Dejan Soković · Christian Huyghe
Jasmina Radović *Editors*

Quantitative Traits Breeding for Multifunctional Grasslands and Turf

Eucarpia 2013

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Preface

Grasslands are among the largest ecosystems in the world and consequently are of great importance to human populations. They constitute a third of all agriculture land in Europe, and have played a major role in the development of agriculture and rural economy in many parts of Europe, from ancient times to the present day.

Grasslands are an important source of animal feed, particularly for ruminants. They provide most of feeds, not only during the grazing season, but also during winter staple housing. In addition, grasslands both protect and utilise marginal soils that are unsuitable for arable cropping. Although there being both permanent and temporary grasslands, they exhibit the best ecosystem quality of all agricultural production systems in Europe. They preserve the biodiversity, including the forage species, and provide undisturbed habitats for species originally present in natural ecosystems. At the same time, grasslands protect soil, improve water quality and recover natural habitat through the reduction of nutrient losses, soil water evaporation and water and wind erosion, as well as by limiting leaching of nitrate and pesticides.

The genotypes of the species which are the main components of the grasslands have great influence on total outcome and successful utilization of grasslands. Therefore, continuous improvement of fodder crop varieties is crucial for adhering to the latest practices in agriculture production and landscape architecture, and for meeting the needs and expectations of end-users. Similarly, turf cultivars contribute to the quality of environment and human life by producing quality swards under adapted management, particularly in urban areas. The large turf and forage seed markets and their high economic value further highlight the importance of the breeding of these species.

A wide range of breeding programs for forage and amenity species have resulted in substantial genetic improvements in herbage yield, quality and disease resistance, in recent years. Furthermore, new breeding methods and techniques (especially selection based on molecular markers) are rapidly expanding the boundaries of cultivars improvement and making it possible to achieve outstanding breeding results.

The *30th Meeting of the EUCARPIA Fodder Crops and Amenity Grasses Section* was held from 12 to 16 May 2013, in Vrnjačka Banja, Serbia. The Meeting was organised and supported by the Institute for forage crops, Kruševac. Additional support was provided by the Serbian Ministry of Education, Science and Technological Development, EUCARPIA, City of Kruševac, the company Rubin (Kruševac) and the dairy farm Lazar (Blace). Among the participants were geneticists, professional plant breeders, molecular biologists and other researchers from institutes, universities and seed companies, but also plant scientists, phytocenologists and gene bank officers from both the private and the public sector. There were scientists whose areas of research ranged from improving fodder and turf breeding methods and implementing new techniques in molecular biology, to cutting edge fodder crops and amenity grasses genetics. There were also professional plant breeders who required practical outputs of this research and the reduction of the overall cost of implementation. Their synergy always ensures huge importance and fruitfulness of EUCARPIA Fodder Crops and Amenity Grasses Section Meetings.

This book includes both plenary and invited papers, but also offered papers that were presented orally or as posters during the conference. Presentations and discussions covered a broad range of themes in fodder and turf breeding and research. Attention was also given to topics that arise from the border areas of presented scientific fields, especially through invited presentations that provided new ideas for integrated and interdisciplinary research.

The title of the Meeting and the book, *Quantitative Traits Breeding for Multifunctional Grasslands and Turf*, focuses on the challenges associated with the breeding of quantitative traits, which directly impact grasslands and fodder crops productivity, profitability and sustainability. It also focuses on a multidisciplinary approach to grassland research and utilisation. The papers are arranged in seven parts to reflect the structure of the Meeting: "Introduction," "Genetic Diversity Among and Within Species," "Practical Breeding of Fodder Crops," "Turf Grass Breeding," "New Biotechnology Methods in Sustainable Breeding: Strategies and Implementation," "Breeding for New Roles of Multifunctional Forage Species," and "Forage and Seed Production." We hope that the included papers offer a unique collection of ideas and breakthroughs in the fields of fodder crop and turf breeding and genetics, as well as in the creative and innovative application of new tools in practical breeding.

The scientific programme of the 30th EUCARPIA Fodder Crops and Amenity Grasses Section conference and selection of invited and proffered speakers were arranged by Christian Huyghe, as chairman of the Scientific Committee, and by members of Scientific Committee: Dirk Reheul, Ulrich Posselt, Roland Kolliker, Jan Nedelnik, Charles E. Brummer, Danny Thorogood, Susanne Barth, Zoran Lugić, Sanja Vasiljević, Jasmina Radović and Dejan Sokolović. The Scientific Committee also carried out the important and demanding, but time-consuming, task of reviewing papers. The editors are sincerely grateful for their contributions and whole-hearted engagement that ensured the success of the Meeting and high standard of the papers in this book.

We would also like to thank all the members of the local organising committee (Snežana Babić, Jasmina Milenković, Snežana Anđelković, Tanja Vasić, Mirjana Petrović, Zoran Lugić, Bojan Anđelković, Milomir Blagojević, Vladimir Zornić and Dragan Terzić from IFKKS; Borisav Kobiljski and Vojislav Mihajlović from IFVCNS; Zorica Tomić from IAHBG and Dušica Delić from ISRBG) who did an excellent job in running the Meeting smoothly and in providing a friendly atmosphere.

We express our gratitude to all participants for their interesting presentations and lively discussions during the Meeting, but also those authors who were not able to attend. Additionally, we would want to thank all the authors for preparing their papers for these Proceedings and for their commitment, hard work and responsibility which made it possible. Finally, we express our appreciation to Jacco Flipsen and Ineke Ravesloot from Springer, for the excellent collaboration and their assistance in the publishing of this book.

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Part I
Introduction

Chapter 1
Forage and Grasslands in a Sustainable Agriculture: New Challenges for Breeding

C. Huyghe and E.C. Brummer

Abstract Grasslands and forage crops are major components of the agri-food systems worldwide and must provide a wide range of ecosystem services, from the production of feed and food to the preservation of the environments. This paper presents the stakes associated to the three pillars of sustainability, with special attention given to the farmers and their expectations regarding farming systems. We suggest that risk aversion of farmers must be especially taken into account for the definition of new production systems and for the implementation of innovations. Implementation of production systems combining annual grain crops and perennial forage crops or associating plant and animal productions either at farm or territory scales make it possible to maintain production and economical performance and to improve environmental performances. The production systems as well as the breeding and registration systems are involved in lock-in situations that are a complex equilibrium of stakeholders and their activity around a series of standards of production and utilisation. These situations hinder the implementation of "disruptive" innovation that could move the systems to higher levels of productivity and environmental service. The conditions to escape from such situations are explained. Innovative conception theory provides a frame for identifying new concepts and for searching knowledge to support those who are creating the innovations. Adoption of innovations by farmers is a critical issue and tools to facilitate the implementation of innovation by farmers are identified, especially work in farmers groups, advisory systems and, on a long term perspective, training and education. It is also necessary to consider the heterogeneity of farmer communities. The early adopters must be targeted as they are opinion leaders who will first implement the innovations and disseminate them to neighbours and partners.

Keywords Production system • Sustainability • Innovation • Conception • Transition • Risk aversion

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Introduction

Grasslands and forage crops are major components of the cropping systems and agri-food systems worldwide. Grasslands and forage crops and the associated animal production systems provide a wide range of ecosystem services, from the expected agricultural production to environmental and cultural services. Their sustainability is continuously under question. The Millenium Ecosystem Assessment (2005) provides a useful tool for characterisation of ecosystem services, including support, provision, regulation and cultural services. As such, production is a primary ecosystem service. Because grasslands and forage crops are part of a long supply chain, any potential improvement or innovation in their management must be questioned according to its impact on these various services. Among possible innovations is the breeding and release of new crop cultivars (varieties), including forages, that could help develop resilient, sustainable agroecosystems (Brummer et al. 2011).

Key Challenges

In the world, as well as in Europe, the acreage of permanent grasslands has declined over the last decades, while the acreage of temporary grasslands, i.e., those included in rotations with annual crops, remained stable. The proportion of grass-legume mixtures in temporary grasslands increased to produce more plant protein with less nitrogen fertilizers. The production per unit area of temporary grasslands is poorly documented but the available evidence suggests small amounts of yield improvement, some of which is possibly due to advanced genetics (Sampoux et al. 2011).

The analysis of the expectations of stakeholders regarding grasslands has been documented in a few reports. The Multisward research project (www.multisward.eu) developed questionnaires that were submitted to various stakeholder groups. When questioned about the relative importance of economic, environmental, and social issues related to grasslands, on average these three compartments were given a nearly similar importance (Fig. 1.1). However, there were differences among groups of stakeholders, with farmers giving more importance to the economic issues than all other groups, not surprisingly (A. Van den Pol pers. com).

In his opening lecture during the Eucarpia 2011 meeting, Reheul (2011) pointed out that more than productivity, it was necessary to focus breeding effort on grasslands towards sufficiency, in a context of limited resources.

This underlines the importance to consider the stakes in a broad sense and several key challenges related to sustainability of production systems are to be discussed.

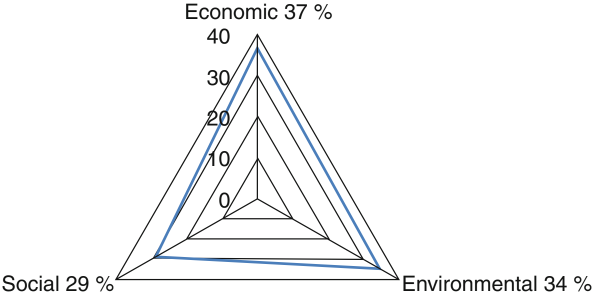

Fig. 1.1 Importance of the three pillars of sustainability of grasslands in Europe, according to a questionnaire run with the Multisward project (From A. Van der Pol, Wageningen University)

Production of Feed and Food

Meeting the worldwide food demand is a major challenge for agriculture and all production systems. According to a FAO report released in 2009, the challenge will be to feed nine billion people in 2050, requiring a 70 % increase in agricultural production. As pointed out by Guillou and Matheron (2011), this major challenge will require (1) a reduction in losses throughout the supply chain, (2) an improvement in the efficiency with which various inputs are used, and (3) an increase in the yield potential on currently available farmlands. Indeed, little unused land is available to be brought into production.

A main reason for this significant increase in demand is the global transition towards a food diet with an increasing proportion of calories from animal products; these systems have production efficiency lower than those producing only plant-based calories. This transition is explained by the very strong relationship between the share of animal calories and the national income per inhabitant (Combris and Soler 2011).

The demand for crop biomass to be used for energy production or chemical products also appears to be increasing, adding competition with feed or food production. This land-use change must be taken into account when environmental balance of biofuels is calculated (Searchinger et al. 2008).

The loss of production in grasslands and forage systems is a very important problem, and it's solution would do much to improve grassland systems. Under commonly used, non-intensive grazing managements, animals do not efficiently use a significant amount of the biomass available for grazing, limiting the amount of animal products that should be generated from a given area. Similarly, forages harvested as hay or silage can also experience significant losses. Poor silage management such as harvesting forage with a high water content that slows acidification or using forages not suited to silage production can result in large losses (Huyghe and Delaby 2013). Silage losses were a major concern of farmers and extension specialists in Sweden and consequently, in the 1980s, applied research was implemented to improve silage production and significantly reduce these losses (Sporndly and Nilsdotter-Linde 2011). Plant breeding programs could address traits of importance for forage conservation. For example, water-soluble carbohydrate content proved to

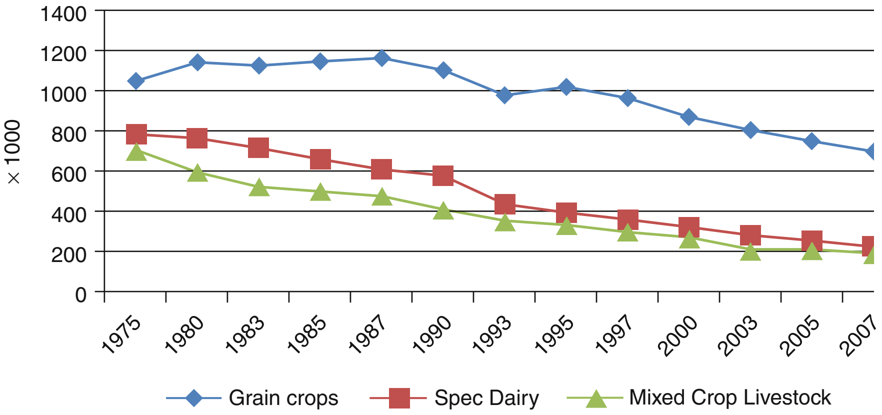

Fig. 1.2 Variation in number of farms involved in three productions in EU-9 since 1975 (Source: Eurostat)

be a limiting factor for the production of good silage in some grasses such as cocksfoot and breeding programs have begun to select for WSC content in response. Breeding can also modify plant architecture to enable swards to withstand cell-grazing or improve stock-piling ability.

Considering the production needed to meet future consumption demands requires taking into account all groups of stakeholders that are involved in this process. Farmers will be especially analysed, as they are likely to implement innovations, including new varieties.

Farmers always have to optimise their production function, where they farm a scarce resource, the land, with an expensive input (labour). The optimisation is achieved by the use of intermediate consumption (inputs), such as energy, fertilizers, pesticides or concentrates for animals. This has been done in a context that has dramatically changed over the last decades, resulting in a decline in the number of farms. This is illustrated in Fig. 1.2 by the number of farms involved in grain crops, specialised dairy production and mixed crop livestock farming in the EU-9 since 1975. This decrease which resulted in larger farms and larger herds was achieved because of huge increases in work productivity, thanks to widespread adoption of mechanization and an increase in mechanical power.

Long and diversified rotations, combining annual and perennial crops are beneficial to both soil fertility and production, yet research shows that such systems tend to disappear. The increasing size of farms has resulted in more simple production systems. This is very visible in annual crops. A survey run in France in 2010 showed that 38 % of the annual cropping systems contained not more than two crops. This was particularly pronounced in the Seine River Basin, where permanent grasslands and lucerne crops progressively disappeared (Mignolet et al. 2012). As a consequence, these systems are fragile and require large amount of inputs, especially pesticides. The increasing concern for reduction of pesticides and mineral nitrogen requires revisiting such systems that maximize functional diversity in order to

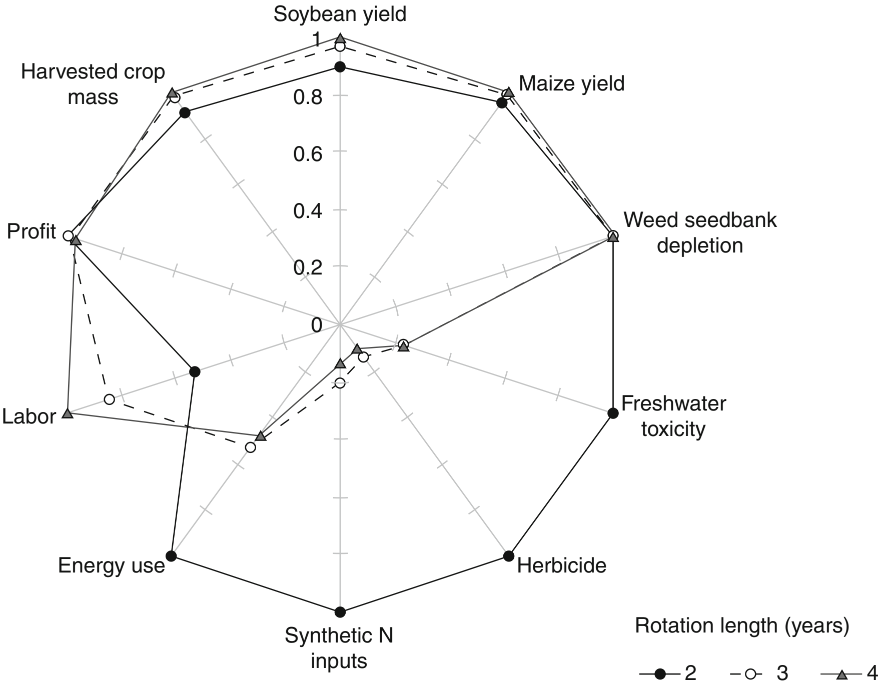

Fig. 1.3 Benefits from long rotations including perennial forage legumes as documented by multiple indicators of cropping system performance. Composition of the rotations: 2 years: Maize + soyabean; 3 years: Maize + soyabean + small grain + red clover; 4 years: Maize + soyabean + small grain + alfalfa (Adapted from Davis et al. (2012))

identify the drawbacks and understand the reasons why farmers tend not to implement them anymore.

As shown by Davis et al. (2012), the introduction of red clover during 1 year or alfalfa during 2 years in rotations with maize and soyabean had no impact on the profitability of the production system and slightly increased production of maize and soyabean. These systems substantially reduced the use of pesticides, especially herbicides, thereby positively affecting freshwater quality. Inclusion of leguminous forage crops reduced the use of mineral nitrogen fertilizers because of the increasing share of legumes in the rotations. But, these longer rotations required more labour, probably due to the multiple harvests of forage crops. Being implemented in large farms, this impact on labour could well explain why farmers are reluctant to use such rotations, even if they are very beneficial from the environmental point of view (Fig. 1.3).

However, risk aversion is another key element of the farmers' behaviour. This aversion leads a farmer to not adopt a practice, a technique, or a production system that induce more risk (more variation) even if the change could potentially result in more profit or production. For example, uncertainties on yield or quality potential of a crop or a variety or concerns about adequate establishment and management of the alternative crop may lead to its rejection. Complexity is also a source of risk and because multi-species crop rotations or mixtures of species are more complex than simple monocultures and require more management decisions, limiting adoption. Short-term objectives are always preferred to long term ones simply because the outcome is more controllable and knowable. However, as shown in experimental sociology, risk averse farmers can still be willing to test new possibilities on very small parts of an overall operation (Meyer and Meyer 2006). Agricultural scientists and extension specialists need to work with farmers to introduce new technologies, like new varieties, in ways that are compatible with a farmer's risk aversion. If the main reason for avoiding new alternatives is the variance it introduced into a farmer's system, then basic and applied research should do a better job of documenting variance, in addition to mean responses.

Environmental Issues: Several Times Scales to Be Considered

The challenge of implementing a sustainable agricultural system is to preserve the environment while ensuring the system is both productive and profitable. Environmental preservation covers a wide range of aspects. Here, we will focus on the various time scales of these environmental issues.

The short-term issues are all related to the reduction of the impacts of production systems on environment. Desirable changes would reduce nitrate and phosphorus leakage and minimize the presence of pesticide residues to improve freshwater quality. A well adapted succession of crops, diversified rotations with more legumes, reduction of pesticides use and a better ecotoxicological pattern of these pesticides are ways to achieve this objective. It is also desirable to preserve biodiversity, which could be uncommon, common or functional. Increasing field sizes and more homogenized landscape mosaics are detrimental to biodiversity. Adding perennial crops and grasslands to annual crop landscapes enhances biodiversity, both because of perenniality and because the elements associated with grasslands, such as hedges or ponds, accommodate a diversity of species not otherwise present.

The mid-term issues are related to crises of resources. Water is likely to be limited in many countries in the near future, if not already, and the increasing demand for agricultural irrigation competes with the needs for human consumption in ever enlarging towns and for industries, including energy industries. Fossil energy is a key issue for agriculture. Indeed, the energy efficiency of agriculture and especially animal production is low and the reduction of available resources or the increasing cost of energy will have a severe effect on production systems. Much of the large increase in productivity per land area achieved over the last decades was due to availability of energy, including its use in producing mineral fertilizers. The date of peak oil has been well documented, but the recent availability of rock (shale) gas postpones the deadline when fossil energy prices become too high to be used in agriculture. Phosphorus is also a critical resource for agriculture, and there are numerous uncertainties on the remaining stocks. But it is an essential fertiliser to achieve high agricultural productivity, with few possible substitutes because very few species can solubilise insoluble phosphorus.

The long-term environmental issues are related to climate change. The concentration of 400 ppm of CO2 in the atmosphere was reached in May 2013 at the Mauna Loa Observatory in Hawaii, this level being the highest over the last 3 million years. While the occurrence of climate change is unquestioned, the ramifications in various countries are clearly unknown. Beyond a large increase in summer temperature in most countries and a change in the rainfall regime, one of the anticipated impacts is an increasing variation among years. Thus, developing resilient production systems is essential. Agriculture could contribute to the mitigation of climate change inducted disruptions by reducing greenhouse gas emissions, including the production of CO2, CH4 produced by ruminant livestock, and N2O produced after application of mineral and organic nitrogen fertilisers. Changes in production systems, especially in ruminant production, may significantly reduce GHG emissions. Agricultural systems offer the potential of carbon storage in soils, but this seems to be a difficult option from a long-term perspective. Although soils have the ability to store carbon when converted from arable land into pasture, when converted back to crops, the carbon is released at a twice-higher rate.

It is essential to design future plant and animal production systems that meet these short-, mid- and long-term issues. When choosing among various options – such as breeding objectives – that meet the short-term environmental issues, consideration should be given to those which will also be beneficial at mid- and long-term horizons.

Lock-in and Transition

Plant breeding in general is often questioned on its capability to meet diverse expectations, with questions on the fact that it mainly targets major production systems and pays little attention to less intensive production systems and the crops that comprise those systems. This point is also raised for forage breeding that could be considered as mainly targeting intensive milk-producing systems while ignoring pasture-based, less intensively managed livestock systems.

Indeed, new varieties developed by plant breeding have been extremely important contributors to the success of intensive farming systems. Varieties bred for these systems were able to efficiently valorise high levels of inputs. This was also the case in forage when plant breeding significantly contributed to the so-called forage revolution. High input agriculture requires substantial technological development, and in some cases, the public is reconsidering whether this development is unequivocally good for society. For example, some people express a wish to see farmers and other stakeholders more involved in the definition of the breeding and variety registration objectives and even being involved in practice, through participatory breeding. Researchers should work to forge links with the society at large in order to be exposed to different sources of knowledge and understand how that knowledge could inform scientific research goals (Stengers 2013).

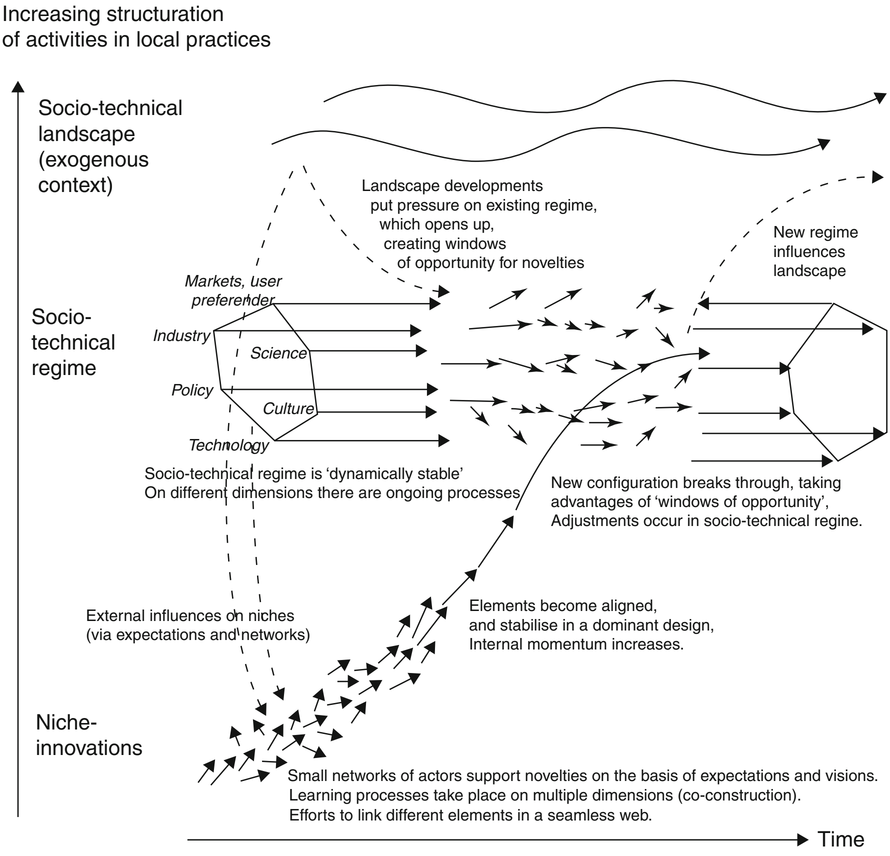

Fig. 1.4 Socio-technical regime and emergence of niche-innovations (Source: Geels and Schot (2007))

Stengers' criticism regarding the gap between researchers and stakeholders could also be considered as valid for plant breeding. For example, a breeder may consider that a new variety of a new species would be valuable, but a farmer viewing his/her existing farming system may not see how it fits. That is, existing systems may be considered as being in a lock-in situation. Lock-in and transition theory was initially developed for industry (David 1985; Arthur 1989) and for the energy sector (Cowan 1990), and later applied to the agricultural sector (Cowan and Gunby 1996), toward implementing agroecological systems (Vanloqueren and Baret 2009), and to the durum wheat supply chain (Fares et al. 2012). This theory defines a framework for assessing the coherent evolution and organisation of production systems (Geels 2005, Fig. 1.4). Lock-in theory identifies a dominant socio-technical regime where players are in equilibrium, organised around a certain production system; in our case, the players include farmers, plant breeders, end-users, and regulators. In such a situation, new technologies and new regulations may either re-enforce the lock-in situations or create the possibility for new equilibria to emerge. Innovations that may initially exist in niches could then be implemented at a much larger scale.

With regard to plant breeding activity, current systems may be locked-in by the application of biotechnology, molecular biology, and molecular marker/genomic selection methods. Because of the very quick progress of technologies and their present cost, they are implemented on a limited number of species, whose commercial value is the largest. These technologies improve genetic gain and/or result in desirable traits not attainable through breeding, increasing the relative merit of these species from those to which technologies are not applied. The implementation of these technologies could re-enforce lock-in situations, maintaining current systems rather than searching for alternatives (Keller and Brummer 2002). For example, the agronomic potential of multi-species temporary grasslands has been clearly demonstrated, offering the possibility to realize acceptable economic and environmental performance and perhaps creating new socio-economic systems as well. However, because of the possible genetic gap, these types of systems will be unlikely to be implemented in lieu of the existing systems.

Regulations can also enforce lock-in systems; for instance, those dealing with variety registration and seed certification constrain cultivar ideotypes to specific criteria. New variety ideotypes may be proposed to the farmers and regulatory statutes should be adaptable to accept them. The history of the registration rules for Festulolium in the various European countries is a good example that illustrates how these possibilities were progressively set, moving from difficulty of registration to acceptance of the new species and varieties of it.

Finally, lock-in systems can often be maintained by government policies. In the Midwestern US, for instance, maize and soybean production is supported by various governmental programs that are not available for alfalfa (or other forage) crops, such as federally subsidized crop insurance. As a consequence, even though alfalfa production is economically viable and environmentally desirable, the increased risk of producing the crop in the absence of government programs likely limits its inclusion in crop rotations (Olmstead and Brummer 2008).

Innovative Conception

We propose that long-term breeding objectives be developed to support a sustainable agriculture. In order to do this, we may use C-K theory (C for Concept and K for Knowledge) proposed by Hatchuel and Weil (2003) and Hatchuel et al. (2003) as a way to conceptualize innovations. This theory describes lineages of conception that must be fed from pieces of knowledge coming from diverse sources. The innovative conception may even induce new research directions.

This approach is very relevant to the frequent partnerships between private breeders and public research in forage breeding. It stresses the need to clearly identify the key objectives and outcomes and not only to focus on technological issues. The C-K theory also underlines the need to identify the fixation effects, i.e., the nodes of concept that are unanimously disregarded. It is necessary to permanently reassess the situation and envisage all possibilities, including those that were previously discarded. This is particularly important when preparing calls for proposals as this may guide future academic research.

Practices in a Coherent System to Combine Economic, Social and Environmental Performance

Designing farming systems received much attention in the recent years with a special attention given to the implementation of economically viable and environment-friendly production systems. Meynard et al. (2012) reviewed some of these approaches, especially the reflexive and Interactive Design and underlined key issues. They especially pointed out the importance to take into account the basic needs of the actors to be summarized in a Brief of Requirements (Bos et al. 2009).

When analysing the possible impact of new varieties, this must be done by defining the optimum production systems where the new varieties will be implemented. Indeed, the choice of a given variety will be made considering the whole production system, where the variety may only contribute to a better efficiency of the whole system, as defined by Hill and MacRae (1995), while other practices may be relevant if the forage and animal production system is thoroughly reconceived.

Adoption of Innovations by Farmers

As stated by Shumpeter in 1911, an innovation is an invention that meets its market. The likelihood that a farmer will adopt an innovation is related to risk. Because farmers are risk averse, we must assess how to reduce the risk associated with a new variety (an innovation), or at least, to reduce the farmer's risk aversion to growing that variety.

Reducing risk or risk aversion first depends on the innovation itself and its impact on farmers' activity. For instance, precision farming and precision cattle production systems reduced risk aversion because they provided precise information that was useful to farmers. Other approaches that are efficient in reducing risk aversion: (i) farmers groups enable sharing experiences among peers; (ii) field days can demonstrate in practice the innovations; (iii) advisory services can provide the right information to support the innovation; and (iv) education and training can help farmers become comfortable with the innovation. Over the long term, education is very efficient for deep, structural changes, but probably not the most relevant and efficient system for adoption of particular new varieties.

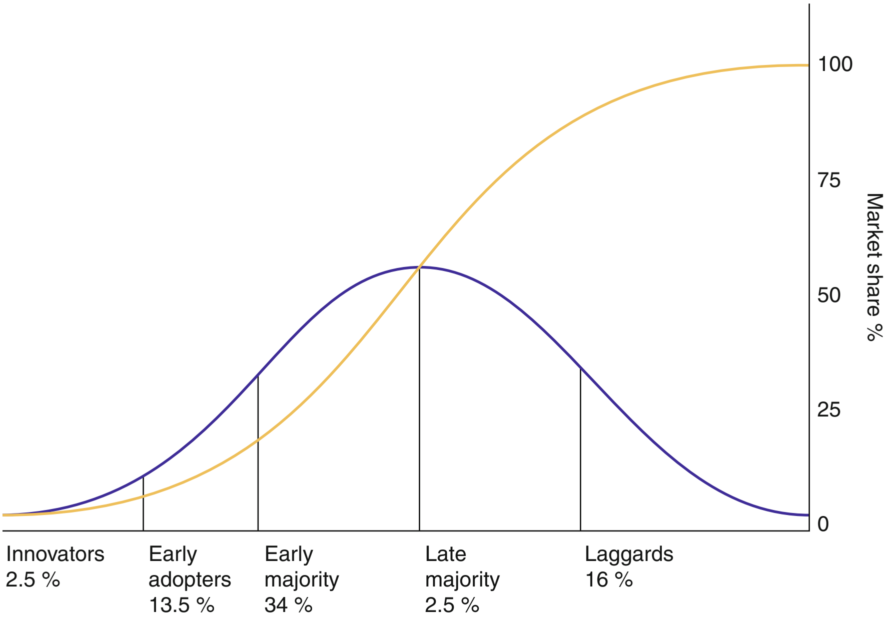

Fig. 1.5 With successive groups adopting a new technology (shown in *blue*), its market share (*yellow*) will eventually reach the saturation level (Adapted from Rogers (2003))

As documented by Rogers (2003), farmers, as other groups of stakeholders, are not homogeneous in their capability to adopt innovation (Fig. 1.5). Early adopters will rapidly test and adopt innovations, while other groups will be followers. It is essential to identify the early adopters, who behave as opinion leaders in the farm community, and to establish trials on their farms, as they can help in disseminating the new practices or the new varieties.

Conclusion

This broad overview of the challenges for variety breeding and the associated animal production systems underlines the need to define short and long term goals of sustainable farming and to define the breeding objectives, and the subsequent registration criteria, to meet production and environmental issues. A combination of annual and perennial crops, e.g., temporary grasslands in annual cropping systems, and the combination of animal and plant production at farm or territory scales are highly relevant to achieve sustainable agriculture.

Varieties are very advanced types of innovations and careful definition of the objectives, technologies, and regulations may offer either the possibility to explore more sustainable production systems or may lock-in the present equilibriums.

This paper also shows the importance to take into account the farmers' expectations and especially their risk aversion, both in the dissemination of the innovations, considering the whole farmer population, as well as in the analysis of experimental data, where variance must be documented.

The improvement of varieties and of production systems must target to increase the mean agronomic production and contribution to environmental services, but must simultaneously reduce their sensitivity to environmental variation, thus contributing to more resilient farming systems.

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Chapter 2
Agriculture, Forage Crops and Grasslands in Serbia: Production and Breeding

Dejan Sokolović, Jasmina Radović, Zoran Lugić, and Aleksandar Simić

Abstract Serbia is a country with great potential and capacities for agriculture. Agriculture is the basis of Serbian economy and it is the backbone of the development of the country. It is the only sector with positive foreign trade balance, representing 21 % of total export. More than 17.3 % of the Serbian population live on farms and their basic income originates equally from crop and animal production. Cattle products constitute 42.6 % of overall animal production. In the last 10 years, the number of farms engaged in raising cattle, as well as the number of dairy cows, has declined. This reduction of approximately 2–3 % annually has resulted in stagnation and reduction of meat and milk production.

Arable fields and gardens account for *ca*. 65 % of all agricultural land in Serbia, and of that 455,000 ha are fodder crops. The total area sown with alfalfa has experienced a slight reduction in recent years to the current level of 183 thousand hectares. Red clover is cultivated on 120 thousand hectares, while fodder corn fields are expanding. Since approximately two thirds of the Serbian territory consists of hilly or mountainous areas, natural grasslands cover about 1.45 million hectares, or almost 29 % of the total agricultural land area. Fodder crop production in Serbia is traditionally based on domestic cultivars and hybrid maize seeds. Since extensive forage production is dominating in Serbia, particularly in the upland regions, domestic breeding programs are essentially focused on genotypes that can survive and produce under the local agro-ecological conditions. This has led to the inclusion of several new objectives in the breeding programmes, with the aim to improve plasticity and resilience to environmental limitations. The main objectives are drought tolerance, root characteristics, field persistence linked to low pH tolerance and symbiotic N-fixation, tolerance to the most important pathogens, *Fusarium* sp., *Coletotrichum* sp. and *Puccinia* sp., and adaptability to different environmental extremes. The forage crop species currently in use in Serbia consist of highly productive cultivars, with improved dry matter quality. These cultivars can achieve and

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sustain a satisfactory level of plasticity under a wide range of climatic conditions, not only in Serbia but also on the Balkan Peninsula.

Future steps to improve fodder crop production and husbandry in Serbia include regionalisation of forage production according to a set of recommendations, stabilisation of subsidy policies, consolidation of fields and farms, improvement of grassland management and production technologies, and creating a better and more efficient system of transfer of knowledge from scientists to farmers.

Keywords Agriculture • Grassland • Fodder crops • Breeding • Serbia

Introduction

Serbia has considerable agricultural capacities and potentials, due to viable farming communities, a substantial animal production and an abundance of processing facilities. Serbia is an agricultural country, and as can be seen from official statistics, has considerable natural resources, extensive areas of agricultural land, a favourable climate, and a secure water supply.

The number of individuals and the proportion of the total population involved in agriculture are substantially higher than in any other EU country. According to census data from 2011, Serbia had a population of 7,186,862 (SORS 2013b). Farmers constitute 17.3 % of the population, which is equal to approximately 1,240,000 individuals (Petrović et al. 2012). They live on 631,122 farms, with an average size of 4.5 ha (SORS 2013a). Although the average farm size has increased in Serbia, 58 % of farms are still smaller than 3 ha (Petrović et al. 2011). The majority of the farms (more than 450,000) can be found in Central Serbia, but these farms are smaller than the farms in the flatter Pannonia region. Most of the agricultural income is generated by animal and crop production (43 and 42 % of total production, respectively), while fruits and grapes contribute to the remaining 15 % (Petrović et al. 2012).

Serbia covers an area of 8.85 million hectares, of which *ca*. 5.1 million hectares (58 %) are agricultural land. Nearly 4.2 million hectares (83 %) of the agricultural land are cultivated and 0.83 million hectares are permanent grasslands (SORS 2013b). This translates to approximately 0.6 ha of agricultural and 0.5 ha of arable land per capita (Aleksić et al. 2010). However, more recent data from the Agriculture Census that took place in 2012 show that only 3,335,859 ha have been utilised (SORS 2013a). Fifty percent of all agricultural land is located in the Vojvodina province and the remaining 50 % is located in Central Serbia. However, most of the arable land (84 %) can be found in the Vojvodina province, which is predominantly lowland. In the hilly and mountainous areas of Serbia, which constitute two thirds of the total territory, the process of depopulation and abandonment of villages has been a considerable problem during recent decades, resulting in a reduction of farms and number of farmers. The quality of the soil and soil pH are different at different altitudes and are very good in lowland areas (chernozem) but only moderately good in upland regions.

Table 2.1 Land uses of agricultural land in Serbia (in 1,000 ha)

Year	Total	Arable crops	Fodder crops	Orchards and vineyards	Meadows	Pastures
2009	5,058	3,301	455	298	625	834
2010	5,051	3,295	460	297	624	836
2011	5,056	3,294	455	296	621	845

Table 2.2 Main subsidies for animal and crop production in Serbia in 2012 and 2013

For registered livestock (per head)	
Dairy cows (min 4 – max 100)	225 € head^{-1}
Sheep, goats and sows (max 150)	36 € head^{-1}
For fattening cattle	90 € head^{-1}
For investments in facilities and machinery	Based on documents
For arable land per hectare	54 € ha^{-1}
For diesel fuel 120 L per ha of arable land	0.45 € L^{-1}
For milk production (min 3,000 L per quarter)	0.063 € L^{-1}

Recent data on the areal extent of different crop groups in Serbia are presented in Table 2.1. Most of the agricultural land is used for arable crops and, of this area, fodder crops are planted on 455 thousand hectares (SORS 2013b).

Sixty percent of the total seeded area in Serbia is used for cereal crops (wheat, barley, rye and oats) and maize (SORS 2013b). Maize is predominant, with more than 1.2 million hectares seeded and Serbia ranks in fifth place in Europe in maize crop production – after France, Ukraine, Romania and Italy (MATFW 2012). Regarding the ownership of land, 87 % of land is private property, while the rest is owned by the state (Petrović et al. 2012). Approximately 80 % of all agricultural land is in the ownership of individuals. Agriculture is the basis of the economy and the engine for development of rural areas in Serbia and it is the only sector with a positive foreign trade balance. Agriculture contributes to 21 % of total export, but only to 6–8 % of total import (MATFW 2012). The most important trade partners of Serbia for agricultural products are the European Union (EU) and Russia.

The Serbian market has become more and more open in recent years, due to the process of association with EU. As a result, the Serbian government were obligated to invest considerably in the agricultural sector by subsidy policy with the aim of making farming profitable (Table 2.2). There are four groups of subsidies, one for registered livestock, one for investments, one for arable land (for fuel or directly) and one for the production of milk.

The absolute values of the subsidies are not high, but the importance of a stable and regular source of income for farmers cannot be underestimated.

Agriculture is considered one of the most important academic disciplines in Serbia. In the strategy of scientific and technological development for 2010–2015, entitled “Focus and partnership”, the scientific field “Agriculture and food” is one of seven fields specified as “strategic”. Contrary to many other countries, the financing of agricultural research by the state have remained relatively stable over recent years and the likelihood of being awarded a state-funded grant is high.

Animal Production

A significant part of Serbian agriculture is the well developed and flexible animal production sector with a long tradition. Also, Serbia has a strong animal feed industry, not only in the country, but in the region as well. Animal production is the most important branch of agriculture in all developed agricultures and its share in total agricultural production indicates the level of development of agriculture in a country. In Serbia animal production is *ca*. 43 % of the total value of agricultural production (Petrović et al. 2012), compared to 60–70 % in the EU.

Pork meat production is the largest animal production sector in Serbia, but in terms of overall animal production, cattle husbandry is the most important one. Cattle production represents 42.6 % of overall animal production and within it beef meat production is *ca*. 17.6 % and dairy production is *ca*. 25 %. However, during the last decades, livestock breeding in Serbia has been facing numerous problems, including the reduction in number of farms, the small sizes of many farms, the low number of animals per farm, an unstable market, fluctuating prices and changes of subsidies (Petrović et al. 2005). In the last 10 years, the number of farms engaged in raising cattle has declined (Perišić et al. 2011). This trend is the same in the whole EU-27 area, but the number of cattle per farm in the EU has been increasing constantly (Huyghe and Tabel 2010; Huyghe 2010). According to the Census in 2002, 97.61 % of farms in Serbia owned between one and five cows and, together, these farms owned 87 % of the total number of cows in Serbia (Perišić et al. 2011). The Census of Agriculture 2012 states that the average number of cows per farm was 1.3, sheep 2.1 and 0.4 goats. The majority of cows were kept on farms that were 5–10 ha in size, whereas the majority of sheep were kept on farms that were 2–5 ha in size and goats on small farms of less than two hectares. Over the last decades, and especially in the first decade of the twenty-first century, there has been a decreasing trend in number of cattle (Aleksić et al. 2010; Petrović et al. 2011), to the current level of 909,000 heads (Figs. 2.1 and 2.2).

There was a decrease in number animals for all other livestock groups during the last decade, but in the last year the number of sheep and goats increased significantly. There are currently 1.7 million sheep, 3.4 million pigs and 236,000 goats in Serbia (Fig. 2.2).

Total meat production has stagnated in last ten years (Table 2.3). Pork meat production (271,000 t) dominates with 56.6 % of total meat production, while beef and sheep meat production account 16.90 % and 5.04 %, respectively (Petrović et al. 2012).

Fig. 2.1 Number of dairy cows and fattening cattle (in 1,000 heads) for 6 years in the time period 1991–2010 (SORS 2013b)

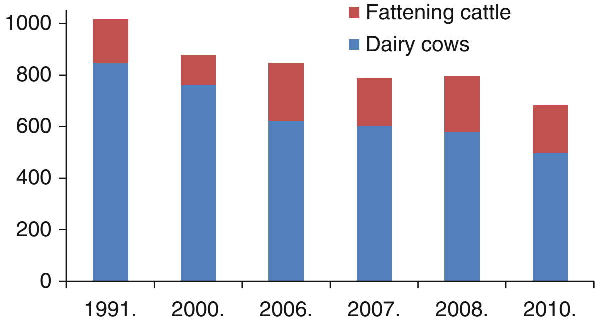

Fig. 2.2 Decreasing trend in number of domestic animals (in 1,000 heads) from the year 2000 to 2012 (SORS 2013b)

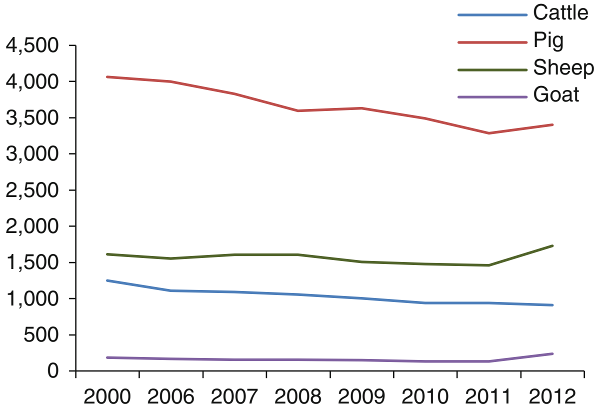

Table 2.3 Dairy and meat production in Serbia (SORS 2013b)

Year	Meat (in 1,000 t)	Cow's milk (10^6 l)	Sheep milk (10^6 l)
1990	548	1,805	20
2000	473	1,585	19
2005	431	1,602	16
2006	–	1,587	15
2007	474	1,549	14
2008	464	1,534	14
2009	457	1,478	10
2010	468	1,462	9
2011	479	1,434	11

Milk production is a very important sector of animal production, with 25 % of total animal production, but it has been decreasing constantly in the last two decades (Table 2.3). Milk is commonly produced on small family farms, which provide 92 % of the total milk production (Petrović et al. 2012). Officially, dairy plants in Serbia process approximately 50–55 % of the total volume of raw milk produced

(SCR 2006). Average milk yield of registered cows of Simmental breed (dominating with 70 % in Central Serbia due to its dual-purpose type) is about 4590 kg and of Holstein Friesian breed about 8,700 kg (Perišić et al. 2011). The consumption of milk and milk products in Serbia is between 150.3 l (FAO 2013) and 200 l (Petrović et al. 2013) per capita, which is far below that of EU countries (280–950 l).

The main reason for this negative trend in milk and meat production in Serbia is the reduction of the number of farms and, especially, the number of animals, without substantial improvement of the production per animal. Another important reason for this reduction is that fields and farms are too small for large-scale production. The average field size in Serbia is only 0.45 ha (1.2 ha in lowland and 0.3 ha in the mountainous regions; SORS 2013a). The reduction of export of animal products has also contributed to the reduction in animal production. For animal products to be competitive on the export market, the combination of three key factors (quantity, quality and continuity in production) is required, but this is currently lacking in Serbian animal production. Low quality forage (with low CP and high ADF), especially in the lowland regions, and consequently high price of animal feed, is one of the main reasons of insufficient production of meat and milk according to experts in animal nutrition.

Fodder Production

Serbia is a country with varied topography, from flat areas in the Pannonia region to hilly and mountainous areas, which constitute approximately two thirds of the total Serbian territory. Different altitudes are linked to different soil qualities (structure and humus content) and acidity (pH), which are very important factors for fodder production. Large areas of land in Serbia, almost 60 %, have acidic soils with pH below 5.5 (Lugić et al. 2010).

The most important forage crops are perennial legumes (alfalfa and red clover) in pure stands, corn for silage, annual legumes, meadows mixtures and natural grasslands. Fodder crops take up 13.8 % (455,000 ha) of the cultivated arable land, or 9 % of the total area of agricultural land (SORS 2013b). The total area dedicated to alfalfa crops has decreased slightly to a current level of 183 thousand hectares, whereas the total area used in red clover production remains unchanged. In contrast, the area used for fodder corn is expanding (Table 2.4). However, silage corn only make up a small percentage (2.4 %) of the total area sown with corn (1.258 million ha).

Most of the forage production is conserved as hay, but also as silage or haylage. Statistics show that the average dry matter yield of major forage crops in Serbia is still too low (SORS 2013b). It increases from below 1 t ha^{-1} on upland pastures, 2 and 3 t ha^{-1} on natural and sown meadows, to 4.5 t ha^{-1} and almost 6 t ha^{-1} in red clover and alfalfa pure stands. However, the genetic potential of local genotypes of the mentioned crops is much higher. Regular dry matter yield for alfalfa and red clover is between 14 and 18 t ha^{-1} (Radović et al. 2007; Lugić et al. 2006), for sown

Table 2.4 Total area dedicated to three main fodder crops in Serbia (1,000 ha)

Year	Alfalfa	Red clover	Corn for silage
1997	192	120	19
2000	191	124	21
2001	193	122	21
2004	190	122	22
2006	188	121	22
2009	188	119	27
2010	187	119	28
2011	183	120	30.2

grass-legume mixtures it is 7–10 t ha^{-1} (Lazarević et al. 2005) and on natural grasslands it is over 4 t ha^{-1} (Vučković et al. 2005). Production of corn silage is between 20 and 23 t ha^{-1} of biomass depending on the year. Natural grasslands cover about 1.45 million hectares, or almost 29 % of total agriculture land in Serbia, whereas sown grasslands cover 155,000 ha (Lugić et al. 2010). Within natural grasslands, meadows cover about 600,000 ha and pastures about 800,000 ha. These grasslands are not only important for forage production, but also in preserving biodiversity, which is very high in Serbia. Some meadow vegetation classes (*Festuco-Brometea* Br.-Bl. et R. TX. 1943) comprise 1,194 species, representing *ca*. 42 % of the total flora in Serbia (Lakušić 2005). Grassland is the best agricultural production system with regards to ecosystem quality, since it provides undisturbed habitats for autochthonous, endemic and endangered species, not only of plants, but also of insects, mammals, birds, reptiles and other animals. Grasslands are also more beneficial for the environment, due to the infrequent need for cultivation, which relaxes the soil, and the reduction in soil losses from ploughing and erosion. Grasslands have high water infiltration capacities and improve water quality due to the reduced need for pesticides and fertilisers.

Genetics and Breeding of Forage Crop Species in Serbia

The fodder crops production in Serbia is traditionalistic and based on domestic cultivars and, for corn, hybrids seeds. Typically, farmers favour local genotypes in fodder production, partly due to their experience of these genotypes and partly due to praxis. However, in extensive forage production systems that generate low revenue, as is common in Serbia and especially so in the upland regions, adopted genotypes or cultivars are of high importance. These forage cultivars have to be flexible, highly adaptable, environmentally plastic, productive and of good quality, as well as tolerant to drought and other unfavourable conditions. Despite it being very difficult to achieve all these traits in a single cultivar, it has been made possible through appropriate approaches in breeding programmes. Domestic breeding programmes are focused on genotypes that can survive and produce under local agro-ecological conditions, which are continuously deteriorating. It is noteworthy that the production seasons in Serbia often experience extreme conditions. Periods of drought are frequent during spring and summer, which lead to reduced yields and can even jeopardise whole crops.

These problems can be solved by two complementary approaches. The first approach involves defining or developing breeding germplasm that has been acclimatised to local agro-ecological conditions. This is achieved by collecting wild plant materials (autochthonous populations and ecotypes) and is regularly practiced by forage breeders in Serbia (Sokolović et al. 2003a, 2012a). These genotypes exhibit a desirable level of adaptability to local agro-ecological conditions and very often have satisfactory yielding performance (Sokolović et al. 2003b, 2004a; Babić et al. 2010), which may lead to direct phenotypic selection and cultivar release (Posselt and Willner 2007). Imported cultivars, on the other hand, are frequently not adapted to local agro- ecological conditions and show weak vigour in subsequent years of grassland utilisation, especially in comparison with local genotypes (Sokolović et al. 2004b, 2010b). The second approach involves establishing forage crops breeding programmes that utilise new breeding methods and have specific breeding objectives, such as drought tolerance, persistency and adaptability under different extreme weather conditions (Sokolović et al. 2010a). The breeding objectives also include breeding for improved root characteristics (Sokolović et al. 2012b), persistency interconnected with low pH tolerance and improved symbiotic N-fixation (Delić et al. 2010), as well as to develop tolerance to the most important pathogens, *Fusarium* sp. (Krnjaja et al. 2004) and *Colletotrichum* sp. (Vasić et al. 2010a, b). The overall aims are to improve the plasticity of the forage crop cultivars and to overcome environment limitation associated with forage crop production in Serbia. Both approaches contribute with some of the desired characteristics to new genotypes and make them adaptable to variable natural and cropping conditions. Clearly, measures could and should also be taken to optimise cropping conditions (e.g. irrigation, land consolidation) and inputs (e.g. fertilisers, pesticides) that further improve the exploitation of plant potential. However, it is unrealistic to expect that large areas of grasslands or arable forages in Serbia can be treated and improved on a short timescale through these methods alone.

In recent years, strong efforts have also been made by plant breeding programmes to meet farmers' needs and expectations. Demands for different grassland mixtures suitable for a range of farming conditions resulted in the breeding of specific cultivar properties for mixtures and pastures, which had the required compatibility, competitiveness and difference in maturity. Significant practical results (i.e. the release of specific cultivars) have not yet been accomplished, but there has been a breakthrough in the breeding of orchardgrass for the specific use in orchards greening (Babić et al. 2009).

As result of intense breeding work, the contemporary assortment of forage crops species currently available in Serbia consist of cultivars that are highly productive and have an improved dry matter quality. They can achieve and sustain satisfactory levels of plasticity under diverse and suboptimal climatic conditions in Serbia, as well as on the Balkan Peninsula.

Cultivars Registration

Due to the high interest in the breeding and improvement of forage crops in Serbia in general, and at the two main scientific and breeding institutes (Kruševac and Novi Sad) in particular, there is a wide range of domestic cultivars that are well-distributed among cultures and species (Đukić et al. 2007; Tomić and Soković 2007). The Serbian market is also open to introduced cultivars, which are subjected to an evaluation process prior to registration, in which they have to exhibit superior properties to local standard cultivars. Despite these measures, the number of actively used cultivars is much lower than the total number of released and registered cultivars, due to the fact that entry on to the official cultivar list is limited to 10 years. After that period, cultivars have to be re-registered or they have to be removed from the market within 2 years. The evaluation process for cultivar registration consists of 3 years of official state trials (VCU and DUS), but only two years of full field production (second and third year) are included in yield calculations. These trials are conducted in multiple locations; how many depends on the species (three for grasses and four for legumes). Even though a sufficient number of cultivars of forage crops have been released in Serbia in recent years (more than 85 since 2003) and the genotypes have exhibited high genetic potential for dry matter production and quality, the production results have been disappointing. Poor management, a lack of capital investment and insufficient education of the farmers, followed by unstable and adverse weather conditions have resulted in unreliable animal feed production on grasslands and fields. However, it is promising that the majority of these factors affecting fodder crops production can be addressed.

Fodder Cultivars Seed Production

The task of providing sufficient amounts of high quality seeds of registered cultivars is an additional challenge for the plant breeding community in Serbia, on top of their continuous work on the genetic improvement of fodder crops. Thus, cultivar seed yield has been given special attention in breeding (Soković et al. 2006). New genotypes have to provide the genetic potential for good seed production, if the process of seed multiplication is to be affordable and attractive for seed producers. Since seed production in Serbia is typically carried out under rain-fed conditions, seed yield is variable and highly dependent on weather conditions. However, if the particulars of seed production of the various perennial fodder grass species are taken into consideration, the Serbian cultivars exhibit high genetic potential for seed yield. In the early phases of local grass breeding programmes, the average seed yields achieved within a 6-year period of production were 482 kg ha^{-1} for cocksfoot, 700 kg ha^{-1} for meadow fescue, 432 kg ha^{-1} for timothy, 581 kg ha^{-1} for tall oat-grass, 751 kg ha^{-1} for tall fescue and 728 kg ha^{-1} for red fescue (Mladenović and Tešić-Jovanović 1983). Among perennial grasses, the highest seed yield was obtained for Italian ryegrass with more than 2 t ha^{-1} (Simić and Vučković 2006). Alfalfa cultivars also displayed extremely large variations in seed yield, due to specific weather conditions in different years. Some alfalfa seed crops can reach yields as high as 1,000 kg, but the average seed yield of local cultivars over a 13 year period was significantly lower, 250 kg ha^{-1} (Karagić et al. 2007). Serbian genotypes of winter vetch gave an average seed yield of 0.8 t ha^{-1}, while spring vetch reached an average seed yield of 1.5 t ha^{-1} (Karagić et al. 2010).

The seed production of forage crop cultivars has been organised by breeding institutions and seed companies, mostly in collaboration with large farms. Legume seed production has mainly taken place in the Vojvodina province, especially in the case of alfalfa, with approximately 2,900 ha of certified seed crops and an estimated 1,000 ha of non-certified seed crops (Karagić et al. 2010). Perennial forage grass seed production mostly takes place in the hilly and arid areas of eastern Serbia or, in the case of *Lolium* species, in areas with medium rain precipitation in western and northern Serbia. Currently, the situation of depopulated villages and a reduced number of animals in upland regions has shifted grass seed production to low-lying regions where fields are larger, mechanisation is better and there is a continued interest in improving animal production. According to Nikitović and Radenović (1996), local grass seed production only covers 30 % of the total seed demand and the remainder has to be imported. Despite favourable agro-ecological conditions for perennial grass seed production, the volumes produced in Serbia are still too low to meet the demand. In contrast, sufficient legume seed are produced in Serbia to supply the local market and to export. Serbian seed companies and research institutes are important exporters of seeds and in recent years they have produced new, globally recognised varieties and hybrids. The foreign trade balance in certified seed trade has changed over time (SORS 2013b), and in 2007–2009 it became positive for certified seeds of local cultivars of alfalfa and red clover (Fig. 2.3).

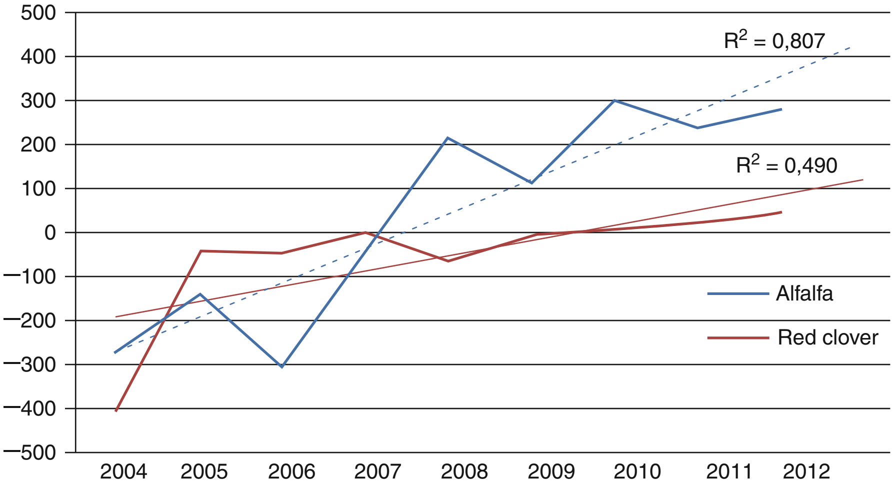

Fig. 2.3 Foreign trade balance data for legume seeds (2004–2012), with separate regression lines for alfalfa and red clover

Fig. 2.4 Foreign trade balance data for grass seeds (2004–2012), with separate regression lines for ryegrass and red fescue

During the same time period, the foreign trade balance for grass seeds has remained negative, with a few exceptions in 2007 and 2009. However, the overall trends are encouraging (Fig. 2.4).

Conclusions

Serbia is a country with great potential and capacities for agriculture. Agriculture is the basis of Serbian economy and it is the backbone of the development of the country. It is the only sector with a positive trade balance and agriculture products represent 21 % of the total export. Animal husbandry, as well as animal production and the feed industry, suffers from serious problems related to a decline in the number of farms and ruminants, especially cattle. This has resulted in a stagnation and reduction of meat and milk production. Fodder crops production in Serbia is largely based on domestic cultivars, which are favoured by farmers, due to familiarity and praxis. The forage crops species cultivar list currently available in Serbia consists of cultivars that are highly productive, have an improved dry matter quality and can achieve and sustain satisfactory levels of plasticity on a range of adverse weather conditions, not only in Serbia but also on the Balkan Peninsula. These cultivars have been bred from autochthonous genotypes that exhibited sufficient levels of adaptability and had satisfactory yielding performances. However, in practice, realised yields have been low due to several limiting factors, but these could be addressed.

In order to improve fodder crops production and animal husbandry in Serbia, some fundamental actions are needed. The implementation of a regionalisation of forage production is necessary. This would open up for regional recommendations on the species, mixtures and cultivars suitable for fodder production in each area. Stabilisation of the subsidy policy will allow farmers to plan ahead. Consolidation of fields and farms, especially in depopulated regions, has become an important task, since the current distribution and sizes of fields are wholly inadequate for profitable and sustainable production. The improvement of production technologies and grassland management, in order to produce better forage, remains the major task for scientists and experts. Finally, it is vital to improve the education of farmers and this requires a more efficient and improved system for the transfer of knowledge from scientists to farmers.

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Part II

Genetic Diversity Among and Within Species

Chapter 3

Ex Situ Conservation of Genetic Resources of Forage and Turf Species in the IPK Genebank at Malchow

E. Willner and K.J. Dehmer

Abstract The IPK Genebank, Satellite Collections North, at Malchow/Poel is responsible for the *ex situ* maintenance of genetic resources of forage plants in Germany. More than 10,400 accessions of grasses (forage and turf species) and 1,300 small grained forage legumes (red clover and alfalfa) belong to the collections, originating mainly from Europe, but partially also from destinations around the world. The main goal is to hold all accessions available for distribution and characterize/evaluate them for different research and breeding purposes as intensively as possible. For this means, a good documentation system is necessary, besides the management of PGR according to European Genebank Standards and a protocol for a quality management system (certificate ISO 9001:2008).

In specific research projects, accessions of the most important species like *Lolium perenne* L. and *Poa pratensis* L. are described for traits concerning morphology and/or relevant for breeding in order to have an assessment on the value of PGR for different uses.

In an overview, we will present which tasks have to be fulfilled and how this is achieved. Some results of characterization/evaluation trials and of specific research projects are provided, setting the stage for a better use of forage genetic resources in Germany, Europe and world-wide.

Keywords Plant genetic resources • Maintenance • Characterization • Evaluation and documentation • Genetic diversity • Valorization for breeding and research

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Introduction

The IPK Genebank with its comprehensive collections of cultivated plants is one of the largest in the world. The predecessor of IPK – Leibniz Institute of Plant Genetics and Crop Plant Research – was already established in 1943 near Vienna. First collections of important crops like cereals were compiled there. A few months later, the collections and the institute were transferred to Gatersleben. In its 70 years of existence since then, comprehensive scientific research was conducted, and parallel to a growing knowledge on cultivated plants, the institute expanded and the genebank collections grew.

Parallel to the development of the institute at Gatersleben and the genebank there, the compilation of special collections of crops at other institutes in Germany was initiated. They were used for breeding or scientific research. In the former Institute of Oil and Fodder Crops Breeding at Malchow, the first collections were established as working collections for breeding purposes by Prof. Hans Lembke, starting in the early 1960s. Here, substantial collections of oil and fodder crops and cultivated grasses were compiled over several decades. Since the German reunification in 1992, these valuable genetic resources belong to the IPK Genebank.

The Malchow oil and fodder crops collections are divided into three groups according to botanical families: *Gramineae, Cruciferae, Leguminosae.* They include more than 14,000 accessions of 17 genera and 140 species. To *Cruciferae* group belong all genera and species which are used as oil plants, e.g. *Brassica* sp*., Sinapis* sp*., Raphanus* sp. As *Leguminosae* fodder crops, only red clover and alfalfa as small grained legumes are maintained at Malchow. In the following, the third group, *Gramineae*, will be the matter of consideration.

To this group, the most important grass species for agriculture and breeding do belong, as forage and turf type. The largest species in the grass collections are *Lolium perenne* L. (3,016 accessions), *Dactylis glomerata* L. (1,885 accs.), *Festuca pratensis* Huds. (1,122 accs.), *Phleum pratense* L. (985 accs.) and *Poa pratensis* L. (727 accs.). But also smaller groups of grass species (*Agrostis* sp., *Festuca* sp., *Holcus* sp. *Deschampsia* sp., *Poa* sp. etc.) belong to the collections, in order to have a wide range of interspecific and intergeneric variability, as well as material for all research and different breeding purposes (as forage, turf or energy grass).

The Value of Genetic Resources: In General

In order to have a really comprehensive collection of grasses with a high level genetic diversity, it is necessary to fulfill following tasks: collection, maintenance, characterization, evaluation and documentation of grass PGR with high working accuracy of curators, which are responsible for the collections.

In the IPK Genebank, collections are permanently but selectively updated in order to close gaps and to enlarge genetic diversity. This happens in two ways, through seed exchange with other genebanks and botanical gardens or via own collecting trips. At the Malchow collections, the second option is preferred. Thus, several collecting trips were conducted since the 1990s in Europe, but also within Germany. In this way, 2,810 accessions from different geographic and climatic regions were newly collected. Intraspecific diversity of some grass species was substantially widened and new species were introduced, enabling the Malchow collections to permanently supply new base material for breeding and research.

The maintenance to secure PGR and to hold seed samples available for users, is affected by two ways: (i) the regeneration of new samples (collected material) or old ones, which have a low germination rate and (ii) the storage of seed samples for medium and long term and as safety duplicate. All processes for regeneration and storage are carried out at the IPK Genebank according to a quality management system (ISO 9001:2008 certificate). After successful multiplication (seed amount is sufficient and germination rate above 80 %), accessions are stored in three seed lots (i) as active collection for users and own cultivations; (ii) as base collection for long term storage and (iii) as safety collection in the Svalbard Global Seed Vault (since 2008: 60 % of whole forage collection, 75 % of grasses and 25 % of legumes).

