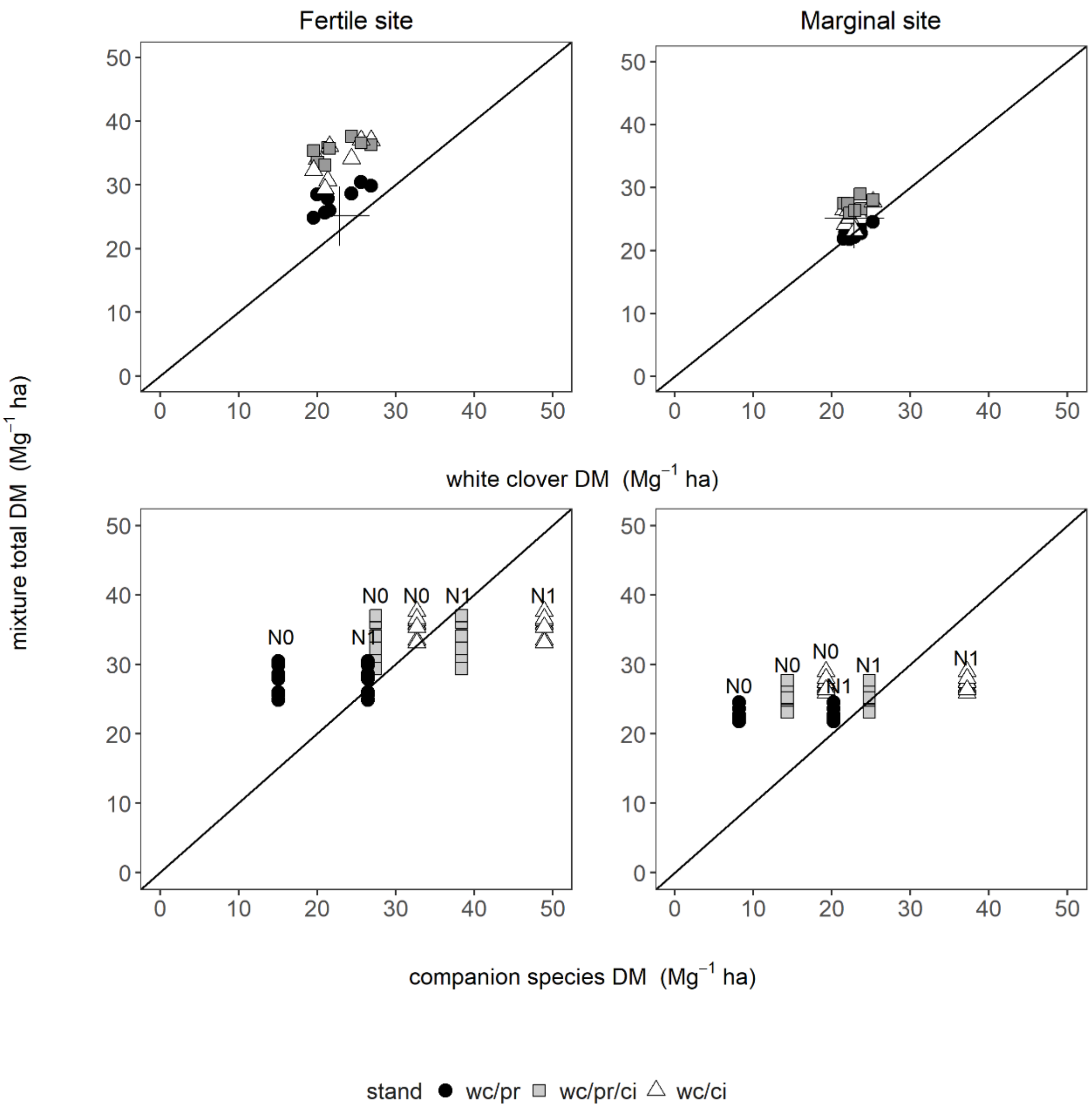

Figure 3.2: Relationship between the accumulated dry matters yields over four years of binary mixtures of white clover with perennial ryegrass (wc/pr), binary mixture of white clover with chicory (wc/ci), and three-species mixture of white clover, perennial ryegrass and chicory (wc/pr/ci) with accumulated white clover dry matter yield (upper row) and with the accumulated dry matter yield of companion species monoculture (bottom row). The companion species monocultures either did not receive any fertilizer (N0) or were fertilized with 240 kg of nitrogen fertilizer per hectar per year (N1). The crosses show the average dry matter yield of mixtures and white clover monocultures.

White clover genotype did not significantly affect the stability of dry matter yield over three years (2015-2017), but crop stand did (Table 3.1, Fig. 3.3). The inclusion of chicory in mixtures increased yield stability at both sites, while the binary mixture of white clover and ryegrass showed a lower yield stability than the corresponding white clover monoculture (Fig. 3.3).

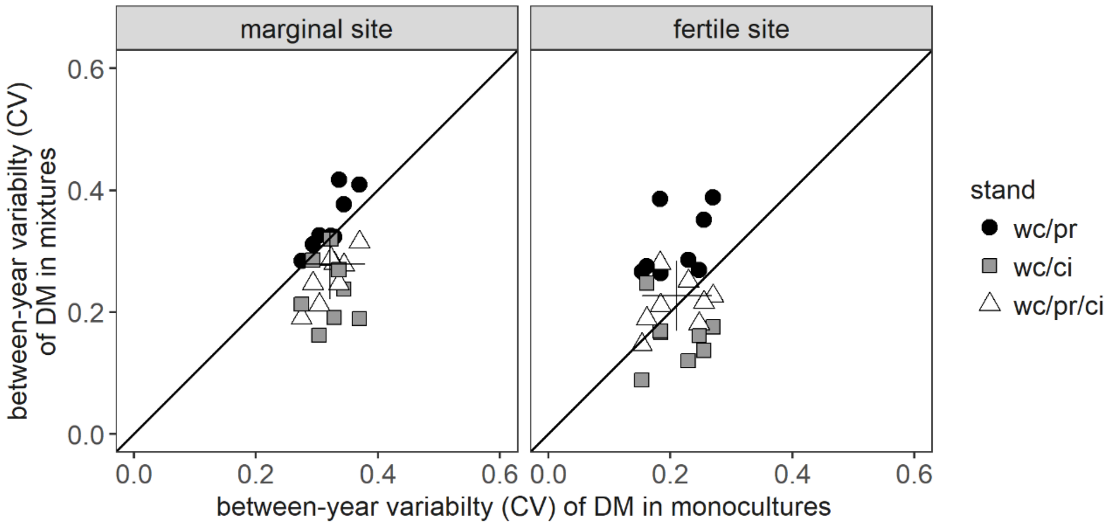

Figure 3.3: Relationship of the coefficients variation (CV) of annual dry matter yield (DM) of mixtures and monocultures of eight different white clover genotypes between years (2015-2017) at two sites. The crosses show the average of mixtures and monoculture yield variability.

3.5 Discussion

White clover genotype did not interact with the crop stands regarding dry matter yield and yield stability; the white clover genotype that performed well in monoculture also did so in mixtures. This finding contrasts with the earlier research on grass-clover mixtures which had shown that the combination of white clover and perennial ryegrass genotypes could substantially affect the performance of mixtures, suggesting genotype-dependent differences in the compatibility of the components (Collins and Rhodes, 1989; Hill and Michaelson-Yeates, 1987; Turkington et al., 1979; Lüscher et al., 1992). Collins and Rhodes (1989) assumed that compatibility between the grass and clover components was of great importance during the establishing phase of grass-clover mixtures. In our experiment, the establishment of all white clover genotypes and partner species was reasonably good and there were no large differences among partner species and white clover genotypes. Thus, the phenomenon described by Collins and Rhodes (1989) did not appear to be relevant here.

We found higher mixture benefits at the fertile than at the marginal site. This contrasts with the expectation that advantages of mixtures should be greater when resources are more limited (Brooker et al., 2015): For example, mixed-cropping can increase crop water use under water limitation because as the mixture components may occupy a larger soil space through their complementary root traits, allowing an improved access to water (Berendse, 1982; Hill, 1990; Frankow-Lindberg and Wrage-Mönnig, 2015; Brooker et al., 2015; Husse et al., 2016; Ravenek et al., 2016). Differences in soil depth between our two sites may explain the difference between our results and this expectation. The marginal site has a rather shallow soil with underlying parent rock material whereas soil depth at the fertile soil exceeds two meter. During the spring and summer months, water availability in the top soil layers is frequently limited at both sites due to the subcontinental climatic conditions. The fertile site, however, maintains higher water availability in deeper soil layers. We therefore assume that the competition for water among the different mixture components of the mixtures was much stronger at the marginal than at the fertile site, where the deep-rooted chicory was able to exploit the water that was available in the deeper soil layers. This assumption is consistent with the fact that site differences in mixture benefit were largest in mixtures that contained chicory.

Interestingly, the highest mixture benefit was found in the binary mixture of white clover and chicory and not in the three-species mixture. The mere number of components, be it species or functional groups, was not explaining higher performance in this experiment. This was unexpected as many studies over the last two decades have demonstrated a positive relationship between species or functional group number and productivity, in particular at a low level of diversity (Nyfeler et al, 2009; Tilman et al., 2001; Finn et al., 2013). Rather, in the present experiment, the identity of the partner species was more important for the mixture benefit. Mixtures containing species with more contrasting traits showed a higher advantage in productivity.

From an agronomic point of view, mixtures are most interesting when they produce higher dry matter yields than the best monocultures (i.e. transgressive overyielding; (Nyfeler et al., 2009; Temperton et al., 2007). Transgressive overyielding occurs when there is a synergetic interaction between species in mixtures, and the net effect of interspecific interactions is sufficiently strong (Cardinale et al., 2007; Kirwan et al., 2007; Kirwan et al., 2009). While overyielding is a rather common phenomenon in agricultural and non-agricultural systems, transgessive overyieldig is rather rare (Cardinale et al. 2007). The probability of transgressive overyielding of mixtures, moreover, appears to increase over time, and only became evident after about five years in the meta-analysis of Cardinale et al. (2007). In contrast to this, Nyefeler et al. (2009) found a high level of transgressive overyielding at the Swiss site of a continent-wide mixture experiment (Kirwan et al. 2007). Which was already significant in the first year of the experiment. In our study, one of the three mixtures at each site had higher yields than the most productive corresponding unfertilized monoculture during the first four years after mixture establishment. This rather high incidence of transgressive overyielding can probably be attributed to the deliberate selection of species for our mixtures, as white clover, perennial ryegrass and chicory are among the most productive species for forage production in temperate-humid grassland systems.

Yield stability, which is also of agronomic importance, was highest in mixtures including chicory and lowest in the binary mixture of white clover and ryegrass. Previous studies reported inconsistent effects of mixed cropping on yield stability. Tilman et al. (2006) found a positive correlation between species number in mixture and stability of yield. On the other hand, Sanderson (2010) reported only weak or no relationships between interannual yield stability and the number of forage species grown in mixtures. Küchenmeister et al. (2012) demonstrated that an increasing contribution of forbs (*Plantago lanceolata* L., *Taraxacum officinale* L.) to the mixture yield even decreased yield stability. The presence of chicory in mixtures substantially increased the between-year yield stability in our study. This benefit may be closely related to the divergent rooting systems of the shallow-rooted grass and white clover on the one hand and the deep-rooted chicory on the other. Therefore, mixtures including chicory were able to exploit the water that was available in the deeper soil layers and produce a more stabe yield.

3.6 Conclusion

The results of this study showed that white clover genotypes did not perform differently in monocultures and mixtures. Although we found no genotype × crop stand interaction, it should not be concluded that there is no scope for breeding to specifically improve the performance of forage mixtures. Our findings are based on a limited number of white clover genotypes and only two partner species and should be verified by a larger range of clover genotypes as well as partner species and varieties. Independent of clover genotype, mixtures clearly showed an advantage over the corresponding monocultures with higher yield production and yield stability. However, these benefits were more related to the species identity than to the number of species in the mixture. Strong effects of the site, i.e., the environmental conditions, on performance of forage species mixtures indicated the need for considering site effects and resource availability in developing productive forage mixtures.

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3.8 References

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

Berendse, F. 1982. Competition between plant-populations with different rooting depths. III. Field experiments. Oecol. 53:50-55, DOI: 10.1007/BF00377135.

Brink GE, Sanderson MA, Casler MD (2015) Grass and legume effect on nutritive value of complex forage mixtures. *Crop Sci* 55: 1329-1337. DOI: 10.2135/cropsci2014.09.0666

Brooker RW, Bennett AE, Cong WF, Daniell TJ, George TS, Hallett PD, Hawes C, Iannetta PPM, Jones, HG, Karley AJ, Li L, McKenzie BM, Pakeman RJ, Paterson E, Schöb C, Shen J, Squire G, Watson CA, Zhang C, Zhang F, Zhang J, White PJ (2015) Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytol* 206: 107. DOI: 10.1111/nph.13132

Caldwell MM (1987) Plant architecture and resource competition. In: E.D. Schulze and H. Zwölfer (Editors), Ecological studies 61. Springer-Verlag, Berlin, pp. 164-179. DOI: 10.1007/978-3-642-71630-0_8

Cardinale BJ, Wright JP, Cadotte MW, Carroll IT, Hector A, Sirvastava DS, Loreau M, Weis JJ (2007) Impacts of plant diversity on biomass production increase through time because of species complementarity. *PNAS* 104(46): 18123– 18128. DOI: 10.1073/pnas.0709069104

Collins RP, Rhodes I (1989) Yield of white clover populations in mixture with contrasting perennial ryegrasses. *Grass Forage Sci* 44:111-115. DOI: 10.1111/j.1365-2494.1989.tb01918.x

Cong WF, Jing J, Rasmussen J, Søegaard K, Eriksen J (2017) Forbs enhance productivity of unfertilised grass-clover leys and support low-carbon bioenergy. *Sci Rep* 7,1422. DOI: 10.1038/s41598-017-01632-4

Dhamala NR, VJ, Carlsson G, Søegaard K, Eriksen J (2017) N transfer in three species grass-clover mixtures with chicory, ribwort plantain or caraway. *Plant Soil* 413: 217. DOI: 10.1007/s11104-016-3088-6

Dodd MB, Sheath GW, Wedderburn ME, Tarbotton IS (2001) Long term performance of white clover cultivars oversown into summer-dry hill country. Proceedings of the New Zealand Grassland Association 63:85-89.

Ergon Å, Kirwan L, Bleken MA, Skjelvåg AO, Collins RP, Rognli OA (2016) Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies. 1. dry-matter yield and dynamics of species composition. *Grass Forage Sci* 71 (4): 667-682. DOI: 10.1111/gfs.12250

Elgersma A, Schlepers H (1997) Performance of white clover/perennial ryegrass mixtures under cutting. *Grass Forage Sci* 52: 134-146. DOI: 10.1111/j.1365-2494.1997.tb02344.x

Evans DR, Williams TA (1987) The effect of cutting and grazing managements on dry matter yield of white clover varieties (Trifolium repens) when grown with S23 perennial ryegrass. *Grass Forage Sci* 42: 153-159. DOI: 10.1111/j.1365-2494.1987.tb02102.x

Finn JA, Kirwan L, Connolly J, Sebastià MT, Helgadottir A, Baadshaug OH, Bélanger G, Black A, Brophy C, Collins RP, Čop J, Dalmannsdóttir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Ghesquiere A, Golinska B, Golinski P, Grieu P, Gustavsson AM, Höglind M, Huguenin-Elie O, Jørgensen M, Kadziuliene Z, Kurki P, Llurba R, Lunnan T, Porqueddu C, Suter M, Thumm U, Lüscher A (2013) Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. *J Appl Ecol* 50: 365-375. DOI: 10.1111/1365-2664.12041

Frankow-Lindberg BE, Wrage-Mönnig N (2015) Light availability is improved for legume species grown in moderately N-fertilized mixtures with non-legumes species. *Basic Appl. Ecol* 16, 403-412. DOI: 10.1016/j.baae.2015.04.007

Frankow-Lindberg BE, Brophy C, Collins RP, Connolly J (2009) Biodiversity effects on yield and unsown species invasion in a temperate forage ecosystem. *Annals of Botany* 103(6): 913–921. DOI: 10.1093/aob/mcp008

Hill J (1990) The three C's- competition, coexistence and coevolution- and their impact on the breeding of forage crop mixtures. *Theor. Appl Genet* 79, 168-176. DOI: 10.1007/BF00225947

Hill J, Michaelson-Yeates TP (1987) Effects of Competition upon the Productivity of White Clover-Perennial Ryegrass Mixtures. Genetic Analysis. *Plant Breed* 99: 239-250. DOI: 10.1111/j.1439-0523.1987.tb01110.x

Hoekstra NJ, Finn JA, Hofer D, Lüscher A (2014) The effect of drought and interspecific interactions on depth of water uptake in deep-and shallow-rooting grassland species as determined by δ18O natural abundance. Biogeosciences 11: 4493-4506. DOI: 10.5194/bg-11-4493-2014

Hooper DU (1998) The Role of Complementarity and Competition in Ecosystem Responses to Variation in Plant Diversity. *Ecology* 79: 704-719. DOI: 10.1890/0012-9658(1998)079[0704:TROCAC]2.0.CO;2

Høgh-Jensen H, Nielsen B, Thamsborg SM (2005) Productivity and quality, competition and facilitation of chicory in ryegrass/legume-based pastures under various nitrogen supply level. *Eur J Agron* 24(3): 247-256. DOI:10.1016/j.eja.2005.10.007

Husse S, Huguenin-Elie O, Buchmann N, Lüscher A (2016) Larger yields of mixtures than monocultures of cultivated grassland species match with asynchrony in shoot growth among species but not with increased light interception. *Field Crops Res* 194: 1-11. DOI: 10.1016/j.fcr.2016.04.021

Küchenmeister F, Küchenmeister K, Wrage N, Kayser M, Isselstein J (2012) Yield and yield stability in mixtures of productive grassland species: Does species number or functional group composition matter? *GRASSL SCI* 58: 94-100. DOI: 10.1111/j.1744-697X.2012.00242.x

Litrico I, Violle C (2015) Diversity in plant breeding: A new conceptual framework. *Trends plant Sci* 20 (10): 604-613. DOI: 10.1016/j.tplants.2015.07.007

Lenth R (2017) emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.0. https://CRAN.R-project.org/package=emmeans

Lüscher A, Mueller-Harvey I, Soussana JF, Rees RM, Peyraud JL (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass Forage Sci* 69: 206-228. DOI: 10.1111/gfs.12124

Lüscher A, Connolly, J, and Pierre Jacquard (1992) Neighbour specificity between Lolium perenne and Trifolium repens from a natural pasture. *Oecol* 91: 404-409. DOI: 10.1007/BF00317630

Nyfeler D, Huguenin- Elie O, Suter M, Frossard E, Connolly J. Lüscher A (2009) Strong mixture effects among four species in fertilized agricultural grassland led to persistent and consistent transgressive overyielding. *J Appl Ecol* 46: 683–691. DOI: 10.1111/j.1365-2664.2009.01653.x

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2017) nlme: Linear and Nonlinear Mixed Effects Models. R package version, 3,1-128, url: http://CRAN.R444. project.org/package=nlme.