The characterization of all samples is another important task in genebank work: A large collection itself is not useful if users cannot find the adequate material for their purposes. Thus, it is essential to have a good description of morphological and breeding relevant traits. This happens at the IPK collections at Malchow on the one hand in combination with cultivation for regeneration. During the vegetation period, accessions are observed for growth development and habit, disease susceptibility, phenology, leaf traits and plant height. On the other hand, in separate trials as so-called comparative cultivations, botanical identity is determined or checked, while in long–term trials over a period of several years overwinter survival/persistence of different species/accessions are checked.

In addition to characterizations, the evaluation of some groups of the grass collection is also necessary and very important for a valorization of PGR. As these evaluation trials are very complex, afford special equipment/labs/methodologies and are time- and cost-consuming, they are often conducted in co-operation with external partners. In recent years, some projects of grass evaluations were initiated on the national level, but also internationally (Nehrlich et al. 2013).

Another very important task is to document all accession information (passport, characterization and evaluation data) as well as possible. For all genebank users, it has to be very easy to obtain all available information of an accession and to search the adequate material for different purposes. IPK's Genebank Information System (GBIS, http://gbis.ipk-gatersleben.de/gbis_i/) was developed to enable users to look for all or special genera/species, accession name, country of origin etc. and several C&E-data, as far as these are recorded. Additional, users can search in further special databases, for forages e.g. in the European *Poa* database (EPDB; Weise et al. 2007), where you can find an overview about accessions of different *Poa* species. Here, the donor genebank or institute is recorded, as well as *Poa* accessions with passport data and where to order them. Also of interest could be information on weather data for the IPK sites at Gatersleben and Malchow (http://www.ipk-gatersleben.de/en/databases/weather-data/).

All handling of PGR at the IPK Genebank with its quality management system is targeted to provide a good service, which means to hold accessions available and send out seed samples upon request in sufficient amounts, with high germination rate and within short time. In the last 18 years, IPK Malchow distributed 1,779 seed samples to 64 users on average world-wide. Users are mainly scientists or breeders in Germany, Europe, USA, Asia, but also other genebanks, botanical gardens, NGOs or private persons. In comparison to earlier years, the last 4 years show a clear increase in user numbers, while also the number of distributed accessions is significantly above average. The reason for this is the better access to IPK's PGR through internet visibility via GBIS/I and an easy handling as users request seeds via this system. Nevertheless, seed deliveries are dependent on the start of new research projects or breeding programs, too.

Another important aspect of good service is data delivery regarding IPK accessions. More and more C&E data are available in GBIS/I. Furthermore, weather data can be downloaded from IPK's homepage for data analyses and better understanding of observation data in a specific year. For special user requests on data/information not available in GBIS/I, the curator can assemble excel sheets from data sources not yet public.

The Value of Genetic Resources: In Particular

At the IPK Genebank, Satellite Collections North, the forage collection was developed in the last 20 years towards the improvement of genetic diversity (more accessions per species/more species available), a better and easier access to seed samples/information and better description/valorization of PGR. With regard to the last topic, it was essential to get into contact with scientists and breeders, in Germany via the GFP and in Europe via ECPGR network or EUCARPIA, in order to agree upon and arrange special research projects for enhanced information on forage PGR. This will be a continuous process, as not all accessions can be included in one project and as new breeding or research goals require new projects.

At IPK Malchow, research projects are divided into projects for primary and secondary evaluation, respectively. The trials for primary evaluation are carried out in the field at IPK Malchow, or together with partners like the Eurograss breeding company (EGB) in order to have a first impression on the traits of accessions (growth, biomass development, disease/stress resistance or susceptibility, phenological data) from multiple sites/environments. Examples for secondary evaluations are the trials for green matter yield tests in forage types of *Lolium perenne* L. or rust evaluations in field and laboratory. Secondary evaluations are carried out together with several co-operation partners (breeders or scientists).

Fig. 3.1 IPK *L. perenne* L. collection sites in Europe (1996–2002)

Examples of Primary Evaluations

A joint plant exploration with EGB was conducted in Romania. The material collected was shared for multiplication in order to have sufficient seeds for characterization and evaluation trials. The main focus here was on *Lolium perenne* L. 455 populations were collected as seeds or living material. From each of these, 24 single plants were investigated in a primary evaluation at Malchow and Asendorf. The most important traits like ear emergence, growth rates at different stages, reaction to diseases, leaf width and persistence were assessed over 3 years. Based on the results, populations with similar traits were unified, totaling in 85 subgroups. These were transferred into the IPK Genebank as new accessions and supplied for further secondary evaluation (green matter yield, crown rust test).

Concerning rust susceptibility, primary evaluations in the field showed no large differences between and within the geographic areas and regions. Worth mentioning is the Crisana region, where all four areas have an average below the mean of this trial. These results correspond very well to results of a subsequent secondary evaluation by Dr. Hans Lellbach, Julius Kuehn Institute Gross Luesewitz, who applied the rust leaf segment lab test to progenies of the above populations. Twenty three of the 85 analyzed subgroups were scored as resistant, which totals 27 %. These subgroups originate mainly from the Crisana region, too (Willner et al. 2010).

In another primary evaluation, 352 newly collected accessions of *Lolium perenne* L. from different European countries (Fig. 3.1) were tested at IPK in special trials over 2 years in order to estimate behaviour over winter, biomass, phenological development and other relevant traits. All collected data were recorded in excel sheets (but not yet in GBIS/I) and used for classification into maturity groups, in order to plant the accessions correctly for a secondary evaluation trial (green matter yield estimation).

Further data analyses were carried out in co-operation with Dr. J.-P. Sampoux, INRA Lusignan. In analyses of variance, least-square means (LSM) of accessions were adjusted between countries by the mean of common standard cultivars included in the evaluation of accessions from each country. Canonical correlations analyses (CANCOR) were performed between phenotypic traits (LSM of accessions) and spatial parameters (latitude, longitude, altitude at collection sites). A first CANCOR analysis was performed at a European scale, using LSM adjusted by standard cultivars and raw spatial parameters. A second CANCOR analysis was performed at country scale after standardizing phenotypic traits, latitude and longitude by setting a null mean within countries. Auto-correlation patterns were investigated at country scale by setting up empirical variograms for standardized phenotypic traits.

The CANCOR analyses showed that latitude and longitude were strongly structuring the spatial distribution of the natural phenotypic diversity of perennial ryegrass at the European scale. The first canonical direction indicated that Northern latitudes and Western longitudes were associated with low heading percentage in the first year, late heading date in the second year, high greenness after winter in the second year, low rust resistance but high resistance to other diseases. The second canonical direction associated Northern latitudes and Eastern longitudes with weak greenness before winter in the first year and high rust resistance.

Results of Secondary Evaluations

Between the IPK Genebank at Malchow and the GFP, the Association for the Promotion of private German Plant Breeding, a good co-operation exists for more than 20 years. Within this framework, several projects were established for better description of PGR of forages and their use in breeding programs.

One of these projects was to evaluate the diversity of *Poa pratensis* L. In a first step, 1,600 seed samples of *P. pratensis* from different European countries, regions and sites were compiled and divided between four partners (three breeding companies and IPK) in order to establish a primary evaluation trial for 2 years. Based on the results, 50 accessions were selected per site (total: 200 entries), showing the largest variability in and between accessions with regard to traits like growth, heading date, leaf morphology, forage or turf type. Seeds harvested from three mother plants per each of the 200 selected accessions (total: 600 entries) were tested in a field trial for 15 traits (characteristic, breeding relevant) per year on 10 single plants per entry in comparison to the clone of the mother plant.

Leaf material of all entries from the field trial was sampled and transferred to the IPK laboratory at Gatersleben. Here, in a GFP/AiF funded project, ploidy levels and diversity were assessed, as well as potential correlations between geographic origin and diversity investigated. According to the results, no clear grouping according to geographical origin was observed, while the examined breeding accessions clustered separately. In addition, material from the Northern European Islands (Great Britain, Iceland, Greenland) is more distant in comparison to the continental ecotypes (Andreeva 2006). Data analyses of field and lab scoring resulted in selection of several best, most variable genotypes for different breeding purposes by the breeding partners of the project.

The co-operation of representatives from 36 European countries within the ECPGR working group on forages is another example for the generation of secondary evaluation data. The partners are responsible for the maintenance of PGR in their country, for keeping PGR available and promoting easy access to and better use of accessions. In regular meetings, working group members discuss up-coming tasks, co-operations and sharing of responsibilities (e.g. Maggioni et al. 2010). At the moment, the main focus is to create An European Genebank Integrated System (AEGIS) as European Collection which unifies the most important PGR of several countries and to maintain these according to the AEGIS quality management system. In the forages working group, there are ten databases on grasses and legumes. IPK Malchow is responsible for the *Poa* database (Weise et al. 2007), especially regarding updates and determination of most original samples and AEGIS accessions. A further task will be to include all available C&E data. At the moment, there is a discussion with experts of the ‚doc and informatics’ network group about the procedure for handling C&E data.

Conclusions and Outlook

In the IPK Genebank, Satellite Collections North at Malchow, a very comprehensive collection of grasses exists, 84 % of which is readily available for users. The collection of grasses, including a high intraspecific variability, is well described in respect to characterization and evaluation data. Most characterization data are online searchable via GBIS/I. Evaluation data are available upon request as excel files from the curator.

The advanced status of the collection was achieved mainly during the last 10 years. This positive development would not have been possible without the contributions from co-operation partners in Germany, Europe and world-wide. Thanks to all partners in past, presence and future.

In the near future, improvement of data analyses and easy access to this information via GBIS/I are the next important task, which only can be completed in co-operation with partners in further projects. Besides the continuation of existing co-operations, we are very interested in finding additional co-operation partners, in particular for data analyses.

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Chapter 4
Genetic Response to Climate Scenarios in *Dactylis* and *Festuca* of Temperate *Versus* Mediterranean Origin

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Abstract Climate change stresses the importance of exploring the genetic adaptation of plants to an increase of both temperature and water deficit. To this end, dense swards of Mediterranean and temperate cultivars of *Dactylis* and *Festuca* were seed harvested after 2 years under artificial climate scenarios (range of summer drought) in two environments (Mediterranean and temperate). To assess possible genetic changes, all populations of the next generation were assessed and compared with their parent population having not been subjected to these climate scenarios. The experiment was a spaced plant design in the temperate location. The results showed that a drought escape strategy through earlier heading time and reduced total plant yield was enhanced by scenarios of greater summer drought. However, the direction and the intensity of the response of the main measured traits suggested that the physiological plant adaptation to water stress differs between species. In *Dactylis*, the phenology appeared to be a very responsive trait in the late heading temperate cultivar and induced a plant size reduction, while no genetic variability seems to be still available for selection within the early flowering Mediterranean cultivar. By contrast, the balance between both traits was not found so crucial in *Festuca* which suggests a possible greater role of the rooting system in drought resistance for this species.

Keywords Population genetics • Perennial grasses • Adaptive response • Phenology • Drought tolerance

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Introduction

Climate change is raising new questions in genetics and breeding of forage crops. Models of plant production and climate projections in France (Terray et al. 2011), predict an increase of temperature beneficial to annual forage production in northern latitude because of growth enhancement in spring and autumn. Conversely, more severe summer drought will threaten more frequently any forage production in southern latitude in particular in the Mediterranean area (Durand et al. 2011). At this scale, Gordo and Sanz (2010) showed that many taxonomic groups are able to tune their phenology according to temperature variation over years as a plastic response. Investigating aerial biomass and senescence among ecotypes of various origins in four grass species submitted to extreme climate manipulations, Beierkuhnlein et al. (2011) underlined the importance of between and within genetic variability against interspecific variability in plant response to climate.

Most forage crops are out-breeding plants. As a consequence ecotypes as well as bred synthetic varieties necessarily harbour a minimum amount of genetic variability which is a prerequisite for selection to occur. However, individual plant reaction to water deficit involves many successive physiological processes over time which relationships and relative effects among and between species are still unclear in terms of genetics. In particular, it is unknown whether climate stressors may act as selection pressures in a dense sward of perennial grasses liable to heritable genetic changes over generations.

Dactylis sp. and *Festuca* sp. are good plant models to investigate these issues. Both complexes of species, as diploids in *Dactylis* and polyploids of high level in *Festuca*, are presently spread all over South-Europe having experienced strong climate variations in the course of evolution. Well-adapted ecotypes which have emerged, in particular in the Mediterranean area, are of great interest as genetic resources for breeding and to untangle the physiologic processes of plant adaptation to water deficit (Lelièvre et al. 2011). By applying experimentally climate scenarios in plots of *Dactylis* and *Festuca* of either temperate or Mediterranean origin, Poirier et al. (2012) showed that an annual water deficit of 500 mm clearly discriminates more between ecotype origins than between species with antagonistic relationships of annual productivity from the temperate cultivars vs. long term persistency from the Mediterranean ones.

In this context, it was interesting to couple the evaluation of agronomic response to climate scenarios with the quantification of possible genetic changes by using the same experimental plant material. This may be carried out longitudinally through plant sampling in a sward at successive periods of climate stress followed by new experimental assessment. However, this would not avoid all artefacts or bias due to plant cloning and limitation in sampling. Furthermore, this does not give access to the heritable part of the response liable to long term species evolution. We hereafter report the results of an evaluation in which all experimental plots were seed-harvested by the end of a 2-years long climate scenario experiment and all next generation populations were compared against the initial batch of seeds of each cultivar as control parent populations.

Material and Methods

In a preliminary experiment, two cultivars of temperate vs Mediterranean origin in *D. glomerata* and *F. arundinacea* were cultivated in Lusignan as a temperate site (lat. 46.4029716 – long. 0.0815821) and in Montpellier as a Mediterranean site (lat. 43.6379542 – long. 3.8623542) under 4 climate scenarios, either by increasing summer water deficit and/or by applying artificial heat waves in July (Poirier et al. 2012). In particular, the S1-scenario consisted in a control, holding water supplies at each site to its annual average (1980–2010). The S2-scenario halved water supplies by rain-shelter covering at night all year long. The cumulated water deficit (water supplies – evapotranspiration in spring and summer) which was achieved over the two first years in the scenario S1 and S2 amounted to −762 and −981 mm respectively in the temperate site and to −1,060 and −1,252 mm respectively in the Mediterranean site. Summer survival rate differentiated strongly between origin and site by decreasing down to 20 % in the temperate cultivars under the S2-scenario at the Mediterranean site. This did not prevent, however, to seed-multiply in-situ each experimental plot in spring of year-3 following almost complete recovery of the swards in the previous autumn. The 64 resulting populations of next generation (4 cultivars × 2 sites × 2 scenarios × 4 replicates (site × scenario)) as well as the 4 parent populations were established in a field nursery of spaced plants in the temperate site. The design was a split-plot in which the species was ascribed to the whole-plot, each of them further arranged in a randomized 3-complete blocks design of 32 next generation populations and 2 parent populations. The single plots were rows of 10 plants; in order to keep the same accuracy of mean estimates between next generation and parent populations, those latter were randomly planted into 3 rows × 10 plants each block. Following establishment in autumn, heading time was recorded in spring of the next year as the date of 2-spikes emergence and expressed in thermal time (°C × day since the 1st of January).

As a parallel second experiment, all next generation and parent populations were transplanted, following germination on March 1st, in 20-cm deep containers filled with compost. After 3-months of growing in glasshouse and no watering limiting conditions, all plants were clipped to 5 cm stubble height and aboveground biomass dried to estimate total plant dry matter yield. Before cutting, the lamina length of the last emerged adult leaf and the total plant height by stretching the leaves were measured. The design was a split-plot similar to the field nursery except that the replications in each site x scenario combination were randomly confounded with the present blocks in the design. Each species as a whole-plot was thus arranged into a randomized 4-complete blocks design of 8 next generation populations (2 cultivars × 2 sites × 2 scenarios) and their 2 parent populations. The blocks consisted of single containers of 10 randomized rows (populations) × 10 plants, each about 10 cm apart, surrounded by bordering plants.

Plant Material

The four cultivars which were used are registered on the French national list of varieties (http://www.herbe-book.org), both dactylis cultivars Ludac and Medly and the tall fescue cv. Centurion in 1997 while the other tall fescue cv. Soni in 2000. Medly is a typical very early flowering tetraploid *D. glomerata* cv. of Mediterranean origin bred from 9 entries from Australia × South France hybrids and Morocco which, however, the contribution between *hispanica vs glomerata* ssp. was not well defined. Centurion is a hexaploid *F. arundinacea* cv of 7 entries from Israel, Tunisia and Morocco, quite genetically distant from its temperate relative as crosses between the two origins give only sterile F1-hybrids (Jadas-Hécart and Gillet 1978).

Statistical Analyses

The data recorded in both trials were computed as 68 populations (4 cultivars × (16 next generation populations + the parent population)) under a mixed model of analysis of variance and holding all bloc interactions and plant within plot variation as random effects. The *proc mixed* procedure of SAS (1999) was used as well as the *lsmean*, *estimate* and *contrast* statements to test significance of the site × scenario genetic response within each cultivar. The responses were expressed as a standardized way (i.e. in standard deviation units, σ) by dividing the contrast of the mean of the next generation populations and that of the parent population by the residual term of Anova as an estimate of the phenotypic variance within cultivar. The responses were also fitted to regression models, nested or not within cultivar, by using the 2-years water deficit in each site × scenario as a regressor.

Results and Discussion

Ecotype differentiation strongly contrasted across species (Table 4.1). Expressed in standard deviation units, heading date differed by 4.28 σ between the Mediterranean and the temperate parent population in *Dactylis* while by 0.36 σ for above ground biomass. Both traits differentiated in a more balanced way within *Festuca* (−1.05 σ *vs* 0.99 σ resp.).

Furthermore, the traits correlated in an opposite way across species; earlier heading time was associated with less above ground biomass in *Dactylis* while in the early flowering Mediterranean *Festuca* all growth traits were higher than those in the temperate cultivar.

It is likely that variable chilling temperature in glasshouse during spring enabled the *Festuca* cv. of Mediterranean origin to exhibit higher plant above ground biomass than the temperate cv. A negative response of heading time in all populations of next generations was found, on average, over site × climate scenarios while

Table 4.1 Mean value of the traits recorded in the parental population of four cultivars of either temperate or Mediterranean origin in *D. glomerata* and *F. arundinacea* (interval of confidence at P<0.05 in brackets)

	Dactylis		*Festuca*	
Traits	Temperate	Mediterranean	Temperate	Mediterranean
Heading date (°C × d)	700	444	755	692
	[682–718]	[426–462]	[737–772]	[674–709]
Above ground biomass (mg DM plant^{-1})	1,319	1,168	819	1,235
	[1,095–1,543]	[945–1,390]	[591–1,048]	[1,012–1,458]
Plant height (mm)	668	563	534	600
	[633–703]	[529–598]	[499–569]	[565–634]
Adult lamina length (mm)	430	395	381	434
	[409–471]	[364–425]	[350–412]	[403–464]

above ground biomass responded positively in the plants from the temperate site and negatively in those from the Mediterranean site (Fig. 4.1). Negative regression clearly suggests that a drought escape strategy is primarily enhanced by increasing water shortage. Because of higher accuracy estimates, the response of heading time was found significantly different across cultivars (F 7:48 df = 2.907; $P<0.0120$). Only a unique linear response ($r^2=0.747$; F 1:14 df = 41.23; $P<0.0001$) was found in above ground biomass while heading time fitted better to a quadratic model ($r^2=0.972$; F 2:13 df = 227.31; $P<0.0001$) emphasizing a particularly strong decrease from the temperate *Dactylis* grown under the S2-scenario in the Mediterranean site. By contrast, the Mediterranean cultivar of *Dactylis* gave no significant response of heading time across all site × scenario combinations.

By plotting the response of both traits in the range of the origin differentiation within species, overall changes from the temperate cultivar of *Dactylis* seem to mimic to some extent the natural evolution which occurred in that species towards earlier heading date and lower growing rate (Fig. 4.2). It appears therefore significant that the Mediterranean cultivar at the extreme of the distribution of heading date did not display any further significant response for that trait as if no genetic variability was still available.

The response in tall fescue appeared more balanced in the same way as the differentiation between parent populations was less pronounced than in *Dactylis*. The Mediterranean cultivar of tall fescue reacted similarly to the temperate cultivar of *Dactylis* by adapting both its phenology and plant size. By contrast, the response within the temperate *Festuca* seems to involve more positively the traits associated with growth rate. Rooting is considered as a major trait controlling persistency in tall fescue. Possibly, positive response of above ground biomass in the temperate *Festuca* results from an indirect response to selection for early deep rooting conferring higher fitness when plants have to cope with water deficits later in summer.

Using polyethylene or cotton wrap to prevent pollen contamination between plots may have induced artefacts by increasing temperature in the days before seed harvest. It is also obvious that harvesting seeds from 3-years old swards do not

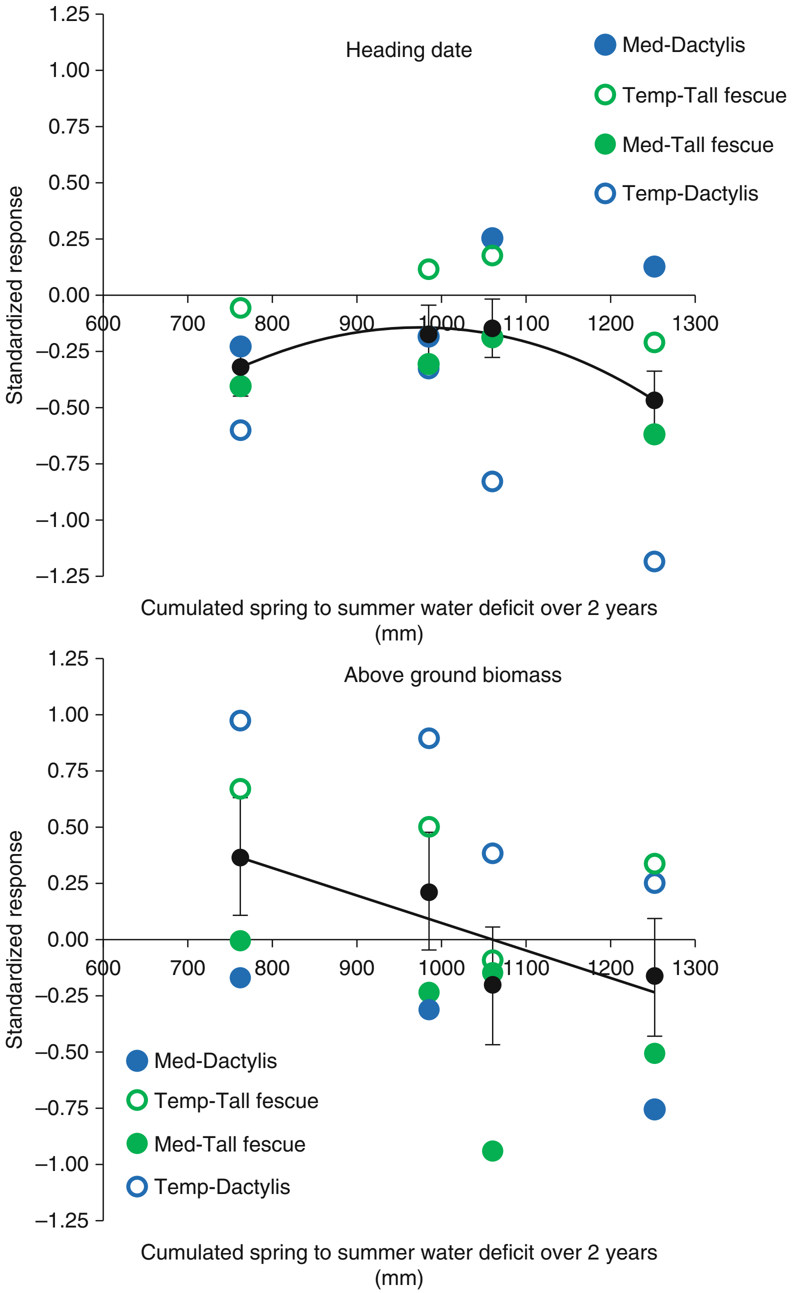

Fig. 4.1 Mean response of heading date and plant above ground biomass to 4 site × climate scenario combinations expressed against 2-years of cumulated spring to summer water deficit in 4 cultivars of either temperate or Mediterranean origin in *D. glomerata* and *F. arundinacea*

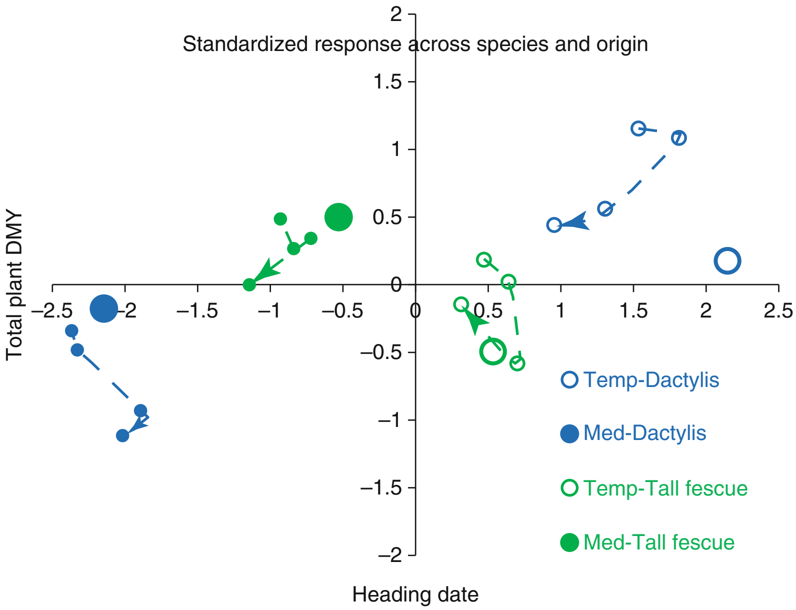

Fig. 4.2 Associated response of heading date and above ground biomass across site × climate scenarios in Mediterranean and temperate cultivars of *D. glomerata* and *F. arundinacea*. The symbol of the parent population of each cultivar is of larger size; the arrows rank the response of the next generation populations across scenario × site according to increasing water deficit, from S1-scenario × temperate site to S2-scenario × Mediterranean site

include only response to water deficit. In this respect, positive response of above ground biomass under the least water limiting scenario in the temperate site could result from a response for competing ability, expressed at early stage of plot establishment and/or during recovery in autumn. In any case, the results show that substantial genetic response can result from genetic variability restricted to a unique cultivar as a parent population. Mean standardized response about 0.5 σ in the absolute implies that true mortality occurred in swards despite apparent overall recovery and that it was accompanied by strong individual selection (e.g. 10 % of rate of selection assuming moderate heritability *s.s.* of 0.30). In some instances, the responses which were observed are orthogonal to species and/or ecotype within species differentiation which suggests that all traits are not of the same importance in plant responses to water stress and they are under complex non-linear control. In particular, it clearly appears that phenology in *Festuca* would not be so crucial for climate adaptation as it is in *Dactylis* or, at least, that it may act secondarily once sufficient deep rooting is achieved through natural selection as seen from the Mediterranean *Festuca* cultivar.

As the response to water deficit prioritizes an escape strategy, to aim at more productivity or, at least, maintaining a same level while maintaining persistency remains a challenge for plant breeders. In this respect, refinement in the climate change models will help to better define forage ideotypes of productivity *vs* persistency alternative in terms of annual climate risk. Future work is presently undertaken to estimate the relative role of phenology in *Dactylis* and *Festuca* drought tolerance from crosses between and within origins. Molecular DArT markers are simultaneously developed to assist the assessment of genetic response as well as to breed for ideotypes (Ghesquière et al. 2012). In this respect, *Dactylis* provides a good genetic model because of total cross-compatibility within the whole complex of species. Moreover, strong origin differentiation and response which were recorded suggest that strong linkage disequilibrium with molecular markers could exist thus facilitating the detection of further genetic changes as well as breeding for a better adaptation from inter-origin crosses.

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Chapter 5
Monitoring of Vegetation Changes in Selected Sinkholes in the Moravian Karst, Czech Republic

Tomáš Vymyslický and Zdeněk Musil

Abstract Sinkholes are very important phenomenon increasing the diversity in karst landscape. Twenty sinkholes in the area of Moravian karst have been monitored since 2000. Plant recording and phytosociological releves have been performed twice a year. The sinkholes are located at karst plateau, with intensive agriculture, resulting in very intensive erosion and underground water eutrophisation and pollution. That is why grassland belts were established along most sinkholes to decrease the level of erosion and eutrophisation. It was partly successful, but especially in the central part of the sinkholes ruderal plant species prevailed. The most predominant ruderal species are *Urtica dioica*, *Agropyron repens* and *Cirsium arvense*. After spraying total herbicide and sowing a special seed mixture in the year 2010 the situation significantly improved. Total number of plant species before the treatment varied from 19 to 86, while the actual numbers are from 30 to 78 per sinkhole. Basing on the results of the monitoring, managements were proposed and applied. The most important management practices are 2–3 cuts per year, removing the biomass, keeping the grassland belt around the sinkhole and planting groups of native small trees and shrubs in order to form natural formations for animals.

Keywords Sinkholes • Grasslands • Monitoring • Management • Seed mixtures

Introduction

Karst is a typical landscape, created by specific characteristics of bedrocks and water regime. It develops in well soluble rocks – limestone, dolomites, and gypsum. In dry desert conditions, salty karst can be found. Karst phenomena, like sinkholes,

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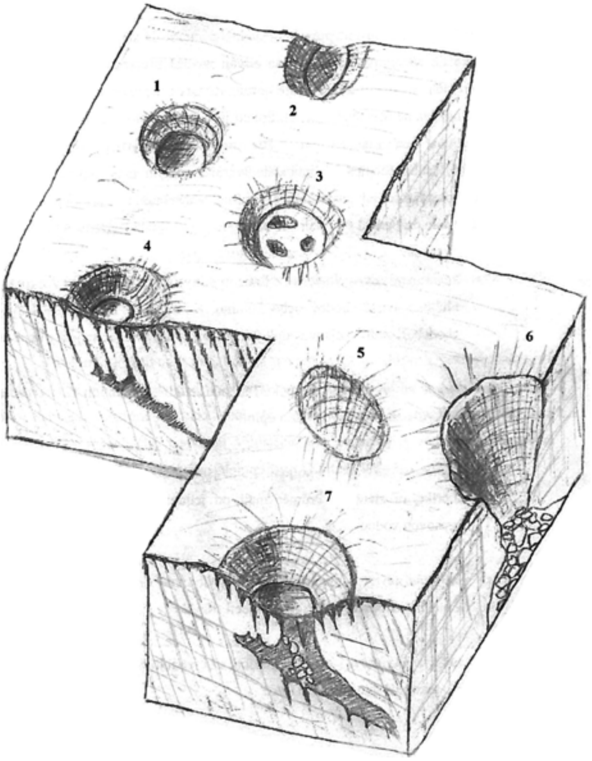

Fig. 5.1 Sinkhole types in Moravian Karst area according to Kudličková (2012): *1*, *2*, *4* – closed bowl-shaped sinkhole; *3* – open bowl-shaped sinkhole with throats; *5* – funnel-shaped sinkhole; *6* crashed sinkhole; *7* – the beginning of creation of crashed sinkhole

are typical and are connected with permeability for water (Bell and Culshaw 2005; Nisio et al. 2007).

Moravian karst, located approximately 30 km to the north of Brno, is the biggest and most developed karst in the Czech Republic. More than 1,100 caves are known, five of them being opened to public visits. Typical relief shapes are sharp, deep and dark valleys and also plateaus with sinkholes occurrence. Moravian karst is valuable not only from the point of view of biodiversity, but also for geodiversity. Sinkholes are surface karst phenomena, surface depressions creating connection between surface and underground. When the bottom is broken down, abyss is created (Fig. 5.1). Main communication agent is water, so its quality is one of crucial factors in Nature protection of karst areas.

In Moravian karst, there are sinkholes on agricultural land that are in some cases intensively used (Kudličková 2012). Their history is longer than history of agriculture, trees were cut down, and pastures and meadows were established. After collectivisation around 1950, surrounding area was ploughed. Sinkholes became inaccessible and lack of management led to ruderal species predominance. Together with intensive agriculture they became entrance place for residua of fertilisers, pesticides and eroded soil into cave systems. In last years extensification of agriculture started, most of sinkholes are now surrounded by grasslands, which reduce soil erosion and water pollution. Grass seed mixture for establishment of grasslands around the sinkholes should be of regional origin. Trees and shrub planting is also desirable. To maintain a sinkhole in good conditions, grass mowing twice a year is essential. So, sinkholes are very typical elements of karst landscape and serve as refuges of biodiversity. The aim of the study was to observe species composition change in the period of 2001–2012.

Material and Methods

Together with Protected Landscape Area Moravian karst administration 20 sinkholes with clay walls and covered with grassland vegetation were chosen in 2000. Floristic composition was recorded in May and June and in September to record as many plant species as possible. Taxonomy was unified according to Key to the Flora of the Czech Republic (Kubát et al. 2002). Vegetation of sinkholes is very variable – according to eutrophication level and management intensity. The most widespread swards are alliances *Arrhenatherion* and *Cirsio-Brachypodion pinnati*. In big sinkholes the vegetation changes according to slope aspect. Southern slopes are familiar with thermophilous vegetation of broad leaved steppe species, like *Brachypodium pinnatum*, *Bromus erectus*, *Carlina acaulis*, *Festuca rupicola*, *Potentilla recta*, *Primula veris*, *Ranunculus polyanthemos*, *Sanquisorba minor*, *Verbascum austriacum*, *Veronica teucrium*, *Vicia tenuifolia* etc. Northern slopes are covered by mesophilous grassland vegetation with species like *Achillea millefolium* agg., *Arrhenatherum elatius*, *Campanula patula*, *Centaurea jacea*, *Crepis biennis*, *Dactylis glomerata*, *Festuca rubra* agg., *Galium album*, *G. verum*, *Hypericum perforatum*, *Knautia arvensis* agg., *Lathyrus pratensis*, *Leontodon hispidus*, *Leucantheum vulgare* agg., *Lotus corniculatus*, *Phleum pratense*, *Plantago lanceoloata*, *Poa pratensis*, *Ranunculus acris*, *Tragopogon orientalis*, *Veronica chamaedrys* agg., *Vicia cracca*, etc. Unfortunately, in most cases the bottom part, called throat, is overgrown with ruderal species like *Urtica dioica*, *Rumex obtusifolius*, *Agropyron repens*, *Calamagrostis epigejos, Taraxacum officinale* agg. etc.

During the year 2009 herbicide was applied in the throat part of sinkholes with ruderal vegetation, dominated by *Urtica dioica* and *Rumex obtusifolius.* After destruction of the vegetation in March 2010 two grass seed mixtures were sown. Both mixtures contained Czech grass varieties. Component selection was based on floristic data and availability of seeds on the market.

Mixture 1: *Agrostis gigantea* cv. Janek (10 %), *Festuca pratensis* cv. Rožnovská (10 %), *Phleum pratense* cv. Skala (10 %), *Poa pratensis* cv. Hetera (20 %), *Festuca rubra* cv. Tradice (30 %), *Lolium multiflorum* cv. Rožnovský (20 %).

Mixture 2: *Agrostis gigantea* cv. Janek (10 %), *Festuca pratensis* cv. Rožnovská (10 %), *Phleum pratense* cv. Skala (10 %), *Poa pratensis* cv. Hetera (20 %), *Festuca rubra* cv. Tradice (50 %).

Results and Discussion

If 13 years of sinkhole monitoring in Protected Landscape Area Moravian karst would be summarised, significant improvements have been observed. This was caused by regular management, especially mowing. In the 90s, grasslands were established around sinkholes. Later, after the year 2000, selected wood and shrub species had been planted in the sinkholes and in 2009 and 2010 herbicide was applied in the throat part and grass seed mixtures were sown. *Agrostis gigantea* cv. Janek, *Festuca pratensis* cv. Rožnovská, *Phleum pratense* cv. Skala were the most successful sown species. In the first year *Lolium multiflorum* cv. Rožnovský quickly occupied the gaps and thus prevented ruderal species germination from the bare soil and subsequent invasion process. Under its canopy seedlings of meadow dicotyledon species could slowly develop and in the second year they replaced the *Lolium multiflorum*.

At present the state of the sinkholes is more or less stabilised. Suitable management is necessary to prevent plant species decrease. It is also necessary to reduce the level of eutrophication and erosion around the sinkholes. It means to establish and manage the grassland belts.

Concerning the number of species (Fig. 5.2), the best state was in sinkhole No. 20, where the species number fluctuated from 60 to 86 vascular plant species. On the opposite in sinkhole No. 1 the number of species varied from 15 to 40. The highest numbers of plant species were recorded in the years 2012 (in 5 sinkholes), 2004 and 2011 (in 4 sinkholes), and in the year 2010 (in 3 sinkholes). This trend positively reflects management connected with spraying the herbicide and subsequent sowing of grass seed mixtures in the year 2010. Total number of plant species before the treatment varied from 19 to 86, while the actual numbers are from 30 to 78 per sinkhole.

Increasing the vascular plant diversity is possible by spontaneous seed dissemination from intentionally un-mown parts of sinkholes with the best vegetation, and

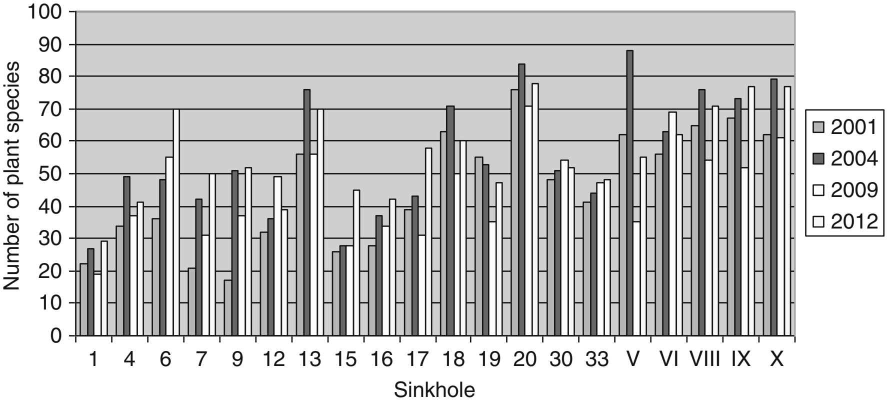

Fig. 5.2 Number of plant species in selected years of sinkholes monitoring

also thanks to occurrence of annual and ephemeral plant species growing on disturbed sites. Planting wood and shrub species in small patches on the sinkhole margins could be evaluated as an extra positive management. It is necessary to protect young woody plants against game browsing. If not, growth delay or withering would be inevitable in the next years. Woody species formations are good refuge and feed source for many insect and animal species. In the future, planting of old landraces of fruit trees could be good, especially along paths and field roads, where people walk.

For the maintenance of current state it is necessary to cut at least once a year, if possible 2–3 times, and to properly remove the cut biomass from the sinkhole. In most sinkholes mowing nitrophilous vegetation of the throat 2–3 times per year is recommended.

If the sinkhole margin is surrounded by intensively managed fields, the level of fertilisation should be reduced as much as possible. The best solution is to establish grassland belt. Newly established grassland belts should be preferably sown with local grass, clovers and herbs varieties or landraces. As the subsequent step it would be good to assemble, test, certify and produce regional species rich grassland mixtures for the purpose of grassing. In the past woody species were very often planted in stripes along the sinkhole, but it would be better to create small groups and to protect the plants against game browsing.

Speleological workers, opening new entrances into caves, represent another problem. They cumulate heaps of soil and stones in the sinkhole for a long time, and destroy all the vegetation. After this material removal there is only bare soil, which is very quickly covered by ruderal vegetation. Sometimes, when heavy machinery is used, all the sinkhole vegetation is destroyed or damaged.

Monitoring grassland vegetation in the sinkholes is a long term activity. Positive changes after management intervention were clearly visible even in the following year (decrease of ruderal species coverage, increase in plant species number). But we do not know how the situation will look like in the next years, so this monitoring should continue.

Management types in karst areas were compared to Veselý and Skládanka (2008). He emphasizes similar management practices like we did in our study. The most important is to prevent eutrophication and to remove regularly biomass of ruderal species. If grassland cutting is excluded, significant negative changes start to occur (higher stand, decrease of moss layer, and lower number of plant species). Mosaic cutting of sinkhole grasslands with good quality is recommended not only because of plant diversity, but also because of insects. Presence of woody species in sinkholes is also very important, but in small groups.

Conclusions

Sinkholes are very important features increasing the diversity in karst landscape. In the karst plateau, sinkholes are the only natural grasslands surrounded by agricultural land. So they have also great importance as source of valuable regional genetic resources. Many species have been collected, stored in the Czech national gene bank and used in breeding programmes.

Based on the monitoring results, managements were proposed and applied. The most important management practices are 2–3 cuts per year, removing biomass, keeping grassland belt around the sinkhole and planting groups of native small trees and shrubs in order to form natural formations for animals.

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Chapter 6
Evaluation of a Diverse Collection of Red Clover Germplasm for Susceptibility to Clover Rot (*Sclerotinia trifoliorum*) and Other Important Traits

Tim Vleugels, J. Baert, and E.van Bockstaele

Abstract Clover rot, caused by *Sclerotinia trifoliorum* or *S. sclerotiorum*, is an important disease in European red clover crops. Little is known about the differences in aggressiveness among *Sclerotinia* isolates from red clover. No completely resistant cultivars are available and few previous studies have screened large red clover collections for resistance. Moreover, the relations between susceptibility to clover rot and architectural and biotic factors were never studied before. In this study we compared the aggressiveness of 30 *Sclerotinia* isolates from 25 locations in Europe. Aggressiveness was assessed on young plants from five cultivars with different resistance. Additionally, we evaluated a diverse collection of 121 red clover accessions. Yield, branching, flowering date, susceptibility to mildew (*Erysiphe polygoni*), rust (*Uromyces trifolii*) and viral disease were scored on the field. Clover rot susceptibility was determined by inoculating young plants with five aggressive *Sclerotinia* isolates. Our 30 isolates differed in aggressiveness. Red clover accessions differed in susceptibility to clover rot, but no accession proved completely resistant. Cultivars were more resistant than landraces and wild accessions. Tetraploid cultivars were more resistant than diploid cultivars. Clover rot susceptibility was not correlated with the degree of branching or plant yield. On the other hand, late flowering and erectly growing accessions were less susceptible to clover rot. Clover rot susceptibility was not correlated with susceptibility to rust or viral diseases, but negatively with mildew susceptibility. The best way to improve resistance may be to recurrently select for resistant plants among diverse cultivars and landraces with lower susceptibility. Tetraploidisation of diploid populations with a higher resistance level can provide an additional level of protection.

Keywords *Sclerotinia trifoliorum* • Red clover • Pathogenicity • Resistance

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Introduction

Red clover (*Trifolium pratense*) is an important perennial forage crop, grown as a pure stand or in association with companion grasses. It is valuable because of its nitrogen fixation, its high quality as a forage crop and its beneficial effects on soil structure (Boller et al. 2010). Clover rot, also clover cancer or *Sclerotinia* crown and root rot, causes severe damage to European red clover crops in regions with mild winters or heavy snow cover. Two pathogens can induce clover rot: *Sclerotinia trifoliorum* Erikks. or *S. sclerotiorum* de Bary., though *S. trifoliorum* is most common on red clover (Vleugels et al. 2012). Symptoms of both pathogens on red clover are similar. In autumn, *S. trifoliorum* develops apothecia on sclerotia in the soil that release large quantities of ascospores. Ascospores infect red clover leaves and infection progresses during winter. The disease outcome depends heavily on weather conditions: under favourable conditions, complete loss of stands can occur (Boland and Hall 1994; Öhberg 2008; Pratt and Rowe 1995; Saharan and Mehta 2008; Taylor and Quesenberry 1996). Breeding for clover rot resistance is hampered by the influence of weather conditions. Natural infection is unreliable so that resistance breeding needs artificial bio-tests (Delclos and Duc 1996; Öhberg 2008; Saharan and Mehta 2008). Yet little is known about the pathogenicity of *Sclerotinia* isolates from red clover, so that is difficult to choose suitable isolates for resistance breeding. Various studies found variation in clover rot resistance within and among populations but no completely resistant populations have been found (Dabkeviènë and Dabkevièius 2005; Delclos and Duc 1996; Marum et al. 1994; Öhberg 2008). Until now, the relations between clover rot susceptibility, architectural factors and biotic factors have never been investigated. It is unknown whether wild populations, landraces or cultivars are the most desirable sources of resistance. Previous studies suggest that tetraploid cultivars are more resistant than diploid cultivars (Öhberg et al. 2008; Taylor and Quesenberry 1996). Growth habit, the level of branching and plant yield may influence clover rot susceptibility, as prostrate, intensively branching populations would be less susceptible (Taylor and Quesenberry 1996). Also late flowering cultivars are suggested to be less susceptible (Öhberg 2008). Finally, nothing is known about the relation between susceptibility to clover rot and other diseases such as mildew (*Erysiphe polygoni*), rust (*Uromyces trifolii*) and viral diseases.

In this study, we compared the aggressiveness of 30 *Sclerotinia* isolates from different European countries. Clover rot susceptibility was studied among a diverse collection 121 red clover accessions, including 83 accessions from the USDA National Plant Germplasm System (NPGS) core collection (Kouame and Quesenberry 1993). Sources of resistance were identified and the effects of variety type, ploidy level, growth habit, degree of branching, plant yield, flowering date and susceptibility to other diseases were investigated.

Material and Methods

Aggressiveness Study

In previous research, 154 *S. trifoliorum* and 38 *S. sclerotiorum* isolates from red clover were obtained in 25 locations in Europe (Vleugels et al. 2012). For this study, a set of 26 *S. trifoliorum* isolates and 4 *S. sclerotiorum* isolates was chosen so that all locations were represented and genetic differentiation was maximised. Aggressiveness was assessed on plants of five cultivars with different susceptibility according to the Swiss variety list (Suter et al. 2008): Astur, Milvus, Global, Suez and Mont Calme. HerkuPlast (QP96T) trays were sown with the five cultivars, two random columns per cultivar, and a border cultivar. Each isolate was inoculated on one tray and the experiment was repeated three times. 12-week old plants were inoculated with 1.5 ml suspension of mycelium fragments and incubated between 15 °C and 25 °C on a daily watered ebb and flow table covered with an opaque plastic cap. After ten days, plants were scored on a scale from 1 (healthy plant) to 5 (dead plant) and the disease severity index (DSI) was calculated (Marum et al. 1994; Vleugels et al. 2011).

Screening a Diverse Red Clover Collection

A field trial was established with 121 red clover accessions and 30 plants per accession, including 83 accessions from the NPGS core collection. Important traits were scored and the average score was calculated for each accession. Growth habit was scored as prostrate or erect. Branching was scored from 1 (sparsely branching) to 5 (extensively branching). A yield index was calculated as the percentage of maximum attainable yield in 3 years. Susceptibility to mildew and rust were scored from 1 (healthy) to 5 (heavily infected) and susceptibility to viral diseases was calculated as the percentage of affected plants after 3 years. A similar collection of germplasm with 79 NPGS core accessions and 34 diploid and tetraploid cultivars was screened for clover rot susceptibility by our bio-test on young plants. Seedlings of each accession were planted in two columns of two HerkuPlast (QP96T) trays, so that four columns (32 plants) were available per accession. Inoculation was done with a mixture of 8000 mycelium fragments ml^{-1} per isolate from four aggressive *S. trifoliorum* and one aggressive *S. sclerotiorum* isolate. The bio-test was executed as described for the aggressiveness study. The average susceptibilities of wild accessions, landraces, and cultivars were compared and the average susceptibility of diploid cultivars was compared with tetraploid cultivars. Correlations were calculated between susceptibility to clover rot and growth habit, branching, plant yield, flowering date, mildew susceptibility, rust susceptibility and susceptibility to viral diseases. Results were analysed with ANOVA and t-tests.

Results and Discussion

Aggressiveness Study

DSIs between 50.8 and 84.1 % were observed and isolates differed significantly in aggressiveness ($p<0.001$). There was a small but significant interaction between isolates and cultivars ($p=0.006$), although no pathotypes were observed.

Screening a Diverse Red Clover Collection

Clover rot susceptibility varied extensively within accessions and among accessions ($p<0.001$), which is characteristic for an outbreeding crop. The tetraploid cultivars 'Tedi' and 'Maro' and the diploid landrace 'No 292' were more resistant, while 15 diploid accessions were more susceptible than the average accession. While the most susceptible accession had an average DSI of 89.6 %, the most resistant accession still had a DSI of 53.8 %, indicating that there were no completely resistant accessions in our collection. Our results indicated that cultivars (73.6 %) were slightly less susceptible than wild accessions (76.0 %) and landraces (75.6 %), but not significantly. Artificial selection in cultivars or natural selection in landraces may have increased the resistance level of cultivars and landraces. Tetraploid cultivars (75.1 %) were significantly less susceptible than diploid cultivars (65.8 %) ($p<0.001$). This is consistent with previous studies (Delclos and Duc 1996; Halimi et al. 1998; Öhberg et al. 2008). Tetraploids may be more resistant because the double genome that allows a higher allelic diversity at immune genes and higher amounts of proteins involved in immune function (King et al. 2012). Furthermore, accessions with a high fraction of erect plants were less susceptible to clover rot ($r=-0.238$, $p=0.015$). The erect growth habit could reduce the relative humidity around the plants, so that the establishment of clover rot is slowed down. Clover rot susceptibility was not correlated with branching ($p=0.061$), nor with plant yield ($p=0.093$). This suggests that selection for resistance to clover rot will not necessarily decrease plant yield, as feared by some red clover breeders. Late flowering accessions were less susceptible to clover rot. Accordingly, Öhberg et al. (2008) also found that late flowering increased the survival rate of red clover cultivars after natural infection in the field. Finally, we found no correlation between susceptibility to clover rot and rust ($p=0.143$) or viral diseases ($p=0.593$), but our data indicated a negative correlation between susceptibility to clover rot and mildew ($r=-0.230$, $p=0.020$). *Erysiphe polygoni*, the causal agent of rust, is a biotrophic pathogen, while *S. trifoliorum* is a necrotrophic pathogen. Poland et al. (2008) stated that resistance to biotrophic pathogens can increase susceptibility to necrotrophic pathogens and our findings may support this thesis.

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Chapter 7
Variability of Alsike Clover (*Trifolium hybridum* L.) Natural Populations from Serbia

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Abstract The aim of this paper is to examine the variability of natural populations of alsike clover (*Trifolium hybridum* L.) from Serbia. The study included six populations of different geographic origin. Collected material was investigated in the fields of the Institute for Forage Crops, Kruševac, in 2009–2011. Morphological traits of individual plants: height, green mass, number of stems, leaf length and width were analyzed on 20 plants per population, and forage quality traits: crude protein, crude fibre and crude fat were analyzed. The highest values of observed morphological parameters were recorded in populations IS 037 (756 g green mass and 86.6 cm plant height, with a 100 % survival rate in the second year), IS 077 (52.2 stems per plant and also 100 % survivals) and IS 086 (2.97 cm leaf length with 75 % survivals). Green mass yield proved to be the most variable trait, where the CV ranged from 32 to 61 %. The importance of the studied traits was estimated using the method of principal components analysis. With regard to chemical composition of dry matter, crude protein content ranged from 18.64 mg kg^{-1} in CS 072 to 20.47 mg kg^{-1} in IS 059, crude fibre ranged from 17.78 mg kg^{-1} in CS 072 to 25.24 mg kg^{-1} in RO 100 and fat ranged from 1.91 mg kg^{-1} in CS 072 to 2.51 mg kg^{-1} in IS 086.

Keywords *Trifolium hybridum* • Morphological traits • Forage quality • PCA • Wild population

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Introduction

Alsike clover (*Trifolium hybridum* L.) is a perennial herbaceous plant, of decumbent to erect growth habit, with trifoliate leaves and with 30–50 florets per inflorescence. It is widespread mainly in the cooler regions of the temperate zone and in the subarctic zones of Europe, Asia and America. It is one of the economically significant species of the *Trifolium* genus (Malaviya et al. 2005) whose nutritive value is slightly lower than on red or white clover (Vanda Paplauskiene and Dabkevičiene 2012) but nevertheless it has certain advantages – it survives longer in grasslands, it tolerates acid and wet soils better than red clover, it gets through the winter well and it is also frost-tolerant (Daugeliene 2000). It is most frequently grown in a grass-leguminous mixture with timothy grass (*Phleum pratense* L.) for use as forage (Kunelius et al. 2006) and as a fertilizer to increase the level of mineral substances in the soil base (Warman 1991). No variety of this species has been developed in Serbia to date, although because of heavier and more acid soils (Warman 1991) there is potential for growing it for use in forage mixtures. The aim of this study is to examine six wild populations of alsike clover, collected in Serbia, by observing their morphological and chemical characteristics. In a long-term plan this could result in the development of a commercially applicable variety.

Material and Methods

The examined material is a part of a collection of seeds of the Institute for Forage Crops, Kruševac, gathered on localities with coordinates shown in Table 7.1.

A field experiment was carried out on land at the Institute for Forage Crops Kruševac, (Globoder). The Institute for Forage Crops is in a region with a temperate continental climate and with an average annual temperature of 10.9 °C and average precipitation of 540–820 mm. The experiment was started at the beginning of October 2009 on a degraded alluvial soil. The seed was germinated in plastic containers and then manually transplanted to plant nursery, at 60×60 cm distance, when plants reached the stage of 3–4 permanent leaves. Plants were harvested in the period of the first cut for 2 years (2009/2010) at the beginning of flowering. The analyses were performed on 20 plants per population. Morphological properties observed were: green mass yield per plant (GMY), plant height of the main stem, number of stems per plant, length and width of middle lamina of the third leaf viewed from the top of the stem. Basic parameters of forage quality – content of crude proteins, crude fibre and crude fat – were analyzed by the Weende system. All analyses were performed using the STATISTICA 8.0 software (StatSoft, Tulsa, OK, USA). For data from the first year, differences amongst particular populations were tested by LSD post-hoc test following one-way ANOVA. For data obtained in the second year, differences were tested by Unequal N HSD test. Differences were considered significant at $p < 0.01$. Differences among populations are presented by letters. Principal component analysis (PCA) was applied to search for a general pattern in the measured morphological variables.

Table 7.1 *Trifolium hybridum* accessions used for analysis of genetic diversity

Abbreviation	Location	Latitude	Longitude	Altitude (m)
CS 072	Željin 1	N43°30.0990′	E020°51.6492′	727
IS 037	Balašević	N43°48.8746′	E021°54.2695′	371
IS 059	Čestobrodica 1	N43°50.5721′	E021°40.5952′	590
IS 077	Crni Vrh 2	N44°08.3621′	E021°58.6408′	770
IS 086	Crni Vrh 4	N44°10.0418′	E021°57.0274′	860
RO 100	Donja Vapa	N43°17.5624′	E020°01.7826′	1,008

Results and Discussion

Analyses of variance revealed highly significant differences between populations for all agronomic traits except leaf width. Average data for significant traits are shown in Table 7.2. The green mass yield represents one of the most important and, at the same time, one of the most variable agronomic traits. In the presented group of populations, IS 037 was singled out for its particularly high green mass yield (756 g). Since yield and height are the most frequently highly positively correlated traits, this population is also characterized by significant plant height – 86.6 cm. Population IS 077 had the largest number of stems – 52.5. Survival rate of single plants from those two populations was 100 %. Population IS 059 also had a survival rate of 100 % in 2011, and values of the examined parameters were similar to the values of two other populations from East Serbia. GMY and number of stems had the highest CV values per population, which is in accordance with results on red clover reported by Asci (2011). Values obtained for height of plants and number of stems were considerably greater than those reported by Lugic et al. (2009) for alsike clover from natural habitats. Population IS 077 is characterized by the highest average values of leaf length (2.97 cm). Leaf width was measured in all populations, but differences were not statistically significant. Leaf width was between 1.42 and 1.61 cm ($p=0.4$) in the first year and between 1.67 and 1.72 cm ($p=0.42$) in the second year. Populations CS 072 and RO 100 recorded the worst results in both years and also their survival rates were considerably lower (60 %).

Relative significance of evaluated characteristics was examined using principal component analyses. The first two principal components accounted for 79 and 12 %, respectively (Fig. 7.1). Populations were clearly separated along the first axis. Populations with high values of the observed traits are on the left side, whereas populations with low values are on the right side. The other axis separates populations with high values of mass and height (IS 037) from those with high values of leaf length and stem number (IS 077 and IS 086).

Certainly the most important indices of forage quality are crude protein, fibre and fat. The population with the highest content of crude protein (204.7 mg kg^{-1}) was IS 059; population CS 072 had the lowest content of crude fibre (177.8 mg kg^{-1}); whereas the content of crude fat ranged from 19.1 mg kg^{-1} in CS 072 to 25.1 mg kg^{-1} in IS 086 (Table 7.3). The obtained values of these parameters are

Table 7.2 The mean values and coefficient of variation of morphological traits and survival rate for evaluated populations

		Population											
		CS 072		IS 037		IS 059		IS 077		IS 086		RO 100	
Trait	Year	Mean value*	CV (%)	Mean value*	CV (%)	Mean value*	CV (%)	Mean value*	CV (%)	Mean value*	CV (%)	Mean value*	CV (%)
Height (cm)	2010	70.0c	20	86.6a	16	81abc	20	84.4ab	15	73.2bc	24	55.4d	17
	2011	41.8b	32	68.6a	19	57.5ab	25	63.7a	16	53.1ab	20	58.8a	11
GMY (g plant^{-1})	2010	448c	47	756a	32	525b	51	676ab	40	680ab	44	234d	57
	2011	87c	61	343ab	49	278b	58	485a	41	217b	60	202b	49
Number of shoots	2010	32.2b	37	42.2ab	32	42.6ab	39	50.6a	26	40.3ab	43	34.5b	38
	2011	21.0b	53	31.3b	38	31.2b	39	52.5a	32	31.7b	45	29.8b	36
Leaf length (cm)	2010	2.72a	10	2.80a	10	2.70a	15	2.75a	13	2.97a	13	2.37b	11
	2011	2.09b	20	2.63a	12	2.59ab	12	2.92a	12	2.85a	17	2.69a	16
Survival rate in 2011		60 %		100 %		100 %		100 %		75 %		60 %	

*Averages of populations presented by different letters were considered significant at $p<0.01$

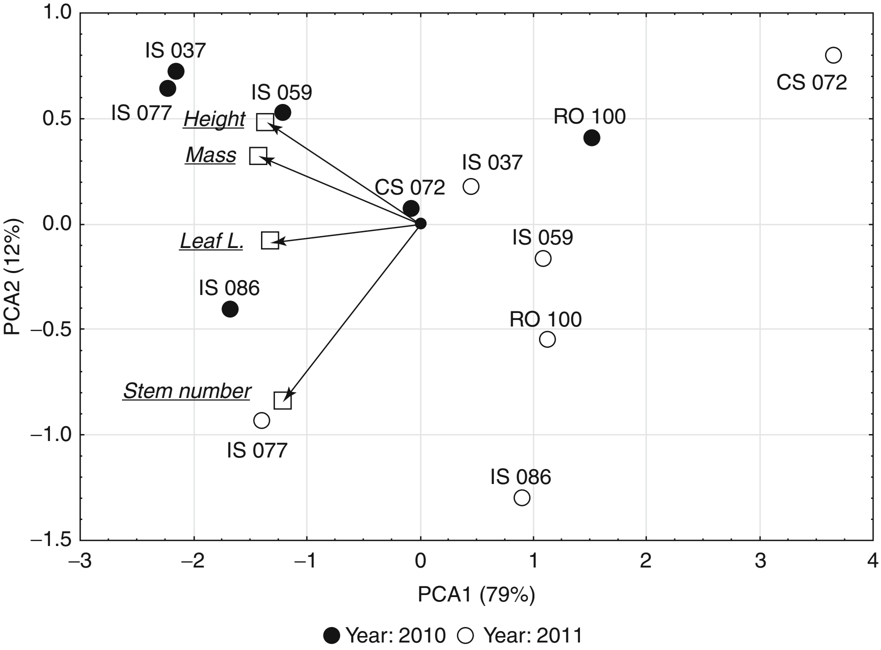

Fig. 7.1 Principal loadings for the first two components of PCA of morphological traits

Table 7.3 Forage quality parameters for evaluated populations (mg kg^{-1} of dry matter)

Trait	Population					
	CS 072	IS 037	IS 059	IS 077	IS 086	RO 100
Crude protein	186.4	188.6	204.7	196.3	195.2	202.1
Crude fibre	177.8	232.1	246.1	241.7	218.7	252.4
Crude fat	19.1	20.3	21.3	18.6	25.1	21.2

reasonable, although values of crude protein and fibre are significantly higher and lower respectively, than the values which Vanda Paplauskiene and Dabkevičiene (2012) got in investigation of alsike clover strains.

Conclusions

It can be concluded from these results that alsike clover material collected in Serbia can be a good source of germplasm for the development of commercially viable varieties. Populations from East Serbia were singled out for their high values of yield characteristics, as well as for satisfactory values of dry matter quality.

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Chapter 8
Characterization of Some Local Populations of *Medicago minima* Collected in the Central Steppe of Algeria

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Abstract The aim of our study is to evaluate some local populations of *Medicago minima* coming from a 2004 survey conducted by INRA Algeria throughout the Djelfa area (Central Steppe). The study is based on quantitative traits of plant, pod and seed. The results obtained show that there is high genetic variability within populations of *M. minima*. Analysis of variance showed highly significant differences between populations for all parameters studied. We noted that the early flowering stage setting was the most discriminating character. Several correlations were observed between the various parameters studied. We noted correlations between various traits related to pods and seeds, but none between vegetative development traits.

Keywords Characterization • *M. minima* • Phytogenetic resources • Variability

Introduction

Legumes of the genus *Medicago* are of special ecological and agro-pastoral importance in the Mediterranean regions. They include forage species expressing high levels of N-fixation and protein production per hectare (Huguet et al. 1994). The annual medics, in the spontaneous state, frequently occupy a prominent place in grazing systems in Algeria (Abdelguerfi 1992). Medics are self-seeding pasture

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legumes distributed throughout Algeria, and a very important source of proteins for ruminants. They improve natural pastures and provide a source of high-quality forage (Chebouti and Abdelguerfi 2004).

M. minima (L.) Bart. is a pasture legume native to the Mediterranean Basin and adjacent temperate regions, and one of the most widely distributed annual medics in both its area of origin and other parts of the world with a semi-arid temperate climate (Fresnillo Fedorenko 2001).

Therefore, the objective of the present study was to evaluate some local populations of *M. minima* using agronomic and morphological traits.

Material and Methods

The plant material consisted of five natural populations of *M. minima*. These populations originated from a 2004 collecting conducted in the region of Djelfa by the National Institute of Agronomic Research of Algeria (INRAA) (Table 8.1). The trial was conducted at the experimental farm of the Research Center of Plant Science (INRAA).

Scarified seeds were sown in pots filled with clay at a rate of five seeds per pot. At emergence, we left two plants per pot. Pots were arranged in a completely randomized design with five replications. At flowering stage, the following traits were measured: earliness of flowering (EF) (in days after emergence), length of the branch carrying the first flower (LB1F) (in cm), number of internodes of the branch carrying the first flower (NIN) and number of primary branches (NPR). For pods and seeds, studied traits were pod diameter (DP) (in mm), thickness of the pod (TP) (in mm), number of coils per pod (NTP), number of seeds per pod (NS/P), seed length (LS) (in mm), seed width (WS) (in mm). These measurements were made on 25 pods and 20 seeds per population using a digital display caliper.

Data collected were subjected to an analysis of variance (ANOVA) and a correlation matrix was calculated using the Discovery Edition of GenStat software.