Ravenek, J.M., L. Mommer, E.J.W. Visser, J. Ruijven, J.W. van der Paauw, A. Smit-Tiekstra, H. de Caluwe, and H. de Kroon. 2016. Linking root traits and competitive success in grassland species. Plant Soil. 407:39-53, https://doi.org/10.1007/s11104-016-2843-z

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rhodes I (1970) The production of contrasting populations of perennial ryegrass (Lolium perenne L.) in monocultures and mixed cultures of varying complexity. *Grass Forage Sci* 25:285-288. DOI: 10.1111/j.1365-2494.1970.tb01205.x

Sanderson MA, Elwinger GF (2002) Plant Density and Environment Effects on Orchardgrass–White Clover Mixtures. *Crop Sci* 42:2055–2063. DOI:10.2135/cropsci2002.2055

Sanderson MA (2010) Stability of production and plant species diversity in managed grasslands: A retrospective study. *Basic Appl Ecol* 11(3): 216-224. DOI: 10.1016/j.baae.2009.08.002

Spehn E, Joshi J, Schmid B, Diemer M, Korner C (2000) Above-Ground Resource Use Increases with Plant Species Richness in Experimental Grassland Ecosystems. *Funct Ecol* 14(3): 326-337. DOI: 10.1046/j.1365-2435.2000.00437.x

Tilman D, Reich PB, Knops J (2006) Biodiversity and ecosystem stability in a decade-long grassland experiment. *Nature* 441: 629-632. DOI: 10.1038/nature04742

Tilman D, Reich PB, Knops J., Wedin D, Mielke T, Lehman (2001) Diversity and Productivity in a Long-Term Grassland Experiment. *Science* 843-845. DOI: 10.1126/science.1060391

Turkington R, Cahn M, Vardy A, and Harper J (1979) The Growth, Distribution and Neighbour Relationships of Trifolium Repens in a Permanent Pasture: III. The Establishment and Growth of Trifolium Repens in Natural and Perturbed Sites. *J Ecol* 67(1): 231-243. DOI: 10.2307/2259347

Chapter IV: White clover genotype affects the forage quality of mixtures with perennial ryegrass and chicory

4.1 Abstract

Grass-legume mixtures often improve the forage nutritive value compared to grass monocultures. Adding more species to grass-clover mixtures might further improve the forage quality. Little is known about the role of the species genotype on the forage quality of mixtures. We established a field experiment to investigate how and to what extent different white clover (*Trifolium repens* L.) genotypes affect the forage nutritive value of different mixtures. Eight novel genotypes of white clover were grown as monocultures, as binary and three-species mixtures with *Lolium perenne* L. and *Cichorium intybus* L. at two sites differing in resource availability. Above-ground herbage was cut twice in the establishment year and four times in each of the following years. Samples were dried and scanned with near-infrared spectroscopy to determine the forage nutritive value. White clover genotypes significantly affected the forage nutritive value. However, the effect of white clvoer genotypes was inferior to the effect of partner species. The range of variation among white clover genotypes regarding all nutritive parameters was higher in white clover monocultures and it got narrower in mixtures. White clover monocultures had a higher concentration of crude protein (CP) and lower concentration of acid detergent fibre (ADF) compared to the mixtures. Forage nutritive value of the crop stands was dependent on the site condition and year.

Keywords: White clover, Monoculture, Mixed stands, Nutritive value, Crude protein, Fibre

4.2 Introduction

Forage nutritive value is defined as the ability of forage to support animal nutrition requirements which is mainly determined by crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and water-soluble carbohydrate (WSC) (Kilcher 1981). Acid detergent fiber (ADF) consists of cellulose and lignin the cell wall and thus limits the digestibility of the whole plant. Forage with a lower value of ADF has higher energy content. Water-soluble carbohydrates (WSC) provide the most readily available source of energy for ruminants and affect forage intake; high WSC concentrations increase both forage intake and the efficiency of protein utilization (Jones and Roberts 1991).

There are marked differences between plant species in inherent nutritional composition. For instance, legumes have higher CP and lower fibre and WSC concentrations than grasses (Brink et al. 2015; Lüscher et al. 2014). Several factors may affect the forage nutritional composition, such as weather conditions (Fulkerson et al. 1998; Smith et al. 1998), soil fertility (Fulkerson et al. 2007), plant functional group (Ergon et al, 2016; Sleugh et al. 2000; Zemenchik et al. 2002) and plant genotype (Casler 1999; Calser 2001). In nutrient-limited grassland systems, grass-legume mixtures often provide greater profitability and improve forage quality compared to grass monoculture (Sturludottir et.al. 2014; Sleugh et al. 2000; Zemenchik et al. 2002). Adding legumes to the mixture of non-legume species showed to have a similar effect on the forage nutritive value as N fertilization; increasing CP while reducing ADF (Buxton 1996: Ergon et al. 2016). This benefit is attributed to the ability of legumes to improve the soil N pool by fixing atmospheric nitrogen and to their high protein content per se (Sleugh et al. 2000; Lüscher et al. 2014). Grass-clover mixtures are widely used in grassland farming because of their many advantages, e.g. higher yields and higher forage quality compared to monocultures. However, they have some limitations: for instance, grass-clover mixtures have higher early summer CP and lower fibre contents than the level required by grazing animals (Høgh-Jensen et al. 2006). Moreover, under intensive management conditions white clover may be almost lost in the system and only the grass component will remain (Høgh-Jensen et al. 2006). Adding a third species to grass-clover mixtures may help to overcome these limitations. Such a species should reduce the competitiveness of the grass, but not of the clover component, and be nutritionally complementary to white clover, e.g. by having its highest fibre content and lowest CP content in early summer. Chicory (*Cichorium intybus* L.) is a perennial deep rooting broad-leafed forage herb with high productivity and forage quality (Rumball 1986). Adding chicory in grass-clover mixture may have a potential to improve the forage nutritive value of mixtures. In this context, selecting plant species or genotypes with more compatible functional traits improve the complementarity among species in the mixture (e.g., Sleugh et al. 2000; Zemenchik et al. 2002). Breeding of the grass species showed an improvement of forage nutritive value (Casler 1999). Genetic variability within species may affect the interspecific interaction in mixture by optimizing plant traits (Casler 1999; Lüscher et al. 2014). Breeding of forage species is almost exclusively conducted in monocultures even though they are often grown in mixtures. So plant species genotype performance in monoculture might be different than inmixtures (Williams et al. 2003; Evans et al. 1995). It has been shown that the improved plant genotype will usually have increased forage quality under most environmental or management conditions to which it is subjected (Casler 1999). No study till to date evaluated the effect of the white clover genotype on the forage nutritive value of mixtures with different partner species.

In the present study, we aimed to assess the effect of white clover genotype on forage nutritive value of different mixtures under different environmental condition. Eight novel genotypes of white clover were cultivated as monocultures in binary mixture with either perennial ryegrass or chicory and in a multi-species mixture with ryegrass and chicory at two different sites differing in soil characteristics and soil depth. We hypothesized that: (i) white clover (*Trifolium repenes*) genotypes vary in forage nutritive value and that (ii) the forage nutritive value of mixtures with different non-legume companion species is affected by the white clover genotype, both because white clover genotypes differ in forage quality and because the proportion of white clover in mixtures is genotype-dependent. We also expected that (iii) the mixture forage nutritive value differs between the two sites since we expect that the fertile site would have a smaller proportion of white clover in the mixture than the marginal site.

4.3 Material and methods

4.3.1 Study area

The present study was part of the IMPAC³ experiment and was carried out during 2014-2017 on two experimental stations of the Georg-August-University Göttingen, Germany. The two sites were noticeably different regarding soil depth, texture, and precipitation. The rather fertile site (51.29 N, 9.55 E) had a low elevation 157 m a.s.l. and the marginal site (51.34 N, 9.58 E) was located at a higher elevation of 342 m a.s.l. The soil type according to the FAO classification system on fertile site was a Gleyic Fluvisol (Ah horizon of 0- 30 cm, 21% of clay, 11% sand, 68% silt) and the soil pH was 7. The soil type on the marginal site was a Calcaric Leptosol and had a shallower Ah horizon of 25 cm depth. Clay content was higher on the marginal site than on the fertile site (34% clay, 2% sand and 55% silt) and the soil pH was 7.5. The monthly average temperature and rainfall per experimental year are given in Appendix (Figure A1 Deutscher Wetterdienst).

4.3.2 Experimental design

In July 2014, eight novel genotypes of white clover (*Trifolium repens* L.), one variety of perennial ryegrass (*Lolium perenne* L., ELP 060687) and one variety of chicory (*Cichorium intybus* L., Puna II) were sown as monoculture, binary mixture of white clover either with perennial ryegrass or chicory (0.4:0.6), binary mixture of ryegrass and chicory (0.5:0.5) and as three-species mixtures of white clover with ryegrass and chicory (0.4:0.3:0.3). The white clover genotypes differed in flowering time, flower intensity and leaf size. The seed rate of each species in a mixture was calculated based on commonly used seed rates in monocultures (1000 seed per m^2). The experimental layout followed a split-plot design with four replications at both sites. In each block, experimental treatments were arranged with five plots, each nested within eight main plots corresponding to the white clover genotypes. In total, each site consisted of 160 plots (5 m length x 3 m width). The between plot spacing was 0.2 m. Plots were regularly treated with glyphosate to prevent the spread of white clover stolon to the other plots. Mixtures including white clover and the white clover monoculture did not receive any fertilizer. Two levels of fertilization, 0 and 240 kg total N ha yr^{-1} (NH_4NO_3+$CaCO_3$) were applied for perennial ryegrass and chicory monocultures and also for mixtures of those two species. As soil phosphorus and potassium contents at both sites were sufficient, no phosphorus and potassium fertilizers were applied. The result presented in this paper focuses on white clover monocultures and mixtures including white clover. All plots were cut twice during the establishment year (2014) and four times per year for the following years (2015-2017). Harvest dates are shown in Table 4.1.

Table 4.1: Harvest dates in the four experimental years. Two sites: fertile (F), marginal (M).

	2014		2015		2016		2017	
Harvest	F	M	F	M	F	M	F	M
1	-	-	11/05	19/05	17/05	23/05	8/05	16/05
2	-	-	01/07	07/07	22/06	28/06	19/06	27/06
3	12/08	12/08	20/08	24/08	08/08	16/08	7/08	14/08
4	14/10	14/10	05/10	13/10	04/10	11/10	26/09	09/10

All plots were cut to a height of 5 cm with a combine harvester (Wintersteiger hd 1500). After each harvest, the biomass from each plot was blended, and a sample of approximately 250 g was taken and dried in the oven at 60° C for 48 hours. Dried samples were grounder to pass through a 1-mm sieve and scanned with a NIR spectrophotometer to determine the concentration of CP, ADF and WSC.

4.3.3 Statistical analysis

The weighted average of the concentration of CP, ADF and WSC over four harvests for each experimental year was calculated:

$$\sum_{i=1}^{h}\left(concentration_i \times \frac{dry\ matter\ yield\ _i}{dry\ matter\ yield\ _{total}}\right)$$

Where *i* is the harvest number, and *h* is the total number of harvest per year. Out of 2560 samples, 15 samples from the last harvest of the marginal site were too small to determine the forage nutritive value and were treated as missing values in the statistical analysis. Statistical analyses of the forage nutritive factors were performed with R version 3.4.2 (R Core Team 2017). All target variables were analyzed by applying linear mixed model (lme) using the software package “nlme” (Pinheiro et al. 2017), including block, main plot within the block, and plot as a random effect, as well as the white clover genotype, crop stand, site, year and their interactions as fixed effects. All models were visually checked for homogeneity of variance and normal distribution of residuals. Because models for ADF and WSC concentrations were unevenly distributed, they were corrected by including an appropriate variance structure. “VarIdent” variance structure was incorporated into the models for both factors to provide better distribution. The most fitted model was simplified using the second Akaike Information Criterion (AICc) to obtain an optimal model that fits the data with an AICc as low as possible. Afterwards, means of the most influential variables and their interactions were compared in the final model using post-hoc pairwise contrasts and tested for significance with the LSD test as implemented in the package “emmeans” (Lenth, 2016). A significance level of $p < 0.05$ for all variables was selected.

4.4 Results:

4.4.1 Forage nutritive value:

The results showed that the white clover genotype, the crop stand as well as their interaction significantly affected the concentration of CP, ADF and WSC (Table 4.2). The range of variation among white clover genotypes was wider in white clover monocultures, and it got narrower in binary mixtures with either ryegrass or chicory and in multi species mixture (Table 4.3). Even though the interaction of white clover genotype x stand significantly affected all nutritive factors, the white clover genotype with an increased forage value in the monoculture also did the same in all mixtures (Table 4.3). The white clover genotype with the highest CP concentration had a lower concentration of ADF both in white clover monocultures and mixtures (wc7).

Table 4.2: *F* and *P-values* to test the effect of white clover genotype, stand, site and year as the main effect and their interaction on the weighted average of the concentration CP, ADF and WSC. Significance level at $P<0.05$.

Factor	CP		ADF		WSC	
	F- value	*P- value*	*F- value*	*P- value*	*F- value*	*P- value*
Genotype	18.1	<0.0001***	12.6	<0.0001***	4.6	0.0001***
Stand	1710.0	<0.0001***	465.5	<0.0001***	1563.8	<0.0001***
Site	0.2	0.6666^{ns}	12.1	0.0130*	165.8	<0.0001***
Year	716.6	<0.0001***	205.7	<0.0001***	4.5	<0.0001***
Genotype: Stand	2.5	0.0007***	2.6	0.0002***	3.1	0.0001***
Stand: Site	130.1	<0.0001***	71.7	<0.0001***	60.0	<0.0001***
Stand: Year	77.9	<0.0001***	93.4	<0.0001***	77.2	<0.0001***
Site: Year	105.8	<0.0001***	37.8	<0.0001***	27.7	<0.0001***
Stand: Site: Year	19.2	<0.0001***	8.6	0.0001***	38.9	<0.0001***

Table 4.3: The concentrations of CP, ADF and WSC in aboveground biomass of different white clover genotypes averaged over all crop stands, years (2015-2017) and sites (marginal and fertile site). Crop stands were binary mixtures of white clover with either ryegrass (wc/pr) or chicory (wc/ci) and three species mixture (wc/pr/ci). Small letters indicate significant differences between white clover genotypes means over sites and years in the same column (crop stand). Capital letters show significant differences between stands means over sites and years in the same row (white clover genotype) ($p < 0.0001$).

	CP				ADF				WSC			
Genotype	wc	wc/pr	wc/ci	wc/pr/ci	wc	wc/pr	wc/ci	wc/pr/ci	wc	wc/pr	wc/ci	wc/pr/ci
wc 1	21.5 ab A	14.8 bc B	14.6 cd B	13.5 bc C	28.3 c D	29.4 bc C	32.5 a A	31.6 ab B	7.5 cd C	12.1 abc A	5.6 c D	8.5 ab B
wc 2	21.5 ab A	15.7 a B	15.2 bc BC	14.7 ab C	28.0 cd C	29.1 c B	31.5 bc A	31.0 b A	7.9 ab C	11.5 cd A	6.1 b D	8.4 ab B
wc 3	20.0 d A	14.3 c B	14.5 d B	13.6 bc C	29.0 b C	29.4 bc C	32.1 ab A	31.4 ab B	7.8 bcd C	12.6 a A	6.1 b D	8.4 ab B
wc 4	21.7 a A	14.9 b B	14.6 cd BC	14.1 bc C	27.6 de D	29.1 c C	32.3 a A	31.5 ab B	8.2 a B	12.4 ab A	5.9 bc C	8.4 ab B
wc 5	21.0 b A	15.5 a B	15.5 b B	14.7 ab C	28.2 cd C	29.0 c B	31.7 b A	31.4 ab A	7.8 bcd B	12.0 abc A	6.1 ab C	8.1 b B
wc 6	20.3 cd A	14.3 c B	14.4 d B	13.9 cd B	29.7 a C	30.2 a C	32.4 a A	31.7 a B	6.9 d C	11.8 cd A	5.8 bc D	8.2 ab B
wc 7	22.1 a A	16.0 a B	16.3 a B	14.9 a C	27.3 e C	29.0 c B	31.0 c A	31.0 b A	7.9 abc C	11.3 d A	6.5 a D	8.8 a B
wc 8	20.9 bc A	14.7 bc B	14.3 d B	13.3 c C	29.1 b D	29.8 ab C	32.5 a A	31.9 a B	7.5 d C	11.8 bcd A	5.9 bc D	8.5 ab B

All nutritive parameters were significantly affected by the interaction of stand x site x year (Table 4.2). In Table 4 the interaction of stand x site is showen for each year separately. The white clover monocultures had a significantly higher concentration of CP (20-24%) and a lower concentration of ADF (26-32%) than the other mixtures at both site in all years (Table 4.4). In all experimental years, white clover monocultures had a notably higher concentration of CP and lower concentration of ADF than the mixtures on both sites. In the first and last year of the experiment, the binary mixture of white clover and ryegrass had a a higher concentration of CP and a lower concentration of ADF than the binary mixture of white clover and chicory and the three species mixture, on both sites (Table 4.4). The presence of chicory in the mixture was more likely to be the reason for increasing the concentration of ADF. The pattern of differences between crop stands in the concentration of CP, ADF and WSC were changed in each year for each site. Regarding the WSC concentration, the presence of ryegrass in mixture showed a rise in WSC concentration. The binary mixture of white clover with ryegrass had the highest WSC concentration among all crop stands (10-16% at the fertile site and 10 to 12% at the marginal site). Including chicory in the three species mixture reduced the concentration of WSC compared to the binary mixture of white clover and ryegrass. This was the same at both sites and in all experimental years. The site condition showed a significant main effect on the concentrations of ADF and WSC, however it had no effect on concentration of CP.

Table 4.4: Concentration of CP, ADFom and WSC in aboveground biomass averaged over eight white clover genotypes on two different sites (fertile and marginal) and three experimental years (2015-2017) for white clover monoculture (wc), binary mixture of white clover with perennial ryegrass (wc/pr), binary mixture of white clover with chicory (wc/ci) and three-species mixture of white clover with perennial ryegrass and chicory (wc/pr/ci). Letters indicate significant differences between values in the same column (year) within each level of comparison ($p < 0.0001$).

Site	Crop stand	CP			ADF			WSC		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Fertile	wc	22.7 a	21.4 a	23.32 a	26.5 d	28.4 e	26.8 e	8.3 c	7.7 d	7.5 cd
	wc/pr	12.4 e	14.1 ef	18.40 c	28.4 c	29.9 d	28.0 d	16.0 a	12.3 a	10.0 a
	wc/ci	11.4 f	15.4 cd	16.1 de	34.9 a	30.9 ab	31.1 a	5.9 d	5.4 f	6.3 e
	wc/pr/ci	10.4 g	14.3 ef	16.5 de	34.6 a	30.2 cd	29.7 bc	8.0 c	8.8 c	7.8 c
Marginal	wc	20.3 b	17.9 b	21.2 b	28.7 c	31.5 ab	28.5 d	8.2 c	7.6 d	6.9 d
	wc/pr	15.0 c	13.7 f	16.6 d	29.2 c	31.7 a	29.1 c	12.2 b	10.9 b	10.3 a
	wc/ci	13.9 d	15.7 c	16.9 d	32.9 b	31.0 bc	31.2 a	6.3 d	6.7 e	5.6 f
	wc/pr/ci	13.3 e	14.3 de	15.7 e	32.5 b	31.5 ab	30.2 b	8.2 c	8.8 c	8.9 b

4.4.2 White clover content

The proportion of white clover was clearly different among crop stands between two sites in each year (*P= 0.0001*). In the first year of the experiment, all mixtures at the marginal site contained a higher percentage of white clover than the same mixtures at the fertile site. However, this difference between sites was not consistent during the experimental years, and it appeared to be reduced over the time. In all experimental years, the grass-clover mixtures contained a higher percentage of white clover than the binary mixtures of white clover and chicory and the three-species mixtures of white clover with ryegrass and chicory on both sites (Figure 4.1). There was a significant difference among white clover genotypes in contribution to yield production (*P= <0.0001*). There was no significant interaction of white clover genotype x stand on white clover content of mixtures. The difference among white clover genotypes might be related to the yield potential of the partner species in the mixture.