Results and Discussion

The results are represented in Table 8.2. We noted a high genetic variability in *M. minima*. The variance analysis showed highly significant differences between populations of *M. minima* in all studied traits. According to Chaulet (1995), species of the genus *Medicago* derive their importance because of their large variability. El Hansali et al. (2007) indicated the existence of a highly significant morphological variability in nine natural populations of *M. truncatula* from Morocco, which is even more important as populations are located in different climates.

For flowering earliness, the population of Charef was the earliest population at 99 days after emergence (Table 8.2). This may indicate that flowering earliness is related

Table 8.1 List of *Medicago minima* populations and original collecting sites

Species	Geographical origin	Altitude (m)	Latitude	Longitude
Medicago minima	Ain Oussera (MmAO)	758	35° 17′ 08″	2° 57′ 37″
	Deldoul (MmDel)	768	34° 15′ 73″	3° 19′ 76″
	M'liliha (MmMli)	806	34° 48′ 89″	3° 48′ 94″
	Charef (MmCh)	960	34° 40′ 31″	2° 43′ 13″
	Oued Touil (MmOT)	718	35° 16′ 43″	2° 33′ 23″

Table 8.2 Traits average values and variance analysis in *M. minima*

Populations	LS	WS	TP	DP	NTP	NS/P	EF	LB1F	NIN	NPR
MmAO	2.44a	1.42a	3.55b	4.14b	3.52a	5.32a	102.33a	16.99a	6.33b	6.33b
MmCh	2.23b	1.14c	3.32c	4.15b	3.12b	5.04a	99.16b	14.06b	5.67b	5.42c
MmOT	1.92c	1.07d	2.99d	3.89c	3.00b	4.56b	102.50a	13.73b	5.92b	5.33c
MmMli	2.25b	1.24b	3.92a	4.52a	3.44a	5.44a	102.16a	16.14a	6.92a	7.25a
MmDel	2.32b	1.25b	3.56b	4.24b	3.52a	5.44a	102.42a	15.25b	5.92b	6.58b
Gen. Mean	2.23	1.24	3.47	4.19	3.32	5.16	101.71	15.23	6.50	6.18
F.obs	20.15	23.87	26.4	11.12	8.35	7.90	56.49	5.44	7.20	14.75
Sign.	***	***	***	***	***	***	***	**	***	***

Gen. Mean General Mean, *F. obs* F. observed, *Significant at $p<0.05$, **Highly significant at $p<0.01$, ***Very highly significant at $p<0.001$
a, b, c Groups average

to environmental conditions, particularly temperature. Sulas et al. (2000) reported that flowering of *Trifolium subterranean* and annual medics occurs in response to day length and temperature, but cultivars vary considerably in their response to these factors. According to Fresnillo Fedorenko (2001), in accessions of *M. minima* adapted to low rainfall environments, plants were smaller, flowered earlier, and had a shorter growth cycle than accessions adapted to wetter environments.

For morphological traits, the population from M'liliha presented the longest branch carrying the first flower (16.14 cm), and the highest number of internodes of this branch and of primary branches (6.91 and 6.58, respectively), whereas the population from Oued Touil presented the lowest values (Table 8.2). For Rotili et al. (2001), the ideal stem for yield and quality should constitute a high number of short and regular internodes, thus optimising leaf to stem ratio.

With regard to traits related to pods and seeds, the largest pods and number of seeds per pod were recorded for the M'liliha population (DP=4.52; TP=3.93; NS/P=5.44, respectively), while the population from Ain Oussera presented the largest seeds with LS=2.44 and WS=1.42. Populations from Oued Touil had the smallest pods and seeds and the lowest number of seeds per pod (Table 8.2).

Several correlations were observed between the various parameters studied. Seed length was positively correlated with seed width (r=0.891*). Thus, populations with a high number of seeds per pod presented thick pods (r=0.914*) and a high coil number per pod (r=0.928*). Pod diameter was positively correlated with pod thickness (r=0.961**) and with the number of primary branches (r=0.881*). We also noted that populations with longer branches had large seeds (r=0.948*) with a

Table 8.3 Correlation between studied traits in *M. minima*

Variables	LS	WS	TP	DP	NTP	NS/P	EF	LB1F	NIN	NPR
LS	1.000									
WS	0.891*	1.000								
TP	0.689	0.608	1.000							
DP	0.529	0.374	0.961**	1.000						
NTP	0.860	0.861	0.808	0.641	1.000					
NS/P	0.870	0.726	0.914*	0.829	0.928*	1.000				
EF	–0.040	0.308	0.159	0.011	0.414	0.120	1.000			
LB1F	0.806	0.948*	0.768	0.567	0.882*	0.772	0.421	1.000		
NIN	0.278	0.460	0.781	0.728	0.530	0.514	0.478	0.715	1.000	
NPR	0.564	0.578	0.943*	0.881*	0.840	0.856	0.461	0.766	0.829	1.000

*significant at $p<0.05$, df = 3; **highly significant correlation at $p<0.01$

high number of coils (r = 0.882*). The number of primary ramifications was positively correlated with pod thickness (r = 0.943*). Laouar and Abdelguerfi (2003) mentioned that the pods of *Medicago intertexta* were heavy (high pod weight) and large (high diameter and thickness), with high numbers of seeds per pod. According to Senhadri-Maamri et al. (2000), it seems that the strong diameter and thickness of the pod vary with the number of seeds per pod. We have not noted any correlation between vegetative traits (Table 8.3).

Conclusion

We noted significant variability in populations of *M. minima* for all the traits examined. This variability offers possibilities for selection of plant material adapted to our soil and climatic conditions. Several correlations were observed between the various parameters studied. We noted significant correlations between various traits related to pods and seeds, but no significant correlation between vegetative traits. As a result of their adaptability and production, annual *Medicago* species, can play an important role in improving forage production and regenerating degraded rangeland in arid and semi-arid environments.

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Chapter 9
Genetic Variability of the Most Important Traits in Meadow Fescue Accessions

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Abstract Meadow fescue (*Festuca pratensis* Huds.) is an important perennial grass for animal feed production on grasslands and pastures. A basic requirement for successful breeding of this species is variability of the initial material. The collection examined consisted of 11 accessions (five breeding populations and six cultivars) of meadow fescue. The trial was conducted as a spaced plant nursery with 30 plants per genotype over 2 years. The aim was to determine genetic variability of the most important traits, specifically heading date (phenological trait), plant height in the first and the second cut, first internodes length, the number of tillers per plant and length, width and number of leaves, (morphological traits), as well as dry matter yield per plant. Statistical analysis of the data (ANOVA) revealed significant variability of genotypes for all properties included in the study. The highest average within-population variability was found for the number of tillers (CV 58 %), while the lowest variability was found for heading date (CV 8.8 %). The between-population variability was statistically significant for all investigated traits. Cluster analysis was used to graphically illustrate the genetic diversity of the meadow fescue accessions included in the study. The high level of genetic variability of this collection highlights the potential for future success in the breeding of meadow fescue.

Keywords *Festuca pratensis* • Genetic variability • Accessions • Breeding

Introduction

Meadow fescue (*Festuca pratensis* Huds.) is a perennial grass species, with high yield potential and biomass quality. It is of increasing agricultural importance, particularly in the Northern Hemisphere, where it dominates in meadows and pastures

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(Casler and Santen 2000). Meadow fescue is often used in intensive production of hay meadows. Pastures dominated by meadow fescue are long-lasting, due to its high tolerance to grazing. It can be grown as a pure crop, but is more often grown in mixtures with other grasses and legumes. The biomass quality is variable and dependent on the stage of maturity, but since it does not flowers until the second half of May, it has a long growth period during which quality remains high. This desirable trait explains why meadow fescue is an important component of many grass-legumes mixtures.

The initial step in the grass breeding process is the determination of genetic variability of the breeding material. The existence of genetic variability within available breeding material (autochthonous populations, breeding populations and cultivars) influences the choice of breeding material and ensures the success of the plant breeding program. Natural populations demonstrate great adaptability to different macro- and micro-habitats, due to the genetic variation of traits of adaptive significance. Therefore, natural populations can often be used for direct phenotypic selection and for cultivars release (Posselt and Willner 2007). Variability within populations and cultivars of meadow fescue has been documented for important breeding traits, forage yield and forage quality (Kanapeckas et al. 2005). Similar to other forage grasses, the primary aim in meadow fescue forage breeding is to obtain drought-tolerant and persistent cultivars with a high yield and quality of livestock feed (Sokolović et al. 2010, 2012). The aim of this paper was to examine the variability within and between breeding populations and cultivars of meadow fescue in order to provide crucial data for future breeding programs and as a basis for further studies.

Material and Methods

A collection consisting of five breeding populations and six cultivars of meadow fescue was investigated during a two-year period (2006–2007). Breeding populations passed two cycles of selection. Four of the breeding populations (OP-1, OP-2, OP-3 and OP-4) originated from local populations, collected mainly in Eastern Serbia, whereas one population (OP-5) originated from the Czech Republic.

The cultivars included in this study were Kruševački 21 (K-21, Serbia), Jabeljska (Slovenia), Premil, Pradel and Preval (Switzerland), and Rožnovska (Czech Republic). The study was conducted in an experimental field with a degraded alluvium soil type at the Institute for Forage Crops, Kruševac, Serbia. The trial was set up in a space plant nursery using a randomized block design, with 30 plants per accession in triplicate, and a plant–to–plant distance of 60×60 cm. Sowing in containers was carried out in the greenhouse in early spring and planting out in the experimental field was completed by April 2005. Heading date (HD), dry matter yield per plant (DMY) and a range of morphological traits, namely plant height (PH) in the first and the second cut, leaf length (LL), leaf width (LW), the number of leaves per tiller (NLT), first internodes length (FIL), the number of tillers per plant (NTP), were recorded during 2 years. Heading date is presented as number of days from the 1st of April. Dry matter yield per plant was measured in two cuts and is presented as annual dry matter yield per plant. All morphological traits, except height in the second cut, were measured at the full heading stage in the first cut. Height in the second cut was measured 30 days after the first cut. All results are reported as mean values over 2 years.

All data were evaluated by analysis of variance (ANOVA). Differences between accessions were tested using the LSD test. Cluster analysis was performed using Ward's method and Euclidean distances (Statistica 5.0, Stat Soft.inc).

Results and Discussion

Highly significant differences between accessions were found in all studied traits. Heading date is a very important trait in grass breeding, because variation in this trait enables the development of cultivars with diverse maturing times. A wide range of available maturing times means that the forage species can be used in a wider range of applications. This is of particular interest for the composition of mixtures for various terms and duration of utilization (Soković et al. 2010). The earliest heading dates were found in cultivar K-21 and breeding population OP-2, while heading occurred last in breeding population OP-4 and cultivar Premil. Overall, heading date exhibited the lowest within-population variability of all traits. The highest coefficients of variability were obtained for the very early (K-21 and OP-2) and the very late (OP-4) accessions, which provides an opportunity to develop varieties with earlier or later maturity. Lemežiene and Kanapeckas (2008) reported that there was no evidence of heading date polymorphism in their meadow fescue collection. In their study, heading dates did not differ more than 3 days between accessions.

The average plant height in the first cut was 89.2 cm, which is substantially lower than was found by Fang et al. (2004), who reported average plant heights of 126–139 cm. However, our results were similar to those presented by Akgun et al. (2008). The average plant height in the second cut was 29.9 cm, which is similar to results published by Lemežiene et al. (2004), who reported plant heights in the second cut ranging from 30.2 to 35.9 cm depending on year.

Breeding population OP-2 and cultivars K-21 and Pradel had the highest leaf length and width, as well as the largest number of leaves per plant. These traits influence the forage quality by increasing the dry matter quality and improving the crude protein content.

First internode length is an important trait from the aspect of plant lodging. Although this trait is positively correlated with plant height, the aim of breeding is to produce plants with sufficient biomass height, but with a shorter first internode. In this collection, Cultivar Pradel had the best ratio between plant height and first internode length (respectively the biggest difference between these traits) (Table 9.1).

In grasses, number of tillers per plant in the first cut (NTP) is the trait which has the highest impact on DMY, but NTP also affect other important traits like seed

Table 9.1 Traits of meadow fescue accessions recorded over two growing seasons, given as the mean of 30 plants. For each trait, the least significant difference (LSD) is given for two significance levels (alpha = 0.05 and alpha = 0.01)

Trait accession		HD	PH 1st cut (cm)	PH 2nd cut (cm)	LL (cm)	LW (mm)	NLT	FIL (cm)	NTP	DMY (g plant^{-1})
OP-1		40.6	88.6	27.7	22.0	7.1	2.5	23.1	81.9	77.0
OP-2		38.7	95.8	33.1	27.7	8.4	2.8	27.9	106.0	115.0
OP-3		41.2	86.5	29.0	23.7	7.0	2.5	24.1	55.8	70.8
OP-4		44.7	74.1	27.7	25.0	7.8	2.5	21.8	38.9	71.0
OP-5		41.8	83.3	28.5	24.1	7.7	2.3	34.5	76.3	85.5
K-21		37.8	98.9	33.4	30.3	7.9	3.0	31.2	132.3	111.8
Jabeljska		41.0	86.1	25.3	23.1	6.9	2.5	23.8	66.6	65.6
Premil		42.7	83.8	29.3	25.2	7.5	2.5	21.9	68.0	84.0
Pradel		41.5	96.4	33.2	26.8	8.1	2.8	25.2	92.8	109.1
Preval		42.0	93.3	31.6	26.3	7.6	2.6	24.6	83.1	103.4
Rožnovska		42.0	93.9	30.8	26.6	7.8	2.6	26.8	72.9	102.7
Mean		41.3	89.2	29.9	25.5	7.6	2.3	24.9	79.5	90.54
LSD	0.05	0.69	3.02	0.84	1.51	0.19	0.18	1.62	3.80	5.52
	0.01	0.95	4.12	1.15	2.06	0.27	0.25	2.15	4.79	7.02

HD heading date (number of days from 1st of April), *PH* plant height in the first and the second cut, *LL* leaf length, *LW* leaf width, *NLT* number of leaves per tiller, *FIL* first internodes length, *NTP* number of tillers per plant, *DMY* dry matter yield per plant

yield. The highest NTPs were found in cultivars K-21 (132.3) and Pradel (92.8), and in breeding population OP-2 (106.0). These are much lower than the NTPs reported by Fang et al. (2004), where the average number of tiller per plant was 232.2. However, large variations in NTP between studies are expected, since the trait is highly affected by environmental conditions.

The same three accessions (OP-2, K-21 and Pradel) scored the highest dry matter yield per plant (115.0, 111.8 and 109.1 g, respectively), which was more than 20 % higher than the average dry matter yield of the collection (90.54 g). The DMY in this study was noticeably higher than that reported by Akgun et al. (2008), where the average dry matter yield per plant was 62.32 g over two years. After examining a large collection of populations and cultivars of meadow fescue over 2 years, Casler and Santen (2000) reported that the average dry matter yield was 105 g plant^{-1}.

The results obtained in the present study demonstrate very high within-population variability in key traits, as indicated by the high coefficients of variation (Table 9.2). The highest coefficients of variation were determined for number of tillers per plant (58 %) and for dry matter yield (47.2 %). The means and coefficients of variation for number of tillers per plant and dry matter yield indicate a high breeding potential of genotypes for these traits.

In order to determine the genetic distances between meadow fescue accessions, a cluster analysis was performed. The resulting dendrogram revealed three groups (Fig. 9.1). Breeding population OP-2 and cultivars Pradel and K-21 were the most

Table 9.2 Coefficients of variation (CV %) of studied traits of meadow fescue accessions

Trait accession	HD	PH 1st cut	PH 2nd cut	LL	LW	NLT	FIL	NTP	DMY
OP-1	7.7	12.7	14.9	12.6	16.4	15.3	16.9	51.7	45.7
OP-2	13.8	20.7	16.8	17.1	16.8	13.3	24.0	49.0	40.6
OP-3	6.1	22.7	21.2	21.1	21.8	18.8	26.0	72.9	71.2
OP-4	15.8	13.7	16.2	15.3	13.6	17.3	19.5	45.8	33.6
OP-5	6.6	14.2	18.8	15.8	13.7	15.8	16.2	66.0	59.9
K-21	11.4	18.3	18.2	18.3	18.4	12.8	26.6	47.5	29.8
Jabeljska	5.7	14.2	19.5	12.7	17.7	16.8	19.0	66.5	56.8
Premil	7.8	14.8	14.3	16.2	18.6	18.5	20.2	67.1	51.7
Pradel	7.5	11.6	17.0	12.2	14.2	14.1	17.2	62.9	40.7
Preval	7.2	12.0	14.6	12.2	12.6	13.1	16.2	51.5	45.2
Rožnovska	7.8	13.7	16.1	13.4	15.0	15.2	16.2	56.9	43.6
Mean	8.8	15.3	17.0	15.2	13.6	15.5	19.8	58.0	47.2

HD heading date, *PH* plant height in the first and the second cut, *LL* leaf length, *LW* leaf width, *NLT* number of leaves per tiller, *FIL* first internodes length, *NTP* number of tillers per plant, *DMY* dry matter yield per plant

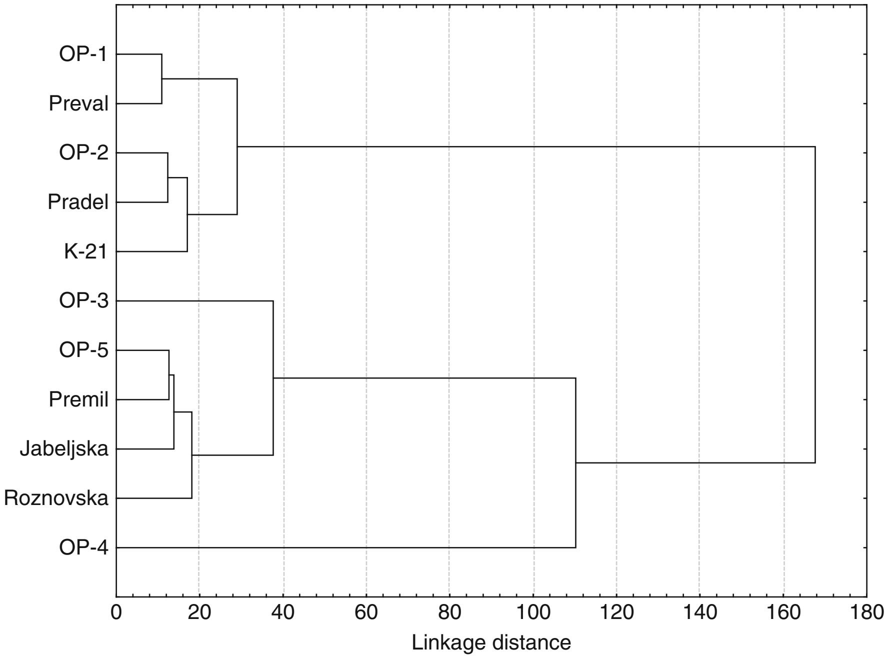

Fig. 9.1 Dendrogram of cluster analysis of meadow fescue accessions based on nine traits

distant from the rest of the collection, due to high number of tillers, dry matter yield per plant, leaf length and width and number of leaves per plant. Breeding population OP-4 was clearly separated from other accessions by its later maturity, low height of plants and the low dry matter yield per plant. Based on these results, superior genotypes (in terms of yield) from the most distanced populations or cultivars will be intercrossed, with the aim to produce superior genotypes of in the F1 generation. This gene pool will form the germplasm for breeding new meadow fescue cultivars.

Conclusion

This study found significant differences in nine key traits within and between meadow fescue accessions. The highest number of tillers per plant and dry matter yield was obtained inbreeding population OP-2 and in cultivars K-21 and Pradel. These three accessions also had the highest leaf length and width, as well as the largest number of leaves per plant, all important components of dry matter quality. The best genotypes from all populations and cultivars, particularly those with the highest genetic distances, will be included in the next step in the meadow fescue breeding process. These genotypes will be intercrossed to form basic breeding germplasm for creating new meadow fescue cultivars.

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Chapter 10
Morphological Variation Between Perennial Ryegrass (*Lolium perenne* L.) Natural Ecotypes

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Abstract Perennial ryegrass (*Lolium perenne*) is a highly important forage and turf grass. The objective of this study was to evaluate the diversity of morphological traits of wild perennial ryegrass ecotypes. Natural ecotypes from Lithuania (5), Poland (2), Russia–Kaliningrad (3), Slovakia (1) and Ukraine (40) were planted in 2011–2012 into the germplasm collection at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry. Winter survival, re-growth in spring, plant height, beginning of heading, flag leaf length and width and dry matter yield per plant of 1st cut were assessed. Variation among ecotypes was high for re-growth in spring and biomass (CV = 22.69 and 22.38 %) and low for winter survival (CV = 6.07). The polymorphism between wild ecotypes averaged CV = 10.57–16.39 % for other traits. Principal component analysis was carried out on morphological traits. The grouping of ecotypes was mostly influenced by plant height, flag leaf length and width, dry matter yield and winter survival.

Keywords Perennial ryegrass • Morphological traits • Diversity

Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important forage (high yield, good digestibility and tolerance to grazing pressures) and amenity grasses. A new variety must be not only stable and uniform, but also distinct from other

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varieties. Therefore, variability of phenotypic traits is very important in breeding. Ecotypes, collected in their natural habitats, usually are not productive, but some genotypes exhibit high resistance to various biotic and abiotic stresses or are of good quality and therefore can be used as parental genotypes in breeding (Lemežienė and Kanapeckas 2008; Kanapeckas et al. 2011). The objective of this study was to evaluate the diversity of morphological traits of natural perennial ryegrass ecotypes.

Material and Methods

Plant Material

The plants of ecotypes of perennial ryegrass (*Lolium perenne* L.), collected in their natural habitats in Lithuania (LT, 5), Poland (PL, 2), Russia-Kaliningrad (RU, 3), Slovakia (SL,1) and north and north-west Ukraine (UA, 40) were planted in 2011 and 2012 into the germplasm collection at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry. The soil of the experimental site is Endocalcari–Epihypogleyic Cambisols (CMg-p-w-can). Eight plants of each genotype were planted at 50 × 50 cm distances.

In the collection trials the following parameters were assessed: winter survival (%), re-growth in spring (score, 1 – very low …9 – very high value), plant height (cm), beginning of heading (date), flag leaf length and width (cm) and dry matter yield (DMY) of 1st cut (g plant^{-1}).

Statistical Analyses

Coefficient of variation (CV) and standard deviation (Sd) of agro-biological traits were estimated. NTSYSpc v. 2.2 (Exeter Software, Setauket, NY) (Rohlf 2005) was used to perform principal component analysis (PCA).

Results and Discussion

High variation for re-growth in spring and biomass (CV = 22.69 % and 22.38 %) and low for winter survival (CV = 6.07), were detected among ecotypes. The average polymorphism between wild ecotypes was estimated for other traits (Table 10.1). Winter survival is one of the most important traits in ryegrass breeding in Lithuania.

Table 10.1 Morphological traits and basic statistics of principal component analysis for morphological traits of natural ecotypes

Characteristics	$\bar{x} \pm Sd$	CV (%)	Eigenvector PC1	PC2	PC3
Winter survival, %	97.67 ± 1.93	6.07	0.532	0.490	−0.028
Re-growth in spring, score	5.3 ± 1.47	27.69	0.730	−0.417	−0.391
Beginning of heading, date	06.02 ± 3.5	10.57	0.588	0.187	0.039
Plant height, cm	45.79 ± 8.11	17.72	0.853	−0.212	−0.327
Length of flag leaf, cm	12.25 ± 2.01	16.39	0.829	0.198	−0.040
Width of flag leaf, cm	0.48 ± 0.07	13.59	0.847	0.276	0.010
DMY of 1st cut, g plant^{-1}	107.2 ± 24.00	22.38	0.381	−0.743	0.469
	Eigenvalue		3.44	1.16	1.00
	% of variation explained		49.11	16.59	14.34
	Cumulative variance		49.11	65.71	80.05

The weather conditions were favourable for perennial ryegrass to grow and develop during the year of experiment. The winter damage was low for most wild ecotypes used in this study (average 2.33 %).

Principal component analysis was carried out based on morphological characteristics. In the first three principal components (PC) accounted for 80.05 % of total variation among studied genotypes (Table 10.1). PC1 accounted for 49.11 % of total variation. According to the corresponding eigenvector values, it was mostly equated with plant height, flag leaf length and width. PC2 accounted for 16.59 % of total variation and was explained by dry matter yield and winter survival. PC3 accounted for 14.34 % of total variation and was mostly defined by dry matter yield and beginning of heading. Factor analyses on different collections have indicated that every collection of genotypes is individual. Plant height, leaf length and width were the most important traits in other authors' experiments (Sokolović et al. 2011) and biomass, beginning of heading and re-growth in spring in our other collection (Statkevičiūtė et al. 2012).

PC1 separated six ecotypes from the majority of ecotypes. However, their origin did not influence the grouping in PCA. No. 3889 (UA) was late and No. 3882 (UA) was medium early heading. Both ecotypes survived winter poorly and had narrow leaves. Genotype No. 3776 (SL) was characterized by the narrowest flag leaf, No. 3828 (UA) by the shortest plant height, No. 3773 (UA) by the poorest re-growth in spring and No. 3774 (UA) by the lowest dry matter yield. No. 3719 (UA) was distinguishable from the rest of ecotypes by the highest dry matter yield of 1st cut (Fig. 10.1).

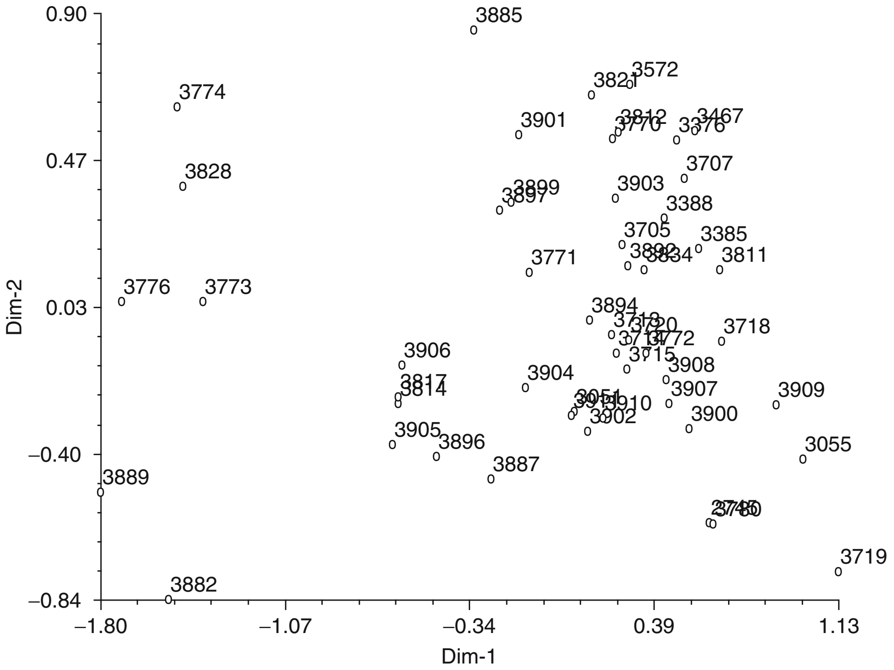

Fig. 10.1 The clustering of natural ecotypes based on morphological traits

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Chapter 11
Variability of Selected Traits in the Czech Alfalfa Core Collection

Jan Pelikán, Tomáš Vymyslický, Daniela Knotová, and Simona Raab

Abstract Within the set of 99 origins (varieties, breeding lines and wild forms) of alfalfa (*Medicago sativa, Medicago* x *varia* and *Medicago falcata*) of the world collection maintained in the Czech national gene bank, 52 characteristics were evaluated. The traits were evaluated on individual plants grown in a field spaced plant nursery. From the mean values of particular weighted, measured and counted characteristics the estimations of correlation coefficients were calculated for 28 of these characteristics. The estimations were used to show the degree of association between measured traits. The results obtained are presented in a correlation matrix and discussed. Furthermore, for each origin and 22 selected characteristics the variation coefficients were calculated from the observed values. These coefficients were used as the measure of within-variety variability. The biggest variability was found for weight of seeds per plant, number of inflorescences per stem and the number of lateral branches. The lowest average variability was observed in the following traits: the height of growth at the onset of flowering, the length and the width of the terminal leaflet and the stem diameter. The main aim of our paper is to give methodological instruction to the practical breeders on how to work more effectively with important traits that could be used in plant breeding.

Keywords *Medicago* spp. • Fodder characteristics • Seed traits • Individual plants • Correlations • Variability

Introduction

Genetic variability is a prerequisite of plant breeding. Primary sources of variability available in breeding are ex-situ collections of genotypes and wild populations. Marshall et al. (2003) refer to extensive use of these collections in breeding

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programmes of red and white clover. Negri et al. (1995) indicated that, for most forage species of agronomic importance, their outcrossing nature, high levels of heterozygosity, and the quantitative nature of most agronomically important characteristics, effective breeding and selection, and subsequent trait evaluation, in order to obtain genetically uniform cultivars is challenging. Genetic resources are evaluated basing on morphological, phenological and agronomical characteristics, which are influenced by environment and plant developmental stage. Kongkiatngam et al. (1996) mentioned that only a few traits are genetically fixed by red clover. The study of correlations among characteristics of plant species is important from the point of view of plant breeding. So, great attention is devoted to the evaluation of selected characteristics on individual plants and their correlations (Fonseca et al. 1999; Julier et al. 1995). The study of yield parameters structure and analyses of relations between particular characteristics influencing yield directly or indirectly has significance during all the stages of breeding work (Holý and Rod 1984). Correlations found among studied characteristics can change in particular years, during plant development, and may depend on the specific populations being evaluated. That is why we pay great attention to the evaluation of obtained materials (Mrázková and Pelikán 1985).

Material and Methods

The genotype collection of alfalfa (*Medicago* sp.) maintained by the Research Institute for Fodder Crops, Ltd. in the frame of "National Programme on Conservation and Utilization of Plant Genetic Resources and Agro-biodiversity" in the Research Institute of Crop Production in Praha-Ruzyně was used for this evaluation. In March 2005 30 plants of all available 99 alfalfa accessions were sown in pots and then planted out on the field. Varieties, breeding lines and wild materials of *Medicago sativa;* varieties and breeding lines of *M. x varia* and one variety of *M. falcata* were included into the evaluation. Only seeds from well defined and natural populations of alfalfa have been collected in the wild. The seeds were collected only once from all available plants in the population according to the gene bank standards, so the seed samples should be representative. Altogether, 52 characteristics on individual plants of these materials were evaluated according to the descriptor´s list for the genus *Medicago* L. (Vacek et al. 1985) in the year 2006. Sampling and characterization methodology (10 plants per origin) may have reduced observed variability. During our evaluation of such a big set of plants it was not possible to increase the number of plants included into evaluation process because of time and work possibilities. We evaluated all the plants in the same phenological phase. Some of the 52 characteristics were only evaluated according to point scale of the descriptor's list and were not included into the analyses. Among all calculated, weighed and measured traits (altogether 28 traits) correlation coefficients were estimated, as the extent of the association of measured traits. The following 28 traits were evaluated: (1) The length of terminal leaflet, (2) The width of terminal leaflet,

(3) Plant weight, (4) Number of stems, (5) Stem length, (6) Stem diameter, (7) Number of internodes, (8) The length of medium internode, (9) Number of lateral branches, (10) Height at the beginning of flowering, (11) Inflorescence length, (12) The weight of thousand seeds, (13) Leaf area, (14) The area of terminal leaflet, (15) Nitrogen content, (16) Fibre content, (17) Nitrogen free extract, (18) Ash content, (19) Digestibility in vitro, (20) Number of ripened inflorescences per stem, (21) Pod – width of the spiral, (22) Number of pods per inflorescence, (23) Number of pods per 100 flowers, (24) Number of seeds per pod, (25) Pod – height of the spiral, (26) The weight of seeds per plant, (27) Number of inflorescences per stem and (28) Number of flowers in inflorescence. Coefficients of variation were calculated on the 22 measured, counted and weighted traits with multiple observations (all the above mentioned except No. 12, 15, 16, 17, 18, 19). From average values of each accession the estimation of basic statistical characteristics among populations within the whole set was calculated. Cluster analysis of the core collection was calculated, discussed and published in a separate paper (Vymyslický et al. 2013).

Results and Discussion

The results of the coefficient of variation calculation of the whole set of accessions are presented in Table 11.1. The coefficients were calculated from the mean values obtained for each accession.

The lowest values of coefficient of variation, calculated based on mean value of the trait in the whole set, were found for the traits growth height at the beginning of flowering (9.9 %), the length of terminal leaflet (10.2 %), the width of terminal leaflet (10.8 %), stem diameter (11.4 %), the length of medium internode (11.4 %) and the width of pod spiral (11.5 %). The highest values of the variation coefficient were found by these traits: the weight of seeds per plant (80.7 %), number of the ripened inflorescences per stem (47.9 %), number of the lateral branches (47.0 %) and the plant weight (33.0 %). High value of variation coefficient indicates non uniformity of accession in given trait. Genotypes having traits with high value of variation coefficient are suitable for future breeding process to be used in different selections. On the other hand, low variability or high uniformity, should be typical for bred varieties. Based on our results it cannot be stated in many characteristics, that bred varieties have the lowest variability. The lowest variability was observed in many characteristics in wild populations. These results reflect well the synthetic origin of many varieties.

The correlation coefficients are presented in the Table 11.2. The correlations are presented for individual pairs of traits. Table value of the correlation coefficient at the significance level $p \leq 0.05$ is 0.21, so all the coefficients higher than 0.21 are significant. The classification among groups: soft, medium, strong and very strong correlations was done arbitrary. From 378 pairs of observed traits the soft positive correlation was found in 41 cases, the soft negative correlation was found in 19 cases, the medium positive correlation was found in 27 cases, the medium negative

Table 11.1 The variation coefficients among cultivars and populations averages of the whole genotype collection

No.	Trait/characteristic	CV (%)	No.	Trait/characteristic	CV (%)
1	The length of terminal leaflet	10.2	12	The area of terminal leaflet	18.4
2	The width of terminal leaflet	10.8	13	Number of inflorescences per stem	33.1
3	Plant weight	33.0	14	Number of flowers per inflorescence	25.0
4	Number of stems	22.2	15	Inflorescence length	27.6
5	Stem length	19.0	16	Number of ripened inflorescences per stem	47.9
6	Stem diameter	11.4	17	The weight of seeds per plant	80.7
7	Number of internodes	13.3	18	Number of pods per 100 flowers	14.7
8	The length of medium internode	11.4	19	Number of pods per inflorescence	22.8
9	Number of lateral branches	47.0	20	Pod - width of the spiral	11.5
10	Height at the beginning of flowering	9.9	21	Pod - height of the spiral	15.5
11	Leaf area	19.3	22	Number of seeds per pod	24.5

Table 11.2 The estimations of correlation coefficients (for explaining the numbers of traits see section Material and methods)

	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2
1	0,09	0.40*	0,02	0,10	0,00	0,00	0,13	0,15	0,05	0,27*	-0,17	0.39*	0,00	-0,24*	0.51**	0.49*	0,02	-0,08	0.37*	0,05	0.32*	0,29*	0.36*	0.42*	-0,14	0,25*	0.65**
2	0,08	0.40*	-0,01	0,09	-0,10	0,01	0,11	0,07	0,12	0.39*	-0,24*	0.45*	0,01	-0,27*	0.62**	0.60**	-0,07	-0,17	0.38*	0,03	0.34*	0,20	0.39*	0.44*	-0,17	0,29*	
2	-0,05	0.57**	0,03	-0,03	-0,06	0,05	0,10	0,28*	0,04	0,26*	-0,15	0.43*	0,21*	-0.45*	0.49*	0.54**	-0,14	-0.40*	0,22*	0,16	0.61**	0.56**	0.59**	0.83***	0.48*		
4	-0,03	0,07	0,03	0,00	0,08	-0,05	-0,07	0,04	0,02	-0,19	0,13	-0,19	0,23*	-0,08	-0,11	-0,08	-0,12	-0,01	-0,11	-0,01	0,16	0,04	-0.45*	0,15			
5	-0,11	0.62**	0,01	-0,10	-0,17	0,08	0,18	0,27*	0,04	0.33*	-0,19	0.60**	0,21*	-0.56*	0.64**	0.68**	-0,14	-0.46*	0.35*	0,19	0.69**	0.66**	0.70**				
6	-0,05	0.49*	-0,05	-0,10	-0,13	0,08	0,18	0,18	-0,04	0.40*	-0,20	0.52**	0,06	-0.36*	0.56**	0.58**	-0,13	-0.32*	0,29*	0,18	0.49*	0.61**					
10	-0,18	0.50*	-0,08	-0,08	-0,09	0,16	0,18	0,23*	-0,08	0,15	0,03	0.35*	0,11	-0.36*	0.41*	0.44*	-0,11	-0.30*	0,24*	0.32*	0.33*						
10	0,01	0.44*	0,02	-0,07	-0,12	0,01	0,06	0,24*	0,07	0.34*	-0,24*	0.43*	0,13	-0.36*	0.48*	0.50**	-0,13	-0.30*	0,23*	0,09							
10	-0,06	0,13	-0,03	0,02	0,03	0,09	0,04	0,12	-0,03	0,07	0,01	0,10	0,02	-0,09	0,07	0,08	0,01	-0,04	0,08								
10	0,08	0,26*	-0,02	0,04	0,05	0,02	0,06	0,04	0,07	0,26*	-0,09	0,27*	-0,05	-0,13	0.33*	0.34*	-0,04	-0,07									
11	0.34*	-0.42*	0,02	0,24*	0,27*	0,05	-0,11	-0,07	-0,06	-0,19	0,22*	-0.50*	-0,26*	0.52**	-0.36*	-0.33*	0,02										
12	0,00	-0,06	0,00	0,02	-0,01	-0,06	-0,09	0,02	-0,04	-0,07	-0,07	0,02	0,04	-0,03	-0,04	-0,04											
13	0,04	0.56**	-0,03	0,01	-0,14	-0,01	0,13	0,18	0,06	0.40*	-0,27*	0.56**	0,08	-0.41*	0.98****												
14	0,06	0.53**	-0,02	0,04	-0,11	0,01	0,11	0,19	0,07	0.40*	-0,22*	0.54**	0,04	-0.37*													
15	0,20	-0.42*	0,02	0,19	0,28*	0,10	-0,17	-0,09	-0,03	0,03	0.45*	-0.69**	-0.78***														
16	-0,15	0,15	0,00	-0,19	-0,12	-0,14	0,04	-0,04	0,00	-0.45*	-0.38*	0,22*															
17	-0,06	0.48*	-0,01	-0,08	-0,28*	-0,04	0,20	0,13	0,07	0.61**	-0.65**																
18	-0,15	-0,16	-0,03	0,01	0,14	0,15	-0,07	0,02	-0,07	-0.37*																	
19	0,10	0,25*	0,01	0,09	-0,08	0,08	0,11	0,11	0,08																		
20	-0,02	0,04	0,05	0,08	0,02	-0,07	-0,08	0,02																			
21	0,03	0,22*	0,06	0.43*	0,09	0,16	0,01																				
22	-0,01	0,09	-0,05	-0,06	-0,14	0,10																					
23	-0,05	-0,07	0,04	0,18	0,06																						
24	0,12	-0,14	0,04	0.31*																							
25	0,21*	-0,03	0,10																								
26	0,04	-0,03																									
27	0,08																										

Table value of the correlation coefficient at the significance level $p \leq 0.05$ is 0.21
No asterisk – no significant correlation, correlation coefficient 0.0–0.2
* – soft correlation, correlation coefficient 0.21–0.5
** – medium correlation, correlation coefficient 0.51–0.75
*** – strong correlation, correlation coefficient 0.76–0.9
**** – very strong correlation, correlation coefficient 0.91–1
Degrees of freedom are the same for all pair-wise comparisons

correlation was found in 2 cases, the strong positive correlation was found in 1 case (stem length × plant weight) the strong negative correlation was found in 1 case (nitrogen substances content × fibre content) and the very strong positive correlation was found also in 1 case (leaf area × the area of terminal leaflet). By couples of traits with high positive correlation coefficients during selection of breeding materials it is enough to work only with one trait. In case of more positively correlated traits the situation is much easier for the breeder. This knowledge leads to simplifications during the breeding process. The obtained results confirm the experiences of many authors concerning the complicated relation of seed (Vachůnová 1979) and biomass production by alfalfa. Nevertheless some correlations seem to be general, because they occur under different conditions and years of trials. For example Pelikán et al. (1987) found medium positive correlation between the traits number of flowers and seed yield, number of pods and seed yield, stem length and number of pods and medium negative correlation between the traits number of inflorescences and seed yield. The same correlations between these pairs of traits were found also in this study, even if the correlations were only low. These above mentioned correlations are suitable for use in practical breeding process.

The main objective of our paper is to give methodological instruction to practical breeders specifically in the Czech Republic and generally in Central Europe how to work more effectively with important traits that could be used in plant breeding. Our results are based on a study of 457 alfalfa origins. The number of evaluated alfalfa materials is well representative for general conclusions.

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Chapter 12
Influence of Management and Habitat on Genotypic and Phenotypic Diversity of *Dactylis glomerata*

Luisa Last, B. Boller, F. Widmer, and R. Kölliker

Abstract Although the importance of grassland habitats as a reservoir of genetic resources is generally recognized, little is known about the effect of environmental factors and agricultural land use on genotypic and phenotypic diversity in grassland ecosystems. This study aimed to investigate orchardgrass (*Dactylis glomerata* L.), a perennial forage grass well adapted to a broad range of temperate environments as a model species to assess the effect of management practices (intensive, extensive) and farming systems (organic, non-organic) on genotypic and phenotypic diversity. Twenty orchardgrass populations were sampled from Swiss permanent grassland habitats, contrasting in respect to organic and conventional farming systems, management intensity and environmental conditions. Genetic diversity was analyzed using 29 simple sequence repeat markers (SSRs) and 10 morpho-physiological traits evaluated in a field experiment. Nei's unbiased gene diversity (H_E) revealed a high genetic diversity (0.51–0.56). Most of the genetic variation resided within populations (96.06 %). Genetic diversity was significantly influenced by intensification of management resulting in a decrease of genotype diversity within sites ($r = -0.50$, $p = <0.05$). Furthermore, phenotype evaluation revealed significant influence of management on *Dactylis glomerata* populations based on selected traits. However, within- and between-population genotypic and phenotypic diversity was only marginally correlated with faming systems. Facing uncertain climatic changes and an increasing need for sustainable management of resources, the insights gained may be particularly valuable for the *in situ* conservation of valuable genetic resources of grassland species. Furthermore, they support targeted selection of ecotypes for breeding programs.

Keywords Genetic diversity • Management intensity • Grassland • Ecotypes

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Introduction

Permanent grasslands are an important source of roughage for milk and meat production in the mountainous regions of Europe. They not only harbor a large number of different plant species, they also serve as important reservoirs of genetic diversity for many important forage species including ryegrasses, fescues and clovers (Kölliker et al. 2003; Peter-Schmid et al. 2008). The value of ecotypes sampled from permanent grassland has long been recognized by plant breeders and many forage crop breeding programs partly rely on these valuable genetic resources (Boller and Greene 2010). Although many of these ecotypes suffer from disadvantages such as reduced forage yield and quality when compared to advanced cultivars, they also often carry alleles for valued characteristics such as disease resistance or early flowering. In Italian ryegrass, certain local ecotypes have even been found to outperform today's cultivars for traits such as persistence, vigor or resistance to snow mold (Boller et al. 2009). In order to optimally exploit genetic resources, a detailed characterization of the genetic diversity present in permanent grassland and the factors influencing it is indispensable. Orchardgrass (*Dactylis glomerata* L.) is an important component of permanent grassland, particularly in higher altitudes, especially valued for its high yield potential under conditions where ryegrass species are limited. The aim of this study was to investigate the influence of habitat and management on genetic diversity and phenotypic characteristics of orchardgrass in order to provide a basis for the targeted exploitation of ecotype populations in plant breeding programs.

Material and Methods

Twenty orchardgrass populations were sampled from permanent grassland habitats, contrasting with respect to organic and non-organic farming systems, management intensity and environmental conditions. Sampling sites were located in Northern Switzerland and spread over an area of 12 km^2 and from 605 to 1,133 m a.s.l. Sixty parental plants per population were cross-pollinated in isolation to produce 20 Syn$_1$ progenies. Genetic diversity of parental populations was assessed using 32 individuals and 29 simple sequence repeat (SSR) markers (Bushman et al. 2011; Hirata et al. 2011). Phenotypic characteristics were assessed in a field experiment located at Agroscope Reckenholz-Tänikon, Research Station ART, Zurich using 20 ecotype Syn$_1$ populations and the two reference cultivars “Beluga” and “Reda” (Agroscope ART). Sixty individual plants per Syn$_1$ population or cultivar were planted in a randomized block design with 0.3 m distance within and 0.5 m distance between rows. Ten phenotypic traits were surveyed in the year after establishment based on UPOV guidelines. The influence of management intensity, farming system and habitat characteristics was assessed using multivariate statistics.

Results and Discussion

SSR analysis revealed high genetic diversity within *D. glomerata* ecotype populations with average heterozygosity (H_E) ranging from 0.51 to 0.56. As expected for an outbreeding species, analysis of molecular variance revealed most of the genetic diversity (96.06 %) to reside within rather than among populations. No grouping of populations was observed based on management intensity and SSR diversity (Fig. 12.1a). This is in agreement with findings in *L. multiflorum* but in contrast to the clear separation observed in *F. pratensis* (Peter-Schmid et al. 2008) and may reflect the high competitive ability of this species in various environments. However, intensive management lead to a significant decrease in genotype diversity and populations from organic farms showed lower average (H_E) than populations from non-organic farms (data not shown).

With one exception, ecotype populations from extensively managed grassland were clearly separated from ecotypes from intensively managed grassland and from cultivars based on ten phenotypic traits (Fig. 12.1b). Plants from extensively managed grassland had significantly shorter stems (Fig. 12.2b), shorter and smaller flag leaves and shorter internodes (data not shown), while growth habit and susceptibility to rust diseases were not significantly influenced by management intensity (Fig. 12.2a, c). Although both cultivars clearly outperformed ecotypes in terms of vigor and disease resistance, diversity in ecotype populations was large and individuals with interesting characteristics were present in several populations. Populations from extensively managed sites showed greater variation for flowering time, while the variability of the other phenotypic traits was neither influenced by

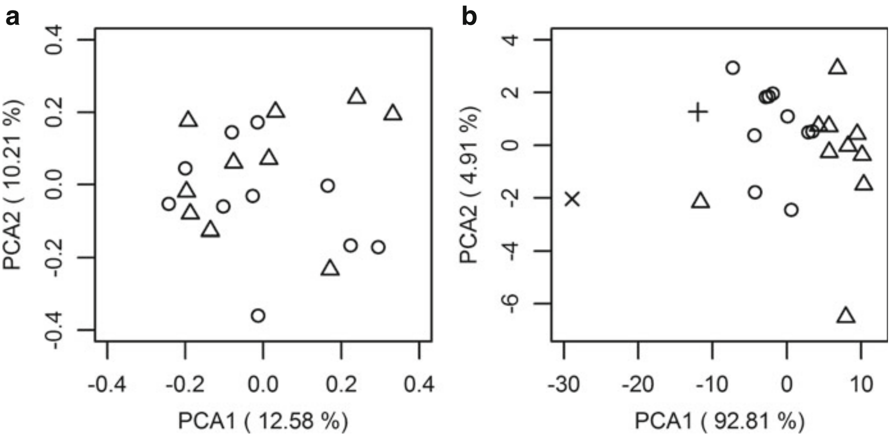

Fig. 12.1 Principle components analysis of (**a**) SSR marker data of 20 parental populations and (**b**) 10 phenotypic traits of 20 Syn_1 populations of *D. glomerata*. *Triangles* and *circles* indicate populations from intensive and extensive management, × and + cultivars Beluga and Reda, respectively

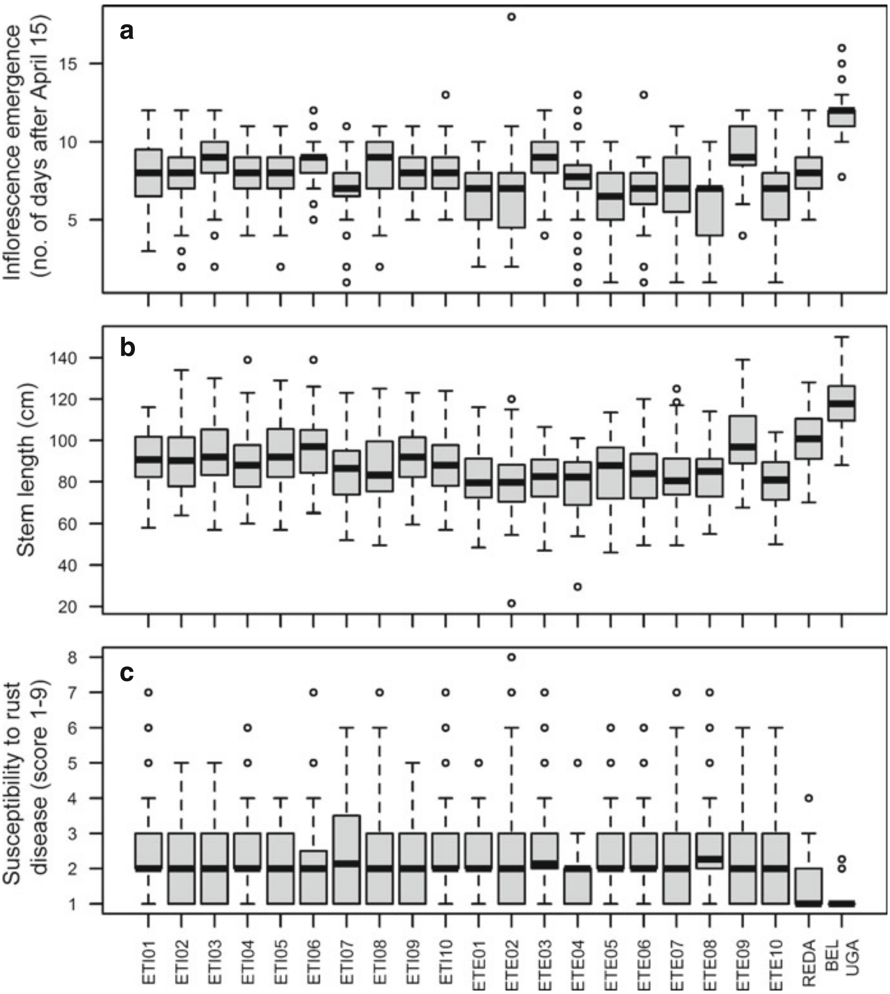

Fig. 12.2 Phenotypic characteristics (**a**) inflorescence emergence, (**b**) stem length, and (**c**) susceptability to rust disease) of 20 *D. glomerata* ecotype populations and two cultivars. ETI1 to ETI10 indicate intensively managed plots on farm 1 to 10. ETE1 to ETE10 indicate extensively managed plots on farm 1 to 10

management or farming system. Our results demonstrate that permanent grassland harbor *D. glomerata* populations with large diversity and agronomically interesting characteristics.

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Chapter 13
The Utilisation of Wild *Fabaceae* Species in Grasslands

Daniela Knotová, Jan Pelikán, and Simona Raab

Abstract Within the period of 2004–2010, the possibility of cultivating some selected *Fabaceae* species as grassland mixture components was tested in field trials performed in Troubsko, Czech Republic. The experiment was established using a standard grass mixture to which 50 seeds of single *Fabaceae* species were admixed. The standard grass mixture contained *Festuca pratensis*, *Lolium perenne*, *Poa pratensis*, *Phleum pratense,* and the hybrid Festulolium. Twenty species of the following genera were tested: *Trifolium*, *Vicia*, *Lotus*, *Lupinus*, *Lathyrus*, *Astragalus*, *Melilotus*, *Medicago*. The experiment was established in a randomised block design with three replicates. The size of each plot was 5 m^2. Each plot was harvested twice per year. Average numbers of plants were calculated on the base of all wild species identified in the standard grass mixture. The survival of tested species was limited by soil-climatic conditions of the locality.

Keywords Diversity • Wild species • *Fabaceae* • Clover-grass mixtures

Introduction

Within the framework of the National Programme of Preservation and Use of Plant Genetic Resources and Agrobiodiversity, our research institute organises seed collecting expeditions. The main objective of expeditions is to collect seeds of not only wild species of cultivated plant species but also of other species of plant families under study for *ex-situ* conservation. Furthermore, we evaluate collected seed materials under field and climatic conditions of the Czech Republic. Already at the beginning of the second half of the twentieth century, Vacek (1963) tested a wide spectrum of wild species belonging to the family *Fabaceae* and recommended some

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of them for further cultivation. Also at present there are efforts to collect and to test wild species of forage plants in different parts of the world (Jansone and Jansons 2007; Martincová et al. 2007). The first report about results achieved within the framework of this project was published by Pelikán et al. (2005). This paper summarises further results obtained within the period of 2004–2010.

Materials and Methods

Some wild species belonging to the *Fabaceae* family were tested with the aim of assessing potential to increase the diversity of clover/grass associations and their use in permanent grassland. The experiment was established in Troubsko, Czech Republic, at an altitude of 280 m a.s.l. In this locality, the average annual temperature and the sum of precipitation were 8.5 °C and 547 mm, respectively. In this experiment a standard grass mixture was used; the composition of this mixture was as follows: *Festuca pratensis* (23 %), *Lolium perenne* (23 %), *Phleum pratense* (15.4 %), *Poa pratensis* (15.4 %), and the hybrid *Festulolium* (23 %). On each experimental plot, a standard grass mixture was sown in the amount corresponding to the normal seeding rate of 26 kg ha^{-1} and with the admixture of 50 seeds of individual tested wild *Fabaceae* species (regardless to the amount of hard seeds). The experiment was established as a randomised block design with three replicates and the size of each experimental plot was 5 m^2. In individual experimental years, numbers of plants of each tested species were counted including those which are reproduced vegetatively by rhizomes, on several different dates in the course of the growing season. Each plot was harvested two times per year and the average number of plants of each tested wild species was calculated on the base of data collected during the year. During the experiment the fertilizers were not used. This experiment was performed within the period of 2004–2010.

Results and Discussion

Results are presented in the following graphs. Average numbers of plants of the genus *Trifolium* counted in individual experimental years are presented in Fig. 13.1 and there was a gradual increase in numbers of plants of individual species belonging to this genus. The highest increase was observed in case of the species *Trifolium medium* mainly due to its spreading via underground rhizomes (runners). In species *Trifolium alpestre*, *Trifolium dubium*, and *Trifolium campestre* the increase was observed till the sixth year of the stand's existence and thereafter a decrease in their numbers took place. In spite of the fact that the species *Trifolium dubium* and *Trifolium campestre* are annual plant species, they persisted in the stand due to the fact that both were relatively low-growing so that the inflorescences were not

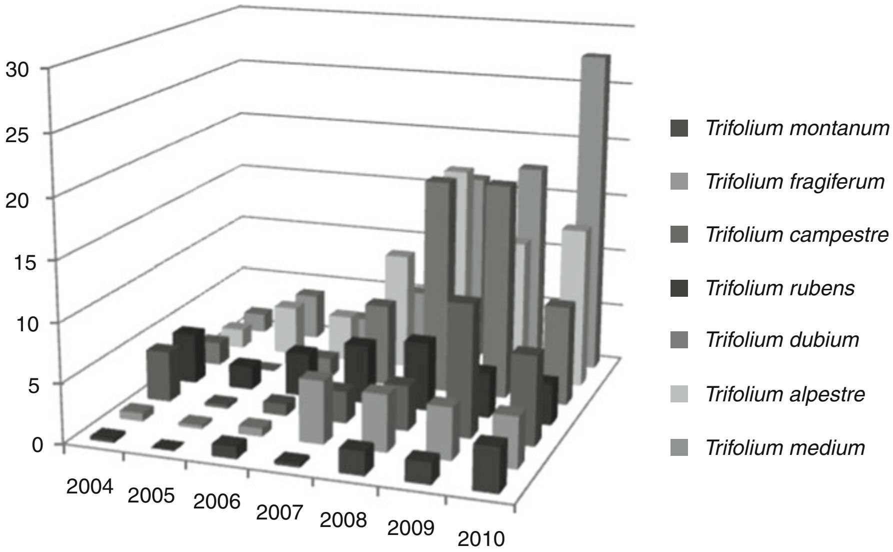

Fig. 13.1 Average number of plant species of the genus *Trifolium*

removed during the first cutting. In this way they produced fully ripened seeds that germinated and established new seedlings. The occurrence of *Trifolium montanum* and *Trifolium fragiferum* was lower, probably due to the fact that they had not quite ideal growing conditions in the experimental locality. It is known that *Trifolium fragifeum* prefers more humid conditions while *Trifolium montanum* likes lime-rich (chalky) soils.

The second group under study involved species belonging to the genus *Vicia.* Obtained results are presented in Fig. 13.2. Among these species, plants of *Vicia cracca* showed to be the best ones. However, its high abundance and long stems caused problems during the harvest of green fodder and, besides, it also suppressed some grass components in the stand. The abundance of species *Vicia pisiformis*, *Vicia angustifolia* and *Vicia tenuifolia* (5–10 plants per 5 m^2) was satisfactory. The species *Vicia villosa* was the most abundant in the year of sowing but in years to follow its numbers gradually decreased.

Results concerning members of the genus *Lathyrus* and of species *Lotus uliginosus* and *Lupinus polyphyllus* are presented in Fig. 13.3. In this group of tested plants, the best results were obtained with the species *Lathyrus sylvestris*, however, its high abundance also caused problems during the harvest of forage, similarly as in case of *Vicia cracca*.

The species *Lathyrus tuberosus* asserted itself very well in the stand. The species *Lupinus polyphyllus* was well represented already in the year of the stand establishment but thereafter it gradually disappeared with the increasing age of the stand. For the species *Lotus uliginosus*, conditions of the experimental locality were not

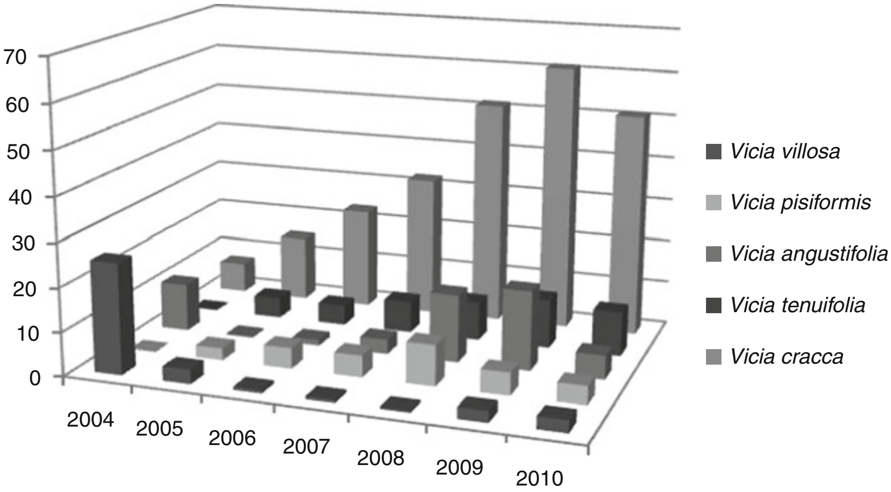

Fig. 13.2 Average number of plant species of the genus *Vicia*

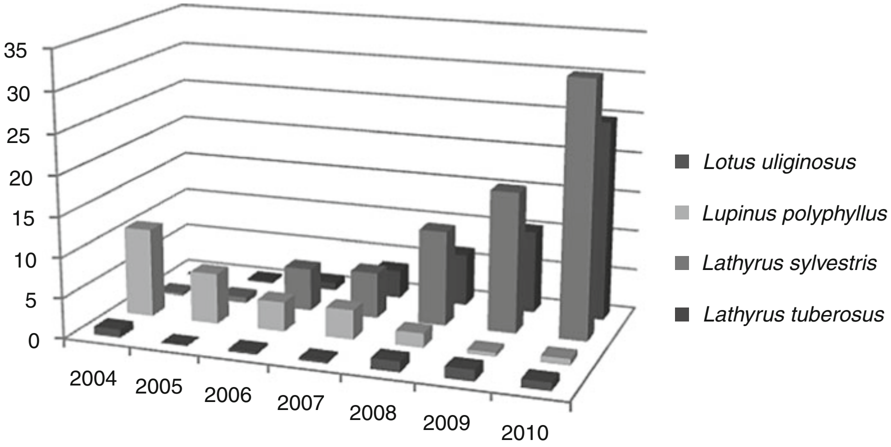

Fig. 13.3 Average number of plant species of the genus *Lotus*, *Lupinus* and *Lathyrus*

favourable (because it also preferred humid habitats); nevertheless, it persisted in the stand for the whole experimental period.

Results concerning the last four species under study are presented in Fig. 13.4. Of members of the genus *Astragalus*, the best results were recorded in stands with the admixture of *Astragalus cicer*, again because of underground rhizomes that enable its secondary spreading.

Although there are references that all species belonging to the genus *Astragalus* are poisonous, the species *Astragalus cicer* is selected and propagated both in the USA and in Canada (Townsend 1988, 1990). This species is used in grazing

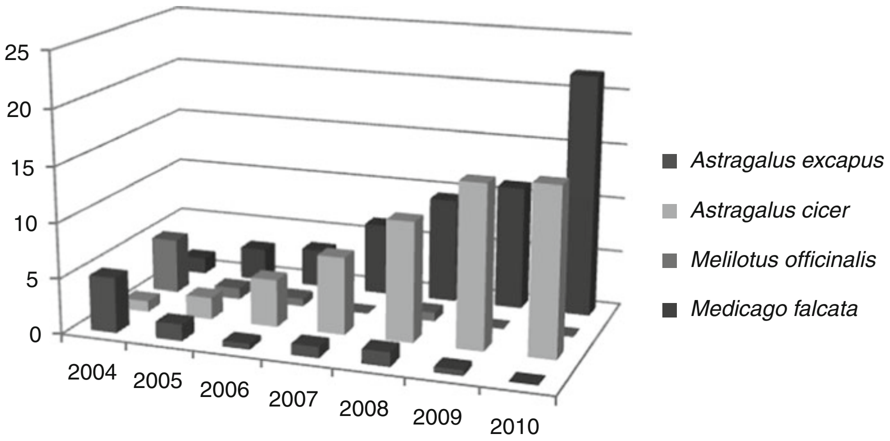

Fig. 13.4 Average number of plant species of the genus *Astragalus*, *Melilotus* and *Medicago*

mixtures for sheep (Johnston et al. 1975). In case of the species *Medicago falcata,* a similar trend was observed as in *Astragalus cicer.* Species *Melilotus officinalis* and *Astragalus excapus* asserted themselves only in the year of the stand establishment and in the year that followed; thereafter, they fully disappeared from experimental stands.

Conclusions

Monitoring of the occurrence of admixed species of wild legumes and the succession of clover/grass associations is a long-term affair. After 7 years of experiments performed in the locality Troubsko it is possible to conclude that the majority of tested wild species belonging to the family *Fabaceae* can be admixed (with the exception of the species *Galega officinalis*) into species-poor grassland stands (i.e. stands with a lower number of plant species). Under favourable environmental conditions, individual members of this family can not only appear in the stand but also survive and persist there. Seeds from the family *Fabaceae* are characterized by production of a proportion of 'hard' seeds, so some seeds germinate in subsequent years. However, when deciding which species is suitable for cultivation under conditions of a given locality and/or habitat it is necessary to consider carefully their properties and parameters. The choice of a good and successful technology enabling production of sufficient amounts of the seeding material and the determination of the seeding rate of a chosen wild species are necessary prerequisites of their practical application. Presumably different cutting frequency and height and fertiliser regime would have a significant effect on the successful establishment of the legume species in the sward and you could expect a completely different result under different management strategies. These factors were not taken into account.