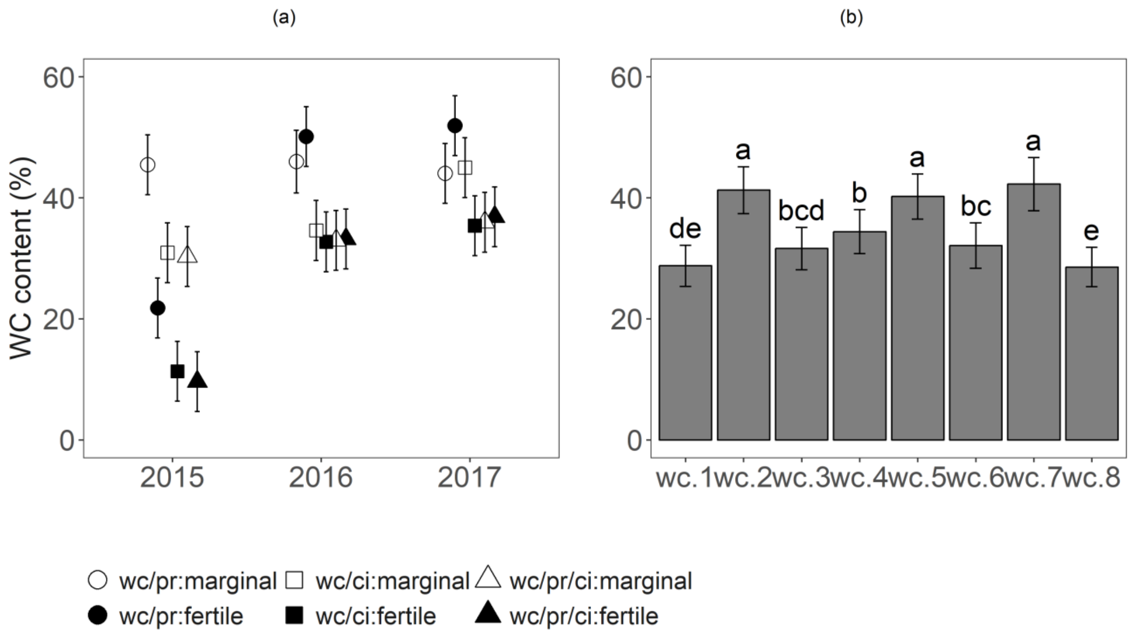

Figure 4.1: The white clover percentage (a) in a binary mixture of white clover with perennial ryegrass (wc/pr), binary mixture of white clover with chicory (wc/ci) and three-species mixture of white clover with both perennial ryegrass and chicory (wc/pr/ci) averaged over white clover genotypes in three experimental years (201-2017), on two different sites (fertile and marginal), (b) of eight white clover genotypes averaged over crop stands, sites and years.

4.5 Discussion

When grown in monoculture white clover genotypes had significantly different concentrations of CP, ADF, and WSC, confirming our first hypothesis. This result is in line with earlier studies that found a significant effect of species genotype on forage nutritional composition of other forage species (Casler 1999; Calser 2001; Smith et al. 2005). Studies of the plant species genotype effect on forage quality are extremely rare for white clover, as the focus has mostly been on traits that enhance persistence and competitiveness of white clover (Annicchiarico and Proietti, 2010; Frankow-Lindberg et al. 2009).

The interaction of white clover genotype x stand significantly affected all nutritive parameters. In our study, the forage nutritive value varied more among crop stands than among the white clover genotypes. Compared to the genotype differences in nutritive value we found in white clover monocultures, the range of variation between white clover genotypes got narrower in mixtures, but remained significant, confirming our second hypothesis.

While our results suggested that the white clover genotype influenced the forage nutritive value of mixtures, it was more strongly determined by the partner species in the mixture. White clover monocultures had notably higher concentrations of CP and lower concentrations of ADF compared to all mixtures. The high concentration of CP in white clover monocultures is probably due to the inherently high protein content of legumes. The lack of a stem fraction of white clover may be the reason for low ADF concentrations of white clover monocultures compared to the mixtures (Brink et al., 1994). The presence of chicory in mixtures increased the ADF concentration which showed the substantial contribution of stem material in altering the nutritional composition of forage. Plant species with erect forms will tend to have a higher concentration of ADF because of their need for stronger structures (Vreugdenhil, 2017). The binary mixture of white clover and perennial ryegrass had the highest concentration of WSC. Presence of perennial ryegrass in the binary mixture of white clover and chicory was likely to be the factor increasing the WSC in the mixture. This could be attributed to the high WSC concentrations commonly found in perennial ryegrass (Van Rossum 2013). Our results thus showed that the identity of companion species had a substantial effect on the forage nutritive value, which is in line with earlier studies (Sanderson, 2010; Zemenchik et al. 2002).

There was no significant interaction of a white clover genotype neither with a site nor with a year that showed the performance of white clover genotype to be independent of site condition and year. The site condition per se did not affect the concentration of CP, while it had a significant effect on the concentration of ADF and WSC. The interaction of stand x site x year interactively affected the forage nutritive value.

Previous results regarding the effect of legume content on the forage nutritive value are inconsistent. While several studies found a positive effect of clover content on the CP content at low levels of N input (Sturludóttir et al., 2013; Elgersma et al., 2018), Jing et al. (2017) found no relationship between the proportion of legume in a mixture and CP concentration. In our experiment white clover coexisted well with the non-legume partner species. The proportion of white clover was higher in the binary mixture with ryegrass compared to that with chicory and the three-species mixture. Chicory is a perennial herb with broad erect leaves that can shade white clover, which has a horizontal stem at the ground level. This might lead to a lower percentage of white clover in the mixture. Observed differences in the white clover proportion of mixtures can help to explain the crop-stand–specific site and year effects on forage nutritional value: In 2015, when mixtures at the marginal site had higher proportions of white clover than those at the fertile site, their CP concentrations were also higher and their ADF concentrations were lower compared to the fertile site. In 2017, when the proportion of clover in the binary mixture with ryegrass was lower at the marginal than at the fertile site, CP concentrations likewise were lower and ADF concentrations were higher than at the fertile site. Due to these annual variations, we cannot confirm our third hypothesis.

Both the differences in forage nutritive value between the white clover genotypes and the genotype-specific white clover proportion in mixtures contributed to the white clover genotype effect on mixture nutritive value: for example, genotype wc7 was characterized by high proportions in mixtures and high CP concentrations together with low ADFom concentrations in both monoculture and mixtures. By contrast, genotype wc3 combined low CP concentrations and high ADF concentrations with comparatively low white clover proportions in mixtures, resulting in an unfavorable forage nutritive value of mixtures.

4.6 Conclusion

The main result of this study was that the white clover genotype influences the forage nutritive value of different mixtures. However, this effect was of minor importance compared to the effect of the botanical composition of the mixture. We found that the effect of thewhite clover genotype on forage nutritive value was diminished with increasing the complexity of mixture. The resource availability affected the nutritive value of mixtures via influencing the share of species in the mixture.

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4.8 References

Annicchiarico P, Proietti S (2010) White clover selected for enhanced competitive ability widens the compatibility with grasses and favours the optimization of legume content and forage yield in mown clover-grass mixtures. *Grass Forage Sci* 65: 318.

Brink G, Sanderson MA, and Casler MD (2015). Grass and legume effects on nutritive value of complex forage mixtures. *Crop Science* 55: 1329- 1337.

Buxton DR (1996) Quality-related characteristics of forages as influenced by plant environment and agronomic factors. *Anim Feed Sci Technol* 59: 37-49.

Casler MD (1999) Accomplishments and impact from breeding for increased forage nutritional value. *Crp Sci* 39: 12-20.

Casler MD (2001) Breeding forage crops for increased nutritional value. *ADV AGRON* 71: 51-107.

Elgersma A, Søegaard K (2018) Changes in nutritive value and herbage yield during extended growth intervals in grass–legume mixtures–effects of species, maturity at harvest, and relationships between productivity and components of feed quality. *Grass Forage Sci* 73:78–93.

Ergon A, Kirwan L, Fystro G, Bleken MA, Collins RP, and Rognli OA (2017) Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies. II. Nutritional quality. *Grass Forage Sci* 72, 333–342.

Evans DR, Williams TA, Jones S, and Evans SA (1995) The effect of blending white clover varieties and their contribution to a mixed grass/clover sward under continuous sheep stocking. *Grass Forage Sci* 50: 10-15. doi:10.1111/j.1365-2494.1995.tb02288.x

Frankow-Lindberg BE, Halling M, Höglind M, Forkman, J (2009) Yield and stability of yield of single- and multi-clover grass-clover swards in two contrasting temperate environments. *Grass Forage Sci* 64: 236-245.

Fulkerson WJ, Slack K, Hennessy DW, and Hough GM (1998) .Nutrients in ryegrass (*Lolium* spp), white clover (*Trifolium repens*) and kikuyu (*Pennisetum clandestinum*) pastures in relation to season and stage of re-growth in subtropical environment. *Aust J Exp Agric* 38: 227–240.

Fulkerson WJ, Neal JS, Clark CF, Horadagoda A, Nandra KS, and Barchia I (2007) Nutritive value of forage species grown in the warm temperate climate of Australia for dairy cows: Grasses and legumes. *Livest Sci* 107, 253-264

Høgh-Jensen H, Nielsen B, and Thamsborg SM (2006) Productivity and quality, competition and facilitation of chicory in ryegrass/ legume-based pastures under various nitrogen supply levels. *Eur J Agron* 24: 247-256.

Jing J, Søegaard K, Cong WF, Eriksen J (2017) Species diversity effects on productivity, persistence and quality of multispecies swards in a four-year experiment. *PLoS ONE* 12(1): e0169208.

Kilcher MR (1981) Plant development, stage of maturity and nutrient composition. *J Range Manage* 34(5): 363-364.

Lenth R (2017) emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.0. https://CRAN.R-project.org/package=emmeans

Lüscher A, Mueller-Harvey I, Soussana JF, Rees RM, and Peyraud JL (2014) Potential of legume-based grassland–livestock systems in Europe: a review. *Grass Forage Sci* 69(2): 206–228.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2017) nlme*:* Linear and Nonlinear Mixed Effects Models. R package version, 3, 1-128, url: http://CRAN.R444. project.org/package=nlme.

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rumball W, (1986) Grasslands Puna chicory (*Cichorium intybus* L.). *NZ J.Exp. Agric* 14: 105–107.

Sturludóttir E, Brophy C, Bélanger G, Gustavsson A, Jørgensen M, Lunnan T, and Helgadóttir Á (2013) Benefits of mixing grasses and legumes for herbage yield and nutritive value in Northern Europe and Canada. *Grass Forage Sci* 69: 229-240.

Sanderson MA (2010). Nutritive value and herbage accumulation rates of pastures sown to grass, legume, and chicory mixtures. *Agron J* 102: 728–733.

Sleugh B, Moore KJ, George JR, and Brummer EC (2000) Binary legume-grass mixtures improve forage yield, quality, and seasonal distribution. *Agron J* 92: 24–29.

Smith KF, Simpson RJ, Oram RN, Lowe KF, Kelly KB, Evans PM, and Humphreys MO (1998) Seasonal variation in herbage yield and nutritive value of perennial ryegrass (*Lolium Perenne L.*) cultivars with high or normal herbage water-soluble carbohydrate concentrations grown in three contrasting Australian dairy environments. *Aust J Exp Agric* 38: 821-830.

Smith HJ, Tas BM, Taweel HZ, Tamminga S, and Elgersma A (2005) Effects of perennial ryegrass (*Lolium perenne* L.) cultivars on herbage production, nutritional quality and herbage intake of grazing dairy cows. *Grass and Forage Sci* 60: 297–309.

Sturludóttir E, Brophy C, Bélanger G, Gustavsson AM, Jørgensen M, Lunnan T and Helgadóttir Á (2014) Benefits of mixing grasses and legumes for herbage yield and nutritive value in Northern Europe and Canada. *Grass and Forage Sci* 69: 229–240.

Van Rossum M (2013) Dry matter production, nutritive value and botanical composition of a perennial ryegrass white clover pasture applied with GA and N in successive periods in autumn and spring. Dissertation, Lincoln University

Vreugdenhil SR (2017) Understanding the nutritive value of pasture mixtures. Dissertation, Lincoln university

Williams TA, Abberton MT, and Rhodes I (2003) Performance of white clover varieties combined in blends and alone when grown with perennial ryegrass under sheep and cattle grazing. *Grass and Forage Sci* 58: 90-93.

Zemenchik RA, Albrecht KA, and Shaver RD (2002) Improved Nutritive Value of Kura Clover– and Birdsfoot Trefoil–Grass Mixtures compared with Grass Monocultures. *Agron J* 94: 1131–1138.

Chapter V: General discussion

Mixtures of grass and clover are widely adopted in temperate regions because they can provide high biomass yield with little or no reliance on artificial nitrogen fertilizer (Finn et al. 2013; Nyfeler et al. 2009). Legume based mixtures have the potential to contribute to the sustainable intensification of grassland management. Compared to fertilized grass monoculture, grass-clover mixtures improve forage quantity and quality with less environmental impact and at the same time improving soil fertility (Kirwan et al. 2007; Nyfeler et al. 2011). The interactions among component species in a mixture are complementary or competitive, and their implications for the agronomic performance have been explored for many years (Hooper 1998; Ergon et al. 2016; Turkington 1989a). However, the effect of the genetic variation within species on interspecific interactions is less well documented (Collins and Rhodes 1989a; Turkington et al. 1979; Turkington 1989b; Suter et al. 2007). There is little information available whether the choice of the plant species genotype affects the performance of a multi-species mixture. In particular, almost no attention has been paid to the variation among genotypes within species that would increase complementarity among component species in a mixture and thereby the mixture performance. Two different experiments were therefore established to evaluate:

I. Whether and to what extent different non-legume species genotype affects the mixture yield and yield stability (Chapter 1).

II. Whether and to what extent different legume species genotype affects the mixture yield and yield stability (Chapter 2).

III. Whether and to what extent different legume species genotype affects the forage nutritive value of mixtures (Chapter 3).

In order to investigate the effect of different genotypes of non-legume species on yield and yield stability of mixtures, we grew four genotypes of perennial ryegrass differing in two traits (growth form: prostrate or upright, phenology: early or late heading) in monoculture, in binary mixture with white clover and in four-species mixture with white clover and two forb species ribwort plantainand dandelion.

The second project was conducted to assess the effect of different genotypes of legume species on yield, yield stability and forage nutritive value in monoculture and different mixture. Eight white clover genotypes were grown as monocultures, in binary mixtures with either ryegrass or chicory and in three species mixture with both ryegrass and chicory. The white clover genotypes differed in phenology, leaf size and yield potential. To assess the effect of environmental condition on performance of mixtures, we stablished the same experiment on two different sites. The two sites were noticeably different in terms of soil depth, soil texture, and precipitation.

In line with earlier studies, the results of both experiments confirmed the positive effect of mixed cropping on forage yield production and yield stability (chapter 2 & 3). The yield advantage of mixtures compared to monocultures could be related to the complementarity of resource use of the component species. Obviously, a major role played by the ability of white clover to fix the atmospheric nitrogen and improve the soil N pool. The inclusion of forbs in mixtures suggested additional pathways of increased resource use of the mixtures compared to the monocultures (Hoekstra et al. 2014; Cong et al. 2017). Including Dandelion and plantain (chapter 2) and chicory (chapter 3) into grass-clover mixtures substantially increased the forage yield. This benefit was assumed to be closely related to the divergent rooting systems of the shallow rooted grass and white clover on the one hand and the deep rooted forbs on the other. Similarly, Hoekstra et al. (2014) demonstrated that the inclusion of the deep-rooting species (chicory) in grass-legume mixtures increased biomass production, especially under drought conditions. Recent research by Cong et al. (2017), however, found different results on the effect of non-legume forbs (chicory, dandelion, and plantain) as a constituent of red clover-ryegrass mixtures. Including plantain into grass-clover mixtures significantly increased herbage yield, while adding chicory and caraway did not show a yield increase compared to the grass-clover mixture. The ability of forbs to take water and nutrients up from deep soil layers that are not available to shallowly-rooted species are likely to be a main reason why the inclusion of forbs contributes to the overyielding of mixtures (Hoekstra et al. 2014; Moloney 1993). The results of Cong et al. (2017), however, suggest that there are further mechanisms that increase the complementarity of resource use.

In chapter 3 we found that all mixtures yielded higher than the white clover monocultures on the fertile site, while at the marginal site, this was only true for the two mixtures including chicory. Many studied have found a positive relationship between the species/species functional group number and the productivity of the mixture (Ergon et al. 2016; Finn et al. 2013; Helgadottir et al. 2008), but in the present study (chapter 3) we found that the identity of the species in the mixture is more important than the number of species for the mixture benefit.

Not only the choice of crop species but also that of genotypes affected the productivity and yield stability of mixtures (chapter 2). The ryegrass genotypes in our study affected the forage production of the mixtures. The upright perennial ryegrass genotypes notably performed better than the prostrate ones when grown in monoculture (chapter 2). However, the effect of the ryegrass growth form was diminished in binary mixtures but still visible and almost completely disappeared in the four species mixture (chapter 2). The phenology of ryegrass did not affect forage yield (chapter 2). We had expected that phenological differences among ryegrass genotypes influence the mixture performance via improving the complementarity in mixture since they take up resources at a different time. Presumably, this was not the case because of temporal limitations in water availability which among other reasons such as nitrogen limitation might have restricted growth even if temperatures were favorable. Evans et al. (1985) found higher compatibility of ryegrass and clover in mixtures when the seasonal pattern of growth was more differentiated.

In contrast to many earlier studies (e.g., Evans and William 1987; Collins and Rhodes 1989; Hill and Michaelson-Yeates 1987; Turkington et al. 1979) and findings of our first experiment (chapter 2), white clover genotypes did not interact with the crop stands regarding yield production and yield stability. The white clover genotype which performed well in monoculture also did so in mixtures (chapter 3).

We found that environmental factors play a role for the relative performance of white clover genotypes as at the fertile site the difference between the white clover genotypes was more visible than at the marginal site (chapter 3). On average over all crop stands, white clover genotypes yielded higher at the fertile site than at the marginal site. However, significant interaction of genotype x site might be due to the difference between crop stands yield production on marginal site and fertile site; partner species yielded higher at the fertile site where the resources were more available than at the marginal site with limited resources. Irrespective of the crop stands, the white clover genotypes affect are stronger expressed where resources are less limited. Although the three-way interaction genotype x stand x site was not significant, this finding indicates that resource limitation is of importance and that in the other research the sites where genotype x stand effects were found had a likely higher resource availability (Collins and Rhodes 1989; Hill and Michaelson-Yeates 1987).

The resource availability also influenced the mixtures productivity notably (chapter 3). Presumably, this happened via alteration in plant-plant interaction (competition and/or complementarity) in the mixture (Brooker et al. 2015; Sanderson et al. 2002). All the mixtures yielded notably higher at the fertile site than their referring mixture at the marginal site, but white clover monocultures did not differ in yield between the two sites (chapter 3). The resource limitation at the marginal site could be responsible for that difference; limited soil depth and available water at the marginal site might have increased the competition among component species in mixture and limited the plants growth which affected the mixtures productivity.

From an agronomic point of view, mixtures are more interesting when they produce higher dry matter yields than the best monocultures, which is called transgressive overyielding (Nyfeler et al. 2009; Temperton et al. 2007). Transgressive overyielding occurs when there is a synergetic interaction between species in mixtures, and the net effect of interspecific interactions are sufficiently strong (Cardinale et al. 2007; Kirwan et al. 2007; Kirwan et al. 2009). Cardinale et al. (2007), in a meta-analysis summarizing 44 diversity experiments including non-agricultural systems, found that 79% of all mixtures had been more productive than the average monoculture. However, in only 12% of the experiments the mixtures had been more productive than the most productive monocultures (transgressive overyielding). They also demonstrated that the probability of transgressive overyielding of mixtures had increased over time and it took about five years for transgressive overyielding to become evident (Cardinale et al. 2007). In contrast to the finding of Cardinale et al. (2007), Nyefeler et al. (2009) found a high level of transgressive overyielding at the Swiss site of a continent wide mixture experiment (Kirwan et al. 2007). In addition, they observed a significant transgressive overyielding in the first year of the experiment. In our study, one of the three mixtures at each site transgressively overyielded the most productive corresponding unfertilized monoculture (chapter 3). It is remarkable that we found such a high incidence of transgressive overyielding occurring within the first four years (chapter 3). That is probably attributed to the deliberate selecting of species for our mixtures, as white clover, ryegrass and chicory are the most important and highly productive grassland species for forage production in temperate-humid grassland systems.