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Chapter 14
Assessment of Susceptibility of Different Alfalfa Genotypes to *Colletotrichum destructivum*

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Abstract Southern anthracnose or crown rot, caused by *Colletotrichum destructivum*, is a disease that has been detected on alfalfa in Serbia. During a 6-year period, especially in summer and autumn, alfalfa plants with anthracnose symptoms were studied in the field. Stem infection resulted in wilting and death of the upper portion of the stem, giving rise to the characteristic 'shepherd's crook' symptom. To examine the level of sensitivity of different alfalfa genotypes in experimental conditions, six isolates: Coll-3, Coll-8, Coll-9, Coll-10, Coll-18 and Coll-75 (*C. destructivum* originating from Serbia) and one reference isolate CC657 *C. destructivum,* were used. Ten commercial alfalfa genotypes of different origins (K-1, K-28, Zajecarska 83, Osjecka 12, NS Slavija, Banja Luka, Affinity 401 + Z, Florida 77, Vernal S and Perry) were examined in this study. Infected plants showed typical symptoms of anthracnose. Necrotic lesions appeared on the plant stems and gentle bending of the top of the upper third of stems occurred on tested plants. Necrotic lesions then spread to the whole plant and in some plants this led to withering. Assessment of damage by pathogen inoculation was based on a scale of 0–5. Results were processed by analysis of variance as one-factor experiment. Investigated genotypes showed varying resistance to different *C. destructivum* isolates.

Keywords Alfalfa • Anthracnose • *Colletotrichum destructivum* • Genotypes • Susceptibility

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Introduction

The fungus *Colletotrichum destructivum* O'Gara, which causes anthracnose disease in alfalfa (*Medicago sativa* L.), is an important limiting factor for alfalfa growth, plant vigour and persistence (Boland and Brochu 1989). During summer and autumn, diseased plants start to be seen in the field. *C. destructivum* was isolated from stem lesions typical of *C. trifolii* (Graham et al. 1976). Conidia form in the acervuli on stem lesions. Rain splash and wind carry the conidia to the growing petioles and stems. The fungus grows down along infected stems into the crown and taproot, causing tissue damage, a predisposition to winter injury, wilting or plant death. Nevertheless, soil infections of alfalfa by *C. destructivum* have also caused considerable yield losses in North America (Boland and Brochu 1989), Europe (Robotic and Klokocar-Smit 1983), and North and South Africa (Koch et al. 1989). The host range of *C. destructivum* is wide and includes legumes such as *Phaseolus lathyroides* L., *Glycine max* (L.) Merr., *Trifolium* spp., *Lotus* spp., *Leucaena leucocephala* (Lam.) DeWit., *Melilotus albus* Desr., *Vigna unguiculata* (L.) Walp., *Coronilla varia* L., as well as tobacco (*Nicotiana tabacum* L.) and dodder (*Cuscuta* spp.) (Latunde-Dada et al. 1996). The aim of this study was to determine sensitivity of commercial alfalfa cultivars to *C. destructivum* in Serbia, and possibilities for breeding resistant alfalfa genotypes.

Material and Methods

The susceptibility of domestic commercial alfalfa genotypes was tested in order to find potential sources of resistance to *C. destructivum*. The domestic alfalfa varieties studied were Krusevacka-1 (K-1), Krusevacka-28 (K-28), Zajecarska 83 and NS Slavija, as well as varieties from Croatia (Osjecka 12) and Bosnia and Herzegovina (Banja Luka). The experiment also included the commercial varieties Affinity 401 + Z, Florida 77, Vernal S and Perry from USA, which show different levels of resistance to species of the genus *Colletotrichum* (Gray et al. 2003).

Isolates included in this study were selected in spring 2010, based on morphological and molecular traits. Six isolates from Serbia (Coll-3, Coll-8, Coll-9, Coll-10, Coll-18 and Coll-75) identified as *C. destructivum* and one referent isolate CC657 (CBS Culture Collection of Fungi, Yeast, Bacteria, Plasmids and Phages, The Netherlands) were used for screening. The experiment was conducted in a greenhouse at the Institute for Forage Crops, Krusevac. Alfalfa seeds were sown at a depth of 2 cm in sterile substrate in plastic containers. Conidial suspension was prepared from cultures of tested isolates grown for 10 days on PDA medium at 25 °C in the dark. The concentration of spores was determined by hemocytometer at $4–6 \times 10^{-4}$ spores ml^{-1}. Seven-week-old alfalfa plants were scarred by cutting prior to inoculation then sprayed with spore suspension. Alfalfa plants inoculated with sterile water were used as a negative control. Testing was carried out on 60 seedlings per isolate of each variety.

Susceptibility of the different alfalfa genotypes to isolates of *C. destructivum* was evaluated 4 weeks after inoculation using the modified method of Ostazeski et al. (1969), according to a scale of 0–5: Plants without symptoms (0), individual watery spots in the base (1), recessed lesions present (2), appearance of lesions along the stem (3), necrosis of the entire stem (4) wilting and drying of the whole stem (5).

Disease severity index (DSI) was calculated by the formula:

$$DSI = \sum \frac{(n \times k)}{N \times K}$$

n – seedlings in class, k – class severity value, N – total no. of seedlings, K – no. class.

Data were analyzed using one-way factorial variance analysis (ANOVA).

Results and Discussion

All the studied isolates caused symptoms of alfalfa anthracnose in the tested plants. Four weeks after inoculation, necrotic spots in the lower parts of stems appeared in infected alfalfa plants of all the tested cultivars (K-1, K-28, Zajecarska 83, Osjecka 12, NS Slavija, Banja Luka, Affinity 401 + Z, Florida 77, Vernal S and Perry). As the disease progressed, stem colour changed to a glossy yellow, the crown bent into the shape of a hook and plants started to wilt (Fig. 14.1).

Fig. 14.1 Anthracnose symptoms on tested plants; cv. Banja Luka inoculated with isolate Coll-3 (*left*) and cv. K-1 inoculated with isolate Coll-18 (*right*)

Table 14.1 Evaluation of susceptibility in commercial alfalfa varieties to isolates of *C. destructivum*

Cultivars	Coll3	Coll8	Coll9	Coll10	Coll18	Coll75	CC 657	Average	CV (%)
K-1	3.7	4.5	3.5	3.6	2.8	4.8	3.4	3.76	38.3
K-28	4.1	2.9	3.5	2.4	2.9	3.7	2.8	3.19	43.5
Zajecarska 83	2.5	3.2	3.5	2.8	2.5	3.2	4.1	3.11	44.3
Osjecka 12	2.7	4.5	3.6	3.1	2.2	4.2	3.9	3.46	40.1
NS Slavija	2.4	3.4	2.4	4.2	3.6	3.7	2.7	3.20	45.0
Banja Luka	4.1	2.4	3.1	3.0	2.4	3.7	2.9	3.09	45.0
Affinity 401+ Z	2.2	3.7	3.5	4.0	2.8	2.4	2.7	3.04	48.5
Florida 77	2.4	4.0	3.3	3.8	3.2	3.9	2.4	3.29	35.8
Vernal S	2.5	3.6	3.1	3.8	2.9	3.0	2.3	3.02	43.0
Perry	2.0	3.0	2.7	3.9	3.2	3.3	3.4	3.07	35.3
Average	2.86	3.52	3.22	3.46	2.85	3.59	3.06	3.22	41.9
Duncan's test 0.05	1.16	1.21	1.12	1.13	1.21	1.15	1.18		

Results showed varying responses of the alfalfa varieties to the pathogen isolates. Using one factor variance analysis (ANOVA), it was determined that differences in the susceptibility of the tested genotypes were statistically significant (Table 14.1). Alfalfa varieties showed a high variation in resistance to different *C. destructivum* isolates. In general terms, almost all varieties indicated a lower tolerance to isolates Coll-8, Coll-10 and Coll 75, whereas tolerance to isolates Coll-3 and Coll-18 was greater.

Results of the inoculation of the alfalfa genotypes with isolate Coll-3 showed variations between the genotypes. Genotypes Affinity 401 + Z and Perry were characterized by greater tolerance to the tested isolates, while genotypes Banja Luka and K-28 showed high susceptibility. The results of the inoculation with isolate Coll-8 separated the alfalfa genotypes into three groups. The most tolerant to Coll 8 was cv. Banja Luka, while the most susceptible were cvs Osjecka 12 and K-1. Isolate Coll-9 caused a medium resistance reaction on tested plants; only cv. Osjecka 12 showed a low DSI for this isolate. A greater tolerance to Coll-10 was observed in cvs K-28 and Zajecarska 83, but other cultivars showed greater susceptibility to this isolate. The reaction of genotypes to inoculation with isolate Coll-18 showed the separation of the three groups; cvs Osjecka, Banja Luka and Zajecarska 83 all showed tolerance to the isolate. All varieties had a low tolerance to inoculation with isolate Coll-75, with Affinity 401 + Z showing the highest tolerance. Comparing the responses of genotypes to inoculation with isolate CC657, genotypes Zajecarska 83 and Osjecka 12 proved to be very susceptible, while the tolerance of other varieties was much greater.

From the results of our experiment with the domestic and foreign commercial alfalfa varieties, it can be concluded that cvs Affinity 401 + Z and Vernal S showed the highest tolerance to the tested isolates followed by cvs Banja Luka and Zajecarska 83. Other investigated cultivars displayed a much lower tolerance, especially K-1 and Osjecka 12.

Vasić et al. (2010) showed that alfalfa clones had different resistance to *C. trifolii*. Comparison of American and Serbian genotypes showed that the American alfalfa genotypes had the highest level of resistance (64.5 % plant survival). The varieties listed in Serbia displayed uneven levels of resistance, as the mean percentage of surviving plants ranged from 0 to 56.4 %.

As a consequence of its synthetic origin and its autotetraploidy, an alfalfa cultivar incorporates a mixture of heterozygous individuals such that within a cultivar there is a genetic diversity nearly as large as that found between cultivars (Julier et al. 2000). It means that every alfalfa cultivar may contain a varying proportion of susceptible and resistant plants. The resistance pool should contain both alleles for resistance and susceptibility, their proportion depending on the frequency of resistance alleles in the variety. The response of individual plants within varieties to inoculation with pathogenic isolates was also very variable. Average coefficients of variation of individual plants within a cultivar for all isolates were very high, from 35.3 % (cv. Perry), to 48.5 % (cv. Affinity 401 + Z) (Table 14.1). A small number of individual plants managed, despite displaying clear disease symptoms, to produce seeds. The progeny of these plants is an excellent source of genetic resistance to the tested pathogen isolates and will be included in the next cycle of selection for increasing tolerance to pathogens. Identification of clones with dominant resistance is an important step in increasing tolerance. Irwin et al. (2006) indicated the importance of increasing the proportion of individual plants resistant to *Colletotrichum trifolii* within alfalfa cultivars.

Until now, recurrent selection has provided an effective way to increase anthracnose resistance levels in breeding populations of autotetraploid alfalfa. Considering the results of Devine et al. (1971), who showed that after several cycles of selection the level of resistance can be increased, we believe that it is justifiable to continue investigations in this direction. The causal agents of alfalfa anthracnose exist in a few distinct races, which makes the breeding process very complex. Three races of *C. trifolii* (races 1, 2 and 4) have been described based on differential responses of alfalfa cultivars (Ariss and Rhodes 2007). Anthracnose resistance is conferred by two separate dominant genes, designated *An1* and *An2*. *An1* confers resistance to race 1 and likely to race 4, whereas *An2* confers resistance to races 1 and 2. It is noteworthy that the race 3 of *C. trifolii* was only reported in 1982 (Allen et al. 1982), but this race was subsequently reclassified as *C. destructivum* (Ameline-Torregrosa et al. 2008; Yang et al. 2008).

Boland and Brochu (1989), O'Neill et al. (1989) and Schubiger et al. (2003), in their studies of pathogenicity, showed that the species *C. trifolii* is more infectious for alfalfa than *C. destructivum*. Boland and Brochu (1989) and O'Neill et al. (1989) reported that *C. destructivum* in a controlled environment (phytotrons) showed a higher level of infectivity and the same level of infection as *C. trifolii* in field conditions.

Selected alfalfa genotypes that showed high levels of tolerance to isolates tested will be used in further field trials in order to assess their level of resistance accurately. This will enable the separate genotypes to be used in the further process of selection and breeding of alfalfa to increase frequency of resistant alleles in the variety and increase level of plant resistance.

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Chapter 15
The Czech Core Collection of *Medicago* spp.

Tomáš Vymyslický, Jan Pelikán, Daniela Knotová, Simona Raab, and Jan Nedělník

Abstract This study evaluated 52 characters from a set of 457 accessions (including newly bred varieties and collected wild forms) of the world collection of the *Medicago sativa*, *Medicago* x *varia* and *Medicago falcata*. Thirty plants of each origin were planted in the field. Ten of these were evaluated in the years 2005 and 2006. All the evaluated characters were included in the analyses. Missing values were replaced by mean values. Cluster analysis was performed using the software Statistica for Windows for all the accessions together. Complete linkage method was used for clustering and Euclidean distance as the measure of distance.

Keywords *Medicago* spp. • Varieties • Wild forms • Yield • Cluster analysis • Core collection

Introduction

The study of genetic resources and their evaluation has a long tradition in the Czech Republic (Vacek 1963; Pelikán et al. 2005). During the last 50 years roughly 2,000 accessions have been evaluated, described and stored in gene banks, but many characters of individual plants have not yet been evaluated. Recently, great attention has been devoted to a detailed evaluation of the world assortment of fodder crops (Vymyslický et al. 2007). However, at present the evaluation of morphological characters alone is insufficient. For this reason resistance evaluation and molecular analyses were performed and the "core collections" were created.

The importance of wild species/forms as input materials for breeding programmes and for use in organic farming and nature protection is increasing. Genetic resources of red clover and the possibilities of their use were studied by Drobná

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(2004). She evaluated 54 varieties from different geographic areas using basic and multidimensional statistics. The set was divided according to individual characteristics and the results were forwarded to breeding workstations. Drobná and Žáková (2001) evaluated a set of 15 characters on 36 genotypes of *Trifolium repens*. After statistical evaluation by cluster analysis they found varieties with similar geographic origin or with similar qualitative traits present in clusters. Boller et al. (2003) studied the collection of old Swiss landraces of the red clover. They evaluated a set of 20 varieties and important variability (among varieties and within varieties) was found. The authors pointed out that old cultivars and landraces are donors of valuable traits. Gubiš (2001) studied the resistance of alfalfa to bacterial wilt. He found most resistant materials amongst wild forms. The study of variability in the collections is mostly based on morphological, phenological and agronomical characteristics of individual genotypes. By the application of the multidimensional statistics the varieties could be differentiated or aggregated according to evaluated traits/characters (Užík and Žofajová 1997). Kouamé and Quesenberry (1993) used the cluster analysis for the classification of the red clover collection as the base for creating a core collection.

Materials and Methods

All the available seed samples of the *Medicago* spp.—newly bred varieties and collected wild forms stored at the Czech national gene bank—were included in the study's detailed evaluation of morphological and yield characters. Seeds from the gene bank were germinated and seedlings were transplanted into grow-boxes. In the spring of 2005 30 seedlings of each accession were planted in the field spaced 50 × 50 cm apart. During 2005–2006, 52 characters from ten plants of each accession were evaluated in the field and in the laboratory:

- Turf – number of stems in the first cut (to the standard cv.);
- Stem – length, thickness, shape in diameter, hollowness, colour, number of internodes, the length of middle internode, number of lateral branches;
- Terminal leaflet – shape, margin, apex, length, width, area;
- Leaf – colour, indumentum, area, polyphyly;
- Inflorescence – shape, length, number per stem, number of flowers, colour, number of pods per stem;
- Infructescence – number of pods;
- Pod – number per 100 flowers, shape, colour, height of spiral, width of spiral, number of seeds;
- Seed – shape, colour, 1,000 seed weight;
- Vegetation period – by the beginning of flowering (to standard cv.);
- Winter hardiness – resistance;
- Alfalfa mosaic virus – resistance;
- Stand – height at the beginning of flowering (to the standard cv.), height 20 days after the first cut (to the standard cv.), foliage (% of leaves in dry matter), regrowth

(at standard height 25 cm), biomass of yield of all cuts in the year (to standard cv.), yield of hay in all cuts (to the standard cv.), yield of absolute dry matter (to the standard cv.), yield share in the first cut to the whole yield per year (to the standard cv.), seed yield (to the standard cv.);

- Plant – yield of green biomass (to standard cv.), seed yield (to the standard cv.);
- Dry matter – nitrogen content, digestibility, fibre content.

Near Infra Red Spectrum (NIRS) analyses were also performed to determine the chemical composition of dry matter samples. Dried mixed samples from ten plants of each accession were grinded and NIRS analyses performed. The method is based on the transmittance of the near infrared rays through the grinded sample. Chemical composition of each sample was derived from the equation, which was created based on the NIRS results of the control sample.

All the evaluated traits were transformed on to a nine-point scale according to the Czech national descriptor's list for the genus *Medicago* L. (Vacek et al. 1985). For the evaluated quantitative characters the point estimations of the mean value were made and the estimations of correlation coefficients among measured characters were performed.

Cluster analysis was performed using the software Statistica for Windows (STATSOFT INC. 2003), both separately for *M. sativa, M. x varia* and *M. falcata* accessions, and for all the accessions together. The complete linkage method was used for clustering and Euclidean distance as the measure of distance.

Results and Discussion

Based on cluster analysis, all 457 evaluated accessions were divided into groups and representatives were selected to establish the core collection. Finally 99 accessions of the world assortment were selected to establish the Czech core collection (Fig. 15.1). Eighty eight of these are *Medicago sativa* materials, 4 are newly bred materials of *M. sativa,* 5 are varieties of *M. x varia*, 1 is a newly bred variety of *M. x varia* and only one variety represents *M. falcata*. The survey of these materials is presented in Table 15.1. Fifty four out of the selected 99 accessions are registered in the Common catalogue of varieties of the European Union.

Good predictive ability of cluster analysis was confirmed. The closest couple from all 457 accessions consists of Slovak materials marked SVK 3/6 and SVK 2/8. In the first case it was hybrid varieties Burgaltaj and Leonicena from the former Soviet Union, in the second case it was material from Kazakhstan. The next couple was created by the French varieties Orca and Omega. Further, clusters of similar origin, i.e. by country of origin, were established. This kind of cluster was formed by Czech varieties from the former breeding station at Libochovice (Li-POP-1, Li-1 and variety Hana, which was bred under mark Li-POP-2), plus two of Czech origin from the Želešice breeding station (ZE-XXVIII and variety Niva, bred under mark ZE-N2-2). It can be supposed that these materials were bred from similar ancestors. The next clusters were all, or partially, created by origins of *Medicago x varia*. For

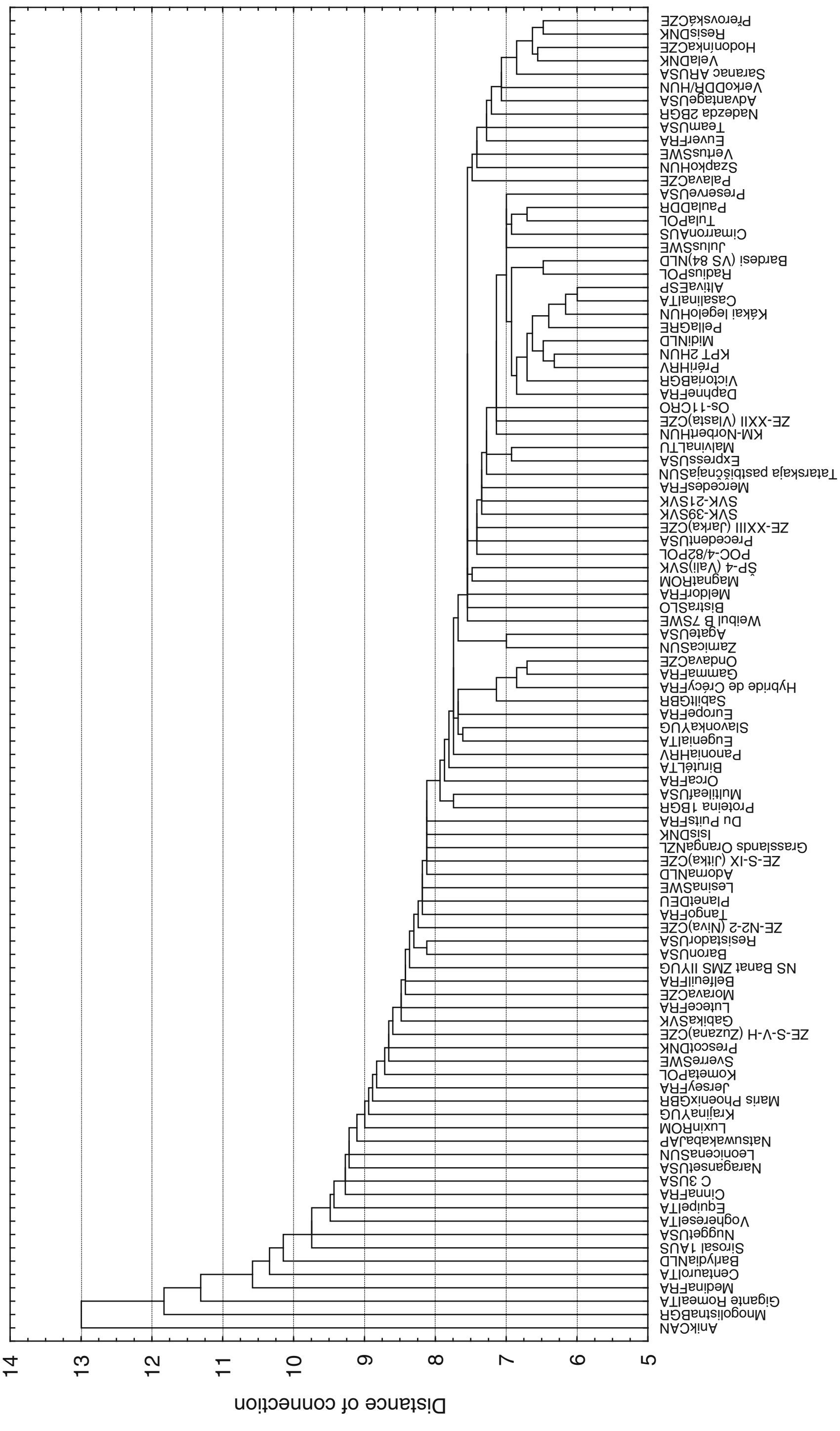

Fig. 15.1 Cluster diagram of the Czech core collection of alfalfa

Table 15.1 The Czech core collection of *Medicago* spp.

1	Mnogolistna	BGR	sativa	34	Malvina	LTU	sativa	67	Nadezda 2	BGR	sativa
2	Victoria	BGR	sativa	35	Os-11	CRO	sativa	68	Vela	DNK	sativa
3	Naraganset	USA	varia	36	Jarka	CZE	sativa	69	Orca	FRA	sativa
4	Kákailegelo	HUN	sativa	37	Jersey	FRA	sativa	70	Europe	FRA	sativa
5	Centauro	ITA	sativa	38	Bistra	SLO	sativa	71	Palava	CZE	sativa
6	Midi	NLD	sativa	39	Daphne	FRA	sativa	72	Zuzana	CZE	sativa
7	NS Banat ZMS II	YUG	sativa	40	Precedent	USA	sativa	73	Preserve	USA	sativa
8	Medina	FRA	sativa	41	POC-4/82	POL	varia	74	Morava	CZE	sativa
9	SVK 21	SVK	sativa	42	Mercedes	FRA	sativa	75	Belfeuil	FRA	sativa
10	SVK 39	SVK	sativa	43	Planet	DEU	sativa	76	Verko	DDR/HUN	sativa
11	Préri	HRV	sativa	44	Biruté	LTA	sativa	77	Hodonínka	CZE	sativa
12	Tatarskajapastbiščnaja	SUN	varia	45	Pella	GRE	sativa	78	Anik	CAN	falcata
13	Paula	DDR	sativa	46	Meldore	FRA	sativa	79	Equipe	ITA	sativa
14	Natsuwakaba	JAP	sativa	47	Kometa	POL	varia	80	Leonicena	SUN	sativa
15	Cinna	FRA	sativa	48	Altiva	ESP	sativa	81	Sirosal1	AUS	sativa
16	Tula	POL	varia	49	Adorna	NLD	sativa	82	Eugenia	ITA	sativa
17	Julus	SWE	sativa	50	Vogherese	ITA	sativa	83	Team	USA	sativa
18	Isis	DNK	sativa	51	KM Norbert	HUN	sativa	84	Hybride de Crécy	FRA	sativa
19	Vali	SVK	sativa	52	Euver	FRA	sativa	85	Du Puits	FRA	sativa
20	Gabika	SVK	sativa	53	Vertus	SWE	sativa	86	Slavonka	YUG	sativa
21	WeibulB7	SWE	sativa	54	Cimarron	AUS	sativa	87	C 3	USA	sativa
22	Tango	FRA	sativa	55	Szapko	HUN	sativa	88	Agata	USA	sativa
23	KPTS 2	HUN	sativa	56	Saranac AR	USA	sativa	89	Lutece	FRA	sativa
24	Barlydia	NLD	sativa	57	Poroteina 1	BGR	sativa	90	Luxin	ROM	sativa
25	Jitka	CZE	sativa	58	Krajina	YUG	sativa	91	Zarnica	SUN	sativa

(continued)

Table 15.1 (continued)

26	Vlasta	CZE	sativa	59	Baron	USA	sativa	92	Sabilt	GBR	sativa
27	Niva	CZE	sativa	60	Prescot	DNK	sativa	93	Gamma	FRA	sativa
28	Bardesi	NLD	sativa	61	Nugget	USA	sativa	94	Ondava	SVK	sativa
29	Radius	POL	varia	62	Sverre	SWE	sativa	95	Multileaf	USA	sativa
30	Casalina	ITA	sativa	63	Resistador	USA	sativa	96	Advantage	USA	sativa
31	Express	USA	sativa	64	Maris Phoenix	GBR	sativa	97	Resis	DNK	sativa
32	Panonia	HRV	sativa	65	Grassland Oranga	NZL	sativa	98	Přerovská	CZE	sativa
33	Magnat	ROM	sativa	66	Lesina	SWE	sativa	99	Gigante Romea	ITA	sativa

example, a cluster created by origins Mnogolistna (BGR), Tulunskaja gibridnaja (SUN), Heinrichs (CAN), Drylander (CAN) and Ufimskaja (SUN). A similar cluster was formed by varieties Bendelebener (DDR), Beloruskaja (SUN), Tula (POL) and von Arnim's Altdeutsche Bastard (DEU). The last cluster was created only from *Medicago x varia* origins, formed by two Polish newly bred materials POC–1/79 and POC–4/82, which both originated from the same station.

An interesting cluster was formed from ten origins from the Slovak Republic, including wild forms collected in the High Tatra mountains. These selections were tested under different stress conditions. Next, interesting clusters were formed by varieties from the same country of origin. For example, a cluster of the American varieties Duke, Mercury, Armor, Liberty and Multileaf; A cluster of the French varieties Warotte, Orca and Omega; And a cluster of the Croatian varieties Posavina, Panonia and Astra or a cluster of American varieties Nuget and Thor. Also many other clusters were created by combining origins from the same country with one or more varieties from another country. For example, a cluster of French varieties Janu, Euver, Jade and Gemini, plus the Swedish variety Alfa II; A cluster of American varieties Resistador, Raidor, Polar I and Arc, plus the Australian variety Paravivo. Another similar cluster consists of American varieties Fortress, Defender, Trident, Voris A-77 and CUF 101, plus the Australian variety Cancreep and English Maris Phoenix; And a cluster of American varieties Mesilla, Lahontan, Agata, Dawson, Dominor and Uinta, plus the English variety Maris Kabul. Unfortunately, we do not know the detailed pedigree of varieties to determine their joint progenitors.

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Part III

Practical Breeding of Fodder Crops

Chapter 16
Variation in Cell Wall Digestibility of Perennial Ryegrass at Heading Stage

Frederik R.D. van Parijs, C. Van Waes, E. Van Bockstaele, G. Haesaert, I. Roldán-Ruiz, and Hilde Muylle

Abstract Perennial ryegrass (*Lolium perenne*) is the dominant forage grass in Europe, because of its high yield level, good persistence, high protein content and energy value. As high levels of water soluble carbohydrates (WSC) lead to rumen acidosis, the energy contained within the cell walls, which is released more gradually, has become a focus in ryegrass breeding. We have assessed the cell wall digestibility (NDFD) of blade, sheath and stem fraction of a set of 13 genotypes harvested at heading stage. Significant genotype-to-genotype differences were found for total plant NDFD, with genotypes explaining 68 % of the total variation. The observed variation in NDFD for sheath and stem was higher than for the total plant, with genotypes explaining 79 and 80 % of the total variation in sheath and stem NDFD respectively. Further, multiple linear regression showed that sheath NDFD, stem NDFD and the weight of the leaf fraction have a significant impact on the total plant NDFD values, whereas blade NDFD does not. Analysis of this limited set of genotypes has revealed limited variation in NDFD at the plant level, but a significant variation in organ fractions and organ-specific NDFD values, indicating potential selection targets to improve cell wall digestibility at the whole plant level.

Keywords Cell wall digestibility (NDFD) • Variation • Perennial ryegrass • Heading

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Introduction

Perennial ryegrass (*Lolium perenne*) is the most abundant forage grass in temperate regions (Sampoux et al. 2013). To reduce environmental and economic costs of animal production, the nutritional value of forage needs to be improved (Wilkins and Humphreys 2003). Improving organic matter digestibility (OMD) increases both intake and energy release during digestion, reducing the release of nitrogen into the environment and increasing milk production (Casler and van Santen 2010; Parsons et al. 2011). A 1-unit increase in OMD of perennial ryegrass was estimated to increase milk yield by 1.5 % (Pacheco et al. 2007).

The total digestibility of forage is affected by both the concentration of water soluble carbohydrates (WSC) and the digestibility of the cell wall (NDFD) (Casler and Van Santen 2010). The latter has become a more relevant selection criterion for three reasons: (1) WSC are subject to extensive environmental variation, (2) WSC are released too quickly in the rumen leading to acidosis when present at high concentrations and (3) a higher NDFD results in a higher energy release (Barrière et al. 2003).

To our knowledge, variation in NDFD has never been studied in detail for *L. perenne* on a genotype basis. By assessing individual genotypes, a larger variation in NDFD values is expected compared to mixtures, where extreme genotypic values are averaged out. In this study, a set of genotypes was harvested at early heading, an agronomically important cut, as total plant digestibility and yield are well-balanced at this phenological stage.

The NDFD of the whole plant is determined by both its cell wall composition and leaf-to-stem ratio (Casler and Van Santen 2010). Here we examined the NDFD of different organs in 13 diverse *L. perenne* genotypes, as well as the leaf-to-stem ratio. In previous studies these two factors were mostly confounded, as total plants were analysed. If genotypic differences in NDFD can be identified at the total plant level, selection response can be improved by selecting for high NDFD at the organ level, or by altering the leaf-to-stem ratio.

Material and Methods

Plant Material

Thirteen diploid perennial ryegrass genotypes were selected from a set of 300 displaying a large variation in total plant NDFD in a preliminary trial in 2008 (Muylle et al. 2013). Plants were vernalised, planted in 10 l containers in four replicates and harvested in spring 2011 at heading (when three spikes were visible). Each pot received an equivalent of 100 kg nitrogen per ha at the beginning of May. The harvested plant materials were dried at 70 °C for 48 h and separated in five fractions: blade, sheath, stem, spike and dead material. These fractions were weighed, ground, and analysed by near-infrared reflectance spectroscopy (NIRS FOSS XDS).

NIRS and Wet-Chemical Analyses

Separate NIRS calibration models were built to predict blade, sheath and stem values, using 72, 40 and 32 diverse samples respectively. For these 144 samples, NDFD was determined as described by Goering and Van Soest (1970).

Statistical Analysis

Plant values for NDFD were calculated using the organ-specific NDFD and NDF values, and organ fractions. The software package R 2.15.1 was used to determine genotype and organ effects by ANOVA (type II) and multiple linear regression (MLR). For pairwise comparison of genotypes, a Tukey test was executed. Finally, the effects of organ-specific NDFD values and the weight fraction of blade on plant NDFD values were estimated by MLR.

Results and Discussion

Inter-genotypic Variation for NDFD

For the 13 diverse genotypes studied, 95 % of the variation in NDFD falls within a range of 7.8 units. Although this measure of variation is not an estimation of the true population variance, it does suggest that there is sufficient variation in NDFD at the plant level. The statistical analysis shows a significant genotype effect ($p < 0.001$), with genotypes explaining 68.0 % of the total variation (R^2).

Compared to the total plant, the variation for blade NDFD is slightly lower, while for sheath and stem, the variation is much higher (Table 16.1). For all organs, a simple ANOVA shows a significant genotype effect ($p < 0.001$). For blade, genotypes explain 76.1 % of the variation, for sheath 79.0 % and for stem 80.4 %. Thus, more variance is explained when organs are evaluated separately. Consequently, by separating organs, more genotypes could be differentiated in their NDFD value, as confirmed by homogeneous grouping (Tukey, not shown).

Differences for Blade, Sheath and Stem NDFD

On average, NDFD is significantly higher for blade than for sheath and stem (95 % C.I. 6.1–7.7 for sheath and 9.9–11.5 for stem). However, a significant genotype by organ interaction is found ($p < 0.001$). For example, Sibasa_235 shows no significant difference between blade and sheath NDFD, while Barata_198 shows no significant

Table 16.1 Summary table of genotype means and variation

	Heading/ harvest date	NDFD (%)				Weight fraction (%)			
		Plant	Blade	Sheath	Stem	Blade	Sheath	Stem	Stem + sheath
Mean	01/06	75.8	78.6	71.7	67.9	57.9	25.8	12.2	38.1
Min.	16/05	72.8	75.7	66.1	59.4	47.2	13.7	4.7	19.4
Max.	14/06	79.4	80.9	78.2	76.6	77.6	33.2	18.2	47.0
95 % range	35.1	7.8	6.2	14.2	18.6	39.1	24.2	17.8	36.7

“95 % range” is the range within which 95 % of genotype values fall, assuming a normal distribution (2*1.96*SD)

Fig. 16.1 *Left*: average NDFD value for each genotype, for separate organs and plant values. *Right*: average weight fraction of each organ for each genotype. Genotypes are sorted in increasing plant NDFD

difference between blade and stem NDFD (Fig. 16.1). However, for each genotype, NDFD for blade is significantly higher than for sheath and stem combined. Thus, selecting higher blade fractions is another option to increase plant NDFD.

Genotypic Variation in Weight Fractions of Blade, Sheath and Stem

On average, 57.9 % of the plant is blade, 25.8 % of the plant is sheath and 12.2 % of the plant is stem (Table 16.1). Although sheath and stem fractions are rather small, together they still comprise 38.1 % of the plant. Significant genotypic differences in weight fractions were identified for each organ, with genotypes explaining 83.2 % of the variation for blade, 82.7 % for sheath and 67.6 % for

Table 16.2 Multiple linear regression model

	Estimate	SE	95 % C.I.	p-value	VIF
(Intercept)	75.78	0.18	75.36–76.20	<0.0001	
Blade NDFD	0.39	0.18	−0.02–0.78	0.0596	2.14
Sheath NDFD	0.35	0.06	0.20–0.49	0.0006	1.42
Stem NDFD	0.16	0.04	0.06–0.26	0.0068	1.25
Blade fraction	0.08	0.02	0.02–0.13	0.0096	1.45

Average plant NDFD is estimated by NDFD of each organ and the blade fraction (which is complementary to sheath and stem fraction). The intercept is the expected NDFD of a plant with an average blade fraction and average organ NDFDs. Variation inflation is limited as shown by the VIF factors (<3)

stem. Indeed, within the set of genotypes, leafy genotypes could be differentiated (e.g. Sibasa_235 and Carillon_91) (Fig. 16.1). Further, the sheath-to-stem ratio is not significantly different across genotypes (2.3 on average), except for Barata_198 (6.2). Excluding Barata_198, the correlation between sheath and stem weight fractions is 61 %.

Strategies to Increase Plant NDFD

In this study, three possible ways of increasing plant NDFD are considered: (1) increasing the blade fraction, (2) increasing NDFD for blade, the largest plant fraction and (3) increasing NDFD for sheath and stem, the less digestible fractions. To assess the extent to which these strategies would affect total plant NDFD, a multiple linear regression was carried out (Table 16.2). Although the blade fraction is high, increasing NDFD for blade has no significant effect on plant NDFD in this experiment ($p = 0.06$), which is due to lack of variation. Increasing sheath and stem NDFD seems to have the highest impact, even though they separately represent a low fraction. Increasing sheath NDFD by 10 units increases plant NDFD with 3.5 units, increasing stem NDFD by 10 units increases plant NDFD with 1.6 units. Together, an increase of 5.1 units in plant NDFD could be achieved.

As blade is better digestible than sheath and stem, changing its weight fraction will also significantly affect plant NDFD. Increasing the blade fraction by 20 units, will increase plant NDFD with 1.6 units. However, as significant genotype by organ interactions are found, the extent to which increasing the blade fraction will affect plant NDFD is genotype dependent. The higher blade NDFD compared to sheath and stem, the larger the effect of increasing blade fractions will be. Simultaneous breeding for a higher sheath and stem NDFD will reduce the difference with blade, and thus, limit the effect of increasing the blade fraction.

The results presented here are still to be confirmed by repeating the experiment for a second year. Further, a larger population ($n = 600$) is being screened for leaf-to-stem ratio and organ-specific NDFD values to estimate the true population variance in diploid *Lolium perenne*.

Conclusion

A high diversity in NDFD plant values was noted for the first cut of perennial ryegrass at heading stage in this set of 13 genotypes. To maximise selection response, we advise selecting genotypes with a high NDFD for sheath and stem, separately or combined. Our results demonstrate that differentiating between different organs is necessary to improve total plant NDFD.

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Chapter 17
Factors Influencing Animal Preference of Tall Fescue Genotypes

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Abstract An important disadvantage of tall fescue (*Festuca arundinacea* Schreb.) is its low voluntary intake, resulting in low performances under a grazing management. Our breeding objective was to relate animal preference with leaf morphology and mechanical characteristics. Sixteen different clones were selected from a breeding programme and cloned to be planted in mini swards grazed by sheep and rabbits. In 2012 we measured leaf blade length, width, shear breaking load, shear strength, pre-grazing sward height, leaf harshness, dry matter content and rumen degraded organic matter. Leaf harshness, pre-grazing sward height and rabbit preference were the best estimators for sheep preference.

Keywords Tall fescue • Sheep preference • Rabbit preference • Shear strength

Introduction

The low voluntary intake and digestibility of tall fescue (*Festuca arundinacea* Schreb.; Fa) compared to ryegrasses (*Lolium* sp.) are limiting the use of Fa in grassland based production systems found in NW Europe. As soft leaved genotypes proved to have a higher animal preference in the past (Gillet and Jadas-Hecart 1965; Jadas-Hecart 1982; Rognli et al. 2010), the softness of the leaf blades, determined by touching the plants, is used to select genotypes with a potentially higher animal preference. Scoring for leaf softness is highly subjective and demands a lot of experience. Moreover leaf softness is a very complex trait influenced by e.g. leaf dimensions and plant architecture. Repeatable, stable and quantifiable methods producing

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results matching with animal preference may advance the breeding of genotypes with improved animal preference.

The objective of this study was to relate animal preference with leaf morphology and mechanical characteristics of leaves. Our research hypotheses were:

- Sheep can be used to distinguish between genotypes of Fa in a consistent way.
- Grazing preference is positively correlated with digestibility and negatively correlated with leaf harshness, leaf shear strength, leaf shear breaking load and dry matter content.
- Rabbits and sheep have a similar preference.

Material and Methods

A grazing trail comparing 16 tall fescue (Fa) genotypes (extracted from ecotypes and from varieties), with contrasting morphogenetic traits (broad vs. fine leaves, dark green vs. light green leaves, soft vs. hard leaves) was established in March 2011 on a sandy loam soil at the experimental farm of Ghent University in Melle, Belgium. Plant material to establish the trial was dug out from a clonal nursery in the spring of 2011. Sixteen selected clones were divided into at least 240 genets of 3–5 tillers. All plots were planted with genets of a single clone spaced 0.20 m between and within the rows, resulting in plots of 2.4 m^2 with genetically identical plants. The trial was planted as a randomized complete block design with four replicates. No measurements were performed in the year 2011, allowing the swards to establish.

In addition, 12 genets of each of the 16 clones were planted in a spaced plants trial with four blocks. In each block, three genets of each of the 16 clones were planted randomly with 0.5 m between and within the rows. The spaced plants trial was fenced to allow grazing by rabbits.

On four occasions in 2012, pre-grazing sward height, leaf blade length, leaf blade width, leaf blade breaking load, leaf blade shear strength and leaf harshness and grazing preferences by sheep and rabbits were measured. Pre-grazing sward height was measured with a falling plate meter on four randomly selected spots per plot (Bransby et al. 1977). Leaf blade length, width, shear breaking load and shear strength were measured on ten randomly selected adult leaves per clone. Leaf blade width was measured with vernier callipers at 1/3 of the distance between the ligule and the tip; leaf blade length from the ligule to the tip was measured using a ruler. Leaf shear breaking load, the maximum load needed to cause breakage at a 90° breaking angle to the length of the leaf, was measured using a Warner-Bratzler meat shear apparatus. Leaf shear strength was calculated by dividing leaf shear breaking load by leaf width. Leaf harshness was scored by two plant breeders on an ordinal scale from 1 (very soft and flexible leaf blades) to 5 (very hard and rough leave blades).

In three of the four grazing periods, apparently rumen degraded organic matter (RDOM) (Demeyer 1991) and dry matter content (DMC) of the clones were measured. One sample of 500 g of fresh material per clone was collected by clipping circa 125 g of grass from each plot (clipping height 5 cm) and by pooling the material over the replicates. This pooling was necessary because the plots were too small to harvest enough material per plot for the lab measurements without jeopardizing grazing observations. The samples were dried for 16 h at 75 °C. Samples were taken prior to the grazing period.

The trial was stocked with Flemish sheep that had been grazing for at least 2 weeks on tall fescue pastures adjacent to the trial. Stocking density was adapted to allow complete grazing of the trial in *circa* 1 week. In accordance with Jadas-Hecart (1982) and Shewmaker et al. (1997), grazing preference was scored every morning on an ordinal scale from 0 (no grazing at all), 1 (between 0 and 10 % of standing biomass eaten) to 9 (between 80 and 90 % of standing biomass eaten). As soon as the best grazed genotypes had reached a score of 9, measurements were stopped.

Parallel with the sheep grazing, four female rabbits (local race) were allowed to graze the spaced plants trial. During each grazing period all individual plants were scored twice on an ordinal scale from 0 to 9. The observation with the best discrimination between the clones was withheld.

Linear regression between sheep preference and the different variables was performed. A multiple linear regression model was fitted to all the data to find out which of variables resulted in the best prediction of sheep preference. The minimum adequate model was obtained by minimizing the Akaike's information criterion (AIC).

Results and Discussion

Significant genotypic differences in preference occurred. The most preferred and least preferred clones were consistently the same over the different grazing periods. The sheep preference observations made at the beginning of each grazing period discriminated more between the genotypes than the observations made at the end of each grazing period. At the end of the grazing periods differences in preference between the genotypes decreased. Obviously, this was a result of the decreasing forage availability of the preferred genotypes.

A significant negative correlation was found between sheep preference and leaf harshness ($Y = -0.36\times$; $p = 0.007$, $R^2 = 0.11$), pre-grazing sward height ($Y = -0.66\times$; $p < 0.001$; $R^2 = 0.42$), leaf blade length ($Y = -0.27\times$; $p = 0.047$, $R^2 = 0.05$), leaf blade shear strength ($Y = -0.30\times$; $p = 0.025$, $R^2 = 0.07$) and DMC ($Y = -0.38\times$; $p = 0.014$, $R^2 = 0.12$). Slopes were not significantly different from 0 for leaf blade width ($p = 0.24$), leaf shear breaking load ($p = 0.073$) and RDOM ($p = 0.014$). A significant positive correlation was found between sheep preference and rabbit preference ($Y = 0.69\times$; $p < 0.001$; $R^2 = 0.47$).

Over all experimental periods, rabbit preference, leaf blade harshness and pre-grazing sward height were the variables that correlated best with sheep preference. This was reflected in the multiple linear regression model. In the minimum adequate model ($R^2=0.73$) the effects of pre-grazing sward height ($p=0.016$), leaf blade harshness ($p=0.001$) and rabbit preference ($p=9.7*10^{-8}$) were significant; whereas the effects of leaf shear strength ($p=0.162$) and leaf breaking load ($p=0.145$) were not significant.

Genotypes with an erect growth habitus had a high pre-grazing sward height and were disliked. Erect growing genotypes may sting the muzzle of the grazing animal more than prostrate growing types. Our sheep preferred genotypes with rather short leaves or long leaved genotypes with flexible (non-erect) leaves that produced swards with small heights.

Rabbit and sheep preference correlated well. So the small rabbits are a good proxy for ruminants which may offer advantages particularly in the early stages of a breeding programme when replicates of plant material are not obvious. Digestibility measurements and dry matter measurements were no more than indicative in this trial, as replicates were absent for these variables. There seemed to be no direct effect of both variables on the preference in our study. The large morphological differences in our trial might have masked the more subtle effect of digestibility. The effect of digestibility on preference of grazed tall fescue should be studied in more detail, with genotypes with a similar morphology. Given the positive relationship found between water soluble carbohydrate content (WSC) and preference in tall fescue varieties found by Mayland et al. (2000), it would be interesting to include the concentration of WSC in further studies.

The results suggested that the preferred clones were soft, fine leaved and low growing, but that not all clones with these properties were preferred. With the knowledge we have now, animal preference trials remain the best method to make progress in animal preference for tall fescue.

Conclusion

Both sheep as rabbits distinguished between the clones in a significant and consistent way. Leaf harshness, pre-grazing sward height and rabbit preference were the best estimators for sheep preference.

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Chapter 18
Fair Evaluation of Yield Potential in Forage Species Evaluation

Ulf Feuerstein and Anita Swieter

Abstract The evaluation of yield and quality in forage species is more laborious and time consuming and yet less precise than in cereals. Some reasons are well known, have been discussed frequently (multi cut and multi year trials) and will be difficult to overcome. In the last years another reason for the low repeatability of forage trials came into focus – the wide range of heading date.

In 2006 five breeding companies (DLF Trifolium, Barenbrug, Deutsche Saatveredelung, NorddeutschePflanzenzucht and Saatzucht Steinach) started a joint project with the Federal Plant Variety Office, Germany (BSA). Its aim was to get more information about the influence of heading date on the yield and quality results in the existing testing systems. The project was carried out between 2008 and 2010 at five locations in Germany and The Netherlands. Yield data collection and processing was done like in a normal yield trial (VCU trial) by the BSA. Additionally, samples for quality analysis were taken and analyzed in an ongoing second project supported by the German Agency for Renewable Resources (FNR).

This paper examines the difficulties of a fair evaluation of yield and quality potential, identifies the problems and points to possible solutions in order to overcome the most dangerous pitfalls.

Keywords Variety evaluation • Heading date • Yield and quality • Ryegrass • *Lolium*

Introduction

The harvest date of forage plants is oriented towards a certain developmental stage. According to this the varieties to be compared should be harvested at the same stage of development. Although this is very important for yield testing and even more

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important for quality assessment it is often disregarded in the current practice. For practical reasons the tester harvests all entries in most forage species at one particular date. Only entries in those species which exhibit a very wide range in heading date are divided into two or three harvesting groups.

In general it can be assumed that grass increases its biomass after start of growing in spring until a certain growing stage after flowering. Parallel to this increasing biomass the quality is reduced. Varieties with an earlier heading date pass through the different stages of development earlier than varieties with a later heading date. As a result early varieties have over a certain time at the same date, during the first growth, a higher biomass yield and lower quality as late varieties.

To overcome this problem many variety tests of perennial ryegrass are divided in three harvest groups: early, intermediate and late. Each of these groups cover a range in heading date between 10 and 18 days. Due to these harvest groups the problem of the different heading dates in a species is reduced but not eliminated.

Therefore already during the 1970th in England and Wales a model was installed to compensate the different heading dates (Walters 1984). This model based on two assumptions:

1. Dry matter yield increases about 133 kg ha^{-1} day^{-1} and
2. Digestibility (D-value) falls per 0.33 units day^{-1}.

This model allows for a given harvest date, where yield and digestibility is assessed, adjustments for every variety. In the case of England and Wales each variety was adjusted to the value of 67 % digestibility (67 D-value). However this model is also based on the assumption that all varieties respond in the same linear way. Some breeders doubt this and this model was not used any more after 2007.

To build up models which are individual it is necessary to get more information for each variety. The simplest linear adjustment requires extra harvests of a minimum of three quality and yield points (1 week before to 1 week after the ideal cutting date). This extra work load in harvesting yield trials is impracticable. Hence the question: How relevant are these differences in yield and quality between actual cutting date and ideal cutting date (e.g. at 67 D-value)?

To get an answer to this question breeders had many discussions with plant variety rights offices. 2006 five breeding companies (DLF Trifolium, Barenbrug, Deutsche Saatveredelung, NorddeutschePflanzenzucht and Saatzucht Steinach) started a joint project with the Federal Plant Variety Office, Germany (BSA). The aim was to get more information about the influence of heading date on the yield and quality results in the existing testing systems.

Material and Methods

Plant Material

A field trial with 14 diploid and 5 tetraploid varieties of perennial ryegrass (*Lolium perenne* L.) was carried out. The following varieties were selected (heading date in days after 1st of April in brackets), including the checks of the official VCU-test in

Germany: Lipresso (check, 41), Liconda (45), Abersilo (46), Trintella (48), Aubisque (check, 50), Respect (check, 51), Fennema (52), Chicago (53), Barata (53), Bargala (53), Eurocity (55), Niata (56), Toledo (57), AberAvon (59), Licampo (60), Stefani (61), Forza (62), Denver (62), Sponsor (64).

The intermediate harvest group ranges from 48 days up to 58 days. Varieties which are up to 3 days earlier or later than this range are accepted for the VCU-test, varieties which are even more early or late are excluded if they were appointed to the intermediate harvest group.

Experiment Set Up

Objective of this project was to calculate the 'real' yield and the 'real' quality from a certain phenological stage in relation to the cutting date.

The experiment was designed as block design with four replications at five locations (Asendorf, Malchow, Steinach, Lelystad and Moerstraten).

The experiment was carried out in two different managements.

1. Standard management: like in official trials – all entries of one harvest group are cut at the same date.
2. Individual management: each entry is cut at its individual heading date

The experiments were carried out like the official variety tests (VCU-trials) in Germany (Bundessortenamt 2008). The harvest was done with forage harvesters.

Statistical Analysis

The data were collected by the Federal Plant Variety Office, Germany (BSA). The calculation was done by the statistical program of the BSA parallel to the calculation of the regular VCU-trials.

Results and Discussion

The yield of early varieties in a harvest group was higher than in the later ones if the cut was done at one harvest date (standard management). For the individual management – each variety was cut at the same phenological stage – the contra was true. In both cases there was a high relationship between yield and heading date. For the standard management a decrease of 0.91 % dry matter yield in the first cut was found for each day later in heading. For the individual management an increase of 1.24 % dry matter yield in the first cut was found for each day later in heading (Fig. 18.1).

For the total dry matter yield this relationship was weaker but still remarkable. For the standard management there was a decrease of 0.32 % day^{-1} and for the individual cut an increase of 0.33 % day^{-1}.

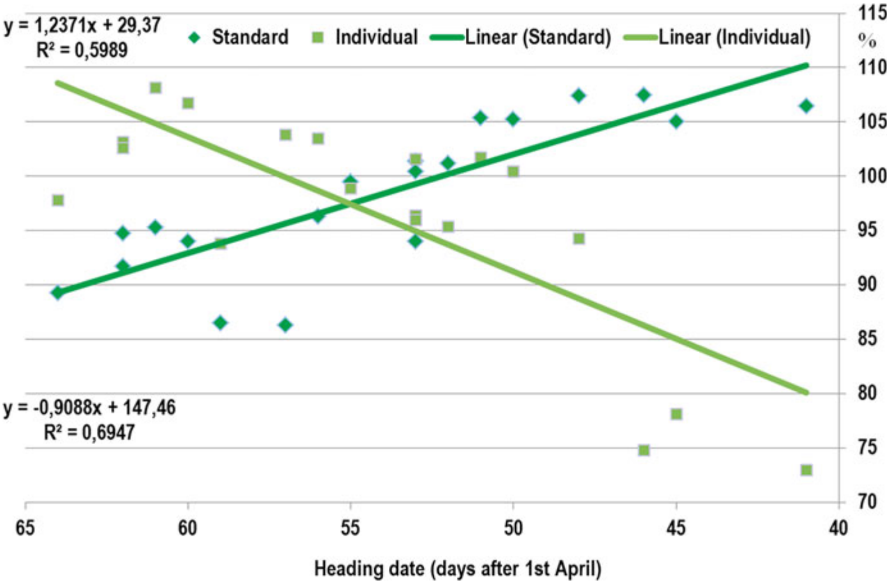

Fig. 18.1 Management comparison of dry matter yield (1st cut – relative to trial mean)

There is a difference of 3.2 % in dry matter yield between early and late varieties of the intermediate harvest group which is exclusively caused by the difference in heading date.

Therefore cutting management has significant influence on the dry matter yield of varieties. This influence is biggest in the first cut, but still remarkable over all cuts. Different varieties react differently to deviating cutting managements.

Conclusions

Today's variety-tests are not fair because of dissimilar heading dates and dissimilar quality characteristics.

The fairness of variety-tests should be improved by means of suitable adjustments.

The D-value adjustment concept is a fine first step but does not take into account residual differences in quality decline or yield increase.

A group of forage breeders has started to collect data to create a model for a better comparability and fairness of variety evaluations in terms of yield and quality.

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Chapter 19
Patterns of Morphophysiological Diversity and Their Implications for Selection of Grazing-Tolerant Lucerne

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Abstract Grazing-tolerant lucerne (*Medicago sativa* L.) has gained popularity for exploitation in pure stands or as a component of legume-grass mixtures in grazing systems of temperate regions. A challenge for breeders targeting mild-winter regions, such as Mediterranean Europe, is the selection of grazing-tolerant germplasm with low cold-season dormancy, because grazing-tolerant materials tend to possess marked autumn dormancy. It is unclear whether the relationship of persistence under grazing with autumn dormancy and low-growing habit is genetically based. In this study, four biparental populations of lucerne were evaluated for a set of morphophysiological traits and tolerance to heavily-stocked continuous sheep grazing. The populations originated from crosses between prostrate, dormant, grazing-tolerant germplasm and erect, non-dormant, little tolerant germplasm. The work aimed at exploring the patterns of morphophysiological variation in the populations, and assessing the relationships of plant morphophysiology with grazing tolerance. Great variation was found for all traits both among populations (except for grazing tolerance) and among F_1 progenies within populations. The correlations between survival after grazing and morphophysiological traits indicated better tolerance of genotypes with more prostrate habit and larger crown. The undesired correlation between grazing tolerance and cold-season dormancy did not occur consistently in all populations. The results can help understand to which extent different useful traits can be combined into a grazing-tolerant variety targeted to mild-winter environments.

Keywords Cold-season dormancy • Grazing tolerance • Growth habit • *Medicago sativa*

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Introduction

Although mainly exploited for hay-making, lucerne is also used as a valuable grazing crop in different countries. Better comprehension has been achieved in the last decades of the morphological and physiological mechanisms underlying the tolerance to grazing in lucerne and several traits have been claimed to contribute to tolerance, including decumbent growth habit, large and deep-set crown and prolific underground budding (Smith et al. 2000). Successful attempts were made to combine the advantages of a suitable morphology with those of the selection under continuous and heavily-stocked grazing (Pecetti et al. 2008).

An additional challenge for breeders targeting mild-winter regions, such as southern Europe, is the selection of grazing-tolerant germplasm with good growth during autumn and winter, to better exploit the potential growing season. A close phenotypic relationship among grazing tolerance, marked cold-season dormancy and low stature emerged in some studies (Pecetti et al. 2008), which may have been determined by the large use of germplasm introgressed with the autumn-dormant *M. sativa* subsp. *falcata*. Various studies also suggested the presence of a trade-off between persistence under grazing and forage yield under mowing (Smith et al. 2000; Pecetti et al. 2008), indicating that these traits are maximized by contrasting morphophysiological types (the prostrate type tending to confer high grazing tolerance but low forage yielding ability). It is unclear whether the relationships between persistence under grazing, autumn dormancy and low-growing habit are genetically based. This study was undertaken with the ultimate objectives of assessing the genetic correlations between grazing tolerance, cold-season dormancy and plant morphology, and acquiring phenotyping data to associate with future genotyping data for the implementation of marker-assisted selection simultaneously for tolerance to grazing and limited dormancy. Aims of the current phase of the work were: (i) exploring the patterns of morphophysiological diversity in each of four biparental populations originated by crosses between prostrate, dormant, grazing-tolerant germplasm and erect, non-dormant, little tolerant germplasm; and (ii) assessing the relationships between grazing tolerance, dormancy, forage yield and plant morphology.

Materials and Methods

Four biparental mapping populations were obtained from all combinations of pairwise crosses of two genotypes singled out from the experimental cultivar Camporegio with two genotypes singled out from the old cultivar Mamuntanas. Camporegio is a prostrate, very autumn-dormant and grazing-tolerant cultivar selected in northern Italy from exotic germplasm (Pecetti et al. 2008), while Mamuntanas is an erect, non-dormant and little grazing-tolerant old cultivar from Sardinia provided with outstanding drought tolerance (Annicchiarico et al. 2013). Each population was represented by over 100 F_1 progenies clonally propagated by rooting stem cuttings. They were replicated three times in a randomized complete block design, with four clonal plants per plot. Plants were transplanted in autumn 2010. All the progenies were evaluated for average spring dry-matter (DM) per plant in one harvest of 2011 and of 2012. Plant crown diameter (on early vegetative regrowth), growth habit (on a 1–6 scale based on shoot erectness), and middle leaflet area were also evaluated in spring 2012. Mean shoot length in late autumn and at the end of winter were recorded, using their mean value per plot as an index of cold-season vegetative activity. From the beginning of June to mid-September 2012 (140 days) the trial was managed under continuous sheep grazing and high-stocking rate (about 50 ewes/ha equivalent). Grazing tolerance of each progeny was estimated in early October as percentage of plant survival per plot.

Data were subjected to analysis of variance (ANOVA) assessing the variation both among populations and among progenies within populations. Frequency distributions of progenies within each population were determined for all traits. Finally, phenotypic correlations were computed, over all progenies and within each population, between grazing tolerance and the morphophysiological characters.

Results and Discussion

Grazing tolerance was very high (over 90 % mean survival) and not different among populations, although the two Mamuntanas parents showed different response to grazing (Fig. 19.1). For all other traits, however, the variation among populations was remarkable according to ANOVA ($P < 0.001$). Progenies within populations differed for all traits ($P < 0.01$). In each population, the values of the two parents for morphophysiological traits tended towards the extremes of the range of the respective progenies (see Fig. 19.1 for the cold-season vegetative activity), confirming their usefulness for establishing highly-variable mapping populations. However, for plant vigour (as average DM per plant summed over the two spring harvests) there was high frequency of progenies exceeding the values of their respective parents (Fig. 19.1), suggesting the expression of some heterosis when crossing such distant parents.

Phenotypic correlations between survival after grazing and morphophysiological traits (data not reported) confirmed the previously observed relationship between persistency, prostrate habit and crown spreading (Pecetti et al. 2006). However, the populations differed for the extent of these correlations. For instance, grazing tolerance was not related to crown diameter in Population C, or to growth habit in Populations A and B. The undesirable negative correlation between grazing tolerance and cold-season vegetative activity was not univocally present in all populations. Plant vigour and grazing tolerance were never correlated, suggesting independent mechanisms underlying the two characters. However, the current relationships of grazing tolerance with other traits await confirmation from ongoing evaluation work aimed to assess the grazing tolerance of the material over a

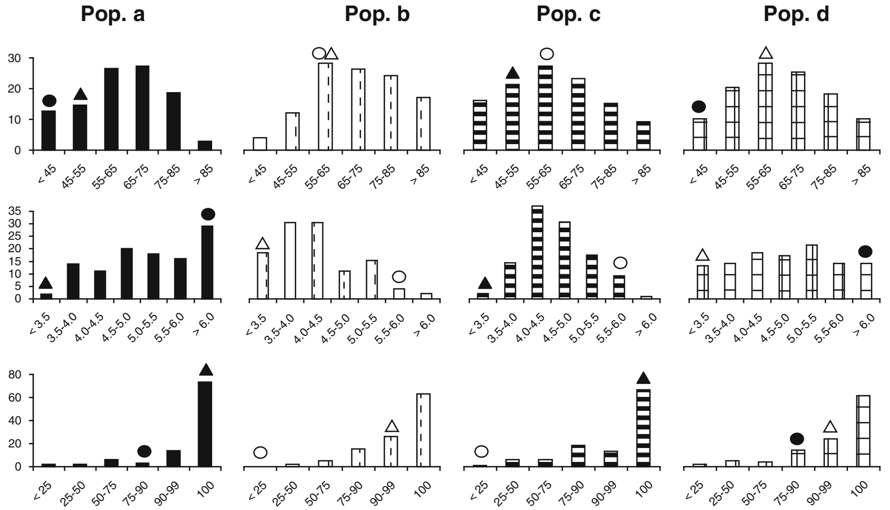

Fig. 19.1 Progeny frequency (%) in four biparental populations (A to D) for different classes of (*top* to *bottom* graphs): (**a**) plant vigour (g DM plant^{-1} over two spring harvests); (**b**) cold-season vegetative activity (shoot growth, cm); and (**c**) survival to heavily-stocked continuous sheep grazing (%). In each population and for each character, the class of each parent is also shown (Mamuntanas parents: *circles*; Camporegio parents: *triangles*)

longer grazing period. Material that underwent greater recombination (e.g. F_2 generation) would be particularly useful in this respect. Based on the present results, combining useful agronomic traits into a grazing-tolerant variety with flexibility of utilization and adaptation should not be precluded, when relying on a wide and diverse genetic base as the present one.

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Chapter 20
Combining Abilities of Different Alfalfa (*Medicago Sativa* L.) Varieties

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Abstract The objective of this study was to test the general combining ability (GCA) of different alfalfa varieties, with the main objective being to improve local varieties through the selection of good parental gene donors. Foreign varieties that in previous studies had been found to adapt well to agro-ecological conditions in Serbia were selected as possible gene donors. The female parental lines from domestic cvs K 22 and K 28 were crossed with the males of seven different alfalfa varieties. Progenies from the crosses were sown in nursery and observed during the second and third year of growth in the experimental field at the Institute for Forage Crops, Krusevac. Results showed significant differences between progenies. Variation among progenies for forage yield and forage yield components (plant height and number of stems per plant) were primarily attributed to general combining effects. The variety Magna had the best GCA with all domestic varieties for forage yield and was an excellent donor for improving this trait. Cultivars Integrity and Magna had the best GCA for increasing the number of stems per plant. These varieties demonstrated a favourable genetic potential that could contribute to the improvement of alfalfa yield in our breeding program. Varieties Vali and Pointer showed negative results for all traits and their poor GCA indicated that they are not suitable for improving domestic selection material. The best progeny will be used as parental lines in a new synthetic variety.