From the agronomic perspective, not only the amount of forage yield is important, the stability of yield between years and within year is also as important. The ryegrass growth form influenced the stability of the yield between years. When grown in monocultures the upright genotypes showed higher yield stability than the prostrate ones. The differences among ryegrass genotypes decreased from the monocultures over the binarymixtures to the four-species mixture; no difference among the growth types was found in binary and four-species mixtures (chapter 2). In contrast to forage production, ryegrass phenology affects the yield stability within year (chapter 2). The late heading ryegrass genotypes showed a lower within-year variation compared to early heading ones when grown in monocultures. This effect declined with increasing complexity of mixture. The companion species in the mixture compensated for the phenology pattern of resource use of the grass. As we expected, the four-species mixture had higher yield stability within years compared to the monocultures and the binary mixtures. White clover genetic variation did not show any effect on the stability of yield between years. However, the botanical composition of mixtures markedly affects the stability of yield production. In contrast to the finding of Küchenmeister et al. (2012) who stated that yield stability decrease with including forbs (plantain and dandelion) in the mixture, in both of our experiments, including forb into grass-clover mixtures increased the yield stability between years (chapter 2 & 3).

The genotype effect of white clover on the forage nutritive value has been rarely studied, as the focus has mostly been on traits that enhance persistence and competitiveness of white clover in the mixture (Annicchiarico and Proietti, 2010). We found a significant main effect of white clover genotype and significant interaction of white clover genotype x crop stand on the forage nutritive value (chapter 4). The effect of white clover genotype on the forage nutritive value was independent of site condition and year. The white clover genotype effect was more visible in white clover monocultures and it got narrower in mixtures, but remained significant. Our results showed that the effect of partner species was superior to the effect of white clover genotype on the forage nutritive value; mixtures' forage nutritive value was more determined by the identitiy of species than by white clover genotypes.

5.1 Conclusion

The quality and the quantity of forage are both important for maintaining livestock and livestock production. As we found in this study, quality and quantity of forage both change substantially based on the botanical composition of the mixture and partly related to the plant species genetic variation. Chicory, ribwort plantain and dandelion are three promising non-legume forage forbs that we found to notably increase the forage yield and yield stability when included in grass-clover mixtures. Supplementation programs are recommended to be designed to specifically address the effect of the interaction of a particular legume genotype with a specific non-legume genotype on quality and quantity of mixtures forage.

5.2 References

Annicchiarico P (2003) Breeding white clover for increased ability to compete with associated grasses. *J Agric Sci* 140: 255-266.

Brooker RW, Bennett AE, Cong WF, Daniell TJ, George TS, Hallett PD, Hawes C, Iannetta PPM, Jones, HG, Karley AJ, Li L, McKenzie BM, Pakeman RJ, Paterson E, Schöb C, Shen J, Squire G, Watson CA, Zhang C, Zhang F, Zhang J, White PJ (2015) Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytol* 206: 107-117.

Cong WF, Jing J, Rasmussen J, Søegaard K, and Eriksen J (2017) Forbs enhance productivity of unfertilised grass-clover leys and support low-carbon bioenergy. *Sci Rep* 7(1): 1422, 2017.

Ergon Å, Kirwan L, Bleken MA, Skjelvåg AO, Collins RP, and Rognli OA (2016) Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: 1. dry-matter yield and dynamics of species composition. *Grass Forage Sci* 71: 667-682.

Elgersma A, Schlepers H (1997) Performance of white clover/perennial ryegrass mixtures under cutting. *Grass Forage Sci* 52: 134-146.

Evans DR, Hill J, Williams TA, Rhodes I (1985) Effects of coexistence on the performance of white clover-perennial ryegrass mixtures. *Oecologia* 66(4): 536-539

Evans DR, Williams TA (1987) The effect of cutting and grazing managements on dry matter yield of white clover varieties (Trifolium repens) when grown with S23 perennial ryegrass. *Grass Forage Sci* 42: 153-159.

Finn JA, Kirwan L, Connolly J, Sebastià MT, Helgadottir A, Baadshaug OH, Bélanger G, Black A, Brophy C, Collins RP, Čop J, Dalmannsdóttir S, Delgado I, Elgersma A, Fothergill M, Frankow-Lindberg BE, Ghesquiere A, Golinska B, Golinski P, Grieu P, Gustavsson AM, Höglind M, Huguenin-Elie O, Jørgensen M, Kadziuliene Z, Kurki P, Llurba R, Lunnan T, Porqueddu C, Suter M, Thumm U, Lüscher A (2013) Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. *J Appl Ecol* 50: 365-375.

Helgadóttir A, Connolly J, Collins R, Fothergill M, Kreuzer M, Lüscher A, Porqueddu C, Sebasti MT, Wachendorf M, Brophy C, Finn JA, Kirwan L, Nyfeler D (2008) The benefits of sward diversity for cultivated grasslands. *Grassland Science in Europe* 22: 39-51.

Hill J. and Michaelson-Yeates TP (1987) Effects of Competition upon the Productivity of White Clover-Perennial Ryegrass Mixtures. Genetic Analysis. *Plant Breeding* 99: 239-250.

Hoekstra NJ, Finn JA, Hofer D, Lüscher A (2014) The effect of drought and interspecific interactions on depth of water uptake in deep-and shallow-rooting grassland species as determined by δ18O natural abundance. *Biogeosciences* 11: 4493-4506.

Hooper DU (1998) The Role of Complementarity and Competition in Ecosystem Responses to Variation in Plant Diversity. *Ecology* 79: 704-719.

Kirwan L, Lüscher A, Sebastia MT, Finn JA, Collins RP, Porqueddduc C, Helgadottir A, Baadshaug OH, Brophy C, Coran C, Dalmannsdottir S, Delgado I, Elgersma A, Fothergill M, Frankow-linderberg BE, Golinski P, Grieu P, Gustavsson AM, Höglind M, Huguenin-elie O, Iliadis C, JØrgensen M, Kadziuliene Z, Karyotis T, Lunnan T, Malengier M, Maltoni S, Meyer V, Nyfeler D, Nykanen-Kurki P, Parente J, Smit HJ, Thumm U and Connolly J (2007) Evenness drives consistent diversity effects in intensive grassland systems across 28 European sites. *J. Ecol* 95: 530-539.

Kirwan L, Connolly J, Finn JA, Brophy C, Lüscher A, Nyfeler D and Sebastià M (2009), Diversity–interaction modeling: estimating contributions of species identities and interactions to ecosystem function. *J. Ecol* 90: 2032-2038.

Küchenmeister F, Küchenmeister K, Wrage N, Kayser M, Isselstein J (2012) Yield and yield stability in mixtures of productive grassland species: Does species number or functional group composition matter? GRASSL SCI 58: 94-100.

Lüscher A, Mueller-Harvey I, Soussana JF, Rees RM, Peyraud JL (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass Forage Sci* 69: 206-228.

Nyfeler D, Huguenin- Elie O, Suter M, Frossard E, Connolly J. Lüscher A (2009) Strong mixture effects among four species in fertilized agricultural grassland led to persistent and consistent transgressive overyielding. *J Appl Ecol* 46: 683–691.

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

Rhodes I (1970) The production of contrasting genotypes of perennial ryegrass (Lolium perenne L.) in monocultures and mixed cultures of varying complexity. *Grass Forage Sci* 25: 285-288.

Sanderson, M. A. (2010). Stability of production and plant species diversity in managed grasslands: A retrospective study. *Basic Appl Ecol* 11(3): 216-224.

Sanderson MA, Elwinger GF (2002) Plant Density and Environment Effects on Orchardgrass–White Clover Mixtures. *Crop Sci* 42:2055–2063

Suter D, Briner, HU, Carlier R (2007) Effect of Lolium perenne L. variety on the botanical composition of grass-clover mixtures. *Grassland Science in Europe* 12: 417–420

Temperton VM. Mwangi PM, Scherer-Lorenzen M, Schmid B, and Buchmann N (2007) Postive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. *Oecologia* 151: 190-205.

Tilman D, Reich PB, Knops J (2006) Biodiversity and ecosystem stability in a decade-long grassland experiment. *Nature* 441: 629-632.

Turkington R, Cahn M, Vardy A, and Harper J (1979) The Growth, Distribution and Neighbour Relationships of Trifolium Repens in a Permanent Pasture: III. The Establishment and Growth of Trifolium Repens in Natural and Perturbed Sites. *J. Ecol* 67(1): 231-243.

Turkington R (1989a) The growth, distribution and neighbor relationships of *Trifolium repens* in a permanent pasture. VI. Conditioning effects by neighbours. *J. Ecol* 77(3): 734-746.

Turkington R (1989b) The growth, distribution and neighbor relationships of *Trifolium repens* in a permanent pasture. V. The coevolution of competitors. *J. Ecol* 77(3): 717-733.

Van Rossum M (2013) Dry matter production, nutritive value and botanical composition of a perennial ryegrass white clover pasture applied with GA and N in successive periods in autumn and spring. Dissertation, Lincoln University.

Summary

Grass-clover mixtures have shown a higher yield, higher yield stability and higher nutritive value compared to individual species in monoculture. The number and identity of species in a mixture are important factors for the mixture's productivity. Species complementarity is assumed to be a key mechanism determining the mixture performance. Not only species but also genotypes might have different complementarity to their mixture partners so that the genotypes' performance might be different in monocultures and mixtures. Moreover, resource availability can be expected to affect the species complementarity.

To study how and to which extent the genotype affects yield, yield stability and forage nutritive value in different mixtures, two different studies were carried out in two experimental stations of Georg-August-University Göttingen, Germany. In the first experiment, we studied the effect of perennial ryegrass (*Lolium perenne* L.) genotypes on mixture yield and yield stability. We hypothesized that the performance of perennial ryegrass genotypes in terms of yield and yield stability would differ between monocultures and mixtures. We also assumed that the effect of the perennial ryegrass genotype on accumulated total and species-specific component yield over four years would be decreased with increasing number of partner species. Four genotypes of perennial ryegrass differing in two traits (growth form: prostrate or upright, phenology: early or late heading) were grown (i) in monoculture, (ii) in binary mixture with white clover (*Trifolium repens*) and (iii) in four-species mixture with white clover and two forb species (ribwort plantain, *Plantago lanceolata* and dandelion, *Taraxacum officinale*). Our results showed that the treatments with the upright growth form of perennial ryegrass were higher yielding than the treatments with the prostrate form, but this effect decreased from monoculture to four-species mixtures. Phenology did not have any effect on accumulated yields over four years. The perennial ryegrass genotype significantly affected the mixture components' yield. Between- and within-year yield stability was generally highest in the four-species mixtures and lowest in the monocultures.

To test the effect of the white clover genotype and resource availability on mixture performance, we established an experiment as a part of the IMPAC^3 project in two sites which were noticeably different in terms of soil depth, texture, and precipitation. We hypothesized that white clover genotypes would perform differently in terms of productivity, yield stability and forage nutritive value in monocultures and mixtures. We also assumed that a lower level of resource availability might increase white clover competitiveness and affect mixtures' performance. Eight genotypes of white clover, one variety of perennial ryegrass and one variety of chicory (*Cichorium intybus* L.) were sown as (i) monoculture, (ii) binary mixture of white clover with perennial ryegrass, (iii) binary mixture of white clover with chicory, (iv) binary mixture of perennial ryegrass and chicory and (v) three-species mixture of white clover with perennial ryegrass and chicory. The white clover genotypes differed in flowering time, leaf size and yield potential. Above ground herbage was cut twice in the establishing year and four times in each of the two following years. Subsamples were scanned with a near infrared spectrophotometer to determine crude protein (CP), acid detergent fiber (ADF) and water-soluble carbohydrate (WSC). Our results revealed that the white clover genotype did not affect the mixtures' productivity; the white clover genotype that performed well in the monoculture also did so in mixtures. One of the three mixtures at each site showedsignificant transgressive overyielding compared to the most productive unfertilized corresponding. Generally, the binary mixture of white clover and chicory yielded notably higher than the other mixtures and monocultures. This shows that the benefits of mixtures are more related to the species identity than to the number of species in the mixture. Site conditions also strongly affected the mixture performance: All mixtures at the fertile site yielded higher than the same mixture at the marginal site.

The white clover genotype significantly affected the forage nutritive. This effect was most visible in white clover monocultures. In mixtures, it was less pronounced, but remained significant. Nevertheless, the forage nutritive value of mixtures was more determined by the partner species than by white clover genotype. White clover monocultures had significantly a higher concentrations of CP and lower concentrations of ADF than the mixtures. Mixtures including chicory had a higher concentration of ADF than the binary mixtures of white clover and ryegrass and white clover monocultures. Presence of ryegrass in mixtures led to a high concentration of WSC.

In synopsis of these two experiments, the effect of plant species genotype was surprisingly small. The white clover genotype that performed well in monoculture also did so in mixtures. Perennial ryegrass genotypes affected yield stability rather than annual herbage yield. On the other hand, inclusion of forbs into the grass-clover mixture improved yield in both experiments.

Keywords: white clover, perennial ryegrass, forbs, monoculture, mixture, yield stability, forage nutritive value, niche complementarit

Appendix

Table A1: Value for cultivation and use (VCU) test scores of the four perennial ryegrass genotypes used in the experiment compared to the mean, minimum and maximum of the total contemporary perennial ryegrass assortment in Germany. Adapted from Bundessortenamt (1995).

Growth form	Pheno-logy	Genotype name	VCU test scores*								
			Date of heading	Average height	Growth form	Suscepti-bility rust	Persistance	Sward density	Herbage yield total	Herbage yield first cut	Herbage yield following cuts
Genotypes used in the experiment											
prostrate	early	Bardonna	1	5	7	6	6	6	5	4	6
prostrate	late	Kerdion	9	3	7	5	7	7	6	5	7
upright	early	Sambin	2	7	4	3	6	6	6	6	5
upright	late	Hercules	8	5	5	5	6	5	6	5	6
Perennial ryegrass assortment (n = 82)											
		min	1	3	3	3	4	4	4	3	4
		max	9	8	7	7	7	7	7	7	7
		mean	5.4	5.9	5.5	4.8	6.1	5.6	5.8	5.3	5.7

*Test scores range from 1 to 9, with the following meaning:
Date of heading: 1… very early, 9 … very late
Average height: 1… very short, 9 … very tall
Growth form: 1… very upright, 9 … very prostrate
Persistence, sward density, herbage yield: 1 … very low, 9 … very high

Table A2: Identity and some features of white clover genotypes for IMPAC³ project.

Entry	Data base and trait identified as outstanding and further, specific features	
WC1 (EGB PX 90305)	Seasons 2008/09, 2009/10, 2010/11	Early flowering, small to medium leaf size, good flowering intensity, moderate yield in mixtures
WC2 (EGB PX 90312)		Intermediate flowering, large leaf size, medium flowering intensity, high yield in mixtures
WC3 (EGB PX90702)		Intermediate flowering, medium leaf size, medium flowering intensity, good yield in mixture
WC4 (EGB PX90710)		Late flowering, medium to large leaf size, medium flowering intensity, low yield in mixtures in D, high yield in F
WC5 (EGB PX90913)		Late flowering, intermediate ripening, medium to large leaf size, high yield in mixtures and pure stands, moderate winter hardiness
WC6 (EGB PX90914)		Very late flowering, medium leaf size, high flowering intensity, low yield in mixtures
WC7 (EGB PX90915)		Early flowering, medium leaf size, medium flowering intensity, high yield in mixtures, medium in pure stands
WC8 (EGB PX90909)	Seasons 2009/2010, 2010/2011,	Early to intermediate flowering, medium to large leaf size, bad winter hardiness, very high flowering intensity, medium yield in mixture

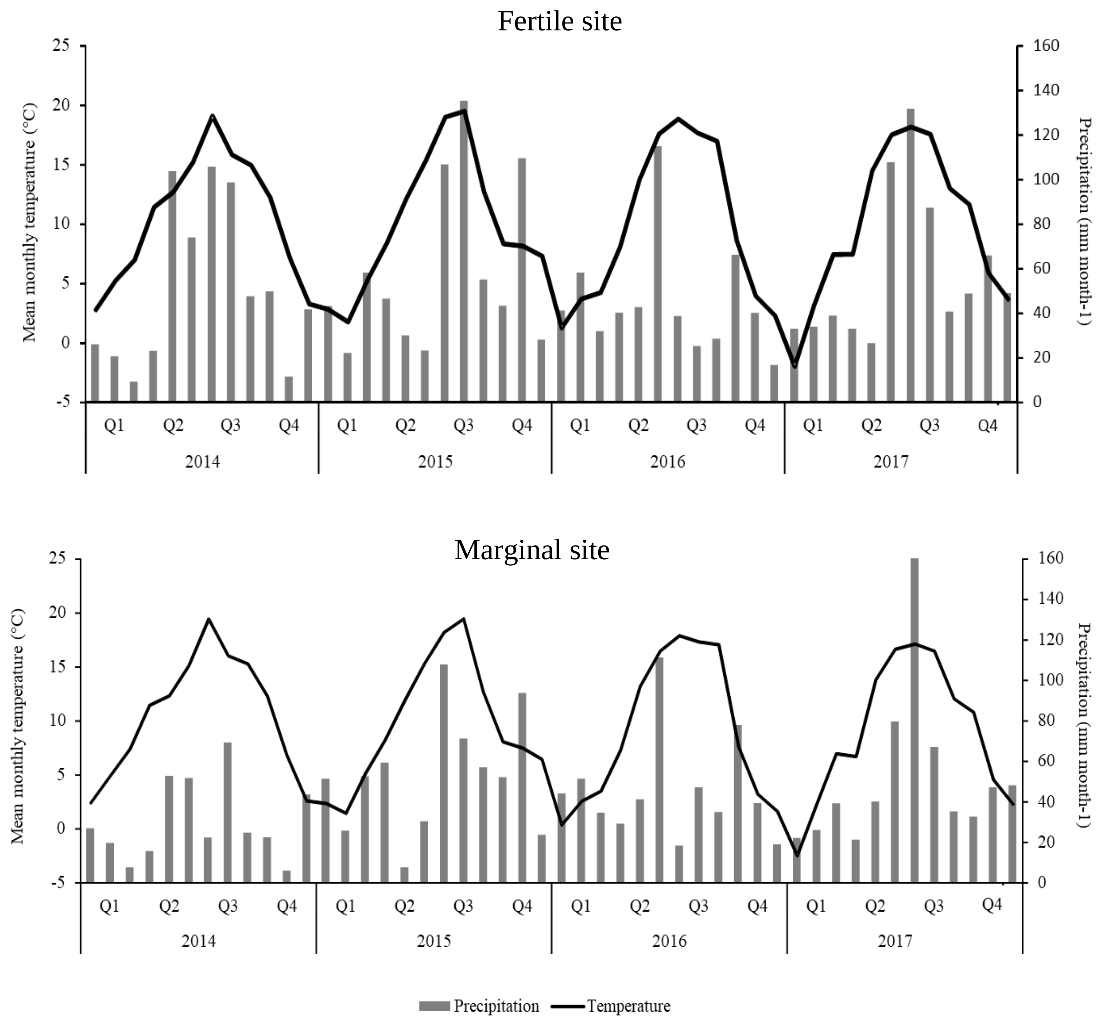

Figure A1: Monthly sum of precipitation (grey bars) and average temperature (black line) during the three experimental years for each site; (a) fertile site and (b) marginal site (data from Deutscher Wetterdienst).

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Declarations

I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

Furthermore, I also affirm that I have not applied for a Ph.D at any other higher school of education.

Göttingen, Sara Heshmati

I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

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