Keywords Alfalfa • Combining ability • Progeny • Hybridization

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Introduction

Results in plant breeding depend primarily on the extent and type of variability in the breeding material and on choosing the appropriate selection methods (Veronesi et al. 2010). Although populations of alfalfa have a broad genetic base for the most important traits, increasing the existing variability by introducing new material is always desirable. Increasing variability in selection material can be achieved by the introduction of genetically divergent material that may be a new source of diversity. It may be possible to develop progenies that are superior in yield by crossing high yielding alfalfa cultivars of different geographic origin (Tucak et al. 2012). They could, in any case, be a new source of variability, especially for some traits that are missing in domestic cultivars (Radović et al. 2009). Before introduction in the breeding process, it is important to test foreign selection material for productivity, adaptation to the new environment, persistence and other important traits. The most economically important characteristics of alfalfa, such as green forage and dry matter yield and quality, persistency, plant height, number of shoots and regrowth, are all quantitative traits (Veronesi et al. 2010). Alfalfa traits whose inheritance is complex in nature (i.e., all the above listed traits) require breeding procedures that include progeny tests (Woodfield and Brummer 2001).

Also, knowing some genetic parameters, such as combining ability, could be useful. The improvement made by a preliminary selection of lines for their GCA comes from the additive variance in the base population. Any further improvement, making use of the non-additive genetic variance, must come from selection for the specific combining ability (SCA) (Falconer and Mackay 1996).

The contributions of both GCA and SCA are important in explaining variation in dry matter yield between alfalfa progenies (Tucak et al. 2012). GCA contributed significantly to genetic variation in forage yield, while SCA (non-additive gene action) was not significant. Also, it is difficult to detect the SCA effect in alfalfa, which is an autotetraploid, due to the existence of both trigenic and tertagenic gene action (Bhandari et al. 2007).

The objective of this research was to assess the GCA of different alfalfa varieties by studying the F1 progeny and to determine their genetic potential for improving local alfalfa varieties.

Material and Methods

Seven varieties from USA (Pointer, Tru Test, Integrity, Magna, G+13R), Croatia (OS 95) and Slovak Republic (Vali), which in a previous study had been shown to adapt well to agro-ecological conditions in Serbia, were chosen as possible gene donors to improve domestic varieties Krusevacka 22 (K 22) and Krusevacka 28 (K 28). The study was conducted during 2009–2012 in a field experiment at the Institute for Forage Crops, Krusevac. Parental female components selected from domestic varieties were crossed with male parent components from seven different alfalfa varieties (14 crosses in total without reciprocals). Plants were hand-crossed, without flower emasculation. The seed from each crossing (F1 progeny) were sown in March 2010 in pots and grown in a greenhouse during the following month.

Table 20.1 Average values of investigated traits and estimates for general combining ability of F1 progenies over 2 years

Cultivar	Pointer	Tru test	Integrity	G+13R	Magna	OS 95	Vali	GCA
Dry matter yield (g plant^{-1})								
K 22	106.25	118.75	173.25	171.75	171.50	162.50	130.50	−1.60
K 28	150.00	197.00	139.50	112.25	170.25	172.25	115.75	1.60
GCA	−21.28	8.48	6.98	−7.40	21.48**	17.98**	−26.28	
Number of stems per plant								
K 22	32.71	32.96	42.80	52.56	42.33	43.07	30.59	1.86
K 28	37.06	45.48	42.00	26.75	41.28	38.00	29.81	−0.52
GCA	−7.52	1.50	4.68*	1.94	4.09**	2.82*	−7.52	
Plant height (cm)								
K 22	95.86	101.88	113.60	102.25	107.67	97.36	97.59	−2.23
K 28	100.22	109.70	98.50	101.25	103.94	97.43	112.46	−1.19
GCA	1.91	2.67	−2.07	−1.37	2.69	−3.73	1.91	

*, ** significant at $P<0.05$ and $P<0.01$ level, respectively

Well-developed young plants were transplanted in the field. The field experiment was designed as a randomized complete block with three replications. Twenty spaced plants were analyzed per replicate (60 per variety). Four cuts per year were made in the second (2011) and third year (2012) (eight cuts in total). The dry matter yield (g plant^{-1}), plant height and shoot number per plant were recorded and presented as average values for all eight cuts. Analyses of variance (ANOVA) for general combining ability (GCA) for the traits being investigated were carried out according to Griffing's (1956) Method 4 (F1 progenies).

Results and Discussions

The results revealed significant differences between F1 progenies (Table 20.1). Average dry matter yields ranged from 115.7 g to 197.0 g plant^{-1}. Estimates of GCA ranging from 21.48 for cv. Magna to −26.28 for cv. Vali were obtained. Significantly positive GCA was recorded in cvs Magna and OS 95, indicating that these two varieties are good combiners and possess the potential to improve dry matter yield in domestic varieties.

Negative estimates of GCA for this trait were observed for cvs Pointer and Vali. Variety Magna had displayed a high yield potential in a previous study and, according to cluster analyses, a large genetic distance from domestic varieties K 22 and K 28 (Radović et al. 2009). Based on that data, cv. Magna was expected to be a source of genes and good traits for improvement of domestic varieties. Varieties Pointer and Tru Test showed lower yield in the second year, suggesting that they do not adapt well to changing climatic conditions, a trait which is very important for stable forage yield during the total growth cycle of alfalfa. It is one reason why these varieties show a low GCA compared with other varieties.

The highest number of stems per plant was recorded in progenies of crosses with cvs Magna, Integrity and OS 95. Those varieties showed significant and positive

GCA for this trait, while cvs Pointer and Vali showed negative GCA. Plant height ranged from 95.86 to 113.6 cm, but there were no significant differences between progenies for average values and estimated GCA.

It is important to note that cv Tru Test showed increased yield and number of stems when crossed with K 28, but crossing with K 22 did not produce good results. Also, the progeny of the cross between K 22 and G+13R showed excellent results for dry matter yield and especially for number of stems per plant.

Several studies reported differences between alfalfa varieties for GCA and the significant effect of GCA on genetic variation of forage yield (Milić et al. 2010; Tucak et al. 2012). This confirmed that alfalfa yield can be improved by exploiting additive and non-additive gene effects. The predominance of GCA in determining alfalfa forage yield has also been documented previously (Bhandari et al. 2007; Milić et al. 2011). In their investigation, Tucak et al. (2012) concluded that the progeny of high yielding parents from the same geographical origin had lower yield than progenies that were obtained from crosses between genetically divergent varieties.

According to estimates for GCA, the best gene pool for improving dry matter yield of domestic cultivars were cvs Magna and OS 95. For increasing the number of stems per plant, the best results were recorded for cvs Magna and Integrity. Dry matter yield improvement can be achieved by the accumulation of favourable alleles and exploiting heterosis effects. The parents of the best progenies will be used as parental lines in a new synthetic variety.

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Chapter 21
Towards Productive Summer Dormant Cocksfoot for Mediterranean Climates

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Abstract There are different strategies for producing fodder crops without irrigation under Mediterranean climate conditions. One strategy is to avoid the drought period by using annual plants such as cereals or Italian ryegrass. Another strategy is to use perennial forage species highly resistance to drought such as *Dactylis glomerata* or *Festuca arundinaceae*. The advantage of the second strategy is a rapid growth when favourable conditions come back after summer and good ground cover, which limits erosion.

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Summer dormancy, defined as an absence of growth in summer despite irrigation, is a very effective adaptation to drought which has been observed in cocksfoot. Nevertheless, summer dormancy in cocksfoot is associated with low vegetative productivity. The objective of this study is to analyse the genetic and eco-physiological determinisms of summer dormancy and vegetative growth in cocksfoot. For this purpose, the progeny between a summer dormant genotype from the variety Kasbah and a summer active genotype from the variety Medly is studied. The first results show that despite an expected negative correlation ($r=-0.40$) between summer dormancy and vegetative plant growth, genotypes exist with the same summer dormancy level as the genotype of Kasbah and a higher vegetative productivity than the genotype of Medly. These results are promising for the development of new cocksfoot varieties combining both summer dormancy and high vegetative productivity. The genomic regions implied in the variation of these traits are under study and should be useful for breeding.

Keywords Drought resistance • Cocksfoot *Dactylis* • Summer dormancy • Genetic determinism

Introduction

Climate change increases the risk of summer drought and the area of Mediterranean climate conditions (IPCC 2007). Under such conditions, one strategy for producing fodder crops without irrigation is to use perennial grasses that are highly resistant to drought such as *Dactylis glomerata* or *Festuca arundinaceae* (Volaire and Norton 2006). Summer dormancy, defined as “an endogenously controlled and coupled series of processes comprising the cessation of leaf growth and senescence of herbage expressed under nonlimiting water conditions in summer” (Norton et al. 2008), has been correlated with superior survival after severe and repeated summer drought in a large range of perennial grasses (Volaire and Norton 2006). However, complete summer dormancy seems to be associated with low productivity (Norton et al. 2008; Shaimi et al. 2009). The objective of this study was to evaluate the possibilities of breaking this correlation between summer dormancy and productivity in cocksfoot.

Material and Methods

A cross between a genotype of the summer dormant cultivar Kasbah and the summer active cultivar Medly was performed under pollen-proof cages at INRA, Rabat, Morocco. The hybrid nature of each sibling was checked with AFLP markers. Two hundred and twenty nine hybrids were cloned and three cloned plants per hybrid were planted in a completely randomized design in a nursery at the Guich experimental

Table 21.1 Basic statistics for all traits and broad sense heritability

Traits	Kasbah	Medly	Plant number	Mediane	Min.	Max.	SD	h^2
Summer biomass (g plant^{-1})	1	5	94	6	1	28	6	0.36
Spring biomass (g plant^{-1})	57	127	94	94	13	202	44	0.48
Summer dormancy	9.7	9.4	94	9.3	8.0	9.8	0.4	0.26
Senescence (%)	87	38	94	46	8	93	20	0.49
Heading date (days)	83	76	94	75	62	92	6	0.62

station of INRA, Rabat in February 2011. The plants were fertilized at the beginning of the experiment (N, P, K) and irrigated in summer. In 2012, hand cuts at 10 cm height were performed on the 31st of January, the 2nd of April, the 15th of May and the 11th of September. 40 U of nitrogen was supplied after the first three cuts.

In 2012, on each plant and in all cuts, dry matter yield (g plant^{-1}) and heading date (when at least three spikes are visible, presented as number of days from the 1st of January) were measured. The percentage of senescence of the plants was assessed the 4th of July (0 = all tissues green, 100 = no visible green tissues). The first harvest was not taken into account and was considered as a starting point. Spring biomass was calculated by adding the biomass harvested after the second and the third cut. Summer biomass was measured by the biomass harvested after the cut in September. Summer dormancy was calculated based on the summer dormancy index of Norton et al. (2008) as S/Sp = (100-[(summer yield/spring yield) × 100])/10, which ranges between 1 = summer active and 10 = fully summer dormant. Only genotypes with no missing data (three replicates) were used. The analyses were performed with the software Statistica StatSoft. Broad sense heritability was calculated as $h^2 = \sigma^2$ geno/(σ^2geno + σ^2error/3).

Results and Discussion

A significant ($p < 0.05$) genotype effect was detected for all traits. The heritability was low to moderate (Table 21.1). For each trait, the variability observed in the progeny was higher than the range of the parents (Table 21.1). The correlations between traits are presented in Table 21.2. It shows: (i) a negative correlation between spring biomass and heading date, as was observed in the parents (Medly was more productive and flowered earlier than Kasbah), (ii) a positive correlation between summer and spring biomass, which was linked to the size of the plant, but (iii) no significant correlation between spring biomass and summer dormancy, taking into account the size of the plant in the calculation, (iv) a negative correlation between the percent of senescence and the spring biomass, although this correlation was not tight and highly senescent and productive genotypes were observed, (v) a positive correlation between summer dormancy and senescence, which show the same physiological process. It was possible to find genotypes in the progeny with

Table 21.2 Correlations between traits

Traits	Spring biomass	Summer dormancy	Senescence	Heading date
Summer biomass	**0.60**	**–0.77**	**–0.52**	0.03
Spring biomass		–0.12	**–0.42**	**–0.32**
Summer dormancy			**0.50**	–0.20
Senescence				0.10
Heading date				

Significant correlations $p < 0.05$ are in bold

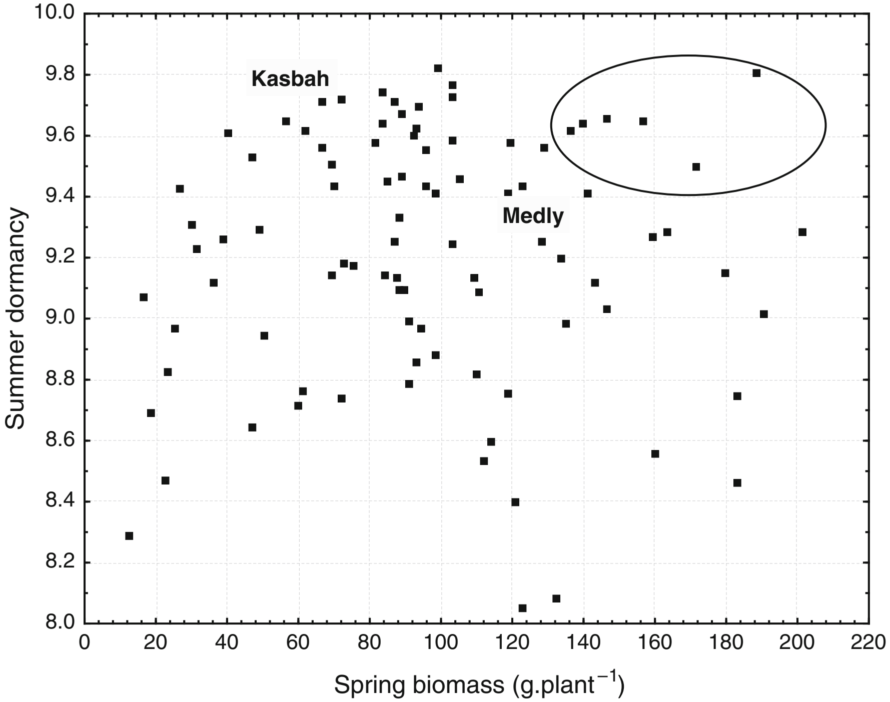

Fig. 21.1 Relationships between spring biomass and summer dormancy. The ellipse identifies interesting genotypes with high summer dormancy and high spring growth

the same level of summer dormancy as Kasbah and a higher spring biomass than Medly (Fig. 21.1).

These results showed that it should be possible to break the negative correlation between summer dormancy and spring yield observed in natural populations, and eventually to create varieties with a high level of summer dormancy and a good level of vegetative production in spring. Currently, genetic determinism of summer dormancy and spring production is being explored in the progenies through QTL analysis, in order to verify the importance of the trade-off between summer dormancy and drought survival.

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Chapter 22
Potential of Some Neglected European Annual Legume Crops for Forage Production

Aleksandar Mikić and Vojislav Mihailović

Abstract There is a general trend of re-introducing currently neglected and underutilised crops into diverse farming systems and providing them with novel roles in contemporary agriculture, which is faced with numerous challenges. One such novel role could be the use of traditional grain legume crops for forage production. One of the essential traits needed for improving the potential of quality forage production in faba bean is a decreased proportion of lignin in forage dry matter, providing more slender stems with a higher nutritional value for feeding ruminants. Hairy vetch demonstrates a wide variability of agronomic traits and may be regarded as having great potential for utilisation in breeding and developing new cultivars for forage production and green manure. The future breeding efforts will emphasize chemical composition, especially crude and digestible protein and fibre content. Despite its relatively short stem length, bitter vetch may serve as one of the best ideotypes in breeding annual forage legumes because of its large number of photosynthetically active leaves and its extremely good utilisation of growing space. The indeterminate stem growth is one of the main obstacles in developing the grass pea cultivars with both high forage yield and reliable seed yield. This trait is equally important in hairy vetch, and needs to be efficiently solved for both forage and grain production.

Keywords Annual legumes • Breeding • Forage production • Neglected crops

Important and Neglected Annual Legume Crops

Legumes (*Fabaceae* Lindl., syn. *Leguminosae* Juss.) are one of the richest and the most important plant families in the world. They comprise annual and perennial, herbaceous, shrubs and trees, and temperate and tropical species (Lewis 2005). Many legume species are of significant economic importance. They are used both for human consumption as immature pods, immature grains or mature grains, and in

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animal feeding in the form of fresh forage, forage dry matter, forage meal, immature grains, mature grains and straw (Mikić et al. 2006). Some legumes are also suitable for grazing and browsing, while some are extensively used as green manure and thus are extraordinarily important in organic farming and sustainable agriculture (Ćupina et al. 2011).

Perennial legume species, such as lucerne (*Medicago sativa* L.), red (*Trifolium pratense* L.) and white (*Trifolium repens* L.) clovers, birdsfoot trefoil (*Lotus corniculatus* L.) and sainfoin (*Onobrychis viciifolia* Scop.) are considered some of the most important crops in feeding ruminants, with advanced and extensive breeding programmes worldwide (Marshall et al. 2008; Annicchiarico and Scotti 2009; Regos et al. 2009; Julier and Meusnier 2010; Riday 2011). On the other hand, annual legume crops are often used as grain in feeding monogastrics, especially chickens and pigs, with pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.), chickpea (*Cicer arietinum* L.), white lupin (*Lupinus albus* L.) and soya bean (*Glycine max* (L.) Merr.) the most significant (Leterme and Théwis 2004; Gabriel et al. 2008).

Despite the fact that pea, lentil, chickpea, bitter vetch (*Vicia ervilia* (L.) Willd.) and grass pea (*Lathyrus sativus* L.) are among the first domesticated plant species of the Old World (Mikić 2012), many of these crops have become heavily neglected and underutilised during the past two centuries. A number of the annual legumes once used in both human diets and animal feeding have become gradually reduced to a few. A good example is faba bean which was replaced by *Phaseolus* beans in eighteenth and nineteenth centuries and is now almost completely forgotten throughout the Balkans (Mikić et al. 2011a). There is a general trend of re-introducing currently neglected and underutilised crops into diverse farming systems and providing them with novel roles in contemporary agriculture, which is faced with numerous challenges (Mihailović et al. 2010). One such novel role for traditional grain legume crops could be for forage production.

Forage Yields and Breeding Targets in Neglected Annual Legumes

As shown in Table 22.1, a preliminary evaluation of forage yields in faba bean spring cultivars of diverse geographic origin showed that this species may produce average yields equal or better than those of forage pea and common vetch (Mihailović et al. 2010). One of the essential traits needed for improving the potential of quality forage production in faba bean is likely to be a decreased proportion of lignin in forage dry matter, providing more slender stems of higher nutritional value for feeding ruminants.

Narbonne vetch (*Vicia narbonensis* L.) is a traditional grain legume crop in South Europe, North Africa and the Near East. An evaluation of its potential for forage production carried out in Novi Sad revealed great variability in the tested populations, and promising genotypes were selected for further work, pre-breeding

Table 22.1 Potential of some neglected annual legumes for fresh forage yield, forage dry matter yield and forage crude protein yield in a long-term trial in Novi Sad (Mihailović et al. 2008, 2009, 2010; Mikić et al. 2009)

Species	Fresh forage yield (t ha^{-1})	Forage dry matter yield (t ha^{-1})	Forage crude protein yield (kg ha^{-1})
Faba bean	46.7	12.7	1,790
Narbonne vetch	39.1	6.2	1,252
Hungarian vetch	48.7	10.7	2,182
Hairy vetch	50.9	11.7	2,351
Bitter vetch	30.7	6.9	1,554
Grass pea	45.3	13.9	3,088
$LSD_{0.05}$	8.6	2.1	402
$LSD_{0.01}$	11.5	2.8	587

activities and possibly developing first forage Narbonne vetch cultivars (Mikić et al. 2009). Such genotypes should be characterised by a large number of internodes and photosynthetically active leaves.

Hungarian vetch (*Vicia pannonica* Crantz) is grown mostly in the countries of the ex-Soviet Union, the Balkan Peninsula, Central Europe and the Eastern Mediterranean. The evaluated populations of Hungarian vetch in Novi Sad show a wide variability of agronomic characteristics related to forage production. The correlation between number of plants before cutting and forage yield requires detailed study to optimise crop density. The populations with high and stable forage yields may be used for developing new cultivars of Hungarian vetch with enhanced forage dry matter chemical composition and seed yield (Mihailović et al. 2009).

Hairy vetch (*Vicia villosa* Roth) is a traditional annual forage legume in both Serbia and the Balkans, along with common (*Vicia sativa* L.) and Hungarian vetches. It is grown as an autumn-sown crop and considered one of the most resistant cultivated species to low temperatures. Hairy vetch demonstrates a wide variability of agronomic traits and may be regarded as having great potential for utilisation in breeding and in developing new cultivars for forage production and green manure. The future breeding efforts will emphasize chemical composition, especially crude and digestible protein and fibre content (Mihailović et al. 2008).

The area of bitter vetch in both wild and agricultural flora in the Balkans is limited mainly to central, southern and eastern regions, such as southeast Serbia, Bulgaria and Greece. It is almost solely an autumn-sown crop, with lower fresh and forage dry matter yields in comparison to other cultivated vetches, but with prominent winter hardiness, earliness and narrow variability of forage yield between years (Mihailović et al. 2006). Despite its relatively short stem length, bitter vetch may serve as one of the best ideotypes in breeding annual forage legumes, since its large number of photosynthetically active leaves and extremely good utilisation of growing space.

A preliminary trial with grass pea accessions, aimed at the evaluation of forage yield components, demonstrated their ability to produce forage yields higher than in other *Lathyrus* species (Mikić et al. 2011b). This potential to produce high yields of fresh forage and hay should be completed by examining all the aspects of the forage chemical composition and cold tolerance. The indeterminate stem growth is one of the main obstacles in developing the annual legume cultivars with both high forage yield and reliable seed yield. This trait is the most prominent in grass pea and hairy vetch and generally requires an efficient and long-term solution in any contemporary breeding programme.

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Chapter 23
New Insights into Alfalfa Forage Quality Through the Research Project Qual&Medica

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Abstract The research project 'High quality alfalfa for the dairy chain' (Qual&Medica), led by Società Produttori Sementi and funded by Fondazione Cassa di Risparmio of Bologna and Regione Emilia-Romagna, focuses on management and genetic tools to improve the feeding quality of alfalfa forage for the dairy chain and to increase the crop profitability in hay-based farming systems. Three workpackages of the project aim to define: (i) breeding strategies to increase the crop feeding value; (ii) mowing strategies to improve hay quality; and (iii) rapid analytical tools for quality classification of harvested forages. The purpose of the fourth workpackage is to communicate the project results and provide technical support to growers that target high quality forages. Qual&Medica pays specific attention to the evaluation of genetic and environmental determinants of alfalfa forage quality parameters, such as protein and fiber fractions. These parameters are investigated in experiments under field and controlled conditions, which exploit the genotypic diversity in a wide set of varieties, landraces and advanced breeding lines. The effect of environmental and harvesting practices, and their interactions with genetic factors, are studied by applying different mowing schedules and water-stress

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levels on different germplasm types. This paper provides preliminary results generated by project experiments carried out in agricultural and controlled-environment experiments.

Keywords Alfalfa • Forage • Heritability • *Medicago sativa* • Quality • Variability

Introduction

Availability of high quality alfalfa forage is an essential requisite to increase the use of locally grown hay in the diet of lactating cows. The recently updated Parmigiano-Reggiano cheese disciplinary indicates that at least 50 % in dry weight of the daily intake of forages must be harvested on land belonging to the dairy farms.

The project 'High quality alfalfa for the dairy chain' is aimed at improving the feeding quality of alfalfa forage, which depends mainly on the protein content and rapid digestibility of the NDF fiber fraction. The purpose of the research is to assure compliance with the Parmigiano-Reggiano production disciplinary, as well as to increase the profitability of the farming system. The workpackages of the project examine the opportunities to improve the quality of alfalfa through breeding strategies, mowing strategies, timely analysis of the hay, communication and technical support.

The first workpackage, targeting effective breeding strategies for improved quality, deals with variability and heritability of quality parameters in Italian and exotic germplasm. Heritable variability for quality parameters is investigated by analysis of the biochemical composition of the forage in different seasons and phenological stages, factors that are known to affect the quality (Palmonari et al. 2011). Morpho-physiological traits, leaf/stem ratio and separate composition of leaves and stems are also taken into account for a better assessment of existing variation (Annicchiarico et al. 2010). This work package includes four field trials and one controlled-environment trial, which are still in progress. In this paper we present results obtained in the first year of the project from the controlled-environment trial on progenies of exotic germplasm and the field trial on clones of Italian varieties.

Materials and Methods

Controlled-Environment Trial

The trial was established at Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie (CRA-FLC), Lodi, under rainout shelters irrigated by sprinklers. One hundred and fifty-four half-sib progenies were obtained from genotypes originally singled out from three cultivars identified for their adaptation to drought in Mediterranean environments. The progenies were grown in microplots of 0.2 m^2 under non-stressed and moderately drought-stressed conditions in an alpha-lattice design, with three replications for each condition. In early July, 20 days after stress imposition by withholding water in the stress treatment, the plots were mown and the dry-matter (DM) yield and the leaf/stem ratio (as the DM ratio of leaves to stems) recorded for both treatments. A second harvest of the non-stress treatment was carried out in early autumn, with the DM yield and leaf/stem ratio again recorded. A subset of 34 progenies was chemically analyzed by Dipartimento di Scienze Mediche Veterinarie, Bologna, for the content of protein, NDF, ADF, ADL and digestible NDF, in order to test and adjust the NIRS calibrations (CRA-FLC and Centro Ricerche Produzioni Animali) for determining the protein and fiber content of all samples from both harvests.

Field Experiment on Clones

Three genotypes, randomly chosen from each of three Italian varieties (La Bella Campagnola, Prosementi, Minerva), were vegetatively multiplied by rooting stem cuttings and used to establish a trial, which included two replications of six cloned plants per genotype. The experiment was carried out in the Società Produttori Sementi experimental farm in Argelato (44° 39′ N, 11° 39′ E). One plant per plot was sampled at two cutting dates (June and July), both at an early (flower bud) and standard (flowering) maturity stage. Each plant was sampled only once to avoid carry-over effects. Dried samples were chemically analyzed by Dipartimento di Scienze Mediche Veterinarie, for the content of protein, NDF, ADF, ADL and digestible NDF.

Results and Discussion

Controlled-Environment Trial

On average, moderate stress *vs* non-stress treatments differed only by $p<0.20$ for leaf/stem ratio (1.029 *vs* 1.089) and DM yield (2.40 *vs* 2.61 t ha^{-1}). The non-stress, autumn mowing exhibited lower DM yield than the non-stress, summer mowing, as well as higher leaf/stem ratio (1.430 *vs* 1.089; $p<0.01$). Neither progeny (genotype) × stress treatment, nor progeny × season interaction were significant. Leaf/stem ratio of the progenies ranged from 0.881 to 1.345 in summer non-stress, 0.823 to 1.284 in summer moderate stress, and 1.031 to 1.832 in autumn non-stress. There was a slight negative trend between DM yield and leaf/stem ratio across conditions ($r=-0.23$, $p<0.01$).

As expected, leaves and stems differed for all traits chemically analyzed ($p < 0.01$). Averaged across summer samplings, leaves showed much higher percent of protein content (27.4 *vs* 11.5) and NDF digestibility after 24 h (51.0 *vs* 19.9), and lower percent of NDF (21.9 *vs* 59.4) compared to stems.

Further investigation is needed to assess the relationship between the leaf/stem ratio and the overall forage quality, to verify the opportunity of selection for the component with lower quality (the stem), and to identify drought-tolerant germplasm retaining good quality under stress.

Calibration equations validated for hay by the laboratory of Centro Ricerche Produzioni Animali (Brogna et al. 2009), were also suitable for dried leaf and stem samples: R^2 values for NIR predictions of chemical determinations were very high for the main quality parameters (>0.95 for protein, NDF, ADF; >0.85 for ADL).

Field Experiment on Clones

The results showed no significant differences in quality parameters among the three varieties. The most relevant factors influencing the quality were mowing date and maturity stage (with no interaction between these). However, the clone effect on protein content was significant, as was its interaction with the mowing date (Table 23.1).

The protein content of the nine clones in the two mowing dates is graphically represented in Fig. 23.1. The correlation for clone values between these dates (r 0.833) highlights the relatively modest interaction effects.

The results so far obtained showed, as expected, that the season and the phenological stage play a major role in determining the variation in quality traits. The study also highlighted the genotypes contribution to the variation in specific traits, which is valuable for selection purposes.

Table 23.1 Analysis of variance for protein content, NDF digestibility (dNDF) and ADL content in alfalfa clones

Factor	Protein (% dry matter)	dNDF (% NDF)	ADL (% DM)
Clone	***	ns	ns
Mowing date	***	***	***
Maturity stage	***	***	***
Clone × mowing date	**	ns	ns
Clone × maturity stage	ns	ns	ns

ns not significant

** $p < 0.01$; *** $p < 0.001$

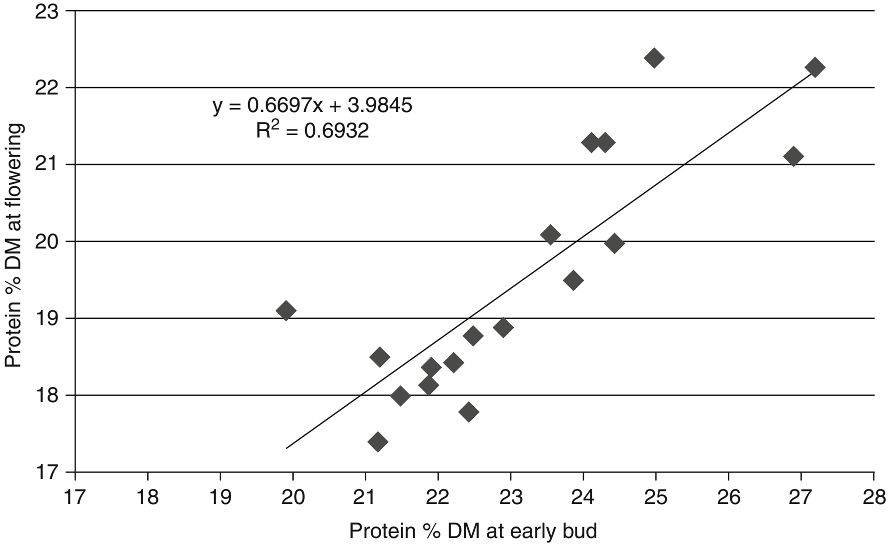

Fig. 23.1 Regression between protein content at two maturity stages for the same clone (each point represents the average of two replicates for one clone at the same mowing date)

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Chapter 24
Certain Aspects of Breeding Forage Brassicas

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Abstract Numerous species belonging to the family *Brassicaceae* Burnett, popularly known as *brassicas*, are multi-purpose crops used not only for various industrial purposes and human consumption, but also in animal feeding. Among the most significant fodder brassicas are those that are grown for forage, such as fodder kale (*Brassica oleracea* L. var. *viridis* L.), rapeseed (*Brassica napus* L. var. *napus*), hybrid Perko PVH (*Brassica napus* L. var. *napus* x *Brassica rapa* subsp. *chinensis* (L.) Hanelt), turnip rape (*Brassica rapa* L. subsp. *oleifera* (DC.) Metzg.), white mustard (*Sinapis alba* L. subsp. *alba*) and brown mustard (*Brassica juncea* (L.) Czern.). All of these play an important role in diverse forage crop rotations, with the autumn-sown ones much appreciated in environmentally friendly production, ecological services, organic farming and sustainable agriculture. Due to their ability to produce abundant above ground biomass, forage brassicas may also be grown as cover crops and for green manure and grazing. Among the traits of great significance for developing new forage brassica cultivars are plant height, number of stems and lateral branches per plant, number of leaves per plant and other forage yield components, fresh forage yield and forage dry matter yield per plant and area unit, earliness and tolerance to low temperatures, drought and other forms of abiotic and biotic stresses. The methods of breeding forage brassicas include mass and individual selection from local landraces, usually characterised by a wide genetic variability of desirable traits, and various methods of selection from hybrid populations, with emphasis upon bulk and pedigree methods.

Keywords Brassicas • Breeding • Forage quality • Forage yield

Introduction

The family *Brassicaceae* Burnett comprises numerous species that are used not only as vegetable crops and for industrial purposes, but also in animal feeding (Erić et al. 2006). Among the most important are rapeseed (*Brassica napus* L. var. *napus*),

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fodder kale (*Brassica oleracea* L. var. *viridis* L.), hybrid Perko PVH (*Brassica napus* L. var. *napus* x *Brassica rapa* subsp. *chinensis* (L.) Hanelt), turnip rape (*Brassica rapa* L. subsp. *oleifera* (DC.) Metzg.), white mustard (*Sinapis alba* L. subsp. *alba*) and brown mustard (*Brassica juncea* (L.) Czern.). All these brassica crops are important in diverse crop rotations, especially those oriented towards forage production, as well as in organic farming and sustainable agriculture (Ćupina et al. 2004).

Basics of Forage Brassicas Breeding

All contemporary breeding programmes and the development of novel cultivars of forage brassica crops are based upon complex characterisation and evaluation of the accessions within one forage brassica collection (Mihailović et al. 2007b). The characterisation results in the description of the most important qualitative traits. The evaluation of the forage brassica accessions includes assessing the data of the most significant quantitative characteristics. The majority of these are forage yield components, such as plant height, number of stems and lateral branches per plant and number of leaves per plant, all of which are positively correlated to fresh forage yield in most forage brassica (Table 24.1). The evaluation also includes fresh forage yield, forage dry matter yield and forage crude protein yield per both plant and area unit (Table 24.2), earliness, tolerance to low temperatures in autumn-sown accessions, drought and other forms of abiotic and biotic stresses and length of growing period, especially in spring-sown genotypes (Mihailović et al. 2008).

The methods used in breeding forage brassicas and developing new genotypes included mass and individual selection from local landraces, characterised with wide genetic variability, and various methods of selection from hybrid populations, with emphasis upon bulk and pedigree methods (Mihailović et al. 2007a).

Table 24.1 Simple correlation coefficients (*r*) between forage yield components and fresh forage yield in some brassicas (Mihailović et al. 2009; Mikić et al. 2009, 2010; Marjanović-Jeromela et al. 2010; Ćupina et al. 2012)

Species					
Forage yield component	Autumn-sown fodder kale	Autumn-sown rapeseed	Spring-sown rapeseed	Spring-sown white mustard	Spring-sown brown mustard
Plant height	0.661*	0.860**	0.780**	0.398	0.074
Number of branches	0.305	0.724*	0.765**	0.891**	0.869**
Number of internodes	0.493	0.834**	0.814**	0.410	0.807*
Fresh stem mass	0.801**	0.976**	0.997**	0.990**	0.953**
Fresh leaf mass	0.839**	0.976**	0.987**	0.857**	0.952**

*significant at 0.05; **significant at 0.01

Table 24.2 Average fresh forage yield, forage dry matter yield and forage crude protein yield in some brassicas (Ćupina et al. 2010; Krstić et al. 2010, 2012; Antanasović et al. 2012; Mihailović et al. 2013)

Species	Fresh forage yield (t ha^{-1})	Forage dry matter yield (t ha^{-1})	Forage crude protein yield (kg ha^{-1})
Autumn-sown fodder kale	51.2	4.1	713
Autumn-sown rapeseed	47.2	4.9	888
Spring-sown rapeseed	33.1	3.0	500
Spring-sown white mustard	22.6	3.4	563
Spring-sown brown mustard	40.9	3.7	644
$LSD_{0.05}$	9.3	1.2	212
$LSD_{0.01}$	12.6	1.7	274

Breeding Most Important Forage Brassicas

Fodder kale is still rather neglected and underutilised in many European countries, but is probably the most important forage brassica crop in temperate regions (Mihailović et al. 2008). This species has great potential for fresh forage yield up to nearly 70 t ha^{-1}. One of the aims of many contemporary fodder kale breeding programmes is to increase the leaf proportion by up to a half of green forage yield per plant (Mihailović et al. 2009). If a typical autumn-sown fodder kale genotype is sown in spring, as a rule, it will not enter the reproductive stage, making developing the cultivars with neutral photoperiod as one of the alternatives. In rainy seasons, fodder kale may produce a second cutting, with average green forage and forage dry matter yields reaching one third of those in the first cutting, thus opening the possibility of selecting genotypes with the ability to regenerate (Erić et al. 2007).

In many European temperate regions, such as the Balkans, the autumn-sown cultivars of rapeseed produce higher forage yields in comparison to the spring-sown ones. Overall, the autumn-sown rapeseed cultivars have a similar forage dry matter proportion to that of fodder kale, with the number of stems and lateral branches the most important forage yield component (Marjanović-Jeromela et al. 2004). The spring-sown rapeseed cultivars are characterised by a higher proportion of stems in the total fresh forage yield (Mihailović et al. 2007a). The rapeseed forage yields in the second cutting, often occurring in rainy seasons, are between one third and one half in comparison to the first cutting (Erić et al. 2007).

In comparison to fodder kale and rapeseed, much less is known about using white mustard as a forage crop. It is sown primarily in the spring, with a growing season from sowing to cutting of up to 60 days. White mustard is generally characterised by a lower leaf mass proportion in fresh forage yield (Mikić et al. 2009). The green and forage dry matter yields in white mustard are lower than in fodder kale or rapeseed (Mihailović et al. 2007a). However, prominent earliness and its potential to produce a considerable amount of fresh forage in a brief period of time represent its main advantage as compared to other forage brassica crops. In favourable conditions, the second cutting in white mustard may reach more than one half of the first cutting (Erić et al. 2007).

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Chapter 25
Evaluation of Dormancy and Winter Hardiness of Alfalfa (*Medicago Sativa* L.) Experimental Lines, Obtained by Crossing Parental Lines Characterized Within the Framework of the PERMED Project

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Abstract The European research project PERMED (Improvement of native perennial forage plants to enhance sustainability of Mediterranean farming systems; 2004 – 2008) has carried out a detailed, large-scale characterization of alfalfa cultivars of different origin in the Mediterranean region, in order to study the genetic variability available for breeding programs. Two cultivars, with superior but contrasting performance in the Mediterranean environment, were chosen as parental lines for a breeding program initiated by Società Produttori Sementi S.p.A. in 2009: the Italian variety Prosementi and the Moroccan landrace Demnat 203. Prosementi is a winter hardy, semi-dormant (Fall Dormancy Index (FDI) 6) cultivar that has a high and constant yield in rainfed condition. It has adaptations to a wide range of environmental conditions. Demnat 203 is a non-dormant population (FDI 11) and has a specific adaptation to frequent cuts under warm, irrigated condition (oasis). The aim of the breeding program is to develop new genotypes with low dormancy, good winter hardiness and high forage yields. The breeding scheme was based on open-pollinated crossing of 84 parent plants for each cultivar in spatial isolation and on subsequent half-sib selection with progeny testing. Three advanced lines derived from this breeding program (PSB QM 4, QM 5, QM 6) are now under evaluation in field trials located in two different Italian areas (Ravenna in Northeast Italy, 44°25′4″44 N, 12°11′58″92 E, and Cagliari in Sardinia, 39°13′24″96 N, 09°7′5″16 E).

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Keywords Alfalfa • *Medicago sativa* • Fall dormancy • Breeding

Introduction

Alfalfa (*Medicago sativa* L.) can enhance the economic and environmental sustainability of crop–livestock system in the Mediterranean basin, but specific adaptations to the climate of the area are required. The main aim of the EU-funded research project PERMED (Improvement of native perennial forage plants to enhance sustainability of Mediterranean farming systems; 2004 – 2008) was to evaluate several alfalfa populations in different environments of the Mediterranean basin. Annicchiarico et al. (2011) evaluated the forage yield responses of several genotypes across a wide range of environments in the Mediterranean basin, providing a useful tool for the breeding program development in this area.

Material and Methods

Following the final output of PERMED project, a new breeding program was initiated by Società Produttori Sementi in 2009, where two different populations of *M. sativa* L. were used as the parental lines: Prosementi, an Italian semi-dormant (Fall Dormancy Index, FDI = 6) and winter hardy variety, with a wide range of adaptations to different environmental conditions, and Demnat 203, a Moroccan non-dormant (FDI = 11) landrace, with a specific adaptation to frequent cuts in warm, irrigated condition (oasis). Eighty-four plants from both cultivars were planted in 2009 in a spatially isolated field nursery at Cervia, RA (44°15′ N, 12°22′ E). They were phenotypically evaluated for fall growth and winter injury. In 2009, the open-pollinated seeds were harvested, while maintaining the identity of each plant, and for 28 parental plants (selected on the basis of previously reported traits) a progeny test was performed. Half-sib families of 1st and 2nd generation (HS1 & HS2) were evaluated in terms of fall growth and winter survival. Fifteen of the HS2 families were selected to supply HS3 seeds for three mixed lines (PSB QM 4, 5 & 6) based on winter survival and fall growth of both the parents and their progenies (Table 25.1). The seed mix was composed of an equal number of HS3 seed from each selected family.

The performance of PSB QM 4, 5 and 6 was evaluated in dense plots of 1.36 m × 7.5 m at the seeding rate of 30 kg ha^{-1} in a randomized block design with four replicates and compared to both standard varieties and advanced lines (Table 25.2). In both trials, varieties Prosementi and Melissa were used as dormancy references, with an FDI of 6 and 9, respectively. The trials were started in 2012 at two different location at Azienda Agraria Sperimentale M. Marani in Ravenna, Northeast Italy (44°25′ N, 12°11′ E) and at AGRIS Sardegna, Dipartimento per la Ricerca nelle Produzioni Vegetali, Cagliari, Sardinia (39°13′ N, 09°7′ E).

In 2012, three cuts were performed in Ravenna (4 Jul, 6 Aug & 3 Oct) and six in Sardinia (20 Jul, 9 Aug, 28 Aug, 25 Sep, 18 Oct & 26 Nov). Additionally, in

Table 25.1 Characteristics of the parental plants of each experimental line: maternal line (Prosementi or Demnat 203), number of constituents, dormancy (semi-dormant = moderate vegetative growth during fall; non-dormant = good vegetative growth during fall), winter survival (% of plants alive after two winters) and quantity of available seeds (gr)

Line	Parental constituents				Quantity of available seeds (gr)
	Maternal plants	Number of constituents	Dormancy	% winter survival	
PSB QM 4	Prosementi	5	Semi-dormant	100	890
PSB QM 5	Demnat 203	6	Non-dormant	100	742
PSB QM 6	Demnat 203	4	Non-dormant	0	810

Table 25.2 Experimental lines and standard varieties included in the field trials in Ravenna and Sardinia, with dormancy type (Fall Dormancy Index, FDI, given where available) and seed source

Cultivars	Dormancy	Source
Prosementi	FDI = 6	Società Produttori Sementi
Miranda	Semi-dormant	Società Produttori Sementi
Buttero	Non-dormant	CRA-FLC
PR57Q53	Semi-dormant	Pioneer Hi-breed
Melissa	FDI = 9	Jouffray–Drillaud
Nuragus	Semi-dormant	AGRIS
Picena	Semi-dormant	CGS Sementi
PSB QM 4		Società Produttori Sementi
PSB QM 5		Società Produttori Sementi
PSB QM 6		Società Produttori Sementi

Sardinia, the first cut in 2013 took place on 21 February. Dry matter yield (DMY; kg ha^{-1}) was evaluated after each cut. For both trials, annual dry matter yield was calculated in kg ha^{-1}. In Sardinia, the dry matter yields of the sixth cut in 2012 and of the first cut in 2013 were used as a measure of fall and winter growth. In Ravenna, where fall and winter growth are not sufficient for cutting, visual inspections of the vegetative activity were carried out in November (26th) and in March (5th) and plants were scored on a scale ranging from 1 (lowest) to 7 (highest). In March, the winter survival was registered in the field trial of Ravenna as number of plants per square meter. Analysis of variance (ANOVA) and Duncan's test (alpha = 0.01) were carried out using the program R version 2.15.2.

Results and Discussion

The analysis of visual inspection scores (Ravenna) and yields (Sardinia) in fall and in late winter revealed highly significant differences between the cultivars (ANOVA, *p*-value < 0.001). For the Sardinia trial, there was a clear separation, where the standard varieties Prosementi and Melissa were classified according to their FDI (Table 25.3). ANOVA analysis of total DMY in the first year also revealed significant differences between cultivars, with two semi-dormant varieties PR57Q53 (Sardinia) and Picena (Ravenna) being the most productive.

Table 25.3 Comparisons between annual yield for 2012 and fall and winter growth of experimental lines and standard cultivars

	Sardinia			Ravenna			
Cultivars	Annual DMY 2012 (kg ha^{-1})	Fall DMY Nov 2012 (kg ha^{-1})	Late winter DMY Feb 2013 (kg ha^{-1})	Annual DMY 2012 (kg ha^{-1})	Fall growth Nov 2012 visual scoring	Late winter growth Mar 2013 visual scoring	Plant density (no of plants m^{-2})
Prosementi	693 ab	380 c	0 d	169 ab	5 b	3 cd	93
Miranda	569 b	355 c	0 d	127 b	3 c	1 d	89
Buttero	847 ab	650 abc	220 bc	187 ab	5 b	5 abc	93
PR57Q53	1091 a	570 bc	0 d	158 a	5 b	3.5 bc	93
Melissa	907 ab	920 a	397 b	145 b	5.5 ab	4 bc	95
Nuragus	699 ab	617 abc	0 d	173 ab	5 b	4.5 bc	89
Picena	906 ab	732 ab	115 cd	215 a	6 ab	5.5 ab	87
PSB QM 4	761 ab	817 ab	77 cd	168 ab	6.5 ab	4.5 bc	93
PSB QM 5	942 ab	852 ab	325 b	176 ab	7 a	7 a	95
PSB QM 6	761 ab	910 a	670 a	160 ab	6,5 ab	7 a	86
ANOVA	**	***	***	*	***	***	ns

For the Sardinia trial, the average of the fall and late winter yields (DMY kg ha^{-1}) are given, and for the Ravenna trial, the average of fall and late winter visual inspection scores (1–7) are reported Letters a to d denotes homogeneous subgroups according to Duncan's test (significance level of alpha = 0.01)

ns not significant

$^{*}p<0.1$; $^{**}p<0.01$; $^{***}p<0.001$

Lines PSB QM 5 and 6 were equally or less dormant than the FDI 9 reference Melissa. The dormancy of line PSB QM 4 was determined to be intermediate between the two references FDI 6 and FDI 9. Further test will be performed to calculate the fall dormancy indices for these lines according to Pecetti et al. (2008). In the Ravenna trial, there were no significant differences in plant density (no of plants m^{-2}) after one winter between standard varieties and experimental lines. These results indicate that the experimental lines PSB QM 4, 5 and 6 could possess a combination of low dormancy and high winter hardiness traits. A weak link between these traits has been reported previously by Brummer et al. (2000), suggesting that concurrent selection for both winter hardiness and higher fall biomass production may be possible. The two field trials are still running, which will allow for more information about dormancy, winter survival and dry matter yield to be collected in coming years.

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Chapter 26
Breeding Perennial Ryegrass with Enhanced Water Soluble Carbohydrate Content

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Abstract A high water soluble carbohydrate (WSC) content in perennial ryegrass leads to a more efficient use of the protein in the grass by the ruminant and hence to reduced nitrogen losses. In our breeding pool we search for breeding material of diploid and tetraploid perennial ryegrass with enhanced WSC content. In 2011 we have sown a yield trial in which we compare high sugar varieties with diploid and tetraploid breeding material with expected high WSC content. Some of the material (families, syn1-populations, induced tetraploids) was not selected for a high WSC content, but showed a high WSC content in previous trials. Other material was based on crosses (pair cross, top cross, polycross) with genotypes selected for their high WSC content. The yield trial was harvested five times in 2012. We took samples at each cut to determine the WSC content by NIRS. The results of the previous trials were confirmed in the experiment. The WSC content of the progenies of the populations with high WSC content was lower than the WSC content of the high sugar grasses although the content of the original population exceeded the content of the high sugar grasses.

Keywords Perennial ryegrass • Breeding • WSC content

Introduction

High sugar grasses provide more available energy for rumen fermentation with reduced levels of excreted nitrogen as a result (Miller et al. 2001; Humphreys et al. 2010). One of the breeding goals in perennial ryegrass is enhancing the WSC content. There is a lot of variation in WSC content among families in breeding populations. Based on family selection with and without single plant analysis, we tried to develop material with improved WSC. This material was tested in a production trial in comparison with high sugar cultivars.

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Material and Methods

In 2011 we have sown a production trial in which we compare high sugar grasses (Abermagic, Aberavon and Aberbite) with control varieties (Rodrigo and Roy) and with breeding material with expected high WSC content. We tested five diploid and seven tetraploid breeding populations.

The diploid population D1 is based on a crossing between two perennial ryegrass families with a high WSC content. In 2008 we carried out three paircrosses with good performing plants of each of the families. The F1 seeds from these paircrosses were multiplied to a synthetic progeny family in 2010. This population has been tested in the experiment.

In 2003 we carried out a topcross of Aberdart with nine clones of diploid perennial ryegrass. The progenies were visually evaluated in a single plant nursery. Half sib seed was harvested on selected plants from seven of the nine parental families in 2006. We obtained enough seeds of 76 half sib families to sow small plots in 2007. We measured the dry matter yield and the WSC content during 2008 and 2009. Based on the results we carried out four multiplications with the best half sib families. Seeds of these multiplications (D2, D3, D4 and D5) have been tested in the production trial.

The tetraploid populations (T1, T2, T3, T4, T5 and T6) were not selected for a high WSC content, but showed a high sugar content in previous trials.

The tetraploid population T7 was selected from a breeding population with a high WSC content. In 2009 we have analysed the yield and the WSC content of 300 plants of this population. The best plants were crossed and the progeny of this multiplication has been tested in the production trial.

The variety Aberavon was being used in all trials were we tested diploid populations and the variety Roy was used in the tetraploid trials. We used Aberavon and Roy as standard varieties to compare the results of the experiment with the results of the previous trials.

The production trial was harvested five times in 2012. We took samples at each cut to determine the WSC content by NIRS. The NIRS calibration is based on chemical analyses by Wiseman et al. (1960).

Results and Discussion

There was a large variation in the mean WSC content ranging from 19.5 for Rodrigo to 26.4 for Aberbite. The tetraploid populations had a higher WSC content than the diploid populations (respectively 23.6 and 22.3).

Figure 26.1 shows the mean WSC content of the parental diploid families and of the progenies, relative to the mean of the WSC content of Aberavon (19.4 and 23.7 in the respective trials). The WSC content of the progenies was lower than the WSC content of the parental families compared to Aberavon but was higher than the

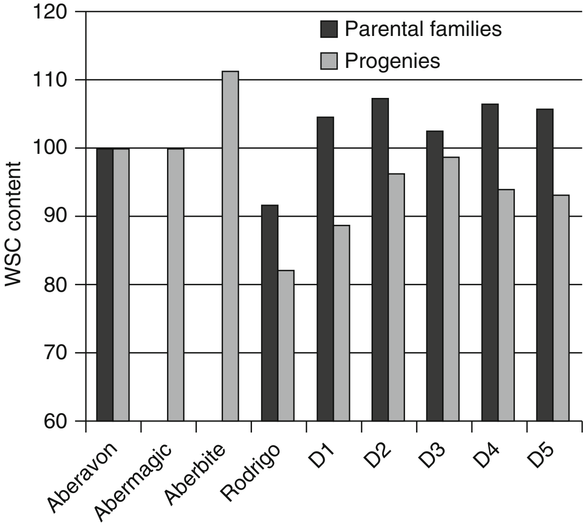

Fig. 26.1 Mean WSC content of the diploid parental families and of their progenies relative to the mean WSC content of Aberavon (100 = 19.4 and 23.7 in the respective trials)

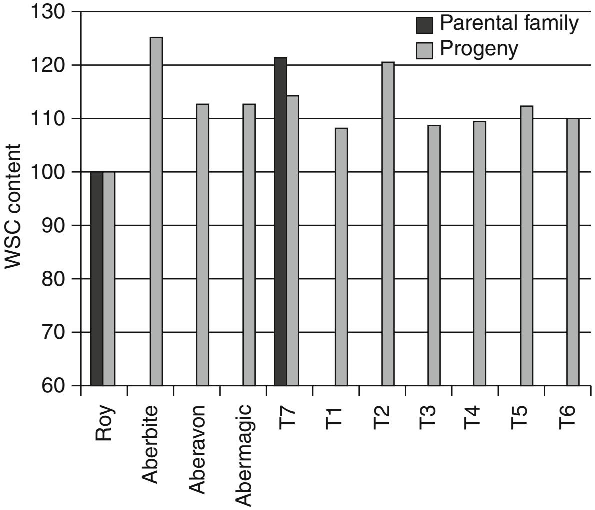

Fig. 26.2 Mean WSC content of a tetraploid parental family and of its progeny (T7) and of 6 unselected families relative to the mean WSC content of Roy (100 = 17.8 and 21.1 in the respective trials)

content of control variety Rodrigo. The parental families of D3 had the lowest WSC content of all the selected families. The progenies of this multiplication had the highest sugar content and only a minor reduction (3.9 %) compared to Aberavon. The population D1 based on two families with a high WSC content had the largest reduction (15.9 %).

Figure 26.2 shows the mean of the WSC content of the tetraploid parental family and the progeny (T7) relative to the mean of the WSC content of Roy (17.8 and 21.1 in the respective trials). The six tetraploid families (T1–T6) show the potential of the breeding pool for high WSC without selection. Population T7 was selected from material with a high WSC content. The WSC content of the progenies was lower than the WSC content of the parental family compared to Roy but the decrease was low.

The progenies of the diploid and tetraploid material selected for high WSC content had a WSC content that was lower than the content of the parental families but still higher than the sugar content of the control varieties Rodrigo and Roy. The diploid population D1 was based on six plants from families with a high WSC content. The WSC content of the six plants has not been analysed. This could explain the low WSC content of the progenies. The other diploid populations were based on 80–300 plants that had not been analysed. The reduction of the mean WSC content of these populations was lower. The WSC content of the parental population of T7 was analysed and the plants with the highest WSC content were multiplied. The progenies of this population had only a slight reduction in WSC content.

Improving the WSC content of perennial ryegrass is possible by combining families with a high WSC content without selection of single plants but the progress is slow. Analysing single plants of populations with a high WSC content and selecting the best plants to create new populations leads to a higher increase of the WSC content.

The WSC content of the tetraploid populations T2 and T7 was higher than the WSC content of the diploid high sugar grasses Aberavon and Abermagic but was lower than the WSC content of the tetraploid high sugar variety Aberbite. None of the tested diploid populations had a WSC content that was higher than the WSC content of the high sugar grasses Aberavon, Abermagic and Aberbite. Further recurrent selection is needed to reach this level.

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Chapter 27
Resistance of Perennial Ryegrass to Crown and Stem Rust Under Field and Greenhouse Conditions

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Abstract The resistance of 59 varieties and three ecotypes of perennial ryegrass (*Lolium perenne*) to crown rust (*Puccinia coronata*) and stem rust (*Puccinia graminis* subsp. *graminicola*) was evaluated under field and greenhouse conditions at the Grassland Research Station in Zubri in 2011 – 2012. The infection pressure of crown rust was higher in 2011 and most of the investigated accessions, with exception of the variety Charger II, showed high susceptibility in the field. The occurrence of stem rust was low in both years. Artificial inoculation of young plants with urediospores of *P. graminis* subsp. *graminicola* in the greenhouse revealed high resistance in the varieties Kokomo and Score.

Keywords Stem rust • Field resistance • Perennial ryegrass

Introduction

Perennial ryegrass varieties are intensively cultivated for seed production in the Czech Republic. Since 2000, some very early occurrences of stem rust (*Puccinia graminis* subsp. *graminicola*) were detected on perennial ryegrass seed crops in a few locations in the Czech Republic. The damage caused by the disease was severe. Seed yields of some perennial ryegrass varieties decreased to 10 % of normal yields, as a result of stem rust infection (Cagaš et al. 2010). Stem rust is a devastating disease, particularly in grass seed production. The severity of crown rust on perennial ryegrass seed production is lower, but it can cause reduced thousand seed weight (TSW) and germination (Urban and Marková 2009). However, the occurrence and severity of rust diseases caused by *P. graminis* and *P. coronata* (crown rust) is decreased in perennial ryegrass cultivated for forage, if grown in mixture with other

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grass or herb species (Roscher et al. 2007). Intensive outbreaks of stem rust appear to be linked to favourable temperatures during spring (Lellbach 2000), which have occurred more frequently in recent years. Stem rust is a disease that has a negative influence not only on seed yield but also on the quality of seed. Relevant information about the response of perennial ryegrass varieties to different levels of rust infection pressure is only sparsely available. Multi-year field observations, supplemented by greenhouse trials of different perennial ryegrass varieties under artificial infection conditions, may improve our understanding of the level of disease resistance present in existing cultivars.

Material and Methods

Field Trial

Sixty varieties and three ecotypes of perennial ryegrass were tested under field conditions at the Grassland research Station in Zubri, Czech Republic. All seeds were obtained from the gene bank of the Crop Research Institute Prague- Ruzyně. Seeds were sown in pots in a greenhouse and later the seedlings were transferred to field plots (11 June 2010) and planted in rows (2 m length, 0.25 m row spacing), in three replications (11 plants per replicate). In total, 33 plants from each variety and ecotype were planted. Seeds of variety no. 53 (Livonne) failed to emerge, so this variety was excluded from further analysis.

Resistance to stem rust based on natural infection was evaluated in the year of sowing (autumn 2010) and in the two succeeding years (2011–2012). However, in 2011, only crown rust (and no stem rust) was observed on the perennial ryegrasses in the field trial. In 2012, the occurrence of stem rust was lower than the occurrence of crown rust. Based on these observations, the occurrence of crown rust was also included in the assessment of rust resistance for the years 2010–11. In the first year, only the presence (+) or absence (−) of rust on plants was evaluated. In succeeding years, rust infections were scored on a scale from 1 to 9, where 1 = more than 75 % of foliage covered with rust, 2 = 75 % rust cover, 3 = 60 % rust cover, 4 = 40 % rust cover, 5 = 25 % rust cover, 6 = 10 % rust cover, 7 = 5 % rust cover, 8 = trace of rust and 9 = no rust.

Greenhouse Test

A greenhouse test using artificial inoculation was carried out in 2011 at the Grassland Research Station in Zubri. Three hundred plants from each variety and ecotype of perennial ryegrass were grown from seed in pots. Young plants were inoculated with a mixture of urediospores of *Puccinia graminis* subsp. *graminicola* and talc at growing stages 13–15 (from three to five true leaves) and assessed as described by Cagaš (1978). Urediospores were obtained in 2011 from multiple-cultivar field plantings of perennial ryegrass in Zubri and other locations nearby. Samples collected from different locations were pooled to form a single population. Plants were inoculated on 29 September 2011 and evaluated for rust resistance from 10 to 13 October onwards.

Results and Discussion

Field Trial

In the year of trial establishment (2010), stem rust was detected on 56.5 % of the tested perennial ryegrass varieties. The remaining 43.5 % did not have any visible symptoms of stem rust. In succeeding years, there was a higher occurrence of crown rust than of stem rust in all of the tested varieties (Table 27.1).

In 2012, stem rust was observed on all three ecotypes, but only on varieties Filip, Jaran, Jonas, Linar, Martha, Patrik, Slavek and Tremolo.

Table 27.1 Crown rust and stem rust disease scores for 59 perennial ryegrass varieties and three ecotypes

	Crown rust				Stem rust	
	2011		2012		2012	
Variety	Score	T_{05}[a]	Score	T_{05}[a]	Score	T_{05}[a]
Advent	5.0	d	7.5	abcd	9.0	a
Amadeus	6.0	e	8.5	ab	9.0	a
Barcredo	6.0	e	8.0	abc	9.0	a
Bareuro	4.0	c	7.0	abcd	9.0	a
Bargold	4.0	c	6.5	bcd	9.0	a
Barminton	7.0	f	8.0	abc	9.0	a
Barnauta	4.0	c	6.0	cd	9.0	a
Barreal	4.0	c	6.0	cd	9.0	a
Boulevard	6.0	e	8.0	abc	9.0	a
Cadillac	6.0	e	7.0	abcd	9.0	a
Calibra	7.0	f	7.0	abcd	9.0	a
Citacion III	8.0	g	6.5	bcd	9.0	a
Darius	5.0	d	9.0	a	9.0	a
Delaware	7.0	f	7.5	abcd	9.0	a
Disco	6.0	e	5.5	d	9.0	a
Figaro	6.0	e	6.5	bcd	9.0	a
Filip	6.0	e	9.0	a	5.5	cd
Flor	5.0	d	7.5	abcd	9.0	a
Fragment	5.0	d	7.0	abcd	9.0	a
Handicap	6.0	e	7.0	abcd	9.0	a
Hannibal	5.0	d	8.5	ab	9.0	a
Henrietta	7.0	f	7.5	abcd	9.0	a
Herbie	7.0	f	8.5	ab	9.0	a

(continued)

Table 27.1 (continued)

	Crown rust				Stem rust	
	2011		2012		2012	
Variety	Score	T_{05}[a]	Score	T_{05}[a]	Score	T_{05}[a]
Charger II	9.0	h	8.5	ab	9.0	a
Jaran	6.0	e	9.0	a	5.5	cd
Jonas	7.0	f	9.0	a	7.0	b
Kelt	7.0	f	8.5	ab	9.0	a
Kentaur	7.0	f	6.5	bcd	9.0	a
Kertak	4.0	c	8.0	abc	9.0	a
Kokomo	5.0	d	7.5	abcd	9.0	a
Linar	3.0	a	9.0	a	6.0	bc
Lipresso	7.0	f	7.5	abcd	9.0	a
Mara	7.0	f	8.0	abc	9.0	a
Marietta	6.0	e	7.5	abcd	9.0	a
Martha	5.0	d	9.0	a	6.0	bc
Mathilde	5.0	d	7.5	abcd	9.0	a
Merci	7.0	f	8.5	ab	9.0	a
Missouri	7.0	f	6.5	bcd	9.0	a
Montreux	6.0	e	8.0	abc	9.0	a
Napoleon	6.0	e	8.0	abc	9.0	a
Numan	7.0	f	8.5	ab	9.0	a
Option	5.0	d	8.0	abc	9.0	a
Patrik	6.0	e	9.0	a	6.0	bc
Plaisir	6.0	e	7.5	abcd	9.0	a
Premium	4.0	c	6.0	cd	9.0	a
Recolta	6.0	e	6.5	bcd	9.0	a
Renoir	5.0	d	7.5	abcd	9.0	a
Respect	5.0	d	8.0	abc	9.0	a
Ritmo	4.0	c	6.0	cd	9.0	a
Sabor	5.0	d	8.5	ab	9.0	a
Sadek	7.0	f	7.5	abcd	9.0	a
Sauvignon	7.0	f	7.0	abcd	9.0	a
Score	5.0	d	6.0	cd	9.0	a
Slavek	7.0	f	9.0	a	6.5	bc
Sponsor	6.0	e	7.0	abcd	9.0	a
Titus	5.0	d	7.5	abcd	9.0	a
Tremolo	6.0	e	9.0	a	5.5	cd
Troubadour	5.0	d	8.0	abc	9.0	a
Twingo	7.0	f	6.5	bcd	9.0	a
14G2000630	4.0	c	9.0	a	5.5	cd
14G2000647	4.0	c	9.0	a	4.5	d
14G2000648	3.5	b	9.0	a	6.5	bc

[a] Homogeneous group according to Tukey's HSD test (significance level of alpha = 0.05)

Greenhouse Test

The greenhouse tests indicated a high predisposition to stem rust in most varieties and ecotypes. These results have greater implications for breeding of perennial ryegrasses than for production. All ecotypes and most of the varieties were susceptible to stem rust, with 75–100 % of the plants displaying disease symptoms. Three varieties (Score, Kokomo and Advent) were resistant to stem rust, with few, or no, affected plants (Table 27.2).

Table 27.2 Occurrence of stem rust on different perennial ryegrass varieties or ecotypes after artificial inoculation, expressed as the percentage of infected plants (%), and correlation between greenhouse resistance and field resistance (r)

Variety	%	T_{05}	r	Variety	%	T_{05}	r
Advent	28	c	0.89	Lipresso	89	ij	0.06
Amadeus	93	klm	0.11	Mara	94	lmn	0.06
Barcredo	99	pr	0.10	Marietta	33	d	0.31
Bareuro	93	klm	0.43	Martha	85	h	0.30
Bargold	94	lmn	0.43	Mathilde	85	h	0.30
Barminton	90	jk	0.06	Merci	81	g	0.07
Barnauta	97	nopr	0.41	Missouri	98	opr	0.05
Barreal	97	nopr	0.41	Montreux	98	opr	0.11
Boulevard	92	jkl	0.11	Napoleon	50	e	0.21
Cadillac	92	jkl	0.11	Numan	96	mnop	0.06
Calibra	98	opr	0.05	Option	78	fg	0.32
Citacion III	86	hi	0.03	Patrik	100	r	0.10
Darius	96	mnop	0.26	Plaisir	95	lmno	0.11
Delaware	93	klm	0.05	Premium	100	r	0.40
Disco	99	pr	0.10	Recolta	98	opr	0.11
Figaro	93	klm	0.10	Renoir	99	pr	0.26
Filip	99	pr	0.10	Respect	98	opr	0.26
Flor	98	opr	0.25	Ritmo	96	mnop	0.42
Fragment	85	h	0.30	Sabor	93	klm	0.27
Handicap	100	r	0.10	Sadek	75	f	0.07
Hannibal	81	g	0.31	Sauvignon	95	lmno	0.06
Henrietta	90	jk	0.05	Score	0	a	–0.01
Herbie	93	klm	0.05	Slavek	100	r	0.05
Charger II	79	g	0.00	Sponsor	80	g	0.13
Jaran	100	r	0.10	Titus	90	jk	0.28
Jonas	99	pr	0.05	Tremolo	98	opr	0.11
Kelt	97	nopr	0.05	Troubadour	98	opr	0.26
Kentaur	96	mnop	0.06	Twingo	99	pr	0.05
Kertak	100	r	0.40	14G2000630	97	nopr	0.41
Kokomo	4	b	–0.16	14G2000647	98	opr	0.40
Linar	79	g	0.76	14G2000648	98	opr	0.61

Correlation between field and greenhouse resistance was only observed in a few varieties (e.g. Advent, Linar, ecotypes), and in some cases the correlation was negative (e.g. Kokomo, Score). However, none of the correlation coefficients were statistically significant (p-values ranged from 0.15 to 0.33). Regression analysis revealed that the link between field resistance and greenhouse resistance was minimal ($R = 0.20$) for all varieties and ecotypes, and varied between 0.89 and −0.16. Therefore, the benefits of using greenhouse tests to assess the resistance to rust infection in perennial ryegrass grown under field conditions are questionable.

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Chapter 28

Ideotypes of Forage Pea (*Pisum sativum*) Cultivars

Vojislav Mihailović and Aleksandar Mikić

Abstract Pea (*Pisum sativum* L.) is one of the most important grain legume crops in temperate regions worldwide. In many countries of Europe, West Asia and North Africa, it is also used in feeding ruminants in the form of fresh forage, forage dry matter, forage meal, silage, haylage and straw. The goals of breeding pea for forage significantly differ from those in breeding pea for grain. A large majority of currently ongoing breeding programmes on forage pea develop cultivars completely or mostly from local or introduced populations of *P. sativum* L. subsp. *sativum* var. *arvense* (L.) Poir. Such cultivars are characterised by long stems with indeterminate growth, long internodes, great number of nodes, large stipules and three pairs of leaflets, purple flowers and low seed yields in comparison to typical feed pea cultivars. They also have prominent winter hardiness, late maturity and large forage losses due to an extremely poor lodging tolerance, being the reason why these cultivars are mostly grown intercropped with cereals. Much of said traits, such as large aboveground biomass resulting from long stems or large leaves are important segments of the ideotypes of a forage pea cultivar. More recent improvements include determinate stem growth and moderately great stem length, in order to reduce withering of at least a whole lower half of leaves on the plant, as well as a leaf proportion in the aboveground biomass higher than 0.50, improved earliness and a forage dry matter proportion of about 0.25, increasing crude protein content and decreasing both neutral and acid detergent fibre and lignin content in forage dry matter. A successful combination of all these desirable traits may result in a forage pea cultivar producing about or slightly more than 10 t ha^{-1} of forage dry matter and about 2 t ha^{-1} of forage crude protein, able to easily fit into diverse cropping systems. The latest trends in forage pea cultivars brought forth the first semi-leafless cultivars with excellent standing ability and enhanced seed production, where numerous and large stipules successfully replace the missing leaflets in providing quality forage.

Keywords Breeding • Forage production • Ideotype • Pea

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What is Forage Pea?

Pea (*Pisum sativum* L.) is one of the most economically important legume crops in the world (Ellis 2007). As many other annual legume species, such as grass pea (*Lathyrus sativus* L.) and other vetchlings (*Lathyrus* spp.), vetches (*Vicia* spp.), faba bean (*Vicia faba* L.) or lentil (*Lens culinaris* Medik.), pea has a wide variability of numerous morphological traits. This peculiarity is the most prominent in pea, significantly progressed and accelerated by various breeding programmes (Ellis 2009). As a consequence, the pea crop may be utilised in diverse ways.

Forage pea is one of the most distinguished agronomic types of the pea crop (Carrouée 1993). In the case of pea and other botanically close cultivated species, green forage, being often synonym of the terms *fresh forage* or *fresh weight*, represents the above ground parts of a plant cut in the stages of full flowering or forming the first pods, as a balance between yield and quality (Mikić et al. 2012). Forage dry matter is usually considered a plant mass remaining after proper drying green forage until constant mass mostly in laboratory and rarer in field conditions, with most part of leaves preserved. Forage pea may be also be used as forage meal, similar to that of lucerne (*Medicago sativa* L.) or red clover (*Trifolium pratense* L.), as silage, together with acidifiers, and haylage, representing a specific way of self-conserving green forage (Karagić et al. 2012).

Forage pea may be cultivated alone, that is, as a pure crop, or in mixtures with cereals, which represents one of the most traditional ways of production in many regions of Europe, Western Asia and North Africa (Mihailović et al. 2011). In the countries as Serbia, a pea-cereal mixture is always cut before the cereal component shoots out its spike, while in, for instance, western Canada, it is typically cut when the cereal crop reaches the soft dough stage. The ratio of pea and the cereal component in a mixture is also an issue of the agricultural tradition, varying between 75–80 %: 25–20 % in the Balkans and 50 %: 50 % in France or Lithuania (Sarunaite et al. 2013).

The results of several long-term pre-breeding trials in Novi Sad, including hundreds of the pea accessions of diverse status in terms of genetic resources and geographic origin, show that, overall, forage pea may be regarded as a crop with a great potential for both forage dry matter and forage crude protein production. The fresh forage yield in pea is 45 and 50 t ha^{-1}, the average forage dry matter yield ranges from 9 to 10 t ha^{-1} and an average forage protein yield varies between 1,500 and 2,000 kg ha^{-1}. Similar forage dry matter yields and crude protein contents have been obtained in spring forage pea cultivar trials in western Canada over the past 5 years (Mihailović et al. 2009).

Diversity of Forage Pea Ideotypes

The main goal of all forage pea breeding programmes is high and stable forage yield and a high quality chemical composition, aiming a green forage yield of more than 50 t ha^{-1} in winter and more than 45 t ha^{-1} in spring cultivars, a forage dry matter

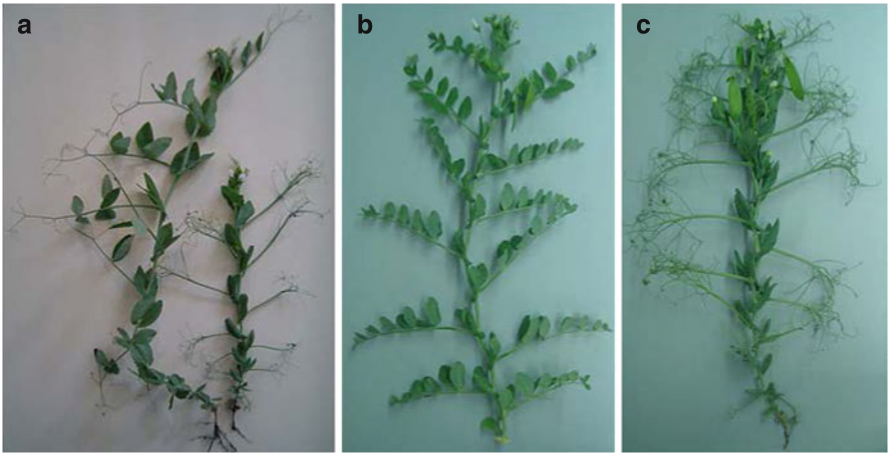

Fig. 28.1 Various ideotypes of a forage pea cultivar: (**a**) classical (*left*), in comparison to a typical grain pea cultivar; (**b**) acacia-leaved; (**c**) semi-leafless

yield of about 10 t ha^{-1}, a variation of forage yields between years less than 20 % and a prominent winter hardiness and earliness (Mikić et al. 2011b).

Stem length is often positively correlated to high forage yield. However, number of internodes has a much more positive impact on it, since it is closely associated with number of photosynthetically active leaves. This trait is regulated by a number of dominant genes and is easy to introgress from one forage pea genotype into another. At the same time, forage yields can be improved by a moderately prominent basal branching (Mikić et al. 2012). The most common ideotype of a forage pea cultivar is unlike the one of a grain pea cultivar (Fig. 28.1a).

A high quality in forage pea cultivars, in terms of a desirable chemical composition, is directly and positively correlated with the proportion of leaves in the total forage yield, since leaves contribute most to forage protein yield. A classical forage pea cultivar has to have large stipules and two or three pairs of large leaflets. One of the alternative types of forage pea cultivars are those with acacia leaf type (Fig. 28.1b), characterised with a large number of leaflets and no tendrils. Although theoretically expected to have an increased leaf proportion and thus better forage quality, they are extremely prone to lodging, suffer either from low seed yields or large seed losses during harvest and make the issue of their successful commercialisation rather questionable (Mikić et al. 2011a).

An additional targeted trait in all modern forage pea breeding programmes is reliable seed yield (Mikić et al. 2013). In such way, a forage pea cultivar, apart from high quality and stable forage yields, may be able to produce medium or high seed yields and thus enable its market commercialisation justified and successful. A seed size of less than 200 g per 1,000 seeds positively affects an increased number of seeds per plant, and thus higher coefficient of multiplication, and a less expensive sowing costs. These efforts resulted in semi-leafless forage pea cultivars (Fig. 28.1c), with a stem length up to 100 cm, a significantly improved standing ability, a greater number of internodes and large stipules providing high and quality forage yields, thicker and more juicy stems making forage more palatable for ruminants and pods grouped in the upper half of a plant increasing seed yield and decreasing seed losses during mechanical harvest (Warkentin et al. 2009).

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Part IV
Turf Grass Breeding

Chapter 29
Recent Achievements in Breeding for Turf Quality Under Biotic and Abiotic Stress

Trygve S. Aamlid and Vincent Gensollen

Abstract The genetic gain from breeding of turf varieties of *Festuca rubra*, *Poa pratensis, Lolium perenne* was assessed using data from 204 candivars entered into Norwegian/SCANTURF variety (VCU) testing from 1986 to 2007, and 675 varieties entered into the French/GEVES variety testing from 1982 to 2010. Among the three subspecies of *Festuca rubra*, ssp. *litoralis* and ssp. *rubra s*howed the strongest improvements in overall turf quality in the Nordic (0.67 % per year) and GEVES programs (0.99 % per year), respectively. The main reasons for these improvements were better winter hardiness and resistance to *Microdochium nivale* in ssp. *litoralis* and better persistency associated with higher tiller density in ssp. *rubra*. For *Poa pratensis* there was no gain in turf quality in France, but a significant improvement (0.94 % per year) associated with higher density, finer leaves and less height growth in the Nordic program, which, unlike the GEVES trials, included trials at low (10–20 mm) mowing height. *Lolium perenne* showed significant improvement in both programs and all characters studied, but the progress was smaller for persistency, overall winter hardiness and resistance to red thread than for tiller density, wear tolerance and tolerance to rust.

Keywords Kentucky bluegrass • Perennial ryegrass • Red fescue • Turf quality • Variety testing

Introduction

Breeding of cool-season grasses for turf started after World War II and was intensified with the introduction of Plant Breeders Rights and the initiation of programs for systematic evaluation and publication of national variety lists in the 1960s (van

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Wijk 1993; Casler 2006; Sampoux et al. 2012). The activity of the publically funded testing programs reached a maximum in the 1980s. Since then, public funding of variety testing has mostly been withdrawn as the European Union only requires testing for Distinctness, Uniformity and Stability (DUS-criteria), and no documentation of the Value of Cultivation and Use (VCU-criteria) for inclusion on the common European list.

When variety testing started in the 1960s, most so-called 'turf' varieties were chosen based on seed availability and agricultural merit. It is therefore not surprising that significant improvements in typical turf characteristics such as tiller density, leaf fineness and height growth (leaf extension rate) were made during the first two to three decades of turfgrass breeding (Van Wijk 1993). For perennial ryegrass (*Lolium perenne* L.), Sampoux et al. (2012) recently documented that this progress had continued for varieties released after 1990. For other species there is less documentation of genetic progress over the past 25 years.

Based on results from the Norwegian/SCANTURF and GEVES testing networks, the objective of this paper is to document and discuss improvements in variety performance of the most important cool-season turfgrasses in two contrasting climatic regions during the period 1982–2012. Although data on disease resistance was not available from the Nordic region, the title of the paper reflects the increasing importance of biotic and abiotic stress tolerance in turfgrass breeding and evaluation programs.

Materials and Methods

The best way to evaluate genetic improvements by plant breeding is to compare in the same field trials the performance of varieties released over an extended period of time. To the best of our knowledge, the only study taking this precise, but expensive approach is the study by Sampoux et al. (2012) on perennial ryegrass. For the purpose of this paper, we took a different, yet commonly used approach (e.g. van Wijk 1993; Duller et al. 2010), i.e. to reexamine data from individual variety trials succeeding one another, but with the same variety/varieties included as control(s).

Data from the Nordic Region

In 1986, 1990, 1995 and 1999, publically-funded turfgrass variety trials in red fescue (*Festuca rubra* L., including three subspecies), Kentucky bluegrass (*Poa pratensis* L.) and perennial ryegrass were established at five sites in Norway. There were also a few entries of colonial bentgrass (*Agrostis capillaris* L.), hard fescue (*Festuca trachyphylla* Hack.), sheep's fescue (*Festuca ovina* L.) and tufted hairgrass (*Deschampsia cespitosa* L.), but too few to evaluate genetic progress. Varieties were rated in the sowing year plus three evaluation years, both in ordinary lawn trials with a mowing height of 3–4 cm and an annual fertilizer input of 120–180 kg ha^{-1} of N (all five sites), and in a short-cut lawn trial with a mowing height of 10–20 mm

Table 29.1 Number of entries (excluding controls) in Norwegian and SCANTURF varieties trials seeded from 1986 to 2007

	Norwegian testing					SCANTURF		
	1986	1990	1995	1999	2003	2005	2007	Total
Chewings fescue	8	15	5	6	5	9	8	56
Slender creeping red fescue	7	2	3	1	1	3	3	20
Strong creeping red fescue	6	6	8	2	3	2	1	28
Kentucky bluegrass	15	15	9	5	7	5	2	58
Perennial ryegrass	7	6	6	8	3	6	6	42
Total	43	44	31	22	19	25	20	204

(one site only). The characters used for calculation of genetic progress were overall turf quality (visual merit, scale 1–9, where 9 is best turf), tiller density (scale 1–9, where 9 is highest density), leaf fineness (scale 1–9, where 9 is finest leaves), summer color (darkness, scale 1–9 where 9 is darkest turf), height growth (cm per season) and winter damage (percent of plot area; this includes both winter diseases and abiotic winter damage). In the last round of this national program seeded in 2003, the number of test sites was reduced from five to two and the low-cut trial was sacrificed for cost reasons.

Since 2005, turfgrass testing for the five countries Finland, Sweden, Denmark, Iceland and Norway is carried out through the joint SCANTURF program. Unlike the former national programs, SCANTURF is funded entirely by entrance fees. In the test rounds starting 2005 and 2007, from which data have been used for this paper, the program included seven trial sites throughout the Nordic region. Two of the seven trials were cut at fairway mowing height (15 mm), the others at 3–4 cm. Species, turfgrass maintenance and assessments were the same as in the former Norwegian program. See www.scanturf.org for more information.

From 1986 to 2007, a total of 204 new varieties (excluding controls) of the five species/subspecies were entered into the trials (Table 29.1). The highest numbers were entered in 1986 and 1990. Unlike in most other European testing programs, the highest numbers were not of perennial ryegrass, but of chewings fescue (*F.rubra* ssp. *commutata*) and Kentucky bluegrass. This reflects the importance of winter-hardy species for the Nordic region.

Control varieties of red fescue and Kentucky bluegrass in all trials were ‘Center’ and ‘Conni’, respectively. These varieties were top-ranked in Norwegian testing from 1981 to 1985 and soon became among the most widely used turfgrass varieties in the Nordic countries. ‘Center’ was used as reference also for slender and strong creeping red fescue (*F.rubra* ssp. *litoralis* (syn. *trichophylla*) and *F.rubra* ssp. *rubra*, respectively) as these subspecies did not have any checks in the trials starting 1986. Unfortunately the same continuity in use of one specific control variety did not exist in perennial ryegrass. In the first trials, candidates were compared mainly with ‘Barclay’, later mainly with ‘Mondial’, and in the SCANTURF trials mainly with ‘Ronja’. Overlaps between control varieties in the different trials nevertheless allowed us to establish a secure reference level for perennial ryegrass.

To estimate genetic gain in various characters, we calculated relative figures with the control variety reference level as 100. These relative figures were related to the

Table 29.2 Number of entries (excluding controls) in GEVES variety trials seeded from 1982 to 2010

	1982–1985	1986–1990	1991–1995	1996–2000	2001–2005	2006–2010	Total
Perennial ryegrass	16	55	78	55	53	58	313
Kentucky bluegrass	7	27	23	9	14	7	87
Chewings fescue	4	23	18	24	16	17	102
Strong creeping red fescue	5	15	24	24	17	16	101
Slender creeping red fescue	3	17	14	13	15	10	72
Total	35	137	155	125	115	108	675

starting year of the different trials using simple linear regression. Regression slopes (=genetic progress) were presented when correlations were significant at $P < 0.05$.

Data from France

Contrary to other national authorities in Europe, GEVES maintains VCU testing as a criterion for inclusion on the French list of turfgrass varieties. For this paper we used results from VCU trials starting every year from 1982 to 2010. In 2008 the GEVES network was extended to include test sites in Spain (Mediterranean zone), Ireland (oceanic zone), Netherlands and Germany (Sub continental zone), Hungary (continental zone) and Norway (Nordic zone) (Lassalvy et al. 2012). Thus, the number of sites varied from six in 1983 to fourteen in 2010. A total 675 varieties were evaluated, almost half of them perennial ryegrass (Table 29.2).

Unlike the Nordic trials, GEVES trials were only evaluated for 2 years after the sowing year. Characters recorded were mostly the same, but there was no assessment of overall winter damage or turf height growth in the GEVES trials. In return, the GEVES protocol included persistency by the end of the each trial, tolerance to football-type wear, establishment rate (turf coverage 2 months after sowing) and tolerance to the most important turfgrass diseases. In this paper, these characters will be emphasized in order to complement data from the Nordic region. Contrary to the Norwegian/SCANTURF trials, where turfgrass height growth and winter damage were recorded in absolute terms (high value = poor performance), disease resistance in the GEVES trials was always evaluated on a scale from 1 to 9, where 9 is the most resistant turf.

The set of control varieties in the GEVES trials was not constant from 1982 to 2010 in any of the species. There were, however, always overlaps allowing us to estimate missing values for control varieties that were not included in a specific year. To estimate genetic gain in various characters, we calculated relative figures with the mean of control varieties used in the 1995 trials as 100. These varieties were chewings fescue ‘Enjoy’, slender creeping red fescue ‘Dawson’ and ‘Manoir’, strong creeping red fescue ‘Ensylva’ and ‘Pernille’, Kentuky bluegrass ‘Broadway’ and ‘Parade’, and perennial ryegrass ‘Numan’, ‘Repell’ and ‘Troubadour’.

Results and Discussion

Red Fescue

Low requirements for fertilizers, irrigation and fungicides makes red fescue an appropriate species for sustainable and Integrated Pest Management (IPM) of turfgrass areas. This is especially relevant in relation to EU directive 2009/128/EG which calls for a strong reduction or even prohibition of pesticide use in certain turfgrass areas (Strandberg et al. 2012). Major constraint limiting expanded use of red fescue has been the species' limited tolerance to heat and wear (Rummele et al. 2003). Among the three subspecies, chewings fescue has traditionally been the most popular in the Nordic countries as it represents the best combination of good winter survival, disease resistance and high density, while strong creeping red fescue has been the most widely used i southern Europe due to better color retention during summer drought.

There are conflicting results among the two test regions regarding genetic improvement in the three subspecies of red fescue. For the Nordic region (Table 29.3a) a significant increase in turf quality was achieved only in slender creeping red fescue and this was accompanied by equally significant increases in tiller density and reductions in winter damage. In 1986, varieties of slender creeping red fescue were, on average, 13 % behind chewings fescue 'Center' in turf quality, but in 2007 they were only 3 % behind.

In contrast to the results from the Nordic region, both the present results from GEVES (Table 29.3b) and those reported for the period 1975–92 (van Wijk 1993), suggest stronger improvements in turf quality and persistency for strong creeping red fescue than for the two other subspecies.

This probably reflects that different aspects contribute to persistency in climatically different regions. In the Nordic countries it is undoubtedly winterhardiness, including tolerance to pink snow mold (*Microdochium nivale*), which is in agreement with Table 29.2b showing significant improvements in this character only for the slender creeping type of red fescue. In France persistency is probably more related to tiller density, as discussed by Sampoux et al. (2012) for perennial ryegrass. The significant reduction in tolerance to red thread (*Laetisaria fuciformis*) among strong creeping red fescue varieties in the GEVES trials is probably also a reflection of the increase in tiller density of this subspecies.

Kentucky Bluegrass

Because of its winter hardiness and persistency, Kentucky bluegrass has always been a widely-used species in the Nordic region. Due to apomixis, breeding of Kentucky bluegrass is, however, more complicated and less predictable than for outcrossing grasses (e.g. Funk 2000), and the falling number of varieties submitted for

Table 29.3a Simple correlation coefficients between year of entry into variety testing and scores for turfgrass characters relative to control varieties in Norwegian/SCANTURF trials 1986–2007

Norway/SCANTURF	Visual turf quality	Tiller density	Leaf fineness	Summer color	Turf height growth	Winter damage
Chewings fescue						
Correlation	0.17 ns	0.30*	–0.14 ns	–0.10 ns	–0.29*	–0.58***
Annual change	–	0.25 %	–	–	–0.29	–5 %
Slender creeping red fescue						
Correlation	0.78***	0.79***	0.39 ns	0.37 ns	–0.44 ns	–0.74***
Annual change	0.67 %	0.76 %	–	–	–	–17 %
Strong creeping red fescue						
Correlation	0.19 ns	0.12 ns	0.07 ns	0.12 ns	–0.30 ns	–0.44**
Annual change	–	–	–	–	–	–7 %
Kentucky bluegrass						
Correlation	0.72***	0.56***	0.43***	0.16 ns	–0.54***	–0.05 ns
Annual change	0.94 %	0.64 %	0.50 %	–	–0.61 %	–
Perennial ryegrass						
Correlation	0.66***	0.68***	0.42***	0.59***	–0.58***	–0.34*
Annual change	0.64 %	0.80 %	0.53 %	0.72 %	–0.83 %	–2 %

Annual changes (%) (=slope of regression) was indicated for correlations significant at $P<0.05$

Table 29.3b Simple correlation coefficients between year of entry into variety testing and scores for turfgrass characters relative to control varieties in GEVES trials 1982–2009

GEVES	Visual turf quality	Establishment rate	Wear tolerance	Persist-ency	Toler-ance to rust	Toler-ance to *M.nivale*	Tolerance to red thread
Chewings fescue							
Correlation	0.67***	0.18 ns	0.43***	0.31**	0.26*	0.12 ns	0.25*
Annual change	0.50 %	–	0.75 %	0.17 %	0.25 %	–	0.12 %
Slender creeping red fescue							
Correlation	0.65***	0.27*	0.42***	–0.23 ns	0.11 ns	0.46***	0.22 ns
Annual change	0.52 %	0.21 %	0.85 %	–	–	0.60 %	–
Strong creeping red fescue							
Correlation	0.82***	0.57***	0.58***	0.71***	0.22*	0.01 ns	–0.40***
Annual change	0.99 %	0.59 %	1.14 %	1.01 %	0.24 %	–	–0.29 %
Kentucky bluegrass							
Correlation	0.07 ns	–0.07 ns	–0.12 ns	–0.24*	0.20 ns	–0.05 ns	–0.28 ns
Annual change	–	–	–	–0.41 %	–	–	–0.10 %
Perennial ryegrass							
Correlation	0.80***	0.61***	0.65***	0.32***	0.62***	0.47***	0.21***
Annual change	0.74 %	0.27 %	0.54 %	0.20 %	0.93 %	0.68 %	0.09 %

Annual changes (%) (=slope of regression) was indicated for correlations significant at $P<0.05$
Significance levels:***: $P<0.001$, **:$P<0.01$, *:$P<0.05$, ns: not significant

testing in the Nordic region (Table 29.1) may perhaps be taken as an indication of a shift in breeding investments to other species. For southern parts of Europe, this is also confirmed by the results from GEVES showing virtually no gain in overall turf quality and even negative trends for some of the specific characters (Table 29.3b). Another explanation for this lack of progress may be the excellent variety 'Cocktail' which was registered by GEVES in 1991 and later included as a control variety.

Unlike GEVES, Nordic testing shows improvements in turf quality, tiller density, leaf fineness and height growth of Kentucky bluegrass on level with those in perennial ryegrass (Table 29.3a). For the best varieties, these improvements seem to be more prevalent in the trials with mowing height 2 cm or lower than in the ordinary lawn trials cut at 3–4 cm. When 'Limousine' was first introduced in Norwegian variety testing in 1990, it was more or less in a class of its own, but since 2005, it has been accompanied by other high-density, fine-leaved varieties such as 'Kaitos', 'Linares' and several others. However, as suggested by the negative trend in persistency in the GEVES trials, it may be speculated that many of the newer varieties are not particularly adapted to the heat, drought and biotic stresses encountered at lower latitudes. From North America, there is evidence that even 'dwarf' varieties of Kentucky bluegrass become more susceptible to *Drecslera* leaf spot and rust if mowing heights are reduced below 2 cm (Ebdon 2008; Jordan and Lyons 2010).

Perennial Ryegrass

With a European seed consumption of 33,000 t (Sampoux et al. 2012) it is not surprising that more efforts are put into breeding of perennial ryegrass than of any other species. Numerous varieties are released every year, and both the Nordic and GEVES testing programs showed significant improvements in turf quality, tiller density, leaf fineness, height growth, wear tolerance and rust tolerance as also reported by Duller et al. (2010), Nijenstein (2010) and Sampoux et al. (2012). Unlike the other species, the Nordic trials also suggest that perennial ryegrass varieties have, on average, become darker, although this trend, inspired from North America (Thorogood 2003) is not necessarily an advantage in lawns, fairways and sports fields that tend to be invaded by light-colored annual bluegrass (*Poa annua*). More importantly, the GEVES trials suggest a significant gain in persistency and tolerance to *M.nivale*, and the Nordic trials suggest a gain in winter hardiness, although these improvements were smaller than for the aforementioned characters. In spite of indications that improved turfgrass varieties with shorter subcrown internode lengths have better frost tolerance than old varieties with longer internodes (Casler 2006), most evidence to date shows little or no improvement in the winter hardiness of perennial ryegrass (van Wijk 1993; Thorogood 2003; Sampoux et al. 2012). Winter hardiness is indeed a complex trait involving tolerance to both abiotic and biotic stresses (Thorogood 2003), but research is underway detecting quantitative trait loci (QTLs) and associated physiological mechanisms that may eventually lead to improved winter hardiness in this species (DaCosta et al. 2011; Hulke et al. 2012; Rognli 2013). A different and perhaps faster approach is the introduction of tetraploid turf type ryegrasses. Such varieties do not have the same density and leaf fineness as diploid varieties, but preliminary SCANTURF observations in spring 2013. showed the tetraploid variety 'Double' to be more tolerant to gray snow mold (*Typhula incarnata*) than its diploid counterparts.

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Chapter 30
Evaluation of Grass Species, Cultivars and Mixtures for Use in High Quality Lawn Tennis Courts

Andrew J. Newell

Abstract Tennis is a popular sport and has been played on natural grass surfaces since its inception in the 1870s. Choosing grasses for this sport was not specifically evaluated until studies were initiated for the ALL England Lawn Tennis and Croquet Club in the 1990s at STRI. Selected data from trials that informed the choice of grass species and cultivars for use in high quality lawn tennis courts are described. These showed that *Lolium perenne*, *Poa pratensis* and *Festuca rubra* (slender creeping) were most tolerant of abrasive type wear designed to simulate intense tournament play. It was also found that choice of cultivar could have a larger effect on wear tolerance than choice of species. In mixtures of grass species the selection of *L. perenne* cultivar appeared to be the dominant factor affecting overall wear tolerance. The performance of the *P. pratensis* component in grass mixtures varied in different trials from being most to least wear tolerant. Monocultures of this grass were comparable with *L. perenne* in all trials. Surface hardness measurements identified differences among species. Prior to and during wear cultivars of *L. perenne* produced a harder surface. This in part led to the decision to limit the grass selection programme to cultivars of *L. perenne*. Results from wear tests conducted in 2010 and 2011 are described. Recommendations are made for selecting cultivars of *L. perenne* for use in high quality lawn tennis courts in the UK.

Keywords Lawn tennis • Wear tolerance • *Lolium perenne* • *Poa pratensis* • *Festuca rubra* • *Agrostis* sp

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Introduction

Tennis has been played on natural grass since the first lawn tennis club was founded in 1872. There has been an international tennis tournament played on grass at Wimbledon since 1877. Choosing grass species for this specialised use was initially determined by chance rather than design. The game was first played on ornamental lawns that were also used for croquet and the fine fescue (*Festuca* spp.) and bent grasses (*Agrostis* spp.) associated with these lawns were the grasses of choice. Advice from the Board of Green keeping Research (Reconstituted as The Sports Turf Research Institute in 1951) some 50 years later continued this tradition. In advice to The Queen's Club in London recommendations were made to sow 1 part *A. tenuis* to three parts *F. rubra* (Anon 1931). At the time the specific suggestion was to use New Zealand Chewing's fescue and browntop bent. Further to this it was strongly advised that *L. perenne* should never be used.

The development of grass breeding for sports and other amenity uses has improved other species that could be considered for and have been included in seed mixtures for tennis; the main species in the UK being *L. perenne*. It is probably fair to say that breeding has changed the turf characteristics of this species more than any other species in the last 40 years. It is now widely used in seed mixtures for tennis. And, it is the only grass species purposely sown to create the tennis lawns at Wimbledon today. It is the intention of this paper to describe the process by which the species and specific cultivars of *L. perenne* were selected to create the playing surface for the largest annual sporting event in the world.

Materials and Methods

Trials to select the best grasses (species and cultivars) for high quality tennis courts in the UK were initiated at the STRI in 1993. Data from four trials sown in 1993, 1994, 1998 and 2009 are included in this paper. Trial management has previously been described for the 1993, 1994 and 1998 sown trials (Newell and Jones 1995, Newell et al. 1996, Newell and Wood 2000). All trials were sown in randomised block designs and managed to simulate a high quality tennis surface. All trials were also subjected to intense abrasive type wear for 2–3 week periods simulating Wimbledon Championship play in the year after sowing. This pattern was followed for the 2009 sown trial. However, this trial was also extended to include a second year of tennis management and intense wear. Wear was applied using the STRI differential slip wear machine fitted with abrasive rotors (Canaway 1982). The management of the 1998 and 2009 sown trials was very similar to the earlier trials. However, for the 1998 sown trial and all subsequent trials a bespoke tennis testing area was used. This was built in 1995 by removing the top 100 mm of soil from an

Table 30.1 Number of cultivars of different grass species and subspecies included in the 1993, 1994, 1998 and 2009 sown grass selection trials for tennis

	Cultivars			
Grass species	1993	1994	1998	2009
A. capillaris	4			
A. Stolonifera	1			
F. rubra(Ch)	4	3		
F. rubra(Sl)	4	4	2	
F. rubra(St)	2			
L. perenne	8	12	41	45
P. pratensis	4	5	4	
D. Cespitosa			3	

The names of different cultivars used are included in the data tables

Table 30.2 Composition of seed mixtures (percentage seed weight) evaluated in the 1993 and 1994 sown grass selection trials for tennis

		Grass species and subspecies				
Trial	Mixture	*A. capillaris*	*F. rubra* (Ch)	*F. rubra* (Sl)	*L. perenne*	*P. pratensis*
1993	M1–M2	10	15	15	60	
1993	M3–M4		40		60	
1993	M5–M6			40	60	
1993	M7–M8		20	20	60	
1993	M9–M10				70	30
1993	M11–M12				30	70
1993	M13–M14		30	30		40
1993	M15	20	40	40		
1994	M1–M2		17	17	66	
1994	M3–M5			40	60	
1994	M6–M7		40		60	
1994	M8–M10				70	30
1994	M11–M13				30	70
1994	M14–M17			30	40	30
1994	M18			60		40

area of the STRI trials grounds. This was replaced with an identical 24 % clay root zone (supplied by Surrey Loams Ltd.) to that used to construct courts at the All England Lawn Tennis and Croquet Club.

Grass species and numbers of cultivars represented from each species in each trial are shown in Table 30.1. The composition of seed mixtures in the 1994 and 1994 sown trials is given in Table 30.2.

Live grass cover measured using an optical point quadrat and visual quality, assessed on a 1 (poor) to 9 (good) scale, are used to assess variation among grass species and cultivars. Where given surface hardness measurements were made using a Clegg impact soil tester fitted with a 0.5 kg weight dropped from a height of 0.3 m (Simon Deakin Instruments). Chlorophyll index was measured using a Filedscout CM100 chlorophyll meter from Spectrum Technologies Inc.

Results and Discussion

This paper draws on previously published data (Newell and Jones 1995, Newell et al. 1996, Newell and Wood 2000) from trials sown in 1993, 1994 and 1998. Unpublished results from a more recent trial sown in 2009 are also included. It is not intended to fully review data already in the public domain. The aim is to describe the key stages followed that resulted in the criteria applied today to select grasses for high quality lawn tennis courts in the UK. The data presented largely describe the effects of intense abrasive wear on the durability and appearance of different grass treatments. Grasses selected for high profile tennis use have to withstand periods of intense wear associated with tournament play. Other characteristics such as disease tolerance, summer colour and winter discolouration will be important in selecting the best grasses. However, these should only be taken into account after grasses are shown to be sufficiently wear-tolerant to survive periods of intense play.

A summary for the main results from the 1993 sown trial are presented in Table 30.3. The destructive nature of the wear is obvious with live grass cover declining from in excess of 90 % cover to less than 10 % for some treatments in the 3 week wear period. The results show that some species and cultivars performed markedly better than others. Among the grass species *L. perenne* and *P. pratensis* appeared most wear tolerant. To a lesser degree some cultivars of *F. rubra* from the slender creeping subspecies group were also wear-tolerant. In addition to the species variation cultivar differences were found within and among the most wear tolerant species. At the other end of the scale *Agrostis* species appeared to be least wear tolerant.

This study also allowed some comparison of the performance of different mixtures of grass species. In this comparison it was apparent that there were better alternatives to the mixture in practical use at the time (M1). Indeed 13 of the 15 mixtures tested had significantly more live grass after wear. The fine grass mixture (M15), which at the time was representative of a very high quality seed mixture for golf green or ornamented lawn use performed equally poorly.

To help understand the performance of the different grass species in the mixtures their relative contributions to live grass cover have been calculated. These results, which have not previously been described, are presented in Table 30.4. In these results the relative proportions before and after wear are given along with the difference between the two. The important point to make is that the performance of different species in the mixtures followed their performance as monocultures, at least to some degree. Also there appeared to be a hierarchy of wear tolerance based around the grass species in the mixture. In all mixtures the bent grasses (*Agrostis* sp.) appeared to perform poorly. Where *F. rubra* was included in the mixture the relative proportions of this species increased relative to *Agrostis* following wear. However, *L. perenne* appeared even more wear tolerant with the relative proportions of this species increasing relative to *F. rubra* in all mixtures that the two species were included in. In terms of the performance in mixtures the most wear tolerant species in this study appeared to be *P. pratensis*. The relative proportion of this grass increased following wear relative to the other grass species tested in mixtures alongside this species.

Table 30.3 Live grass cover (%) just prior to, during and after abrasive wear simulating tennis play for different grass species, cultivars and mixtures in the 1993 sown grass selection trial for tennis

Grass	Species/subspecies	Pre wear	Wear day 11	Wear day 21
Barclay	Lp	85.8	47.0	21.8
Danilo	Lp	84.5	41.5	26.0
Elka	Lp	86.2	53.5	30.5
Lorina	Lp	89.8	54.5	33.8
Master	Lp	84.8	42.0	20.0
Meteor	Lp	86.0	43.5	26.2
Superstar	Lp	82.5	36.0	20.8
Troubadour	Lp	79.8	46.0	27.0
Cocktail	Pp	91.5	58.5	37.0
Julia	Pp	90.0	57.0	30.2
Limousine	Pp	86.2	61.0	31.0
Rocardo	Pp	85.5	39.5	16.5
Egmont	Ac	88.8	5.0	2.8
Highland	Ac	81.0	2.5	1.8
Lance	Ac	92.5	12.5	6.8
Sefton	Ac	90.8	9.0	4.8
Penncross	As	89.5	14.0	7.8
Bargreen	Fr-ch	80.5	26.5	8.0
Bingo	Fr-ch	75.0	20.5	9.8
Center	Fr-ch	82.8	21.0	8.8
Waldorf	Fr-ch	80.2	13.0	6.0
Barcrown	Fr-sl	93.8	41.0	21.2
Jupiter	Fr-sl	89.2	33.5	15.8
Oriflamme	Fr-sl	91.0	23.5	9.0
Smirna	Fr-sl	90.8	29.5	16.2
Cindy	Fr-st	77.5	20.0	4.5
Hollywood	Fr-st	82.5	16.5	8.2
M1*	Lp/Fr-ch/Fr-sl/Ac	88.8	22.5	11.8
M2	Lp/Fr-ch/Fr-sl/Ac	90.0	41.5	22.8
M3	Lp/Fr-ch	88.2	41.5	18.2
M4	Lp/Fr-ch	88.2	41.5	28.0
M5	Lp/Fr-sl	90.0	47.5	24.8
M6	Lp/Fr-sl	88.0	44.5	26.8
M7	Lp/Fr-ch/Fr-sl	86.2	42.0	24.2
M8	Lp/Fr-ch/Fr-sl	91.2	43.5	27.8
M9	Lp/Pp	91.2	47.0	25.8
M10	Lp/Pp	88.5	53.5	32.5
M11	Lp/Pp	88.2	57.5	33.0
M12	Lp/Pp	91.0	56.5	33.5
M13	Pp/Fr-ch/Fr-sl	90.2	37.0	26.0
M14	Pp/Fr-ch/Fr-sl	91.0	50.5	30.5
M15	Ac/Fr-ch/Fr-sl	91.8	21.0	10.5
LSD		5.9	11.1	5.9

*Wimbledon seed mixture

Table 30.4 Proportions of different species sown (percentage seed weight) and species proportions as a percentage of live grass cover before and after abrasive wear for seed mixtures in the 1993 sown grass selection trial for tennis

	Sowing proportion				Before wear				After wear				Difference			
Mixture	Lp	Fr	A sp	Pp	Lp	Fr	A sp	Pp	Lp	Fr	A sp	Pp	Lp	Fr	A sp	Pp
M1	60	30	10		52	7	42	0	94	0	6	0	42	−7	−35	0
M2	60	30	10		61	14	25	0	88	10	2	0	27	−4	−23	0
M3	60	40			72	28	0	0	82	18	0	0	10	−10	0	0
M4	60	40			80	20	0	0	89	11	0	0	9	−9	0	0
M5	60	40			69	31	0	0	81	19	0	0	12	−12	0	0
M6	60	40			81	19	0	0	93	7	0	0	12	−12	0	0
M7	60	40			75	25	0	0	87	13	0	0	12	−12	0	0
M8	60	40			80	20	0	0	92	8	0	0	12	−12	0	0
M9	70			30	83	0	0	17	74	0	0	26	−9	0	0	9
M10	70			30	77	0	0	23	58	0	0	42	−19	0	0	19
M11	30			70	65	0	0	35	62	0	0	38	−3	0	0	3
M12	30			70	61	0	0	39	50	0	0	50	−11	0	0	11
M13		60		40	0	71	0	29	0	40	0	60	0	−31	0	31
M14		60		40	0	31	0	69	0	12	0	88	0	−19	0	19
M15		80	20		0	38	62	0	0	64	36	0	0	26	−26	0

The change following wear (Difference) is calculated as the proportion after wear minus the proportion before wear

The important point to make following the first trial was that choice of grass species and cultivars could markedly influence the performance and appearance of natural grass tennis surfaces.

A second trial was sown in 1994. A subset of results, showing the effects of wear on live grass cover, from this study, are presented in Table 30.5. This work again showed that species and cultivar selection was very important. However, apart from the three *F. rubra* cultivars from the chewing fescue group, choice of cultivar was as important as species choice. The ranges for the *L. perenne*, *P. pratensis* and *F. rubra* (slender creeping) cultivars overlapped, with marked significant differences within and among the species groups.

Table 30.5 Live grass cover (%) just prior to, during and after abrasive wear simulating tennis play for different grass species, cultivars and mixtures in the 1994 sown grass selection trial for tennis

Grass	Species	Pre wear	Day 11	Day 22
Barclay	Lp	67.8	25.0	13.5
Barlinda	Lp	70.8	19.0	13.0
Danilo	Lp	73.0	29.8	12.8
Elegana	Lp	75.8	26.5	15.0
Elka	Lp	77.8	35.2	20.5
Lorina	Lp	72.5	34.0	23.5
Merci	Lp	66.8	30.5	20.0
Meteor	Lp	76.2	31.2	15.8
Queens	Lp	68.8	28.5	15.0
Rambo	Lp	75.2	22.5	11.8
Superstar	Lp	68.5	25.5	11.0
Troubadour	Lp	71.0	28.0	14.5
Barvictor	Pp	89.5	38.2	20.2
Cocktail	Pp	93.5	45.8	24.2
Julia	Pp	75.8	39.8	17.2
Limousine	Pp	79.0	47.5	21.5
Miricle	Pp	90.5	55.5	22.5
Barcrown	Fr-sl	92.8	29.0	18.8
Jupiter	Fr-sl	83.8	29.0	13.0
Mocassin	Fr-sl	94.5	28.2	19.0
Smirna	Fr-sl	89.2	25.8	14.8
Center	Fr-ch	78.8	16.8	8.2
Lobi	Fr-ch	76.8	18.5	8.8
Nimrod	Fr-ch	83.0	20.5	9.8
M1*	Lp/Fr-sl/Fr-ch	77.0	28.5	10.5
M2	Lp/Fr-sl/Fr-ch	79.5	32.0	22.8
M3	Lp/Fr-sl	85.2	34.0	20.8
M4	Lp/Fr-sl	84.2	42.8	25.0
M5	Lp/Fr-sl	79.8	28.0	18.8

(continued)

Table 30.5 (continued)

Grass	Species	Pre wear	Day 11	Day 22
M6	Lp/Fr-ch	78.2	29.8	19.8
M7	Lp/Fr-ch	74.5	32.2	15.8
M8	Lp/Pp	70.5	31.8	14.0
M9	Lp/Pp	70.2	36.5	20.8
M10	Lp/Pp	82.5	31.0	21.0
M11	Lp/Pp	71.0	37.0	15.8
M12	Lp/Pp	77.2	31.8	18.5
M13	Lp/Pp	78.2	43.0	22.2
M14	Lp/Fr-sl/Pp	71.8	21.8	16.8
M15	Lp/Fr-sl/Pp	77.0	30.2	19.2
M16	Lp/Fr-sl/Pp	81.2	37.5	20.8
M17	Lp/Fr-sl/Pp	79.0	32.2	15.5
M18	Fr-sl/Pp	94.5	39.8	22.5
LSD		9.1	8.8	5.3

[*] Wimbledon seed mixture

The mixtures tested also demonstrated that the commercial seed mixture being used in 1994 (M1) could be improved. This was replaced with mixture M4 following the trial. This mixture was a 60:40 blend of Lorina (*L. perenne*) and Barcrown (*F. rubra*). Like the first trial the performance of the different grass species in the mixtures can be compared. Relative proportions of different grass species in the mixtures before and after wear have been calculated for this paper (Table 30.6). These results contrasted somewhat with the previous 1993 sown study. Overall, *L. perenne* appeared to survive better (relative proportion of live grass cover increased) than the other grass species that it was tested alongside in the different mixtures. The relative *P. pratensis* composition declined in the majority of mixtures following wear. This was despite comparable performance with other grass species when sown as a monoculture. One of the differences between trials that may have contributed to this finding was the time of sowing. The 1993 trial was sown in mid-August, whereas the 1994 trial was sown in mid-September. It should be noted here that the main renovation period for lawn tennis courts in the UK would start in September after the season finishes (normally mid-September).

Selected results from the 1998 sown trial showing measurements made just prior to, during and at the end of the wear period area are presented in Table 30.7. The emphasis for this trial changed somewhat with the principal aim being to identify improved cultivars of *L. perenne*. Breeding for the grass species was moving at a much faster pace at the time (as it still is today) than for other amenity species, with large numbers of new cultivars being released. It was also apparent from the previous studies that this grass species would tend to dominate when included in mixtures with other grasses.

Table 30.6 Proportions of different species sown (percentage seed weight) and species proportions as a percentage of live grass cover before and after abrasive wear for seed mixtures in the 1994 sown grass selection trial for tennis

	Sowing proportions			Before wear			After wear			Difference		
Mixture	Lp	Fr	Pp	Lp	Fr	Pp	Lp	Fr	Pp	Lp	Fr	Pp
M1*	66	34		81	19	0	82	18	0	2	−2	0
M2	66	34		74	26	0	82	18	0	7	−7	0
M3	60	40		70	30	0	68	32	0	−2	2	0
M4	60	40		69	31	0	78	22	0	9	−9	0
M5	60	40		74	26	0	73	26	1	−1	0	1
M6	60	40		88	12	0	87	13	0	−1	1	0
M7	60	40		81	19	0	89	11	0	8	−8	0
M8	70		30	85	0	15	90	1	9	5	1	−7
M9	70		30	73	0	27	80	0	20	7	0	−7
M10	70		30	91	0	9	94	0	6	3	0	−3
M11	30		70	63	0	37	76	0	24	13	0	−13
M12	30		70	44	0	56	64	0	36	20	0	−20
M13	30		70	55	0	45	79	0	21	24	0	−24
M14	40	30	30	70	23	8	61	28	10	−8	6	3
M15	40	30	30	71	22	7	73	24	3	2	2	−4
M16	40	30	30	56	25	19	65	24	11	9	−1	−9
M17	40	30	30	64	22	14	76	16	9	12	−7	−5
M18		60	40	0	72	28	1	82	18	1	9	−10

The change following wear (Difference) is calculated as the proportion after wear minus the proportion before wear

*Wimbledon seed mixture

Table 30.7 Live grass cover (%) just prior to, during and after abrasive wear simulating tennis play for different grass species, cultivars and mixtures in the 1998 sown grass selection trial for tennis

		Pre-wear			Day 8			Day 21	
Grass	Species	Live grass	VM	Hardness	Live grass	VM	Hardness	Live grass	VM
Aberelf	Lp	69	5.9	221	33	5.8	224	24	5.2
Aberimp	Lp	73	6.1	188	34	6.0	228	21	4.5
Ace	Lp	64	4.9	222	31	5.0	218	20	4.0
Advent	Lp	72	4.0	259	32	3.5	253	18	3.0
Amadeus	Lp	74	3.8	245	37	4.5	224	19	3.0
Aphrodite	Lp	72	5.0	223	37	4.5	215	19	4.0
BA 11972	Lp	67	5.9	188	32	5.2	232	17	4.0
BA 13207	Lp	72	3.1	222	33	3.8	204	20	2.2
Ballet	Lp	75	5.5	225	34	5.5	221	33	5.5
BAR ER 6628	Lp	72	4.5	243	40	5.2	226	24	3.5
BAR ER 6738	Lp	75	5.5	226	36	5.5	225	24	4.5
BAR ER 7711	Lp	73	4.8	225	33	5.0	203	22	4.0

(continued)

Table 30.7 (continued)

Grass	Species	Pre-wear			Day 8			Day 21	
		Live grass	VM	Hardness	Live grass	VM	Hardness	Live grass	VM
BAR ER 7825	Lp	75	5.6	228	29	4.5	233	14	3.2
BAR ER 7841	Lp	79	6.6	222	42	6.5	234	30	5.8
BAR USA 94-11	Lp	66	3.0	220	28	3.0	238	15	2.0
Bellevue	Lp	70	5.2	242	35	4.8	230	22	3.2
Brightstar	Lp	69	4.2	227	35	4.0	241	17	3.0
Dali	Lp	75	4.5	225	37	4.5	230	25	3.5
Dancer	Lp	70	4.4	231	33	3.5	209	22	2.8
Darius	Lp	71	6.0	217	36	5.8	227	25	5.2
Disco	Lp	71	4.9	231	33	4.8	222	23	3.5
DPL 9603	Lp	70	5.0	222	36	4.2	232	18	3.2
Eden	Lp	60	2.1	227	27	2.5	203	16	1.5
Elegana	Lp	76	5.1	218	36	5.5	232	24	3.8
Elka	Lp	65	4.1	246	28	4.2	204	19	3.5
Envy	Lp	72	3.2	231	33	4.2	227	19	2.8
Evita	Lp	72	6.4	198	40	5.8	224	28	5.0
Fragment	Lp	69	4.4	226	33	4.5	221	22	3.0
Garant	Lp	64	5.0	240	33	4.2	213	18	3.0
JPR 277/4	Lp	69	4.2	269	32	4.0	237	20	3.5
Lex 86	Lp	73	3.0	250	33	3.5	228	20	3.2
Livonne	Lp	73	5.4	240	31	5.2	224	21	4.8
Lorina	Lp	74	5.0	223	40	6.0	242	29	4.8
LPR 96086	Lp	71	4.2	209	33	5.0	228	20	3.5
Merci	Lp	69	5.0	238	39	5.5	221	29	4.2
Mom Lp 3276	Lp	67	4.4	226	34	5.0	216	19	4.0
Plaisir	Lp	71	5.1	234	33	4.8	221	19	3.5
Rambo	Lp	69	4.4	234	31	4.0	207	11	2.2
Ritmo	Lp	79	5.9	223	37	6.2	231	25	4.5
Titus	Lp	74	6.0	213	34	5.5	235	21	4.0
Vienna	Lp	70	4.5	252	37	5.0	226	24	4.5
Barcelona	Pp	70	3.9	172	35	5.8	161	14	4.8
Cocktail	Pp	71	3.8	188	34	5.2	191	18	4.8
Limousine	Pp	72	3.9	163	35	6.2	174	23	4.8
Unique	Pp	69	3.1	187	26	3.5	188	10	2.5
Barcrown	Fr	81	4.9	179	34	5.0	165	21	5.0
Mocassin	Fr	70	4.2	180	32	5.2	155	15	4.0
BAR DC 710-2	Dc	56	4.1	155	22	4.0	157	9	2.5
BAR DC 980	Dc	69	4.1	180	25	4.2	163	10	2.0
SR 6000	Dc	73	4.1	122	30	5.2	140	18	3.0
LSD		n.s.	1.0	29.0	7.8	1.2	29.0	9.1	1.3

Visual merit scores (VM; 1 = poor to 9 = good) and surface hardness (gravities) are also given

Selecting the best cultivars of *L. perenne* appeared to be the principal factor affecting the performance of different seed mixtures. The 1998 study also included limited numbers of *P. pratensis* and *F. rubra* cultivars, most of which had been tested previously. Three cultivars of *Deschampsia cespitosa* were also included. At the time this species was being promoted as a new grass type for use in a range of sports and general amenity situations.

This work again demonstrated significant variation in wear tolerance among the grasses tested. However, we also introduced a measurement of surface hardness which showed that choice of species could influence this important characteristic. At the time it was desirable to find ways of increasing surface hardness and associated with this ball bounce, without compromising the durability or appearance of the surface. These results clearly demonstrated that it was possible to get a firmer surface from *L. perenne* than the other species tested without compromising on durability or appearance if the better cultivars were selected. This was somewhat of a "game changer" and meant that going forward the sole aim was to identify improved cultivars of *L. perenne*. Practically courts at Wimbledon were also converted to pure *L. perenne* surfaces.

A further point of value is to note the correlation between objective live grass cover measurements and subjective visual assessments. During and at the end of the wear period the two assessments were significantly and positively correlated ($r = 0.60$ and 0.68 respectively). It is also of value to mention that in a statistical sense it was easier to separate the visual assessments into more distinct groups.

Bringing things up to date, data from the most recently completed grass selection trial for tennis are included in Tables 30.8 and 30.9. This study continued to evaluate new cultivars of *L. perenne* alongside good cultivars identified in previous trials. Visual merit scores, chlorophyll index measurement and surface hardness values are presented for measurements made just prior to, during and towards the end of wear applied in the summer of 2010 and 2011. The chlorophyll index is a quick method for objectively measuring surface greenness. The values obtained were strongly correlated with the visual merit scores for measurements made during and at the end of the wear periods. At the end of the wear periods the correlation coefficient between these two assessments was 0.81 in 2010 and 0.70 in 2011.

In the context of surface properties and appearance it is appropriate to note that there was a negative association between hardness and visual merit for cultivars of *L. perenne* after wear had been applied. The correlation coefficient between visual merit and surface hardness was −0.58 in 2010 and −0.70 in 2011. This should not be confused with findings from the 1998 trial where cultivars *L. perenne* produced a harder surface than other grass species prior to and during wear. What this demonstrates is that soils dry out quicker and as a consequence become harder if the grass cover is worn away.

Results from the 2009 sown trial have been used to draw up a preferred list of *L. perenne* cultivars for use in lawn tennis courts in the UK. This list in alphabetical order is Aberimp, Barsignum, Carnac, Chardin, Cleopatra, Ennyo, Malibu,

Table 30.8 Visual merit scores (VM; 1 = poor to 9 = good), chlorophyll index and surface hardness (gravities) just prior to, during and after simulated tennis wear for different cultivars of perennial ryegrass in the 2009 sown grass selection trial for tennis

	Pre wear			Day 6			Day 11		
Name	VM	C-Index	Hardness	VM	C-Index	Hardness	VM	C-Index	Hardness
Aberimp	7.0	261	127	6.3	180	167	6.0	133	158
Abersprite	6.8	228	128	5.3	163	176	6.3	127	166
Ace	4.8	217	138	4.3	153	195	4.8	125	185
Barfloyd	4.8	225	143	3.8	154	207	3.3	124	207
Barlady	5.5	220	137	4.8	149	180	5.5	123	184
Barsignum	6.5	246	132	5.5	166	186	5.8	134	177
Brightstar	1.8	213	136	2.3	148	192	2.3	119	209
Cachemire	3.8	217	137	3.3	145	185	2.8	116	190
Carnac	5.0	219	130	4.5	158	187	3.8	124	191
Cassiopeia	5.5	223	136	4.3	154	192	4.3	123	187
Citation Fore	2.3	215	134	2.3	151	205	2.3	118	213
Cleopatra	6.3	242	127	5.5	169	178	5.8	130	184
Conrad 1	5.5	215	134	4.5	160	181	4.5	125	189
Corelli 1	6.0	230	136	4.5	160	194	5.0	122	187
Dickens 1	5.3	220	137	4.5	150	186	4.3	128	196
Dylan	6.5	242	134	4.8	162	180	5.3	126	178
Ennyo	6.5	235	132	5.5	165	178	5.0	129	181
Escapade	5.5	220	136	4.0	155	180	3.3	119	188
Eurodiamond	4.3	218	136	3.8	152	192	4.0	122	183
Evita	5.3	213	131	3.5	158	185	4.0	122	203
Flamenco	5.5	239	134	4.8	163	186	4.5	126	187
Funk	5.5	219	134	4.0	149	195	3.3	116	185
Gray Fox	2.3	221	135	2.0	152	206	2.0	116	209
Greencup	5.0	227	141	3.5	158	207	4.0	120	215

Greenflash	5.0	250	138	4.8	175	189	5.5	129	185
Greensky	5.5	229	138	3.5	159	179	3.8	126	181
Greenway	5.3	226	136	4.3	155	191	4.5	130	194
Himalaya	5.3	226	136	4.5	153	184	5.0	123	187
Jessica	5.8	232	135	4.8	159	176	6.0	129	179
Libero	6.0	240	134	4.8	162	184	4.0	127	180
Ligala	5.8	218	137	5.0	151	191	4.5	120	194
Lourega	4.3	241	132	3.0	164	179	2.5	121	187
Madrid	5.5	229	136	4.8	155	193	4.3	127	182
Malibu	6.0	242	135	4.5	162	184	4.8	125	188
Matisse	6.8	229	127	5.8	162	176	5.5	128	169
Melbourne	5.8	230	134	4.5	160	180	5.3	125	193
Merci	3.5	225	137	4.0	160	197	5.5	124	198
Mercitwo	6.5	251	129	6.3	173	164	6.5	138	175
Monaco	4.8	203	138	3.0	142	196	3.0	117	192
Pontiac	4.8	234	136	5.0	159	190	5.8	130	190
Sirtaky	4.8	203	138	3.8	148	196	2.3	117	193
Venice	6.0	226	138	5.0	163	192	5.8	131	193
Vesuvius	2.0	216	124	3.8	154	174	4.0	122	168
Wimbledon	4.5	221	137	4.3	152	179	5.0	124	192
Zebu	5.5	239	135	5.0	168	188	6.0	129	184
LSD	1.60	23.4	7.2	1.11	14.3	15.4	1.16	9.0	15.6

The measurements shown were made during the summer of 2010

Table 30.9 Visual merit scores (VM; 1 = poor to 9 = good), chlorophyll index and surface hardness (gravities) just prior to, during and after simulated tennis wear for different cultivars of perennial ryegrass in the 2009 sown grass selection trial for tennis

	Pre wear			Day 6			Day 11		
Name	VM	C-Index	Hardness	VM	C-Index	Hardness	VM	C-Index	Hardness
Aberimp	5.5	228	128	5.0	156	132	4.8	110	262
Abersprite	5.3	213	130	4.5	147	137	4.0	104	264
Ace	5.0	210	145	4.8	148	142	3.8	105	282
Barfloyd	4.8	228	143	2.8	146	141	1.8	99	299
Barlady	4.5	212	143	3.8	144	146	3.3	102	302
Barsignum	5.5	223	140	5.5	153	136	5.0	103	274
Brightstar	2.5	217	141	2.3	144	143	2.0	101	305
Cachemire	5.0	215	136	3.5	137	141	3.0	100	297
Carnac	6.5	229	133	5.8	156	135	6.0	106	263
Cassiopeia	5.5	224	141	5.0	148	143	4.3	107	282
Citation Fore	2.8	233	141	2.8	151	140	2.5	103	309
Cleopatra	6.3	226	134	5.0	155	140	6.0	107	262
Conrad 1	5.8	217	144	5.0	142	143	4.0	99	299
Corelli 1	6.5	220	141	5.3	151	142	4.3	106	298
Dickens 1	5.8	212	144	4.5	146	138	4.8	105	284
Dylan	6.3	225	135	4.3	147	140	4.0	106	272
Ennyo	5.8	220	143	5.3	148	138	4.5	104	286
Escapade	5.5	231	142	4.0	145	143	3.5	101	301
Eurodiamond	4.5	228	147	3.5	145	140	3.8	100	291
Evita	6.0	214	143	4.5	144	139	3.8	104	287
Flamenco	5.3	229	143	3.8	143	140	3.5	101	279
Funk	5.3	219	140	4.0	142	141	3.3	100	283
Gray Fox	3.0	234	143	2.5	146	143	2.3	101	316
Greencup	5.3	221	145	4.3	146	144	3.0	101	276

Greenflash	3.8	225	144	4.3	148	141	3.5	102	283
Greensky	5.5	222	139	4.3	144	138	3.5	102	280
Greenway	5.3	226	142	4.8	149	142	3.8	100	291
Himalaya	5.0	217	136	4.0	145	142	4.0	106	276
Jessica	4.8	209	141	3.8	144	139	5.0	109	276
Libero	4.8	231	145	3.3	149	141	2.5	108	286
Ligala	5.5	229	142	4.3	147	139	3.3	107	305
Lourega	3.3	219	141	2.8	143	138	1.5	100	297
Madrid	3.5	207	141	2.5	135	143	1.8	95	303
Malibu	5.8	229	143	5.5	153	142	4.8	107	278
Matisse	6.8	223	138	5.5	145	140	4.5	110	277
Melbourne	6.3	220	136	5.3	149	143	4.8	101	285
Merci	3.8	209	143	3.5	142	142	3.3	104	294
Mercitwo	5.8	223	131	5.3	156	138	4.3	107	267
Monaco	5.8	223	146	4.0	142	141	3.3	99	308
Pontiac	5.5	237	140	5.8	162	143	6.5	115	262
Sirtaky	6.0	219	143	3.8	139	142	3.0	100	296
Venice	6.5	233	138	5.8	149	140	5.5	108	293
Vesuvius	3.8	217	133	3.8	139	133	3.3	98	283
Wimbledon	4.3	219	139	4.3	145	142	3.8	106	292
Zebu	6.0	225	137	5.5	155	142	6.0	113	271
LSD	1.29	16.4	8.7	1.22	10.8	5.5	1.46	7.8	25.7

The measurements shown were made during the summer of 2011

Melbourne, Mercitwo, Pontiac and Venice. We advise that mixtures of two or three cultivars are selected from the list to be used in practice and that characteristics such as seed purity, germination, disease tolerance and colour are taken into account before a final decision is made.

Work is continuing to identify new improved cultivars at *Lolium perenne* to replace grasses in the current preferred list.

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Part V

New Biotechnology Methods in Sustainable Breeding: Strategies and Implementation

Chapter 31
Characterisation of Genetic Diversity for Resistance and Quality Traits Using Molecular Tools

Hilde Muylle, Susanne Barth, and Tom Ruttink

Abstract Several decades of molecular studies in forage crops like *Lolium* revealed to some extent the genetic control of traits such as crown rust resistance, flowering time, vernalisation response and water soluble carbohydrates. The first studies focused on only a very limited genetic diversity, up to a dozen of alleles in a series of diverse linkage mapping populations and identified major genes or QTLs with a major effect. In more complex traits such as dry matter yield (DMY) and fodder quality where multiple physiological processes contribute to the phenotype, a greater number of genes are involved and the environment often has a masking effect on the genetic component of the trait. Unravelling the genetic control of these complex traits requires the use of populations with more genetic diversity and with shorter linkage disequilibrium (LD). It further requires the development of high resolution phenotyping methods and molecular tools to discover and screen a wider range of genetic diversity. The different research strategies used in these highly heterozygous cross-pollinating species shifted from constructing mapping populations with a very restricted number of alleles towards association mapping in a diverse collection of germplasm, or linkage mapping in highly structured and interlinked populations. With recent developments in high-throughput genotyping, phenotyping and advanced statistical approaches, more genetic diversity can be exploited to understand the genetic control of agronomic important complex traits for highly heterozygous and genetically heterogeneous species such as *Lolium*. This shift towards high resolution molecular studies on complex traits using wide genetic diversity opens new strategies for innovative breeding in forage crops.

Keywords *Lolium perenne* • Genetic diversity • Resistance • Quality traits • Molecular tools

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Introduction

Several studies have analysed the progress in *Lolium* breeding over the past decades (Sampoux et al. 2011; Chaves et al. 2009). Improvements in total dry matter yield (DMY) and in disease resistance were 3 and 11 % per decade, respectively, and aftermath heading has been greatly reduced. PCA analysis of the phenotypic trait variation in 21 cultivars that have been released in the past four decades (Sampoux et al. 2011) shows that improvements of the cultivars are positively correlated with higher water soluble carbohydrate (WSC) content, in vitro neutral detergent fibre digestibility (IVNDFD), and in vitro dry matter digestibility (IVDMD). Furthermore, improvement in cultivars is negatively correlated with lignin and neutral detergent fibre (NDF) content, spike emergence and aftermath heading. A positive correlation is observed between improved spring and summer DMY, early spring vigour, leaf and lamina length, while these are negatively correlated with tiller number (Sampoux et al. 2011).

Heritability

Heritability accounts for the proportion of observable differences in a trait between individuals within a population that is due to genetic differences. The bigger the influence of the genotype and the less the influence of the environment on a trait, the more heritable is such a trait. Studying genetic diversity of a trait and genetic selection for traits requires sufficient heritability of the traits in order to be able to identify causative underlying genetic factors, quantitative loci and genes. Phenotypes with a high heritability, e.g. plant height (Alves de Araújo and Coulman 2004) and heading date (Armstead et al. 2004), have been the predominant phenotypes in more detailed molecular genetic analysis. Usually forage species are grown in swards and their phenotypes grown in single spaced or potted plants are not comparable to their phenotypes as grown in sward conditions (Conaghan and Casler 2011). Only for very few traits like heading date data derived from spaced plants and plants grown in swards are comparable. DMY is one of the most important traits of perennial ryegrass and is measured in nearly every cultivar evaluation trial (Conaghan and Casler 2011). Estimates of narrow-sense heritability in sward plots for dry matter yield are highly variable but typically low to moderate (0.20–0.50) (Frandsen 1986; Jafari 1998). Dry matter yield is subject to substantial genotype-environment (G × E) interaction (Conaghan et al. 2008). Traits with higher heritability are e.g. leaf width (Barre et al. 2009).

Trait-Locus Association Studies

To aid *Lolium* breeding, much efforts were invested in the hunt for the genetic factors controlling agronomically important traits. Most studies that aim to identify genetic polymorphisms leading to phenotypic variation have been based on forward genetics: it starts with analysing the phenotypic variation in a given population and identifies statistically significant associations with genetic polymorphism at one or more genetic loci by QTL or association mapping. The genetic polymorphisms used for locus-trait associations can be anonymous markers that are genetically linked to the causative loci due to LD, or in a more targeted approach, they can be particular alleles identified in candidate genes.

Early trait-locus association studies focused on linkage mapping populations with or without known parents or grandparents in a range of segregating population structures. Parental genotypes were chosen with contrasting phenotypic expression of agronomic traits (e.g. susceptible x resistant genotype, late x early heading). For a given locus, a maximum of four alleles could be studied and in a two-way pseudo-testcross design, only four allelic combinations can be studied. In outbreeding species only a smaller proportion of alleles is informative compared to a segregating F2 population with inbred parental genotypes. Initial population sizes for mapping studies have often been rather small. Small population sizes were used to produce initial genetic maps and gave only a rough idea on the position and size effects of QTL for certain traits in a given mapping population. Since recombination rates differ in different genetic backgrounds and genomic regions, it is also difficult to predict how large populations need to be to facilitate detailed QTL mapping and positional cloning. A recent review of Shinozuka et al. (2012), summarized that 560 QTLs have been reported for a range of traits, with 60 % of the QTLs explaining less than 15 % of phenotypic variance. For pathogen resistance, a metaQTL analysis on three mapping populations revealed consistent QTLs on LG2 and LG6. These QTLs cover regions with 5.9 % up to 15.3 % phenotypic variance explained. With the aim to exploit genetic diversity in ryegrass breeding by means of molecular tools, it was necessary to confirm identified QTL in different backgrounds or mapping populations. To exploit this knowledge in synthetic varieties, which are artificial populations created by free pollination of a varying number of parental genotypes, more knowledge is needed on the allelic diversity of these loci and the percentage phenotypic variance explained and the mode of action of each allele. Subsequently, marker assisted selection can be used to control the frequency of desired alleles in a variety.

To identify trait-associated loci and the allelic diversity of these loci, various approaches have been reported. Association mapping studies in a highly unstructured population using collections of wild accessions have revealed the allelic diversity of loci and trait allele association for e.g. heading date (Skøt et al. 2011). Given the high LD decay in unstructured populations, Auzanneau et al. (2011) used a more structured population to identify alleles associated with leaf elongation. Furthermore, using connected populations as described by Pauly et al. (2012) enables to study more alleles, exploiting lower LD decay than in unstructured populations. The identified regions are still too large to identify the causal gene/locus within the region, but it offers the opportunity to identify with a limited number of markers, a higher number of associated loci than in linkage mapping populations (too few alleles studied) and unstructured AM populations (high LD decay). Brazauskas et al. (2013) exploited a divergent selection process to identify trait-allele associations for crown rust resistance. A polycross of five parental clones was subjected to two rounds of divergent selection (resistant versus susceptible) and differences in allele frequencies at given loci were studied between the contrasting divergent selections.

Higher Resolution Phenotyping Through Decomposition of Agronomic Traits to Plant Traits

Many traits are the result of the combined activity of several underlying biological processes, and therefore can be considered as 'composite traits'. By taking into account confounding effects, a composite phenotype may be partitioned into relevant sub-characters. For instance, in order to analyse quality traits during the course of plant development, variation in dry matter digestibility can be split into the effects of chemical composition, plant architecture, or plant developmental stage. Hence, through dedicated phenotyping of these sub-traits, the contribution of each individual sub-trait (e.g. lignin quantity and composition, WSC, leaf to stem ratio, leaf length, harvest time relative to heading date) can be estimated.

Given a certain heritability, each sub-trait (e.g. lignin content, tiller number) is controlled by a minimum number of genetic loci involved in this biological process (e.g. lignification, axillary meristem development) and locus-trait associations should identify the underlying genes controlling the activity of that biological process as well as possible (anti-)correlations between the biological processes underlying a composite trait.

Studies on the genetic control of disease resistance in ryegrasses were mainly based on phenotyping using a categorical scale, from 0 to 6 for crown rust (Muylle et al. 2005; Brazauskas et al. 2013) or from 0 to 4 for gray leaf spot (Curley et al. 2005) and using spore mixtures. Scoring traits as categorical data is a rapid and efficient method and often the only feasible approach in large scale phenotyping projects in terms of human and instrumental resources. However, refining the phenotyping by using single strains and looking at various sub-traits of disease resistance will result in more continuous phenotyping data. Continuous data clearly cover a broader phenotypic range and resolution compared to categorical data and therefore enhance the resolution of the trait-locus associations, and may be required to detect small and additive effects underlying composite traits.

The calculation of statistically significant associations between genotypic and phenotypic traits requires phenotyping of large numbers of individuals in order to cover a broad phenotypic diversity, and replication of experiments within and across environments to unravel GxE interactions. In addition, a given phenotypic character may be linked to a developmental plant stage. For example, lignification differs between young or mature leaf or flowering stem tissues (Tu et al. 2010), hence the plant organ composition is a major determinant of biomass digestibility. In this case, accurate phenotyping may require a time series with repeated observations throughout the season to monitor the temporal dynamics of the process, and to discriminate between inherent genotype-specific digestibility and genotype-dependent differences in plant development. Moreover, in perennial crops, observations across years are relevant for the evaluation of temporal changes in agronomic traits during prolonged production periods in the field. For instance, production in the year of establishment may differ from subsequent years. So-called staggered start designs help to dissect environmental effects and age-of-crop on the field (Loughin 2006), but require at least a duplication of the experiment in subsequent years. Therefore, the precision of phenotyping, and hence trait-locus associations, is restricted by the cost and time needed per phenotypic observation, and is dependent on the availability of human resources and equipment.

Because of the large numbers of observations, the automation of phenotyping is desirable. At the field scale, forage quality parameters like dry matter digestibility, WSC and crude protein content have been captured with a mounted near infrared reflectance spectroscopy (NIRS) device (Xiong et al. 2007). Quantification of specific components such as fatty acid composition may progress towards global metabolic profiling combined with QTL analysis, also known as genetical metabolomics. Genetical metabolomics of flavonoid biosynthesis has been demonstrated in poplar (Morreel et al. 2006) and is gaining attention in other crops. Riedelsheimer et al. (2012) performed genome-wide association mapping using 56,110 SNPs and 118 biochemical compounds including lignin precursors from the leaves of young plants, as well as agronomic traits of mature plants in field trials, in a population of 289 diverse maize inbred lines. For 26 metabolites strong correlations with SNPs were identified that together explained 32 % of the observed genetic variance.

Also, whole plant imaging technologies have been developed to phenotype complex traits like plant architecture and photosynthesis efficiency in the field, as reviewed by Walter et al. (2012). An array of automated imaging methods for phenotyping purposes is becoming available. At present they are still most successful in laboratory and greenhouse environments (Walter et al. 2012), however they have also been proven successful in open field on a medium throughput scale (Saracutu et al. 2010). Moving from ‘low resolution’ phenotyping of composite traits to high resolution phenotyping of sub-traits resulting from underlying biological processes will lead to a better understanding of the genetic control of these complex traits. In addition, it is expected that suppressing confounding effects through dedicated phenotyping will increase the percentage of phenotypic variance explained.

From QTL to Candidate Genes

Once an initial QTL mapping study has been conducted and QTL with reasonable LOD scores and thus associated certainties have been detected, further fine mapping of the QTL regions is required, often associated with an increase in the number of individuals in mapping populations. This is only meaningful if a higher density of markers can be applied to the population. One option is using a candidate gene approach to narrow down the QTL flanking regions. Molecular markers can be developed for these areas by identifying BAC clones in the QTL area and develop further marker from identified BAC clones to narrow down the genomic regions. This opens the opportunity to develop candidate gene-anchored markers.

Disease Resistance Traits

Disease resistance traits have improved by recurrent phenotypic selection after artificial or natural infection. A recent metaQTL analyses summarizes all disease resistance. QTL studies, totalling 27, 9 and 7 QTLs for crown and stem rust and grey leaf spot, respectively (Shinozuka et al. 2012). In QTL analysis the number of alleles is limited and the genetic resolution is in the order of several cM, corresponding to a few hundred genes. Several hundred R-genes have been cloned and some have been mapped in *Lolium*, or as comparative mapping approach across various species (Muylle et al. 2005; Dracatos et al. 2008, 2009). These molecular studies have revealed that these R-genes typically occur in clusters in the genome, so that a QTL genomic region contains several neighbouring R-genes that each may contribute to the phenotype, thus hampering the identification of causative family members. Comparative genomics approaches do not seem to discriminate between R-gene family members because there is no consistent genome-wide co-localisation of QTLs and candidate R-genes between *Lolium* and cereals. In addition, the LRR domain known to be involved in pathogen recognition, appears to be highly variable, so that it is unclear which part of the genetic diversity is actually essential for a given pathogen race. The question remains whether multiple minor alleles provide resistance to a single dominant pathogen race, or whether a few major resistance genes provide resistance to a few different pathogen races. So, there is currently a lack of resolution, due to the fact that the genes are clustered and the long LD in QTL populations prohibits the discrimination of single genes or causative polymorphisms.

Quality Traits

The genetic dissection of herbage nutritive quality has been done using various approaches. QTL studies using a few mapping populations revealed various QTLs for dry matter digestibility (DMD), cell wall content (Cogan et al. 2005; Xiong et al. 2007) and fructan metabolism (Turner et al. 2008). Association studies of candidate genes in the fructan metabolism revealed interesting associations between WSC, DMD and nitrogen content and allelic variants within an alkaline invertase gene (Skøt et al. 2007).

Linkage disequilibrium in herbage nutritive quality genes have been studied and revealed that LD decay to r^2 values of 0.2 occurs over 500–3,000 bp (Ponting et al. 2007). Further analysis of the allelic diversity of these candidate genes, as well as the full interpretation of haplotypes is an interesting approach to identify defective alleles prone to be associated with interesting phenotypes, especially for genes which are highly linked and co-localize with QTLs for nutritive value (Xiong et al. 2007).

Comparative Genomics and Candidate Gene Identification

Molecular studies in ryegrass have often benefitted from the available genetic and genomic resources in closely related grass species. It has long been recognised that the grass genomes exhibit a relatively high level of synteny, i.e. a conserved genomic gene order across species, and grass genome evolution has been well characterised (Gaut 2002; Salse et al. 2008). A high degree of synteny and colinearity, combined with the notion that orthologous genes likely perform similar physiological functions in closely related species, is key to transfer knowledge on the function and genomic position of a particular locus (e.g. a candidate gene or QTL) back and forth between ryegrass and related crops of the *Poaceae*. In early studies, molecular markers with a sufficient degree of sequence homology were transferred between grass species to generate comparative genetic maps. Such comparative maps were used to reveal global synteny patterns, to identify orthologous QTL regions across species (Armstead et al. 2004; Jensen et al. 2005), and/or to identify candidate genes in ryegrass using the orthologous genome sequence and predicted genes of model species as described above for disease resistance and quality traits (Shinozuka et al. 2012).

In recent years, classical molecular cloning of individual genes based on sequence homology and/or BAC sequencing is being replaced by EST sequencing and, more recently, RNA-Seq for gene discovery (Sawbridge et al. 2003; Asp et al. 2007) and bioinformatics data-mining. For instance, Ruttink et al. (2013) assembled 19.345 *Lolium perenne* transcript sequences, annotated them based on orthology to the *Brachypodium* gene set, and assigned them to gene families. Phylogenetic analysis of these gene families, in combination with experimentally determined functions of orthologous genes from the model crops allows the comprehensive identification of *Lolium* candidate genes within gene families and it provides a link to integrated comparative genomics databases, such as PLAZA2.5 (van Bel et al. 2012). Studer et al. (2012) used SNPs identified by Roche 454 cDNA sequencing of the parents of the VrnA mapping population to create a genetic map with 767 transcript-anchored markers, which subsequently served as scaffold for the perennial ryegrass GenomeZipper (Pfeifer et al. 2013). The perennial ryegrass GenomeZipper is an extension of the existing genome zippers (Mayer et al. 2011) build upon conserved synteny between barley, rice, *Brachypodium* and sorghum, and it projects 4.035 *Lolium* transcripts in a putative linear order onto seven virtual *Lolium* chromosomes. Together, these tools can be used to screen for candidate genes in orthologous genomic regions across species and can support the scaffolding of a *Lolium* genome sequence.

The success of comparative genomics for candidate gene identification in *Lolium* depends on the evolutionary conservation of genome structure and gene content with respect to the other grass genomes. Two types of data are being generated that together provide an increasingly detailed picture of genome evolution and genome diversity. First, current genome sequencing efforts focus on the completion of a reference sequence of additional grass species, including *Lolium perenne*, barley and wheat. Comparative analyses of the currently available full genome sequences of five grass species has revealed a rich repertoire of small and large scale genomic rearrangements, insertions and deletions, and gene duplication or gene loss events that disrupt micro-synteny. For instance, 30–40 % of the genes in wheat and barley do not reside in conserved gene order (Feuillet et al. 2012) and similar observations have been made for other grass species comparisons (Gaut 2002; Mayer et al. 2011; Massa et al. 2011).

Second, the huge increase in sequencing throughput has spurred population scale genome resequencing to investigate the genetic diversity within species. Genome resequencing and comparative genome hybridisation (CGH) has revealed a high degree of structural polymorphism and substantial genome sequence diversity among diverse maize inbred lines (Llaca et al. 2012) and rice accessions (Goicoechea et al. 2010). The abundance of various classes of intraspecies genome sequence polymorphisms (nucleotide substitutions, small or large scale insertions or deletions, segmental duplications, gene copy number variations, presence-absence variations, genome rearrangements) has two important implications for future genetic analyses in *Lolium*. First, it reveals the limitations of using genome structural organisation of a single reference genotype of a related grass species in comparative mapping studies. The establishment of one or more annotated genome sequences for *Lolium* is therefore urgently needed, as well as subsequent large scale resequencing of ryegrass populations to investigate the level of genome sequence conservation in comparison to other *Poaceae* and to identify species-specific, or even population-specific genome sequences. Second, intraspecies gene duplication or gene loss events change the stoichiometry of regulatory or metabolic pathways and represent a type of genetic diversity with potentially large phenotypic effects (Maron et al. 2013) that is currently overlooked in trait-allele association studies in *Lolium*, which mainly focus on SNP molecular markers.

Molecular Markers for Association Studies

With the advent of sequencing technology, the type of markers used for locus-trait association studies are changing. In classical QTL studies, anonymous markers were used, while the continuous increase in throughput and the strong drop in sequencing cost per base have trigged a shift towards sequence based analysis of nucleotide polymorphisms, typically SNPs and small indel markers. Several advantages as compared to ‘classical’ markers exist.

First, sequencing based methods allow to map markers *in silico* onto a reference with nucleotide resolution and thus provide a bridge between genetic maps and physical maps. This allows to position markers with respect to flanking genes and their functional annotation.

Second, the number of marker loci screened per individual can be varied depending on the library preparation methodology. This is important because the population structure determines the LD and therefore the minimum number of markers required for locus-trait associations, as described above. Sequencing can be performed as whole genome scans (shotgun libraries), or at a randomly distributed but reproducible subset of the genome (reduced representation libraries), or targeted to a specific set of candidate loci (targeted resequencing). Examples of reduced representation libraries are Restriction-site Associated DNA sequencing (RAD-seq, Pfender et al. 2011; Hegarty et al. 2013) or genotyping-by-sequencing (GBS, Elshire et al. 2011; Byrne et al. 2013). Species with relatively large genomes such as *Lolium* often contain a high percentage of repetitive DNA sequences, which can be discriminated from gene-enriched euchromatic regions by the level of DNA methylation. Low coverage random shotgun sequencing is expected to be ineffective in *Lolium*, because repeat-derived reads cannot be unambiguously mapped and are discarded during the calling of polymorphisms. In GBS or RAD-seq sequencing methods, random insert libraries are prepared with methylation-sensitive restriction enzymes such as *Ape* KI in an attempt to avoid hypermethylated repeat sequences and enrich for the gene-rich euchromatic regions. Transcriptome sequencing (RNA-Seq) provides a potentially interesting type of genotyping data, as transcript-based polymorphic markers can be combined with expression values to estimate eQTLs, although population scale RNA-Seq is currently prohibitively expensive due to the library preparation cost (Ingvarsson and Street 2011). Examples of targeted sequencing include amplicon sequencing for a relatively small number of targets, or hybridisation based sequence capture for a target sequence of up to a few million base pairs. The use of amplicon sequencing of the FT genomic sequence has led to the identification of heading date associated SNPs and short indels in the promoter region of FT (Skøt et al. 2011). Using indexed amplicon or sequence-capture libraries for pooled NGS sequencing instead of molecular cloning and Sanger sequencing will strongly reduce the cost and increase the throughput of genotyping. Taken together, sequencing based genotyping methods provide orders of magnitude higher numbers of markers to be assayed in parallel as compared to classical methods.

Third, sequencing based genotyping methods allow de novo discovery of polymorphisms during genotyping, while dedicated SNP panels only screen a priori known markers. In several grass crops, including maize, barley, wheat and rice, high-throughput dedicated SNP genotyping panels have been established that screen tens to hundreds of thousands of SNP markers in parallel. These were used for QTL studies in barley and for GWAS and the reconstruction of the domestication history of rice. In *Lolium*, SNP genotyping panels are effective for populations with a relatively narrow genetic base (or long LD), but their transferability is compromised depending on the genetic distance between the genotypes of the SNP discovery panel and the actual association population. For instance, Ruttink et al. (2013) showed that about 40 % of a set of 1.2 million transcript-anchored SNPs was unique to a given genotype in a discovery panel of 14 divergent genotypes. This suggests that many novel SNPs should be discovered by sequencing an entire divergent AM population and that a substantial fraction of the genetic diversity will effectively be ignored by using predefined SNP panels from discovery panels, even with >1 million SNPs. Furthermore, SNP assay design typically requires that the 50 bp flanking region of each SNP is free of neighbouring polymorphisms that potentially interfere with primer or probe binding and thus may lead to false negative detection. With an expected high prevalence of novel undiscovered SNPs in populations of divergent genotypes (i.e. AM populations), genotyping accuracy may be compromised using predefined SNP panels. It is estimated that as low as 3 % errors in genotyping may interfere with association mapping approaches (Ingvarsson and Street 2011). In conclusion, sequencing based genotyping methods are crucial for the discovery of low abundant polymorphisms in divergent populations. Although often ignored in association studies, rare alleles may account for a substantial fraction of the unexplained heritability typically found in association mapping studies.

Approaches for Reverse Genetics Studies in *Lolium*

Targeted resequencing of candidate gene loci in all individuals of a given population (natural accessions, association mapping population, or breeding genepool) serves two purposes. On the one hand, polymorphic markers can be used to identify locus-trait associations as described above. Alternatively, using bioinformatics tools, the same genotyping data can be queried for polymorphisms that are predicted to severely disrupt gene function, such as frame shift-inducing insertions or deletions, or premature stop codons. According to population genetics theory, in outbreeding species defective alleles occur in natural populations at low frequency and usually in a recessive heterozygous state. Maronni et al. (2011) identified such rare defective alleles in a collection of poplar natural accessions using NGS sequencing. Two independent heterozygous plants can be crossed to yield a homozygous line without inbreeding effects, in which the effect of a loss of function mutation in a specific candidate gene can be studied. This is the basis of Breeding by Rare Defective Alleles (BRDA). Van Holme et al. (2013) subsequently demonstrated that poplar plants homozygous for a defective allele in the HCT gene involved in lignin biosynthesis have reduced lignin content and altered lignin composition. This reverse genetics approach can be used to identify or validate causal polymorphisms and provides an interesting alternative for TILLING or tDNA insertion libraries, which are difficult to make in outbreeding species such as *Lolium*. This is largely due to the difficulty to generate homozygous lines through selfing, and/or the requirement to generate and maintain a large population of mutant lines to saturate the gene space with induced mutations. Ruttink et al. (2013) analysed *Lolium* RNA-Seq data in a SNP discovery panel and *in silico* identified 5782 SNPs that cause a putative premature stop codon leading to truncated proteins in a total of 3,820 genes. These include some genes involved in signalling pathways and the C4H, CCoAOMT and 4CL genes of the monolignol biosynthesis pathway. This demonstrates that targeted resequencing of candidate gene families across diverse *Lolium* populations can lead to the identification of rare defective alleles and provides the possibility to generate homozygous mutant lines by dedicated crossings.

Conclusions

In conclusion, the high level of genetic diversity that is inherent to outbreeding crops is both a burden and a benefit. It is a burden because it complicates comparative mapping (inconsistent behaviour of markers), ortholog identification (asymmetric orthologous pairs due to gene duplications or gene loss), positional cloning (chromosomal or segmental rearrangements) and it limits the use of a reference sequence from a single genotype (genotype-specific chromosomal content and organisation). However, it should be recognised and appreciated that the rich and unexplored resource of genetic diversity underlies the phenotypic diversity in natural and breeding populations and that this is the core currency of breeding. While SNPs are currently the predominant molecular markers used in NGS-based association studies, more attention should be paid to gene copy number variation and to rare defective alleles because these represent underexplored, yet highly valuable types of genetic polymorphism potentially underlying major phenotypic effects. The technological and computational tools are available for targeted or whole genome sequence analysis, and we can learn from the model species how to analyse, visualise and interpret genomic diversity data, to estimate allele-trait correlations and to use it for the construction or selection of superior genotypes.

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Chapter 32
Genome Sequencing of Model and Forage Legume Species: Consequences for Genetic Studies

Bernadette Julier, Philippe Barre, and Frédéric Debellé

Abstract The main forage legume species (alfalfa, red clover, white clover) belong to the same clade than the model species *Medicago truncatula* whose genome has been sequenced. Attempts for sequencing forage legume crops, even if they are heterozygous and may be polyploid, are also reported. In addition to sequence information, new methods of genotyping are being developed. These methodologies offer new possibilities to conduct genetic analysis of traits of agronomic interest. The traits can be studied in model species, with QTL or genome-wide association studies that end to the identification of candidate genes. The genetic studies can also be carried out in the crop species, with analysis of genetic diversity and structure, QTL studies, association studies based on a candidate gene or a genome-wide approach, bulk-segregant analysis or genomic selection in a near future. The new possibilities of high throughput genotyping increase the power of these studies. It is expected that breeding program on forage legumes will benefit from these studies.

Keywords Alfalfa • Clover • Gene • *Medicago* • *Trifolium* • QTL

Introduction

The three main forage legumes grown in temperate climate (alfalfa – *Medicago sativa*, white clover – *Trifolium repens*, red clover – *Trifolium pratense*) belong to the Galegoid clade that also includes the model legume *Medicago truncatula*. The cultivated forage legumes have biological characteristics which hamper genetic and genomic studies: they are allogamous, perennial and alfalfa and white clover have a

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polyploid genome. By contrast *M. truncatula* has biological characteristics favorable for genetic and genomic studies: it is a diploid, autogamous and annual species. These characteristics explain why *M. truncatula* has been chosen in the 1990s as a model species for legumes.

In this paper, we describe the sequencing effort which has been made to end up with the genome sequence of *M. truncatula*. We also show how new sequencing technologies allowed to start sequencing programs directly on legume crops. Then we describe how genetic studies on a model species could be useful to target candidate genes for the crops. Finally we show that all this information is useful to improve the efficiency of the genetic studies in forage legumes through the introduction of genomics tools.

Sequencing of the Model Legume *M. truncatula*

Numerous tools have been developed on *M. truncatula* (markers, genetic resources, mutants, mapping populations, genetic maps, EST sequences, etc.) and the gene-rich portions of its genome have been recently sequenced (Young et al. 2011). *M. truncatula* genome sequencing was started in 2003 by an international consortium using the same strategy than for the Arabidopsis and rice genomes: BAC by BAC sequencing by the Sanger technology combined to physical and genetic mapping. This resulted in a high quality genome sequence assembly anchored to chromosomes and covering about two thirds of *M. truncatula* genes (Young et al. 2011). However nearly a third of the genes was then only represented by short DNA sequences unanchored to chromosomes. In the last 2 years, efforts in France and in the USA aimed at completing the genome assembly by whole genome shotgun sequencing (Debellé et al. 2012; Krishnakumar et al. 2013). Next generation sequencing, in particular paired end and mate pair Illumina technology, was used to sequence DNA libraries carrying inserts of various sizes (from a few hundreds bp to more than 10 kbp). These new resources, together with data previously obtained by the consortium (BAC end sequences, genetic and optical maps etc.) and improved bioinformatic tools led to the development of new genome assemblies anchoring to chromosomes nearly 95 % of *M. truncatula* genes. Improvements were also made in the annotation of the genome using new evidence from ESTs, RNA seq and proteomic data. These improved resources now represent a sound basis for functional and comparative analysis. In recent years similar efforts led to the sequencing of other legume genomes such as those of *Lotus japonicus*, *Glycine max* (soybean), *Cajanus cajan* (pigeon pea), *Cicer arietinum* (chickpea) or *Phaseolus vulgaris* (common bean). Comparative genomics showed an extensive conservation of synteny between legumes, with syntenic blocks sometimes reaching the size of chromosome arms. These observations open the way to reconstructing the ancestral legume genome. They also open new perspectives in translational genomics since findings in model legumes can help genetic analysis of plants such as forage legumes for which less genomic resources are currently available. A major discovery brought in particular by legume comparative genomics was the identification of a whole genome duplication event that occurred early (around 58 Mya) in the evolution of the papilionoid subfamily of legumes, which includes most important legume crops. This duplication was soon followed by important genome rearrangements and gene loss that occurred before speciation in this subfamily. The fractionation, subfunctionalization, and neofunctionalization of gene pairs originating from the whole genome duplication likely contributed to the evolution of legume specific features such as the establishment of nitrogen fixing symbioses with rhizobia (Fig. 32.1).

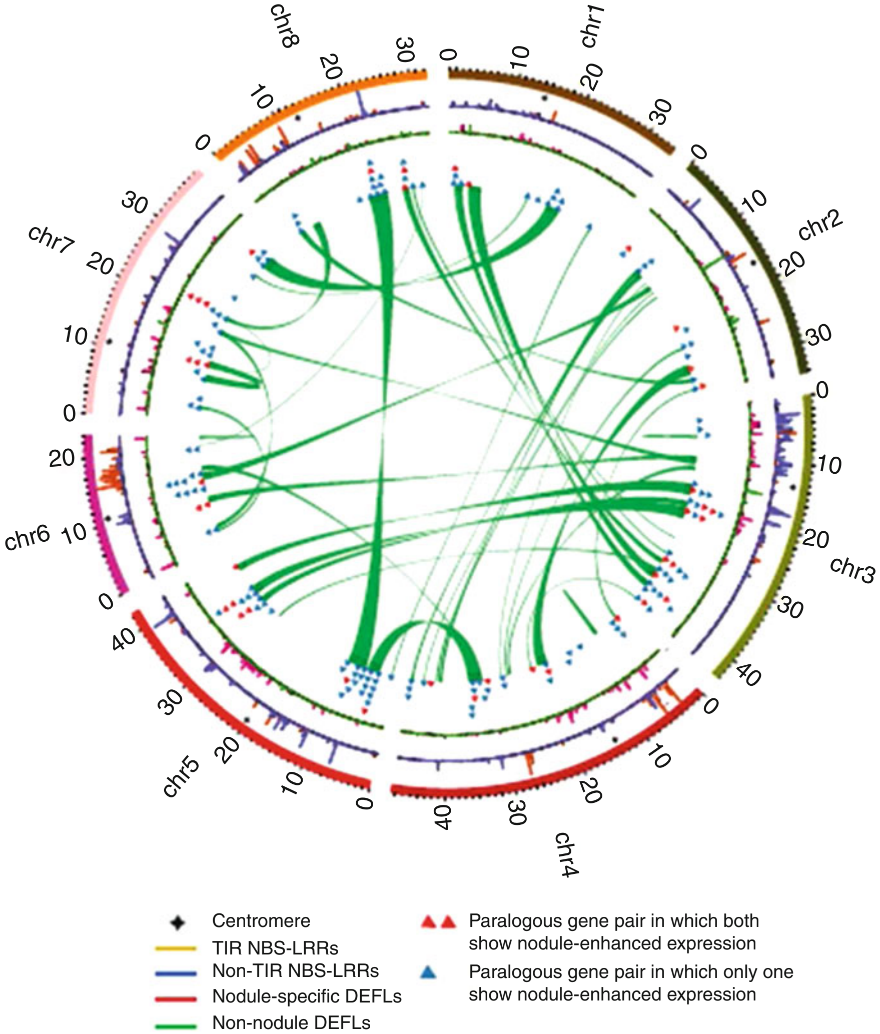

Fig. 32.1 Circos diagram illustrating the *Medicago* genome wide duplication (Young et al. 2011)

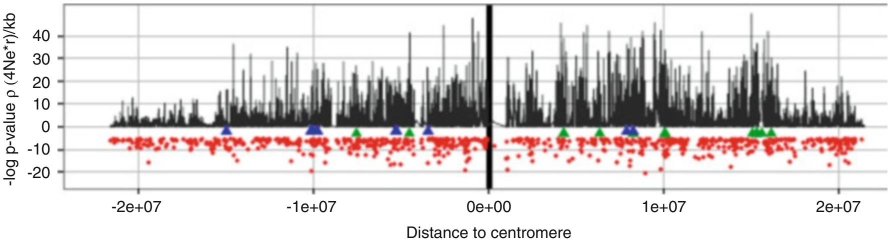

Fig. 32.2 Recombination rate along chromosome 5 (Paape et al. 2012)

This *M. truncatula* reference genomic sequence was established on A17 genotype. The intraspecies sequence polymorphism is under study by an international consortium in order to discover single nucleotide polymorphisms (SNPs), insertions/deletions (INDELs) and copy number variants among 384 genotypes (http://www.medicagohapmap.org/?genome). The database of sequence variation will be useful to describe population structure but also to develop a genome-wide association (GWA) mapping resource. Indeed, GWA is a powerful tool to identify genes involved in traits related to genetic evolution of the species or traits of agronomic interest in crop species. Resequencing efforts have already given promising results: the development of a SNP array (Loridon et al. 2013) that can be used to understand the factors that shape the genetic diversity of *M. truncatula*, the identification of large number of SNP (1.5 million) and the analysis of linkage disequilibrium (Branca et al. 2011), the analysis of recombination rate along the genome depending on gene density and gene function (Fig. 32.2) (Paape et al. 2012).

Phylogeny of *Medicago* genus was investigated, again using resequencing and mapping on *M. truncatula* genes (Yoder et al. 2013). The phylogeny obtained with the genome-wide polymorphism (almost 90,000 sites) could not even be recovered with 5,000 polymorphic sites. Interestingly, some regions did not support the genome-wide phylogeny, as the consequence of coalescence or introgression events in *Medicago* history.

Sequencing of Forage Legume Species

Due to the complexity of their genomes (heterozygosity and sometimes polyploidy), forage legumes lag behind grain legumes with respect to the development of genomic resources. Several genomic and/or cDNA sequencing programs have been launched on alfalfa and white clover. In France, alfalfa genomic sequencing is based on paired-end and mate-pair data obtained on Illumina Hi-Seq from a non-inbred autotetraploid genotype. Up to now, *de novo* assembly of these data was not very successful in this autotetraploid species, probably because of important allelic diversity (up to four different haplotypes), with large indels. Additional resources have to be developed for a better *de novo* assembly, together with improved

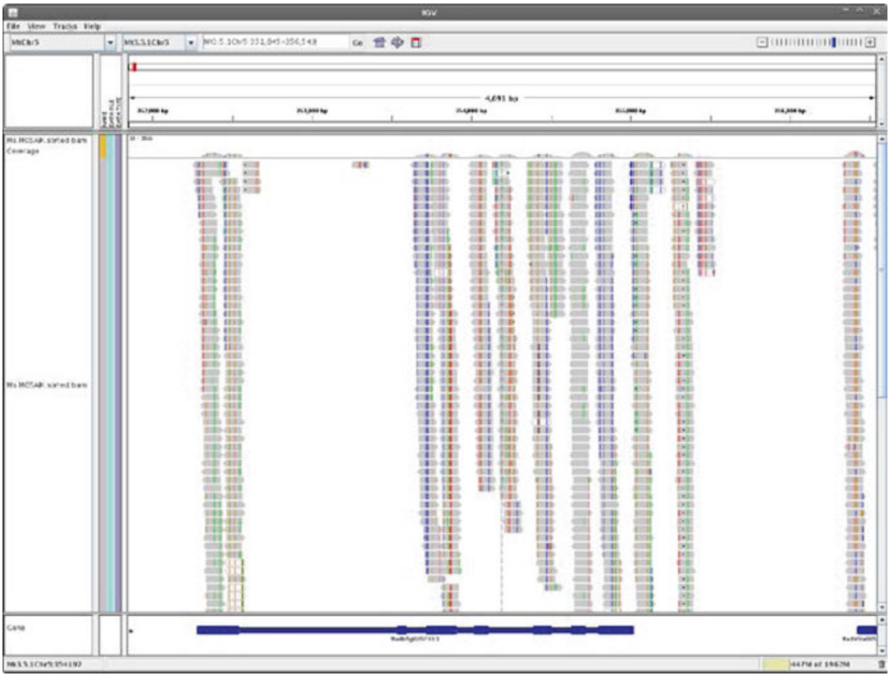

Fig. 32.3 Microassembly of alfalfa reads on *M. truncatula* gene sequences

bioinformatic programs to overcome the diversity encountered among the four haplotypes of a single genotype. Current sequence assembly thus relies on the *M. truncatula* sequence, considering alfalfa sequencing as a complex resequencing of the model species. Microassembly, based on mapping alfalfa reads on *M. truncatula* gene sequences, was thus attempted. With available alfalfa data, about 47 % of *M. truncatula* genes were recovered (Fig. 32.3). However, if exon regions were highly conserved, non-coding regions were not conserved in the two species. In addition, some of the genes may not have been recovered because of large sequence polymorphism in the two species. Transcriptome sequencing has been carried out on a set of ten individuals coming from cultivated or wild accessions in order to analyze the effect of domestication on genome evolution (Chantret N, personal communication). These data will be useful to annotate the genes. However, development of markers does not require full genome assembly. Indeed, transcript sequences obtained from alfalfa genotypes were assembled to obtain a list of several thousand SNP that were used to develop a set of HRM markers (Han et al. 2011). In white clover, the strategy was to generate transcript sequences of an inbred line and to establish a reference transcript set that separated the two homeologous genomes (Nagy et al. 2013). Additional sequencing of transcripts of several white clover genotypes were mapped to the reference transcript set and more than 200,000 SNP were detected. Griffiths et al. (2013) also reported *de novo* genome sequencing on an inbred line of white clover.

Genetic Studies in the Model Species

Genetic studies in *M. truncatula* produce lists of genes that are good candidates to explain phenotypic variation for traits of agronomic interest in the legume crop species. Mapping populations have been developed and mapped, and those composed of RIL may be used to detect QTL for any segregating traits. The small size of *M. truncatula* genome and the possibility to obtain many seeds in a few months offer the possibility to carry out fine mapping methods and to identify genes or small genomic regions involved in trait variation when a QTL with a strong effect has been detected. For example, after a QTL analysis in four connected mapping populations, a fine mapping strategy and a transcriptomic analysis, a Constans-like gene on chromosome 7 was proved to explain variation for flowering date and stem length in *M. truncatula* (Pierre et al. 2011). By using a similar method, the RCT1 gene on chromosome 4 was found as being involved in the resistance to anthracnose (*Colletotrichum trifolii*), a major disease for alfalfa (Yang et al. 2007).

Association genetics is another way to detect genomic regions or genes involved in trait variations. A genome-wide association study was recently published (Stanton-Geddes et al. 2013). The genome of 226 accessions was sequenced and the genotypes were evaluated for phenotypic traits such as plant height, trichome density, flowering time or nodulation. SNP in candidate genes, either uncharacterized or already known, were identified as explaining variation for the phenotypic traits. Transcriptomic studies can also help to identify genes that are differentially expressed depending on constraints (Zahaf et al. 2012). The large collections of induced mutants are also useful to test the role and function of a gene on a phenotypic trait (Pislariu et al. 2012).

Consequences for Genetic Studies on Forage Legumes

Genetic studies in forage legumes benefit from new genomic tools and knowledge in both model and crop species. Even if no draft genome sequence of forage legume crops is available, partial sequence data together with *M. truncatula* genome sequence offer opportunity to better understand species evolution, genetic structure of populations and determinism of traits. All these methods are conducted with the objective to include molecular tools into breeding programs (marker-assisted selection). They require the development of numerous markers, genomic and transcriptomic sequence data and if possible assembled genome sequences.

Sequencing different genotypes from *Medicago* genus is a way to study genome structure or species evolution. Sequencing pairs of genes originating from duplication in alfalfa, Ho-Huu et al. (2012) found that functional divergence between gene copies originates from different mechanisms: change in the level of purifying selection between paralogous genes, occurrence of positive selection, or transient relaxed purifying selection following gene duplication.

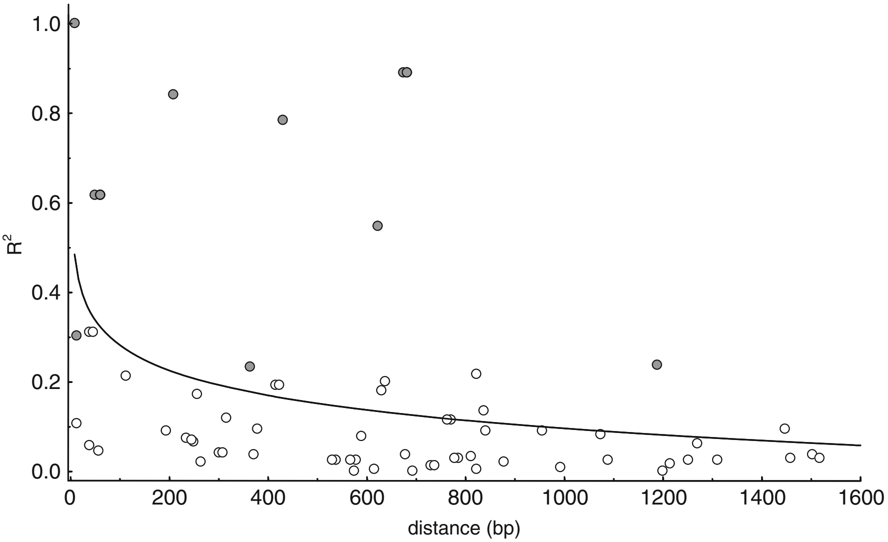

Fig. 32.4 Linkage disequilibrium decay in Constans-like gene as a function of distance among SNP in bp (Herrmann et al. 2010). *Grey dots* indicate significant linkage disequilibrium

Even if genetic structure of populations have already been studied with molecular markers (for example, Muller et al. 2006; Flajoulot et al. 2005 in alfalfa), the analysis of species diversity with larger set of markers covering the whole genome would give a more extensive view of population structure and the part of allelic variation present in a species that is not currently used into breeding programs. Linkage disequilibrium has been estimated in alfalfa (Herrmann et al. 2010). As expected for this allogamous species, it declines very rapidly (no significant LD beyond 1,200 bp) in the gene that was studied (Fig. 32.4). Evaluation of linkage disequilibrium across the genome depending on the population structure would help to define adequate strategies for genetic analyses.

In forage legumes, the analysis of genetic determinism of traits of agronomic interest relies on several methodologies: genetic mapping and QTL detection on biparental populations, association mapping, bulk segregant analysis. The development of genomic selection has not been reported yet on forage legumes, and would probably require theoretical developments to take into account the polyploidy level. QTL mapping has been used in all three main forage legume species, for traits having moderate or high heritability. The current need is to develop high throughput and cost-effective genotyping methods. The use of several connected biparental populations would contribute to extent the genetic diversity and to detect positive alleles that could be used for genetic selection within the crossing design (Pauly et al. 2012). Because of the short linkage disequilibrium, association mapping is based on a candidate gene strategy or requires either a huge number of markers or to create populations in which LD has been increased for genome-wide studies. The genes that were shown to explain variation for traits in *M. truncatula* are good candidates for association mapping in legume crop species. With the assumption that these genes could also explain variation for the same traits in the crop species, their sequence can be studied in the crop species. In the case of Constans-like gene (see above), sequence polymorphism was associated to phenotypic variation for flowering date and stem length in alfalfa (Herrmann et al. 2010) and a marker-based divergent selection conducted to two contrasted populations for these traits (Julier 2012). Contrastingly, we failed to find an association between RCT1 sequence polymorphism and anthracnose resistance in alfalfa (Julier et al. 2012). In bulk segregant analysis, contrasted populations are created from phenotypic selection, and markers that have different frequencies in the populations are supposed to explain the phenotype. This strategy was efficient to detect markers linked to cold tolerance in alfalfa (Castonguay et al. 2010). This method also requires high throughput genotyping methods.

Sequencing information is growing very fast, in both model and crop legume species and should quickly give rise to whole genome sequences, even for heterozygous and polyploid species. Several results have shown that molecular tools may be useful for genetic studies by giving more information on genetic diversity, structure and genetic determinism of breeding traits. New efforts are still needed to extent these tools so that broad access to both genotyping and sequencing is possible. Methods to carry out sequencing, genome assembly, detection of polymorphism should become democratic. Some theoretical aspects on the use of these molecular tools for heterozygous and polyploid species require new developments. We can predict that all these methods will be soon implemented into breeding programs.

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Chapter 33
A Genetic Association Between Leaf Elongation Rate and Flowering Time in Perennial Ryegrass

Daniel Thorogood, Tina Blackmore, Rosalind Mathews, Debra Allen, James MacDuff, and Matthew Hegarty

Abstract Leaf elongation rate (LER) is a key component of forage grass dry matter yield. Leaf lamina initiate rapid expansion during the winter at a time that coincides with lengthening days but this extension is not associated with any large environmental shifts such as an increase in light intensity or temperature. The rate increase precedes any anatomical indication of floral initiation in apical meristems (double ridge formation) by around 2–3 weeks. We measured LER in a perennial ryegrass (*Lolium perenne* L.) pair-cross mapping family that has been extensively mapped with more than 1,200 SNP markers generated by Illumina's Infinium platform. These have been anchored to the seven *Lolium* linkage groups as defined by Triticeae nomenclature with seven micro-satellite markers originally developed by ViaLactia Biosciences. Measurements were made in the winter/spring during extending day length, after full natural vernalisation. The plants' leaves were beginning to expand rapidly with leaf extension rates varying between approximately 0.4 mm and 1.1 mm h^{-1}. We obtained a significant correlation between LER and both apical meristem elongation and ear emergence (the date at which inflorescences emerge above the flag-leaf ligule) measured in two separate seasons. A QTL for LER was revealed with a peak some distance away from the major floral initiation and ear emergence date QTL on linkage group 7. This is the first evidence of genetic linkage that determines the often observed association between LER in herbage grasses in the spring with floral initiation determined in large part by the action of *Arabidopsis* flowering initiation gene homologues, Flowering Time Locus (*FT*) and Constans (*CO*).

Keywords *Lolium perenne* • QTL • Reverse genetics • Spring growth • Forage yield • SNP genotyping • Floral initiation • Ear emergence • Photoperiod • Phenology

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Introduction

Although being able to maximise forage yield is important for animal production, it is also important for farmers to understand and control seasonal production of forage grass yield in order to determine optimal animal stocking rates, and to minimize the necessity to produce and use home-produced or bought in silage and animal feed supplements. Temperate grass growth follows a seasonal pattern where there is rapid growth in the spring, followed by reduced growth in the summer and a secondary, smaller growth peak in the late summer, early autumn. Growth is minimal in the winter, limited by low temperature conditions until growth commences the following spring.

The timing of seasonal fluctuations in grass growth is genotype dependent. One of the major determinants of grass growth is leaf extension rate (LER) as demonstrated by Sartie et al. (2011) who reported that plants with high LER tended to produce higher dry matter yields. This preliminary study attempts to give an insight into the genetic control of variation in LER in the spring period in a perennial ryegrass (*Lolium perenne* L.) population.

The timing of ryegrass leaf expansion in the spring is associated with reproductive development (Davies 1971; Peacock 1976) and Parsons and Robson (1980) and Kemp et al. (1989) showed that the increase in leaf growth coincided with floral initiation well before any observable signs of stem elongation leading to inflorescence development and flowering. Furthermore it was shown that elongation was not entirely temperature dependent as elongation rates were far greater than at equivalent temperatures experienced in the autumn.

A number of regions of the genome accounting for genetic variation for ear emergence date in ryegrass have been identified as quantitative trait loci (QTL). Of these QTL, flowering gene homologues of other grass species are known to underlie two QTL: on linkage group 4, vrn1 of wheat (Jensen et al. 2005) and on linkage group (LG) 7, hd1 and hd3 of rice (Armstead et al. 2008). Ear emergence is highly heritable in ryegrass but represents a late stage of floral development. Genetic variation for the early stages of floral initiation in ryegrass appears not to have been investigated.

A number of studies have been made to identify regions controlling plant growth characteristics including leaf extension rate in ryegrass (see for example Barre et al. 2009; Sartie et al. 2011).

This study seeks to determine whether there is a genetic link between the rapid increase in LER in ryegrass observed in the spring with floral initiation. Determining the timing of floral initiation is destructive and time-consuming so the study also investigates the association between floral initiation and, ear emergence date to validate the latter as an indirect measure of the earlier floral initiation process.

Material and Methods

The perennial ryegrass mapping population used in this study was derived from a cross between two contrasting parental genotypes. A plant from an ecotype collection from Cardigan Island (Wales, U.K.) (Ba12142) was crossed with a plant of the turfgrass variety 'AberSprite'. One of the 'F1' plants was then crossed to another 'AberSprite' genotype and 85 progeny plants were established.

A custom SNP array assay was developed at IBERS generating ~3800 SNPs based on transcribed sequence from mature leaf tissue, using a HiScanSQ platform. Of these SNPs we were able to map using Joinmap v.4 (Van Ooijen 2006) 1,234 polymorphisms distributed on seven distinct linkage groups (Fig. 33.1). These were anchored to the seven Triticeae linkage groups using seven SSR markers of known linkage association (rv0746, LG1; rv0062, rv0188, LG2; rv0515, LG3, rv0380, LG4; rv0184, LG5; rv0311, LG7), developed by Vialactia Biosciences, New Zealand (Gill et al. 2006).

Plants were grown in 15.0 cm diameter pots of John Innes No. 3 compost in a frost-free green-house with supplementary lighting during daylight hours such that natural day length was unaffected. Leaf extension rates were measured on 85 plants at weekly intervals from early January through to the beginning of May. This was done by marking the part of the youngest visible leaf that had reached the leaf sheath/blade interface of the next oldest leaf with non-toxic acrylic paint on three shoots per plant. After 48 h, the distance between the paint mark and the leaf sheath/blade interface of the subtended leaf was measured and the rate of leaf elongation (a product of cell division and cell elongation) was calculated as mm h^{-1}. It was assumed (from previous studies) that leaves measured at this stage of their development were in their linear growth stage.

Apical meristems of single shoots taken from each plant were dissected under a low power dissecting microscope (x2–x6) on five dates (25th March, 3rd, 11th, 16th and 23rd April) in order to assess development. Although *Lolium* apex developmental stages can be recognized, transition to a floral apex is a continuous process (see Perilleux et al. 1991) so it is difficult to pinpoint the timing of the double-ridge stage that is the first visible indication of floral initiation. Instead, because the apex elongates as it develops, development was assessed by measuring the length of the apex at the four measuring dates and also as the number of days after 1st April for the apex to measure 5 mm in length.

Ear emergence dates, determined when the tips of at least three inflorescences could be seen emerging above the leaf sheath/blade junction of the flag leaf, were recorded at two locations (Aberystwyth, UK and Edinburgh, UK.) in 2001 and 2002 respectively, on three clonal plants of each genotype planted in randomized block 0.75 m-spaced field plant trials with three replicates.

QTL analyses of all phenotypic characterisations were done using MapQTL v.5 (Van Ooijen 2004).

Results

Ear emergence dates were correlated with estimates of floral initiation (Table 33.1). Furthermore, QTL analyses indicated a coincident major QTL on linkage group 7 for ear emergence at Aberystwyth and Edinburgh, apical meristem length at all five measuring dates, and the number of days taken for the meristem to reach 5 mm in length (for representative examples, see Fig. 33.2). The maximum LOD scores and % variance accounted for by the QTL for ear emergence was 8.4 (36.6 %) for

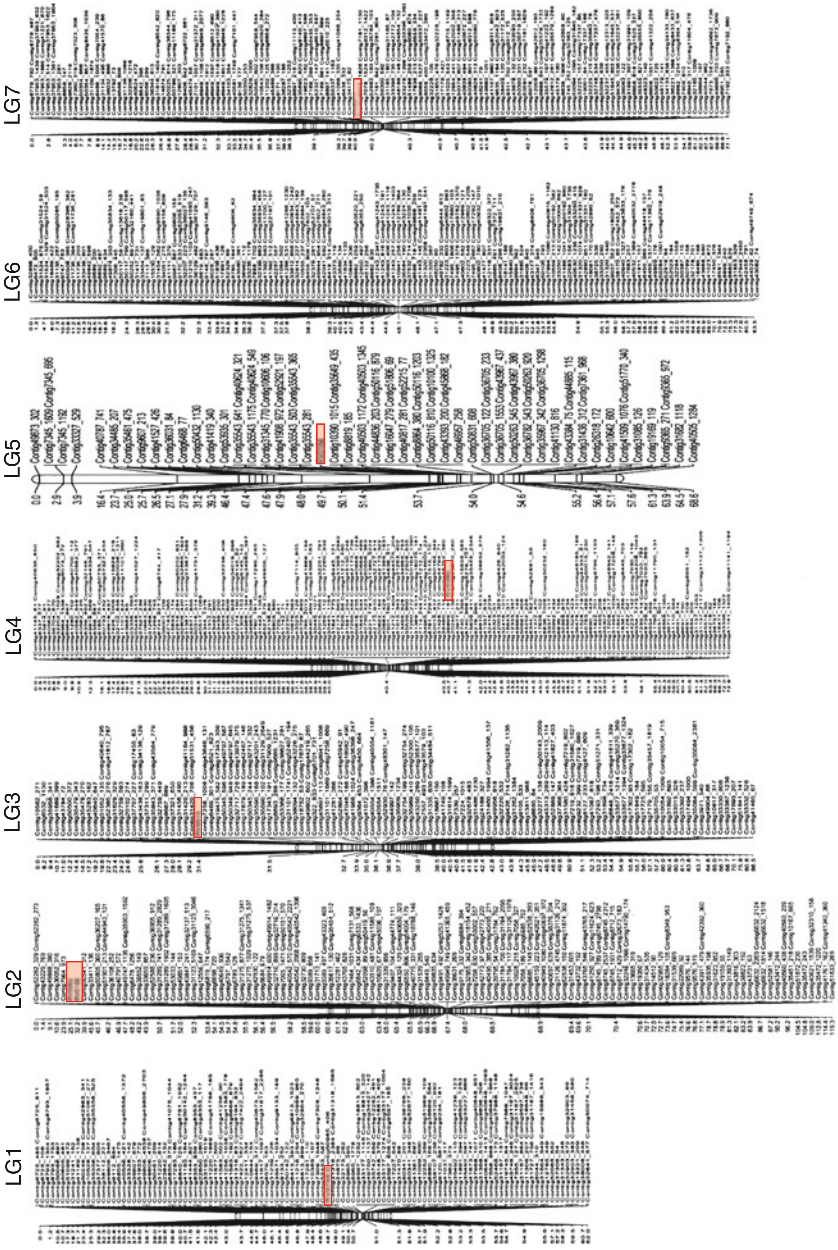

Fig. 33.1 Recombination map of SNP markers with SSR anchor marker positions highlighted on expected Triticeae linkage groups

Table 33.1 Correlation coefficients between ear emergence scores and floral initiation

Ear emergence date[a]		Apical meristem length (mm)					Days to 5 (mm)[a]
	Aberystwyth	25/03/2013	03/04/2013	11/04/2013	16/04/2013	23/04/2013	
Aberystwyth	0.79*** (83)	–0.22* (82)	–0.45*** (80)	–0.47*** (83)	–0.34** (82)	–0.63*** (82)	0.52*** (73)
Edinburgh		–0.31** (70)	–0.36** (68)	–0.36** (71)	–0.31** (70)	–0.55*** (70)	0.47*** (65)

Degrees of freedom in brackets

NS, *, **, *** not significant, significant at $P > 0.05$. 0.01 and 0.001

[a] Days after 1st April

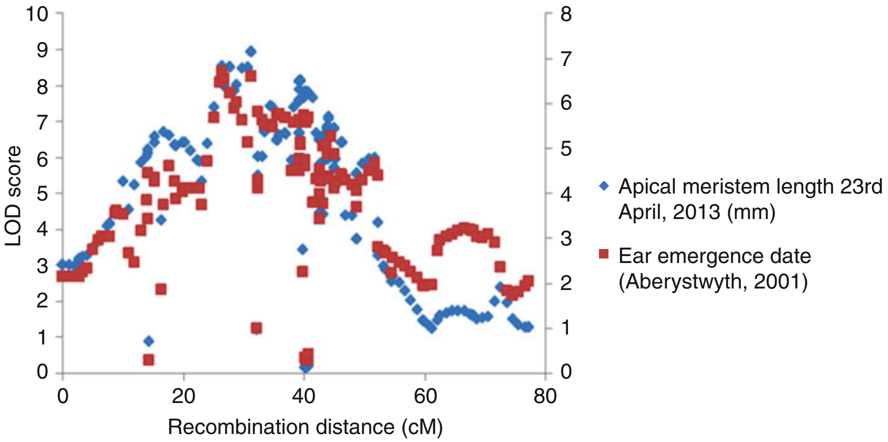

Fig. 33.2 Co-location of a QTL region on linkage group 7 for ear emergence date (days after 1st April) at Aberystwyth (2001) and apical meristem length on 23rd April, 2013

Aberystwyth and 6.7 (34.2 %) for Edinburgh. The maximum LOD scores and % variance accounted for by the QTL for apical meristem length varied from 6.0 (25.3 %) on the 25th March to 8.9 (38.7 %) on the 23rd April. The LOD threshold was not exceeded for either ear emergence date or apical meristem length at any of the measuring dates on any other linkage group.

Significant correlations between ear emergence date and leaf extension rates were found throughout the measuring period (Table 33.2). QTL analyses of LER also revealed a consistent, large QTL on linkage group 7 with a peak around 50 cM. This appears not to coincide with the QTL for floral initiation and ear emergence that has a broad peak at a distance of between 25 and 40 cM. These QTL were by far the biggest, and the only ones found consistently across measuring dates/locations for floral initiation-related and LER traits. The QTL for the mean LER over all measuring dates is illustrated in Fig. 33.3 alongside the QTL for apical meristem length measured on 23rd April.

Discussion

A correlation between estimates of floral initiation and ear emergence was not unexpected especially in this population as the major QTL on LG7 found to control both traits is known to be underlined by two flowering time genes, hd1 (constans (*CO*) homologue) and hd3 (flowering time (*FT*) homologue) (Armstead et al. 2005). This provides good justification to use ear emergence date as an indicator of the relative initiation of the, more difficult to measure, earlier floral initiation process.

Table 33.2 Correlation coefficients between ear emergence scores and leaf extension rates

Ear emergence date[a]	Leaf extension rate ($\mathrm{mm\ h^{-1}}$)					
	09/01/2013	16/01/2013	23/01/2013	30/01/2013	06/02/2013	13/02/2013
Aberystwyth	−0.19NS (83)	−0.08NS (83)	−0.21* (80)	−0.1NS (82)	−0.26* (83)	−0.24* (83)
Edinburgh	−0.23* (72)	0.01NS (72)	−0.12NS (69)	−0.11NS (71)	−0.27* (72)	−0.19NS (72)
	20/02/2013	27/02/2013	07/03/2013	14/03/2013	21/03/2013	04/04/2013
Aberystwyth	−0.28** (83)	−0.08NS (83)	−0.14NS (83)	−0.23* (83)	−0.25* (83)	−0.17NS (83)
Edinburgh	−0.24* (72)	−0.17NS (72)	−0.21NS (72)	−0.27* (72)	−0.27* (72)	−0.28** (72)
	12/04/2013	17/04/2013	24/04/2013	02/05/2013	max_LER	mean_LER
Aberystwyth	−0.39*** (83)	−0.45*** (83)	−0.34** (83)	−0.2NS (83)	−0.22NS (83)	−0.32** (83)
Edinburgh	−0.4*** (72)	−0.38*** (72)	−0.28* (72)	−0.21NS (72)	−0.27* (72)	−0.32** (72)

Degrees of freedom in brackets

NS, *, **, *** not significant, significant at $P>0.05$. 0.01 and 0.001

[a] Days after 1st April

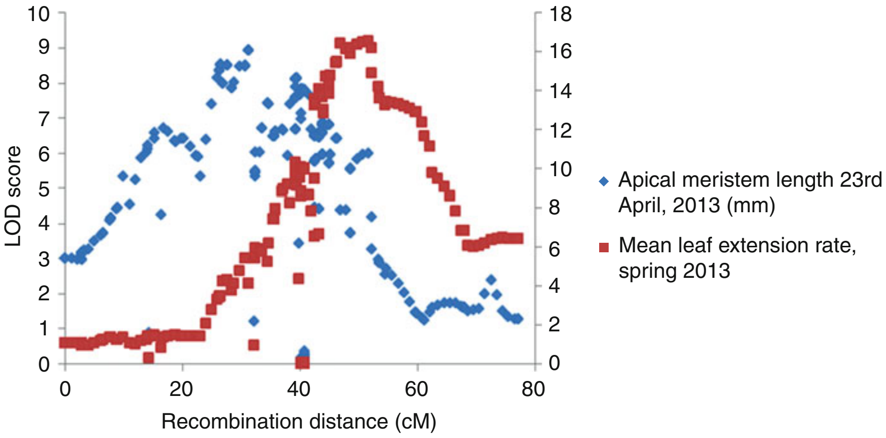

Fig. 33.3 Location of QTL for apical meristem length and mean leaf extension rate

Our results show a strong genetic association between spring LER and floral initiation and that this association is at least in part due to linkage of (as yet unknown) genes controlling LER with photoperiod responsive genes controlling floral initiation. This implies that the association between spring LER and early flowering can be broken through recombination and DNA markers would be able to facilitate breeders' efforts to do this in their attempts to extend the growing season and to combine early spring growth with later flowering habit. It is self-evident that breeders have succeeded in doing this through phenotypic selection already in the creation of late flowering varieties with early spring growth.

The creation of a saturated map created on a SNP array consisting of polymorphisms of expressed gene sequences makes it possible to identify underlying candidate genes that generate variation in LER.

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Chapter 34
Inheritance of Crown Rust Resistance in a Perennial Ryegrass Genotype of the Cultivar Arvella

Franz Xaver Schubiger and B. Boller

Abstract Crown rust, caused by *Puccinia coronata* f. sp. *lolii* is a common disease of perennial ryegrass (*Lolium perenne*) in Europe. To study the inheritance of resistance to crown rust, a resistant genotype of the diploid cultivar Arvella was crossed with a susceptible genotype of the cultivar Aurora to generate a F_1 population. Out of this population, two parents were selected on the basis of crown rust reaction and backcrossed with a susceptible Aurora genotype to generate two sets of F_2 populations. All parents, the F_1 and the two F_2 populations were screened for their response to three single-pustule isolates (SPI) of crown rust in a detached-leaf segment test under growth chamber conditions. Genetic analysis of resistance led to the identification of two dominant resistance genes. The first gene, LpPc4, conferred resistance to SPI A and K, but not to SPI F. The second gene, LpPc5, proved to be effective against SPI F and K, but not against SPI A.

Keywords Perennial ryegrass • *Lolium perenne* • Crown rust • *Puccinia coronata* • Resistance breeding

Introduction

Crown rust, caused by *Puccinia coronata* f. sp. *lolii* is a common disease of perennial ryegrass (*Lolium perenne*) in Europe. Resistance breeding is the most efficient method of controlling this disease. Resistance of ryegrass cultivars to crown rust has been reported in several studies (Schubiger et al. 2010) and the genetics of resistance was comprehensively reviewed by Dracatos et al. (2010). On the other hand, there is evidence of different pathotypes of crown rust which differ in their virulence on particular perennial ryegrass genotypes (Aldaoud et al. 2004). However,

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Table 34.1 Single-pustule isolates of *Puccinia coronata* f. sp. *lolii* used in this study

Isolate	Origin	Year of collection	Accession no.
A	Switzerland, Zurich	2004	04 12 08
F	Belgium, Merelbeke	2004	04 531 01
K	Switzerland, Zurich	2003	03 01 02

information about the relationship between ryegrass resistance genes and crown rust pathotypes is largely lacking.

The aim of the present study was to test the hypothesis that dominant resistance genes determine the inheritance of resistance exhibited by a genotype of the perennial ryegrass cultivar Arvella.

Materials and Methods

Single-Pustule Isolates

The three single-pustule isolates (SPI), used in this study, derived from uredinial collections from Switzerland and Belgium in 2003 and 2004, respectively (Table 34.1). Spores of each collection were sprayed separately on susceptible plants of the cultivar Aurora. Following 24 h of darkness, infection occurred in a dew chamber with a 16 h photoperiod and a day/night temperature of 18 °C. Urediniospores were collected from a single uredinial pustule (presumed to originate from a single urediniospore) and inoculated on fresh detached leaves on water agar complemented with benzimidazole. Urediniospores of individual developing rust pustules were then used to inoculate plants of Aurora to produce urediniospores of a SPI for further use.

Plant Material

As resistant donor, the genotype Arv was used which originated from a cross between a resistant and a susceptible genotype of the diploid cultivar Arvella. Genotype Arv (resistant to the three SPI A, F and K) was crossed with a genotype A11 from the cultivar Aurora (susceptible to the three SPI A, F and K) to generate an F_1 population. Out of this population, two parents were selected on the basis of crown rust reaction: Arv1 (resistant to SPI A and K and susceptible to F) and Arv2 (resistant to SPI F and K, and susceptible to A). Each of the two parents was crossed with a susceptible Aurora plant (A10) to generate two sets of F_2 populations. All parents, the F_1 and two F_2 populations were screened for their reaction to three SPI of crown rust in a detached-leaf segment test. A total of 112 plants were tested from each cross. The plants were grown in the greenhouse for 7–8 weeks (a cut was done 5 weeks after sowing) prior to use in the detached-leaf segment test.

Table 34.2 Segregation ratios for resistant (r) and susceptible (s) plants in a F_1 generation of a cross involving a resistant genotype (Arv) and a susceptible genotype from cultivar Aurora (A11) after inoculation with single-pustule isolates A, F and K, n = 112

Cross	Generation	Isolate	Arv	Number		Ratio tested	Chi-square	P-value
				r	s			
Arv × A11	F_1	A	r	53	59	1:1	0.32	0.57
	F_1	F	r	58	54	1:1	0.14	0.71
	F_1	K	r	82	30	3:1	0.19	0.66

Detached-Leaf Segment Test

Detached-leaf segments of 2–3 week old regrowth were placed on water agar complemented with benzimidazole (35 mg l^{-1}). SPI were applied to the detached-leaf segments using a compressed air duster. The goal of inoculation density was 400–600 urediniospores cm^{-2}. Leaf segments were incubated for 24 h at 16 °C in the dark and then transferred to a growth chamber at 16 °C with continuous light (fluorescent lamps, 20–30 μ Mol m^{-2} s^{-1}). Assessments of disease response were carried out at 14 days after inoculation, using a 0-to-4 scale (Schubiger et al. 2007). Infection types 3 and 4 were regarded as compatible (virulent SPI/susceptible host) and all others as incompatible (avirulent SPI/resistant host).

Statistical Analysis

The observed ratios of resistant to susceptible plants in the segregating populations were compared to theoretical ratios using Chi-square tests. A P-value greater than 0.05 indicates that the progeny does not deviate significantly from the presumed ratio.

Results

The F_1 population derived from a cross between the genotype Arv (resistant to SPI A, F and K) and the susceptible genotype A11. The frequency of resistant and susceptible plants within the F_1 population resulted in a ratio of 1R:1S for isolates A and F (Table 34.2).On the contrary, a 3R:1S segregation ratio was observed within the progeny when inoculated with the isolate K. A genotype, resistant to SPI K, was either resistant to SPI A or F as well or to both SPI: The proportion of the plants that was resistant to SPI K and A (and susceptible to SPI F), was 22 %. And 26 % of the plants were resistant to K and F but susceptible to SPI A. A quarter of the progeny (25 %) was resistant to all of the three SPI tested. Moreover, each of the genotypes susceptible to SPI K (27 %) was also susceptible to SPI A and F.

Table 34.3 Segregation ratios for resistant (r) and susceptible (s) plants in a F_2 generation of a cross involving a resistant genotype (Arv1) derived from F_1 and a susceptible genotype from cultivar Aurora (A10) after inoculation with single-pustule isolates A, F and K, n = 112

Cross	Generation	Isolate	Arv1	Number		Ratio tested	Chi-square	P-value
				r	s			
Arv1 × A10	F_2	A	r	60	52	1:1	0.57	0.45
	F_2	F	s	0	112	–		
	F_2	K	r	60	52	1:1	0.57	0.45

Table 34.4 Segregation ratios for resistant (r) and susceptible (s) plants in a F_2 generation of a cross involving a resistant genotype (Arv2) derived from F_1 and a susceptible genotype from cultivar Aurora (A10) after inoculation with SPI, n = 112

Cross	Generation	Isolate	Arv2	Number		Ratio tested	Chi-square	P-value
				r	s			
Arv2 × A10	F_2	A	s	0	112	–		
	F_2	F	r	59	53	1:1	0.32	0.57
	F_2	K	r	59	53	1:1	0.32	0.57

F_2 families were shown to segregate for resistant versus susceptible responses to SPI depending on the parent used in the cross. Genotype Arv1, resistant to SPI A and K and susceptible to F, was crossed with the susceptible plant A10. The progeny had a good fit to the segregation ratio of 1R:1S for resistant and susceptible genotypes, when inoculated with SPI A and K but all of them were susceptible to F (Table 34.3). Each of the 60 genotypes resistant to SPI A was also resistant to SPI K.

Genotype Arv2, resistant to SPI F and K and susceptible to A, was likewise crossed with the susceptible plant A10. A segregation ratio of 1R:1S was observed within the progeny for the SPI F and K (Table 34.4). The progeny was susceptible to SPI A. The identical genotypes were resistant or susceptible, respectively, to the two SPI F and K.

Discussion

The segregation of resistant versus susceptible offspring in the F_1 family suggests that two unlinked genes for crown rust resistance must have been transferred from resistance donor Arv into the F_1 family: Gene LpPc4 conferred resistance to SPI A and K but not to SPI F. Gene LpPc5 conferred resistance to SPI F and K, but not to SPI A. That is to say, both genes conferred resistance to SPI K, but differed in terms of resistance response to SPI A and F, respectively. Moreover, these two genes acted dominantly and independently from each other to confer resistance.

Segregation analyses of the F_2 families confirmed this suggestion. In the two F_2 families, supposed to have either LpPc4 (Arv1) or LpPc5 (Arv2), the segregation fitted a 1R:1S ratio only to those SPI which were avirulent to the corresponding resistance gene.

Chances are that there are other resistance genes in the resistance donor Arv. However, these unknown genes are ineffective to the three SPI tested and the three SPI must be virulent to these genes.

Several QTL's involved in crown rust resistance are reported in the literature Dracatos et al. (2010). The two resistance genes LpPc4 and LpPc5 have to be studied to determine whether they are different or identical to previously reported loci.

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Chapter 35
High Density Array for SNP Genotyping and Mapping in Tetraploid Alfalfa

Yuanhong Han, Dong-Man Khu, Xuehui Li, Andrew Farmer, Juanita M. Martinez, E. Charles Brummer, and Maria J. Monteros

Abstract The development of an Illumina iSelect Infinium array that includes approximately 9,277 SNPs identified from sequencing 27 diverse alfalfa (*Medicago sativa* L.) genotypes can be used to accelerate genome-wide association, mapping and molecular breeding efforts in alfalfa. Individuals from a mapping population obtained from a cross between Altet-4 and NECS-141 segregating for aluminum tolerance, fall regrowth, lignin content and other agronomic characteristics were genotyped using the developed array. The dosage status (nulliplex, simplex, duplex, triplex, or quadruplex) of an individual was successfully distinguished using GenomeStudio software for most of the SNPs evaluated. A total of 3,701 SNPs were polymorphic and segregating in the progeny of the mapping population. The number of SNP with segregation ratios suitable for mapping in tetraploid alfalfa were 1,738 SNPs for simplex × nulliplex, 582 SNPs for duplex × nulliplex, and 426 SNPs for simplex × simplex. TetraploidMap software was used to integrate SNPs from the array with an existing framework map. The colinearity between the resulting linkage map and the *Medicago truncatula* physical map was evaluated. The resulting high-density linkage map in alfalfa generated from this study can be useful to guide the ongoing assembly of the tetraploid alfalfa genome sequence and to identify genic SNPs in relevant loci associated with key traits of agronomic importance. The iSelect Infinium assay represents a platform for high-throughput SNP genotyping in alfalfa that greatly improves genotyping efficiency and enables detection of allelic dosage in a tetraploid species.

Keywords Alfalfa • Aluminum tolerance • Infinium • iSelect chip • SNP

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Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forage legumes worldwide. Recently, transcriptome sequences from 27 genetically diverse alfalfa genotypes including 16 elite breeding lines from four U.S. alfalfa breeding companies and 11 parental genotypes of existing mapping populations were generated (Li et al. 2012). These sequences were used to identify 872,384 SNPs and 31,760 InDels in alfalfa that were used to generate optimal SNPs for an Illumina iSelect Infinium array. The alfalfa Infinium iSelect chip contains 9,277 biallelic genic SNPs identified from the alfalfa transcriptome sequences (Li et al. 2013). SNPs included in the iSelect chip are targeting candidate genes associated with biotic and abiotic stresses (aluminum tolerance, drought tolerance, disease resistance and insect resistance) and agronomic traits (dormancy, flowering, capacity for re-growth, heterosis, persistence, winter-hardiness and lignin biosynthesis). Because alfalfa is an out-crossing autotetraploid ($2n=4\times$) species, SNP genotyping offers unique challenges due to the number of possible genotypes based on the number of alleles (Han et al. 2012). Therefore, the ability to detect allelic dosage is a key feature for high-throughput SNP genotyping systems in tetraploid species.

Non-optimal soil conditions for crop growth include either nutrient deficiencies or toxicities due to metal ions including aluminum (Al). Al is the third most abundant element on the Earth's crust. Al toxicity is a global problem for crop production. Al is soluble in acidic soils, which make up 50 % of the world's arable land. Alfalfa is very sensitive to soil acidity and Al toxicity. To identify genomic regions associated with Al tolerance in alfalfa, a mapping population was developed from a cross between the Al-tolerant genotype (Altet-4) and the Al-sensitive genotype (NECS-141). This population was used to generate a microsatellite-based linkage map and used to identify several quantitative trait loci (QTL) associated with Al tolerance (Khu et al. 2013). The objectives of this study were to evaluate the effectiveness of the Infinium iSelect chip to determine allelic dosage in tetraploid alfalfa at multiple loci and to identify or refine the position of Al tolerance QTLs.

Materials and Methods

Genotyping

DNA from the parents and progeny of the mapping population was extracted using Promega's Wizard® Genomic DNA Purification Kit. All samples were genotyped using the alfalfa iSelect Infinium Chip at the National Center for Genome Resources, located in Santa Fe, NM, U.S.

Phenotyping

Each individual genotype from the population (185 F_1 plants with three replicates) and the parental genotypes were evaluated for their performance in response to Al using three methods: callus bioassay, whole plant assay in media and soil-based assay (Khu et al. 2012, 2013).

Data Analysis

Genotyping results and SNP data points were analyzed using Illumina's Genome Studio software (Illumina 2011) and the available option for tetraploid species. TetraploidMap (Hackett and Luo 2003) was used to generate SNP-based linkage maps and for Al tolerance QTL identification.

Results and Discussion

The Infinium genotyping platform enables detection of the five SNP allelic dosages in tetraploid alfalfa (Fig. 35.1). Each of the five clusters includes all of the individuals (each of which is represented by a dot) with the same allelic

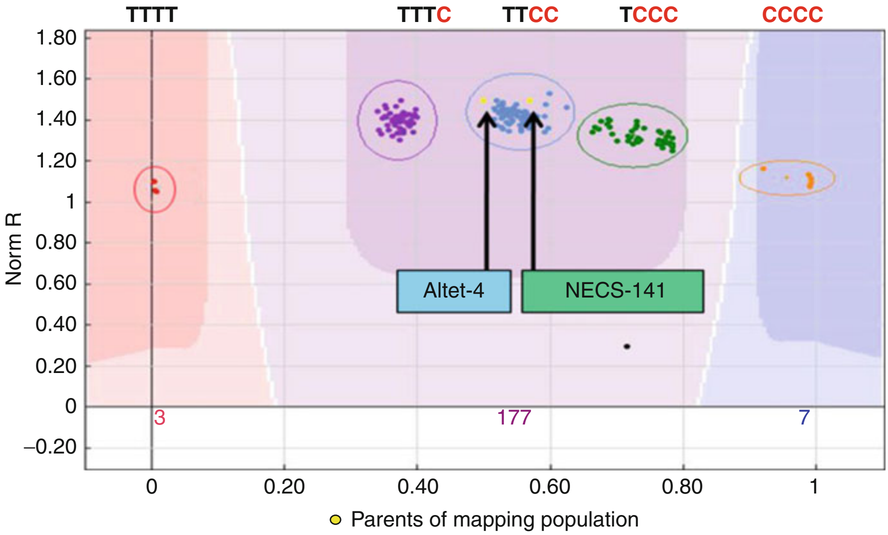

Fig. 35.1 SNP genotyping using an Infinium iSelect chip and identification of the corresponding allelic dosage in tetraploid alfalfa using the transcriptome sequences

Table 35.1 Interval mapping with SNP markers used to identify Al tolerance QTLs in the NECS-141 × Altet-4 population

Trait	Contributing parent	LG	Position (cM)	LOD	Percent variance
Whole plant assay	Altet-4	4	52	3.7	25.2
Whole plant assay	NECS-141	7	54	4.5	21.3
Callus bioassay	NECS-141	1	18	3.6	18.7
Callus bioassay	Altet-4	3	24	4.7	26.7

composition: TTTT, TTTC, TTCC, TCCC and CCCC. A total of 3,701 SNPs were polymorphic and segregating in the progeny. A total of 2,746 SNPs showed segregation patterns suitable for mapping in tetraploid alfalfa: these include 1,738 SNPs for simplex × nulliplex, 582 SNPs for duplex × nulliplex, and 426 SNPs for simplex × simplex. A SNP-based linkage map with eight LGs was generated for each parent: Altet-4 with 348 mapped SNPs and 377 mapped SNPs in NECS-141. The linkage maps showed high levels of colinearity with the *M. truncatula* genome V. 3.5.

Phenotypic evaluations using a whole plant assay and a callus bioassay resulted in the identification of four QTL associated with Al tolerance (Table 35.1). Two of the Al tolerance QTLs identified using the SNP markers are located near QTLs previously identified using microsatellites (Khu et al. 2013). In addition, the enhanced marker coverage obtained from the SNP chip-based genotyping efforts enabled the identification of additional Al tolerance QTL.

The alfalfa iSelect Infinium assay is an efficient platform for SNP genotyping that enables detection of allelic dosage in tetraploid alfalfa. Ongoing efforts include integrating microsatellites and SNPs to enhance molecular marker coverage in the linkage map, refine the location of Al tolerance QTLs and identification of genomic regions of relevance to stress responses and agronomic traits segregating in the Altet-4 × NECS-141 population. The identified markers will be used to enhance Al tolerance and other agriculturally-relevant traits in alfalfa breeding programs.

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Chapter 36
Variation of Cell Wall Digestibility in Fodder Grasses with Particular Focus on a Perennial Ryegrass Breeding Pool

Joost Baert, An Ghesquiere, and Chris Van Waes

Abstract Improved digestibility of neutral detergent fibre (NDFD) of fodder grasses may contribute to a higher dry matter intake and a better grass protein use efficiency by the ruminant. We determined NDFD of several fodder grass species (perennial and Italian ryegrass, meadow and tall fescue, cocksfoot and timothy) and families of a perennial ryegrass breeding pool by NIRS (near infrared reflectance spectroscopy). Among the grass species, perennial ryegrass had the highest and timothy the lowest mean annual cell wall digestibility. Tetraploid ryegrass varieties showed a higher NDFD than diploids. The variation of the NDFD among families of perennial ryegrass is similar to the variation of the organic matter digestibility (OMD) but much lower than the variation of the content of water soluble carbohydrate (WSC). The mean annual NDFD is significantly correlated with the NDFD of all single cuts but best with the NDFD of the last cut which also showed the largest variation. NDFD is positively correlated with OMD and negatively with the dry matter content. There was no correlation with WSC content. Because of the low variation of NDFD among families of perennial ryegrass when frequently cut, selection for higher digestibility by increasing NDFD will be hard.

Keywords Cell wall digestibility • Fodder grasses • Perennial ryegrass

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Introduction

Increase in organic matter digestibility (OMD) of fodder grasses leads to a higher dry matter intake by the cows and to a higher milk yield. The digestibility may be improved by increasing the sugar content or the accessibility of cell wall carbohydrates. Thanks to the large genetic variation in water soluble carbohydrate content (WSC) in forage grasses, high sugar grasses have been bred. However, a very high WSC content in the grasses may cause rumen acidosis and warming up of the silage when opening. Therefore, it might be better to improve the digestibility of the cell wall in order to further improve the OMD. Based on a near infrared reflectance spectroscopy (NIRS) analysis, we studied the variation in cell wall digestibility among grass species. We further looked at the variation of the cell wall digestibility of families in our breeding pool of perennial ryegrass on an annual basis and for single cuts. We determined the correlation between NDFD and yield and quality parameters. These data are discussed with regard to the prospects of breeding perennial ryegrass for an improved cell wall digestibility.

Materials and Methods

To study the variation of NDFD among fodder grass species, a strip plot experiment in three replicates with two levels of nitrogen application rate (350 and 230 kg N ha^{-1} year) and 24 varieties belonging to six species was sown in September 2006 in Merelbeke (Belgium). In 2007 and 2008 the grass plots (size: 8.1 m^2) were cut five times a year. The species (cultivars) were: diploid perennial ryegrass: early heading (Rebecca, Indiana), intermediate heading (Aberdart, Premium), late heading (Barnhem, Merks); tetraploid perennial ryegrass: early heading (Merlinda, Lacerta), intermediate heading (Aberglyn, Graciosa), late heading (Abercraigs, Floris); timothy (Comer, Motim); meadow fescue (Merifest, Pradel); tall fescue (Barolex, Barelite, Bariane); cocksfoot (Barmoral); diploid Italian ryegrass (Bellem, Davinci) and tetraploid Italian ryegrass (Gemini, Barmega).

To study the variation in perennial ryegrass, 30 accessions (families and populations) of either diploid intermediate (DI), diploid late (DL) or tetraploid (T) perennial ryegrass were sown in two replicates in 2010. The trials were mown five times in 2011, the first cut being taken at the end of April, the second half of June, the third end of July, the fourth mid of September and the fifth end of October. The trials received 300 kg of nitrogen (N) fertilizer per ha per year.

At each cut, we determined dry matter yield (DMY) and dry matter content (DMc). A sample of about 300 g fresh weight was taken per plot and dried at 75 °C. The dried samples of the replicates were mixed and ground. We estimated by NIRS the WSC content, the OMD, the crude protein (CP) content and the NDFD. The NIRS calibration of the NDFD is based on in vitro digestibility (Goering and Van Soest 1970) and NDF analysis (Van Soest et al. 1991) of 384 samples. R^2 of the calibration was 0.84 and the standard error of calibration was 2.7 %.

Results and Discussion

Variation Among Fodder Grass Species

There were significant effects of the harvest year, the cuts within each year, the N fertilization level, the species and the varieties on the NDFD. At the high N application rate, the NDFD was slightly (0.8 %) but significantly ($p<0.05$) higher than at the low rate. The mean NDFD of the varieties ranged from 64.1 for the timothy cv. Comer to 73.5 % for the late tetraploid perennial ryegrass cv. Floris (Fig. 36.1). On average, the tetraploid perennial ryegrass varieties had the highest and the timothy varieties the lowest cell wall digestibility. For both perennial and Italian ryegrass species, tetraploid varieties had a higher NDFD (2.2 and 1.5 %, respectively) than the diploid varieties.

Variation in a Perennial Ryegrass Breeding Pool

Table 36.1 shows the mean annual NDFD, WSC and OMD of the three trials and the variation of the entries within each trial. The NDFD variation among entries of all trials was similar to the variation of the OMD, but much lower than the variation of the WSC content. When selecting the 5 % best entries, the overall mean NDFD

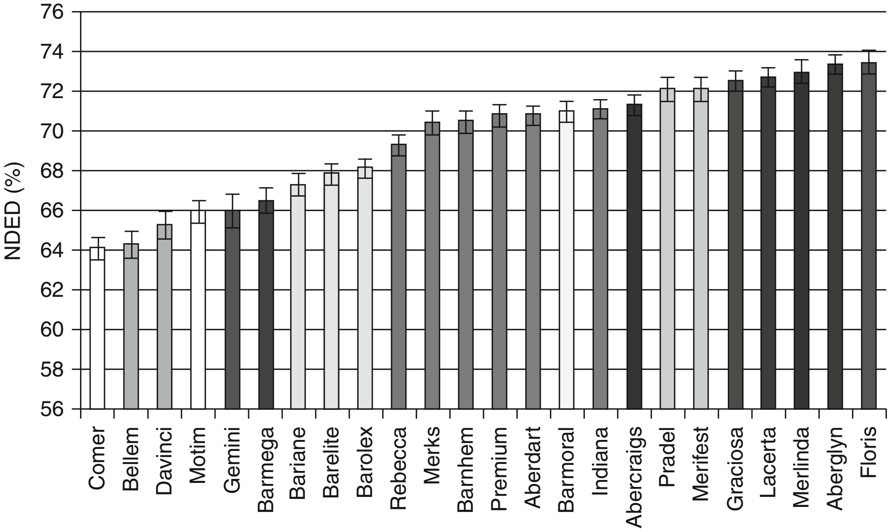

Fig. 36.1 The NDFD (average ± s.e. of 2 years, 5 cuts a year, 2 N rates) of 24 varieties belonging to the grass species perennial ryegrass (*very dark grey*; *with shadow*: tetraploid), Italian ryegrass (*dark grey*; *with shadow*: tetraploid), meadow fescue (*grey*), tall fescue (*light grey*), cocksfoot (*very light grey*) and timothy (*white*)

Table 36.1 Mean annual NDFD, WSC content and OMD (%) and coefficient of variation

Trial	NDFD		WSC		OMD	
	Mean	Cv (%)	Mean	Cv (%)	Mean	Cv (%)
DI	68.9	1.3	18.7	6.1	80.1	1.7
DL	68.7	1.3	20.0	7.1	80.7	1.3
DT	71.3	1.5	22.4	6.3	82.0	1.3

Table 36.2 Correlation between average NDFD on an annual basis and NDFD of single cuts or combination of cuts

Trial	Cut1		Cut2		Cut3		Cut4		Cut5		Cut3+5	
DI	0.62	***	0.45	*	0.47	**	0.49	**	0.62	***	0.83	***
DL	0.58	***	0.43	*	0.66	***	0.33	ns	0.59	***	0.85	***
T	0.51	**	0.54	**	0.66	***	0.62	***	0.63	***	0.82	***
Mean	0.57		0.48		0.60		0.48		0.61		0.83	

*, **, *** significance at respectively $p<0.05$, $p<0.01$ and $p<0.001$

Table 36.3 Mean NDFD (%) and variation at each cut

	Cut1		Cut2		Cut3		Cut4		Cut5	
	Mean	cv %	Mean	cv %	Mean	cv %	Mean	cv %	Mean	cv %
DI	75.2	1.3	64.9	2.6	64.4	2.8	74.4	1.6	65.9	4.3
DL	74.3	1.5	67.0	1.7	62.4	3.7	74.0	1.7	66.0	3.1
T	75.1	2.2	69.1	2.7	66.5	2.6	75.9	2.4	69.7	2.7
Average	74.9	1.7	67.0	2.3	64.4	3.0	74.7	1.9	67.2	3.3

increased from 69.6 to 71.9 %. This increase was less than the standard error of the calibration. The average NDFD (in %) of the tetraploids was 2.5 units higher than that of the diploids.

The NDFD on an annual basis was significantly correlated with the NDFD of all single cuts except the 4th cut (Table 36.2). The fifth cut showed the highest correlation to the annual mean followed by the third and the first cut, but differences were small. Taking the two most correlated cuts (3 and 5) together, the correlation coefficient increased from 0.61 to 0.83.

The coefficient of variation of NDFD among entries within a single cut was highest for cut 5 (3.3 %) and lowest for cut 1 (1.7 %) (Table 36.3). This makes cut 5 a recommended cut if one can analyse only a single cut. Reheul and Ghesquiere (1994) advised to consider also the last cut for the determination of the digestibility if only 1 cut could be analysed. The same advise was given by Ghesquiere et al. (2008) for the determination of the WSC content. The NDFD was slightly positively correlated with the OMD and slightly negatively correlated with the DMc and DMY (Table 36.4). There were no significant correlations between NDFD and WSC content.

Table 36.4 Correlation between NDFD and DMY, WSC content, CP content, OMD and DMc

	DMY		CP		WSC		OMD		DMc	
DI	–0.46	**	0.30	ns	0.07	ns	0.42	*	–0.20	ns
DL	–0.22	ns	0.46	**	–0.10	ns	0.40	*	–0.35	ns
T	–0.16	ns	–0.05	ns	–0.06	ns	0.24	ns	–0.60	***
Mean	–0.28		0.24		–0.03		0.35		–0.38	

*, **, *** significance at respectively $p<0.05$, $p<0.01$ and $p<0.001$

Conclusion

Among the tested accessions of the most relevant NW-European fodder grass species, perennial ryegrass had the highest NDFD. When cut frequently, the variation of NDFD among families is low and breeding progress will be slow. Probably breeding for higher NDFD is more obvious for species with lower digestibility such as tall fescue and timothy or in ryegrass production systems where more stems are harvested (e.g. Italian ryegrass for hay and silage).

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Chapter 37
QTL Identification for Leaf Senescence in Perennial Ryegrass (*Lolium perenne* L.)

Meraluna Canunayon and Daniel Thorogood

Abstract In plants, lifespan is agronomically important as it is a major determinant of productivity. The onset of leaf senescence is associated with the decrease in photosynthetic capability that reduces herbage yield and biomass production. This research aims to identify genes/quantitative trait loci (QTL) that can prolong photosynthesis and delay the senescence processes in *Lolium perenne*. This study is vital for understanding the mechanism of senescence and also for practical purposes such as improvement of productivity, silage and hay quality and stress tolerance. A linkage map of an F2 *Lolium perenne* population derived from a cross between genotypes of the cultivars Aurora and Perma was developed by IBERS consisting of seven linkage groups. This same population was used for the characterisation of leaf senescence related traits. Four different phenotyping activities were conducted, namely: (1) detached leaf senescence under natural light; (2) dark-induced senescence of detached leaves; (3) attached leaves grown in a controlled environment and (4) attached leaves grown under unheated green-house conditions with natural light.

One major QTL for leaf senescence was identified on linkage group 2. This QTL is robust and consistent as it was detected in four different conditions. The traits on visual color index, chlorophyll fluorescence and chlorophyll loss were mapped with an LOD of >3.0. There were also three minor QTLs for leaf senescence related traits on linkage in linkage group 3, 4 and 6.

Keywords QTL • Leaf senescence • *Lolium perenne*

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most economically important forage grasses due to high digestibility (Frame et al. 1989), palatability, good tolerance to grazing and adequate seed production (Wilkins 1991). It is also widely used in amenity grassland including sports turf (Wilkins and Humphreys 2003). It is a diploid species with seven pairs of chromosomes ($2n=2x=14$) and a relatively large genome with an estimated size of about 1,660 Mbp (Forster 1999). It is a wild undomesticated species with inherent outcrossing characteristics thus subject to natural selection under highly diverse environments over many generations. It is a member of the *Triticeae* tribe of grasses and has high levels of synteny with major crops species such as barley, rice and wheat and also with the model grass genus *Brachypodium*.

The goal of this research project was to identify molecular markers and QTLs associated with leaf senescence. The interest was on developing and/or identifying molecular marker systems for perennial ryegrass which can be used for marker-assisted selection (MAS) to improve the current of breeding approaches used for lifespan improvement of forage grasses.

Materials and Methods

Plant Material

The mapping family used for genetic and phenotypic characterisation was an F2 *Lolium* population consisting of 188 plants which were produced by self-pollinating a single F1 hybrid plant, from a cross of two genotypes derived from inbred lines produced from selfing plants of the varieties Perma and Aurora.

Detached Leaf Experiments

Leaf segments were taken from the youngest fully expanded leaf, placed in a petri dish on moistened filter paper and stored for 10 days in a chamber with the following conditions: 12 h day length; 21 °C temperature and 85 % relative humidity.

Attached Leaf Experiments

Fully expanded leaves attached to the plants were measured every 10 days.

Data Gathered

The senescence related trait parameters gathered were: (1) total chlorophyll content using a Konica-Minolta SPAD 502 Plus chlorophyll meter; (2) colour index (visual color assessment) was converted into a numerical value using a scale ranging from 0 to 10 where 0 equals no senescence and 10 equals completely senesced leaves; (3) leaf colour expressed in L, a, b values, measured at around 2 cm from the leaf base using a Konica Minolta Flourescent Spectrodensitometer FD-5; (4) chlorophyll fluorescence parameters (Fo, Fm, Fv/Fm and Plant Performance Index-PI) using a Hansatech Handy PEA; (5) Chlorophyll a and b content using a spectrophotometer and (6) Chlorophyll a/b ratio.

QTL Analysis

A genetic map of the F2 *Lolium* population was constructed by Armstead et al. (2002) with seven linkage groups that covered a total of 515 cM. The same genetic map used for QTL analysis using Joinmap® 3.0 (Van Ooijen and Voorrips 2001). QTL analysis was carried out using MapQTL 4.0 (Van Ooijen et al. 2002). Each trait was analysed by interval mapping. A QTL was declared when the LOD score was >3.0 and the QTL position was supported by Kruskal–Wallis nonparametric single-locus analysis.

Results and Discussion

A major QTL region for leaf senescence related traits was detected on linkage group 2. This QTL region was very consistent and robust being detected in four different environmental conditions. Percentage variation explained by individual QTL ranged from 11 to 27 % and these QTL were considered as major QTL. The traits on visual color index (CI), color (b and L values), chlorophyll fluorescence (Fo/Fm/Fm and PI), and chlorophyll degradation (chl loss) were mapped (Fig. 37.1). The presence of the chlorophyll fluorescence QTL indicates that the putative genes underlying the QTL region influencing leaf senescence, rather than being merely cosmetic stay-green variants, are associated with variation in actual photosynthetic function during delayed leaf senescence.

There were also four minor QTL regions detected in linkage group 3, 4 and 6. These QTLs were found in one environmental condition hence considered as minor QTLs. One QTL was found for chlorophyll content on linkage group 3 explaining 10.3 % of the total phenotypic variation. The two QTLs for chlorophyll fluorescence for attached leaves grown in the controlled environment were located on linkage group 4 explaining 14.9 % (Fm) and 15.6 % (Fv) of the phenotypic

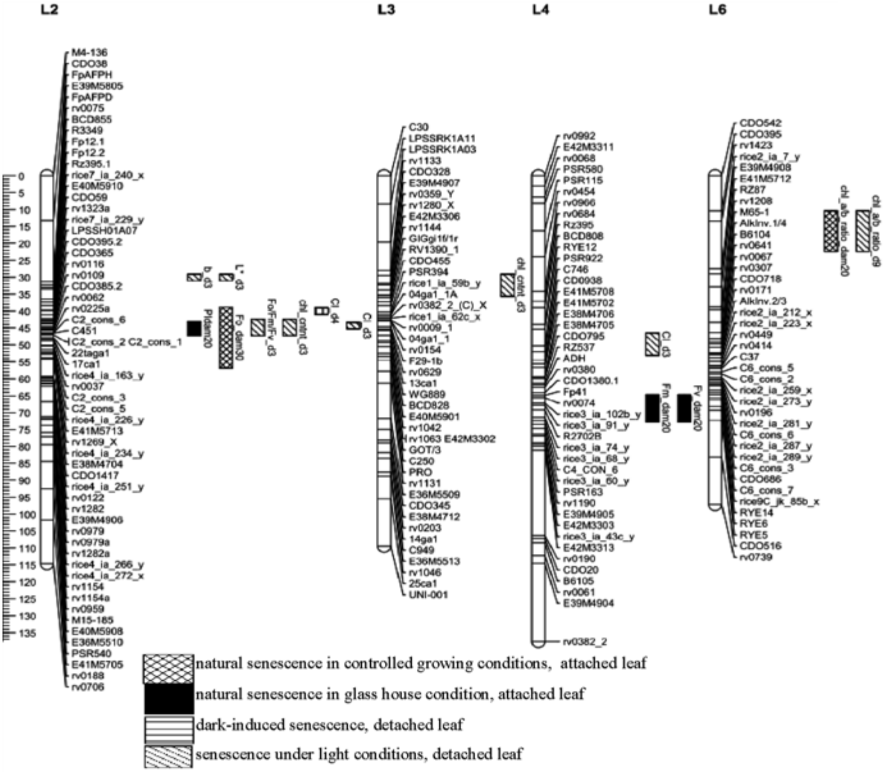

Fig. 37.1 Genetic map of F2 *Lolium perenne* population showing the QTL positions for leaf senescence related traits

variation, while another QTL for color index was found for detached leaves senescing under the presence of light explaining 11.5 % of the variation. Two QTL regions were mapped on linkage group 6 for the same trait on chlorophyll a/b ratio on attached leaves grown under glass house conditions explaining 8.6 % of the variation, and detached leaf senesce in the light explaining 11.2 % of the variation.

Further work is needed to obtain consistent QTL by repeating the phenotyping experiments. This will include a detailed phenotypic characterisation of the flag leaves and vegetative leaves and adding more markers to the map (particularly SNP markers), to have a more dense genetic map. Finding the candidate genes for leaf senescence and tracking a reasonably close molecular marker in *Lolium* populations is feasible with the ongoing development of RAD and SNP markers in this F2 mapping family and the use of substantial genomic information of model crops specifically rice and barley. In addition, we have initiated a gene expression study by RNAseq, to identify senescence associated candidate genes that map to our QTL regions.

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Chapter 38
Validation of Candidate Genes Expression Profiles During Cold Acclimation and Freezing in Perennial Ryegrass

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Abstract Sixty perennial ryegrass (*Lolium perenne* L) plants were cold-acclimated and then subjected to freezing at −10 °C. Quantitative real-time polymerase chain reaction was performed to analyse the expression of six candidate genes during cold-acclimation and freezing periods. Four genes responded similarly in two investigated genotypes during both hardening and freezing periods, while the cold-response genes *LpIRI* and *LOS2* distinguished high induction level. These genes represent valuable candidates for further SNP analysis to identify functional markers for cold tolerance in perennial ryegrass.

Keywords Freezing tolerance • Perennial ryegrass • Relative gene expression • qPCR

Introduction

High forage quality makes *Lolium perenne* L. the predominant forage grass used in temperate agriculture. However, abiotic stress, such as cold, not only limits the geographic distribution of the species but also adversely affects its development and yield. Therefore, breeding of ryegrass varieties with improved tolerance to abiotic stress is an important task. Since the genomic resources in perennial ryegrass have

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been rapidly accumulated over the recent years (Studer et al. 2010) it could serve as a model species for studying and improving tolerance to abiotic stress (Jonavičienė et al. 2012). A large number of genes have been suggested to be involved in the cold response, but identification of the promising targets for breeding cold–resistant crop varieties remains a significant technical challenge. The study aimed at (i) examining gene expression levels of six candidate genes during the freezing treatment in the two parental genotypes of the perennial ryegrass VrnA mapping population (Jensen et al. 2005), (ii) selecting candidate genes involved in cold-acclimation and freezing tolerance for identification of Single Nucleotide Polymorphisms (SNPs) and (iii) performing association studies of the candidate-gene in a perennial ryegrass association panel.

Materials and Methods

Plant Material and Freezing Test

The two parental genotypes, 'Veyo' and 'Falster', of the perennial ryegrass VrnA mapping population (Jensen et al. 2005) were propagated vegetatively from a single mature tiller in a greenhouse. When the plants reached the booting stage, they were placed in a growth chamber for 2 weeks at 6 ± 1 °C and 16/8 h (day/night) with an irradiance of 200 μmol m^{-2} s^{-1} for cold acclimation. After hardening, the plants were subjected to −10 °C for 30, 60, 90 and 150 min, respectively.

RNA Extraction and cDNA Synthesis

Perennial ryegrass leaf and root tissues were grounded in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Residual genomic DNA was removed by on-column DNAse I digestion (Qiagen). Synthesis of the first-strand cDNA was done using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations.

Quantitative Real-Time PCR (qPCR)

Primers for six candidate abiotic stress response genes (cold: ice recrystallization inhibition (IRI) protein – *LpIRI,* low expression of osmotically responsive genes 2 – *LOS2;* drought: DRE-binding protein – *DREB1A,* drought induced gene – *Ti_Con_113;* multiple stress: SNF1-related protein kinase 2.4 – *SnRK2.4,* multicopy suppressor of *ira7* – *MSI1*) were designed using the Primer3 software (Table 38.1).

Table 38.1 Candidate genes and corresponding primers used in qPCR

Candidate gene	Accession number	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product length, bp
SnRK2.4	GQ384359.1	ATGCTGCTGCTCCTCATGTA	GCAATCCAACAAAGAGAATCAC	121
MSI1	EU961955.1	CATTTCTCGCTAGGGTTCCA	GACGTTAGCAGGATCGGAGA	128
Ti_Con_113	P12782.1	GCAACAGCCTCAGTTCCAA	CCCAGATTCGGTCAAAACAT	117
DREB1A	DQ012941.1	ATGGCTGTCGAATGTATGC	CTTGCGTACGAACAAAGCAG	173
LpIRI	EU680848.1	ATTGGTGAGCTTGACCACCT	TTGGTTGTATGGCGAGAGTTC	184
LOS2	GQ144341.1	GTCTGCGCCTTCTACCAGAG	ACGTATCTAGCTCGATCCTGGAC	197

Primer specificity was confirmed by performing a blastn analysis against the perennial ryegrass genome available at Aarhus University. For normalization of target gene expression, the *YT-521-B*-like protein family gene was used (Lee et al. 2010). All qPCR reactions were done using three biological and three technical replicates. PCR amplification was monitored and analyzed on a 7,300 Real Time PCR System (Life Technologies, Carslbad, CA, USA). Primer efficiencies were calculated in standard curve dilutions. Expression ratio was determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results and Discussion

The expression profiles of six abiotic stress response genes were investigated by qPCR in root and leaf tissues of two perennial ryegrass genotypes contrasting in vernalization requirements (Jensen et al. 2005). Distinct expression profiles and different induction levels were observed among the cold-acclimation and freezing periods, as well as between the two genotypes (Figs. 38.1 and 38.2).

The *MSI1* gene was induced progressively in tillers of both genotypes and reached the highest expression level in the 'Falster' genotype at freezing temperatures (Fig. 38.1), while in roots it had a very low expression level (Fig. 38.2). In contrast, the expression level of *SnRK2.4* in tiller tissue was similar at cold-acclimation and freezing (Fig. 38.1). In roots, the expression level contrasted between both periods, reaching the highest expression level in both genotypes at cold-acclimation, while no expression could be observed during freezing (Fig. 38.2). The drought response gene *DREB1A* in root and tiller tissue reached the maximum expression level during the cold-acclimation period and was up to twofolds higher in the 'Falster' genotype compared to the 'Veyo' genotype.

The results showed contrasting gene expression for the cold response genes *LpIRI* and *LOS2* in tiller tissue (Fig. 38.1). The transcription of *LpIRI* peaked at cold-acclimation temperatures and was twofolds higher in the 'Veyo' genotype compared to the 'Falster' genotype. The transcript level of *LOS2* reached the maximum at the cold-acclimation period and was highly expressed in the 'Falster' genotype while there was no expression in the 'Veyo' genotype (Fig. 38.1). Similar expression levels and profiles were observed for cold response genes in root tissue (Fig. 38.2). Highest difference in expression between the two genotypes at the cold-acclimation period was detected for *LOS2*, where its expression in the 'Veyo' genotype was 300–8,000 fold higher than in 'Falster'. These results suggest that freezing stress levels and pathway activation may vary considerably between the two genotypes under investigation. Some candidate genes evaluated herein, especially the cold-response genes *LpIRI* and *LOS2* could be ranked as the best candidates for further analysis to identify SNPs for association studies in a perennial ryegrass association mapping panel in order to develop functional markers for cold tolerance.

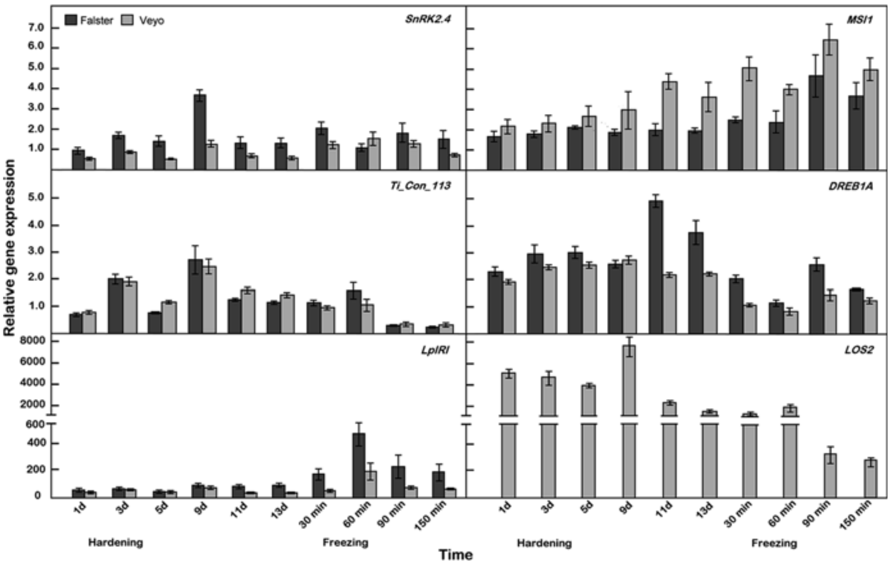

Fig. 38.1 The relative expression of candidate genes involved in freezing tolerance during cold-acclimation and freezing in perennial ryegrass tiller tissue. The bars represent two parental genotypes of VrnA population: Veyo – with no vernalization requirement, Falster – with strong vernalization requirement. Error bars denote standard error

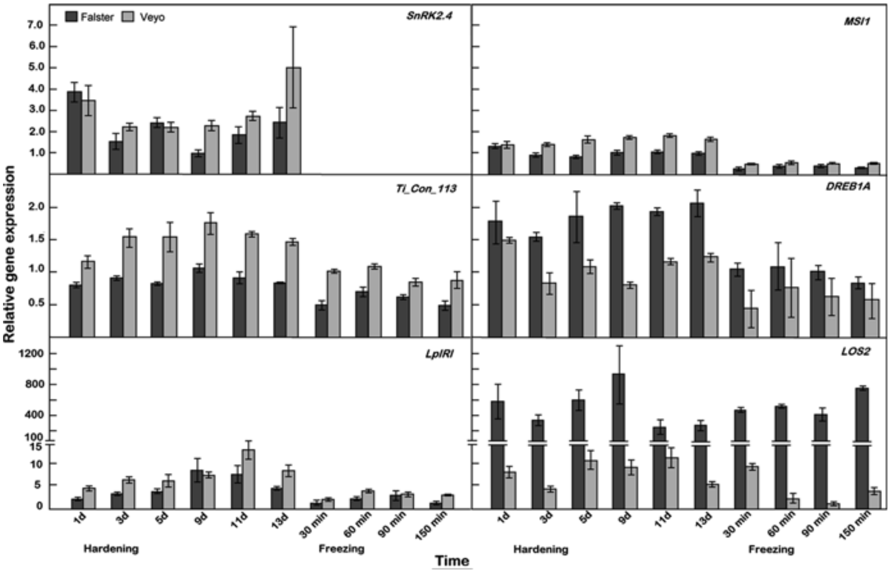

Fig. 38.2 The relative expression of candidate genes during cold-acclimation and freezing in perennial ryegrass root tissue. The bars represent two parental genotypes of VrnA population: Veyo – with no vernalization requirement, Falster – with strong vernalization requirement. Error bars denote standard error

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Chapter 39
Different Aspects of Shoot Branching in Red Clover

Annemie Van Minnebruggen, I. Roldán-Ruiz, E.Van Bockstaele, and G. Cnops

Abstract The aerial architecture of red clover plants and their response to mowing may have a strong influence on agronomic traits such as forage yield, seed yield, regrowth capacity and persistence. Here, we present an in-depth study of the aerial architecture of six red clover genotypes with contrasting branching phenotypes. A detailed morphological analysis revealed significant inter-genotypic differences for the number of nodes and the number of branches, indicating that both bud formation and bud outgrowth contribute to different architectural types. In addition, clear differences for regrowth response after repeated cutting were found among these genotypes, stressing the relevance of branching characteristics for agronomic performance. As both bud formation and bud outgrowth are known to be under strong hormonal control, we determined the response of buds in these six genotypes to the application of strigolactone and auxin. Isolated single node stem fragments were used, to avoid having to deal with the complex architecture of a complete red clover plant, in which the response of an individual bud is influenced by signals from other plant parts. These results were linked to expression levels of genes involved in strigolactone biosynthesis (D27) and auxin transport (PIN1). The results demonstrate that in red clover both the strigolactone and auxin pathways are relevant candidates for further analysis and molecular breeding for high yielding and more persistent red clover cultivars.

Keywords Red clover • Shoot branching • Morphology • Physiology • Genetics

Introduction

Traits such as forage yield, seed yield, regrowth capacity and persistence in red clover may be influenced by architectural characteristics. In agricultural systems, red clover is grown either in monoculture or in multispecies stands that are mown or

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grazed several times throughout the growing season. Consequently, the stand ideally shows an extensive vegetative growth resulting in a high biomass yield, a strong capacity to regrow after cutting or grazing, and a high level of persistence (Taylor and Quesenberry 1996). Branching differences will likely affect plant performance in the stand. However, this species has a relatively short history of systematic breeding and has not directly been selected for architectural traits. Knowledge of the basic mechanisms regulating branching in red clover will contribute to the identification of new breeding targets for a higher and more stable biomass yield. Branch development can be separated into two processes – bud formation and bud outgrowth (Kebrom et al. 2013). Bud outgrowth is regulated by complex hormonal feedback loops involving auxin, cytokinin and strigolactone. Auxin and strigolactones negatively regulate bud outgrowth, while cytokinins promote bud outgrowth (Ongaro and Leyser 2008; Domagalska and Leyser 2011).

Our aim is to understand the processes of bud formation and bud outgrowth in red clover by studying six different architectural red clover types. Here, we describe the phenotype of these genotypes, both in free growth and after cutting. We also describe physiological experiments in which the influence of exogenous auxin and strigolactone application is analyzed. Finally, on a molecular level, the expression levels of two genes involved in the strigolactone and auxin pathway are studied.

Material and Methods

One genotype of each of the following six accessions were chosen for analysis: Crossway, Diplomat, Rubitas, Lemmon, Hungaropolyloid selection (Hp) and No. 16 (Cnops et al. 2010). Clonal replicates of each genotype were grown in a growth chamber (19 °C, 16 h light).

The branching pattern of these genotypes was analyzed as described in Van Minnebruggen et al. (2012). In short, the number of nodes and the number of branches were counted at specific moments, and the bud outgrowth percentage of each plant was calculated by dividing the number of branches by the number of nodes. To determine the regrowth capacity of this set of plants, five clones per genotype were grown in the growth chamber till 4 weeks after the start of flowering, and subsequently cut to a height of 7 cm. At this time point and 2 weeks later, the number of nodes and branches, and the bud outgrowth percentage in the bottom 7 cm were determined.

The response of the six genotypes to hormone treatment was determined according to Van Minnebruggen et al. (2012) with minor modifications. Per treatment, 15 stem fragments containing one not-outgrowing bud were isolated and placed on water solidified with 0.7 % plant tissue culture agar MC29 supplemented with 5 μM GR24 (strigolactone) or 0.5 μM NAA (auxin). Bud lengths were measured at the start of the experiment and 7 days later.

For the analysis of gene expression, similar stem fragments were grown on medium for 24 h. Total RNA was extracted using the RNeasy Mini extraction kit

(Qiagen). A DNAse treatment was done using the DNA-free kit of Ambion, and was followed by a LiCl precipitation. cDNAs were prepared using iScript cDNA synthesis kit (Bio-Rad). Q-PCR was performed with SensiFast SYBR No-ROX kit (Bioline) and was run on a LightCycler 480 Real-time PCR system (Roche). Per genotype and per tissue, three biological repeats were analyzed. The expression of the genes *TpD27* and *TpPIN1* was normalized using *TpEF1A* and *TpUBC2*.

Results and Discussion

Analysis of the shoot architecture of six red clover genotypes resulted in significant differences for both the total number of nodes and the total number of branches (Fig. 39.1a). The difference in the total number of nodes indicates the importance of bud formation in explaining phenotypic differences of the genotypes. Additionally, differences in the total number of branches and the bud outgrowth percentage indicate the importance of bud outgrowth. In the bottom 7 cm, plenty of buds remain for regrowth after cutting or grazing (Fig. 39.1b – at cutting). Also, for regrowth 2 weeks after cutting (Fig. 39.1b), large genotypic variations were observed for the number of remaining nodes, branches and bud outgrowth percentage. For both

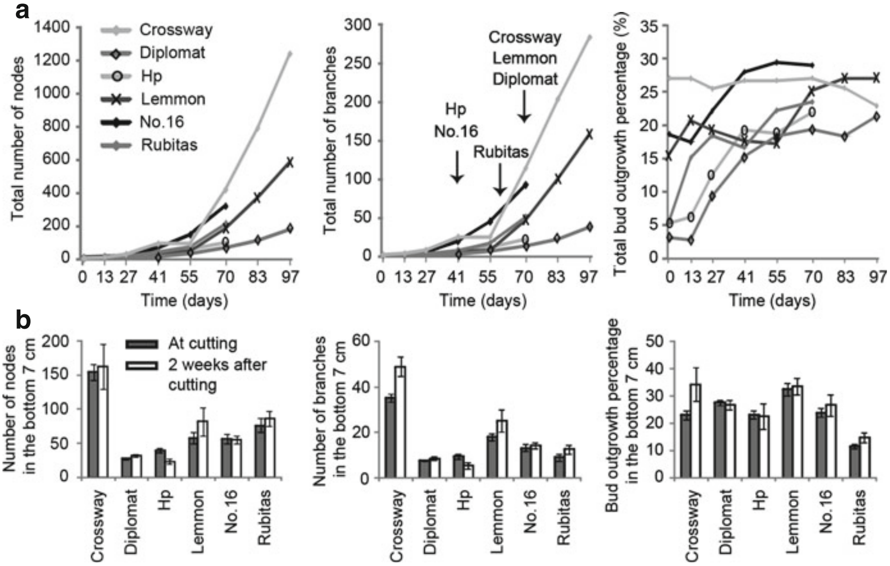

Fig. 39.1 Branching pattern (**a**) and regrowth (**b**) of six red clover genotypes. (**a**) The total number of nodes and branches and the bud outgrowth percentage for clonal replicates grown in the growth chamber until 4 weeks after flowering. (**b**) Subsequently, the plants were cut to a height of 7 cm. The number of nodes and branches, and the bud outgrowth percentage in the *bottom* 7 cm were determined at cutting and 2 weeks later. Flowering time is indicated with an *arrow*. The mean of five clones per genotype is shown. *Bars* are standard errors

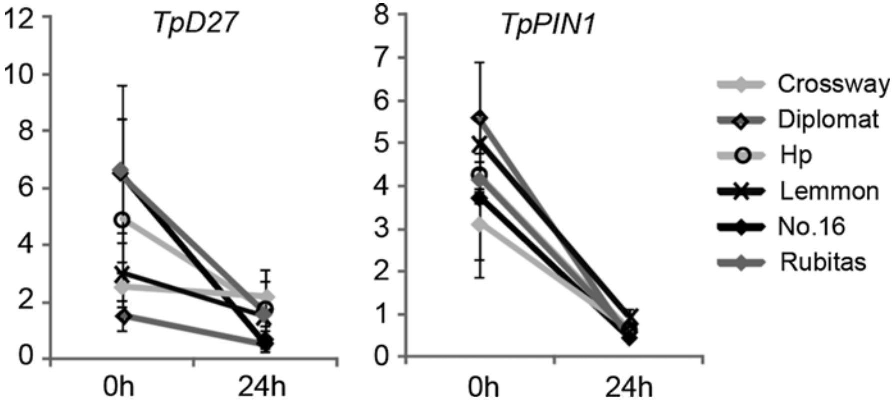

Fig. 39.2 Relative expression pattern of genes in the strigolactone and auxin pathway in red clover isolated non-treated buds 0 h and 24 h after isolation (comparable to decapitation). Expression levels are relative to *TpEF1A* and *TpUBC2*. *D27* = strigolactone biosynthesis gene and *PIN1* = auxin transport gene. Data are from three biological repeats with standard errors as *bars*

experiments (Fig. 39.1a, b), Crossway and Lemmon had the highest number of branches.

In a second experiment, the influence of the branching hormones strigolactone and auxin on bud outgrowth was analyzed by growing stem fragments on medium supplemented with these hormones. The mean relative increase in bud length after 7 days was 5.4 for the control buds, and 1.3 and 1.0 for the GR24 and NAA treated buds, respectively. Thus, in all genotypes, the bud outgrowth was inhibited compared to control buds. We could conclude that there is no defect in the response to these hormones in the six genotypes. However, variations in hormone biosynthesis or transport were not investigated.

In a final experiment, the importance of the strigolactone and auxin pathway in red clover branching were analyzed through expression analyses. The expression of the genes *TpD27* and *TpPIN1* decreases after bud outgrowth (24 h), as expected since strigolactones and auxins inhibit bud outgrowth (Fig. 39.2). Variations in expression levels of these two genes between the six genotypes (0 h) can correspond to variations in the inhibition of bud outgrowth.

As a summary, we conclude that both bud formation and bud outgrowth differ between the studied genotypes, and that both are important for branching in red clover. Physiological experiments show that strigolactones and auxins are involved in bud outgrowth. However, variations in hormone biosynthesis and transport and more complex interactions need to be investigated for their role in branching differences in red clover.

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Chapter 40
AFLP Analysis of Genetic Diversity in an Association Mapping Panel of *Lolium perenne* L.

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Izolda Pašakinskienė, and Gintaras Brazauskas

Abstract Amplified fragment length polymorphism (AFLP) was used to measure genetic diversity in a panel of perennial ryegrass genotypes. The panel consisted of 90 cultivars, 38 ecotypes of Lithuanian origin and 65 ecotypes of Ukrainian origin. The three primer combinations used in the AFLP analyses amplified 227 fragments within the size range of 50–350 bp. Ecotypes formed separate clusters in a principal coordinate analysis (PCoA) according to their origin, while cultivars did not show any clustering pattern. Genotypes were separated into three groups (cultivars, Lithuanian ecotypes and Ukrainian ecotypes) to perform analysis of genetic variation within and between these groups. As expected, most of the variation (96.7 %) was detected within groups. Nei's gene diversity (h) and Shannon's information (I) indices had similar values (ranging from 0.2047 to 0.2094, and from 0.3247 to 0.3329, respectively), indicating similar amount of genetic diversity in all groups. The greatest genetic difference according to population pairwise F_{ST} and Nei's genetic distance (D_N) indices was detected between Lithuanian and Ukrainian ecotype groups. Nevertheless, both genetic distance indices had rather small values ($F_{ST} = 0.019–0.059$; $D_N = 0.0056–0.0147$). In general, high genetic diversity and low differentiation between groups indicate that acquired panel of perennial ryegrass genotypes is suitable for further association studies.

Keywords Perennial ryegrass • Wild ecotypes • Variability

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important grass species in temperate regions. Genetic variation in plant architecture traits is of practical importance in perennial ryegrass breeding because of the potential of these traits in vegetative production enhancement as well as the association with seed yield (Elgersma 1990). However, plant architecture control in perennial grasses is less understood despite high levels of variation and versatile application of these plants as fodder or in amenity grasslands. Association analysis is a powerful tool used to identify genes responsible for variation of complex traits (Ersoz et al. 2007). Estimation of genetic diversity of the population under study is a prerequisite for association analysis. The objective of this study was to measure genetic diversity in a panel of perennial ryegrass genotypes as an initial step in developing functional markers for five perennial ryegrass architecture genes (Brazauskas et al. 2010).

Material and Methods

A total of 193 perennial ryegrass genotypes were analysed in the study: 90 cultivars, 38 ecotypes of Lithuanian origin and 65 ecotypes of Ukrainian origin. One genotype per each cultivar/ecotype was used. All genotypes were diploid. About 100 mg of leaf material was harvested for genomic DNA extraction. DNA was isolated according to modified protocol of Lassner et al. (1989) – additional wash step with chlorophorm and RNA digestion with ribonuclease A were included. 500 nanograms of genomic DNA were digested with Fast Digest (FD) *Taq*I and FD *Ase*I (Thermo Scientific Lithuania). Three primer combinations were used for selective amplification in AFLP. Each primer had three selective nucleotides. PCR products were separated on 6.5 % poly-acrylamide gels in DNA Analyzer 4300 (Licor). Fragments were scored using SAGA Generation 2 software (Licor).

Software package STRUCTURE (Pritchard et al. 2000) was used to infer population structure. Five independent simulations were run by setting the number of populations (K) from 1 to 10, with 10,000 burn-in time and 10,000 iterations of Markov chain convergence for each run. The maximum likelihood was determined using method developed by Evanno et al. (2005).

Principal Coordinate analysis (PCoA) based on Jaccard's coefficient was performed using NTSYSpc 2.2. Analysis of molecular variance (AMOVA) and F-statistics (F_{ST}) were performed using Arlequin v.3.5 (Excoffier and Lischer 2010). Nei's gene diversity (h), Shannon's information (I) and Nei's genetic distance (D_N) indices were calculated using POPGENE v.1.32.

Results and Discussion

A total of 227 fragments (224 detected as polymorphic) were amplified using three primer combinations across 193 accessions. In the PCoA Ukrainian and Lithuanian ecotypes formed separate clusters, while cultivars interspersed and did not comprise any clusters (Fig. 40.1).

Genotypes were separated into three groups (cultivars, Lithuanian ecotypes and Ukrainian ecotypes) and analysis of molecular variance was carried out. Most of the variation (96.7 %) was detected within groups, variation among groups comprised only 3.3 %. High levels of diversity within groups were further confirmed by estimating genetic variation indices (Table 40.1).

The greatest genetic difference according to the population pairwise F_{ST} and Nei's genetic distance indices was detected between Lithuanian and Ukrainian ecotype groups. Cultivar group genetically was slightly more distant from Ukrainian ecotypes than Lithuanian ones. Nevertheless, all genetic distance indices had rather small values (Table 40.2). Pre-defined population structure of the panel was alternatively tested using STRUCTURE. Two groups were identified based on the maximum ΔK value. Majority of Lithuanian ecotypes (25 out of 38) fell into the first

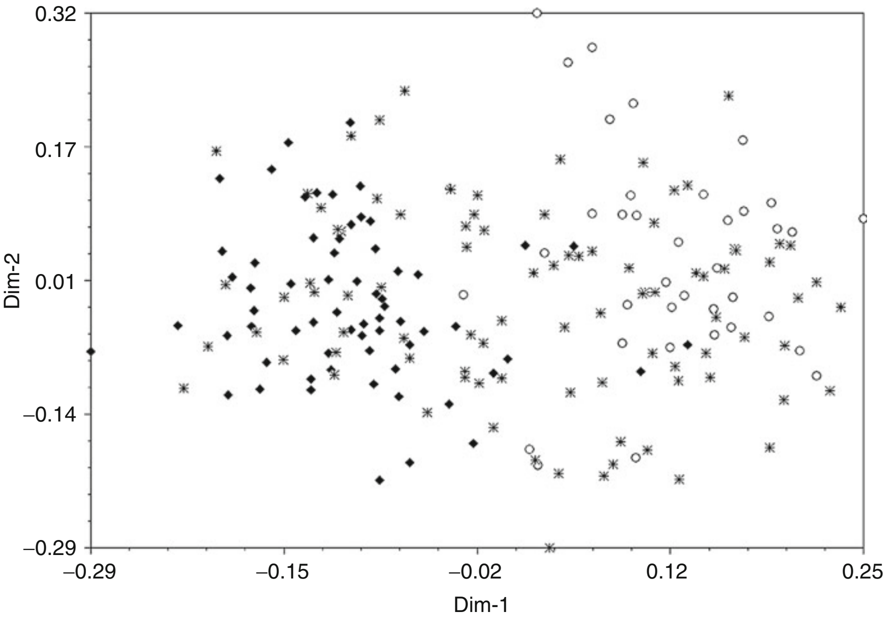

Fig. 40.1 Association among 193 perennial ryegrass genotypes based on Jaccard coefficient, calculated from 227 AFLP bands. *Stars* represent cultivars, *circles* – ecotypes of Lithuanian origin, *black diamonds* – ecotypes of Ukrainian origin

Table 40.1 Nei's gene diversity (h) and Shannon's information (I) indices within groups of cultivars and ecotypes of Lithuanian and Ukrainian origin

	No. of genotypes	h	I
Lithuanian ecotypes	38	0.2047	0.3247
Ukrainian ecotypes	65	0.2092	0.3289
Cultivars	90	0.2094	0.3329

Table 40.2 Nei's genetic distance (above diagonal) and population pairwise F_{ST} (below diagonal) among groups of genotypes

	Lithuanian ecotypes	Ukrainian ecotypes	Cultivars
Lithuanian ecotypes	–	0.0147	0.0056
Ukrainian ecotypes	0.05921	–	0.0072
Cultivars	0.01922	0.02996	–

group, the rest were identified as intermediate with the average membership probability of 0.8. Ukrainian ecotypes comprised the second group (average membership probability 0.84), while the cultivars were mainly intermediate. These results were in concordance with PCoA.

In conclusion, wide genotypic diversity was identified in a panel of 193 perennial ryegrass genotypes. Ecotypes comprised two clusters according to their origin, while cultivars did not reveal any internal subpopulation structure. Perennial ryegrass populations are usually highly heterogeneous due to its outbreeding nature. The separation of both natural ecotypes and cultivars into groups corresponding to the geographic origin was also reported earlier (Brazauskas et al. 2011; Yu et al. 2011). The results obtained in the study will be used for developing functional markers in five perennial ryegrass genes (Brazauskas et al. 2010), which orthologs in related species were shown to control plant architecture traits.

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Chapter 41
Allele Frequency of Local Maize Inbred Lines (*Zea mays* L.)

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Abstract The objective of this study was to screen the genetic divergence of 32 inbred lines in the F4 generation of inbreeding, originating from domestic populations of Eastern Serbia. In order to speed up the selection process, it is important to select genotypes with desirable properties at an early stage. Isoenzymes, as a direct product of genes, can be used as genetic markers in the selection process. Different numbers and distribution of certain alleles in inbred lines of maize indicate their mutual distance and genetic divergence. Differences in the investigated inbred lines can be shown by the presence or absence of specific alleles in loci and their frequency. The highest number of alleles (14) was found in line 5, 14 inbred lines had 13 alleles each, while line 9 had 12 alleles. Three lines each had 10 and 11 alleles, while one line had nine alleles (line 30). Only eight lines were homozygous for all loci (3, 7, 10, 21, 24, 26, 28 and 30). In accordance with allele frequencies, all lines were allocated to three groups with a different number of lines. The first and second groups joined at a distance of 4.2 and formed a new group connected to the third group at the furthest distance of 7. The third group of lines was clearly separated from the first two groups. Lines 26, 28 and 32 can be viewed as independent genotypes, joined to the third group at a distance of 2.25. These results permit a clearer evaluation of the lines during the process of selection and, together with other desirable traits, faster choice of material for further selection.

Keywords Allele frequency • Early evaluation • Inbred line • Maize

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Introduction

For the genetic identification of crops in which polymorphism is very high, analyses of a large number of enzyme systems and their isoenzymes have been developed and these can be used as genetic markers (Taski-Ajdukovic et al. 2010). Corn, as an open-pollinated plant species, must be genetically identified at various stages of the selection process (Tie et al. 1998). Very often during the selection process we need a rapid evaluation of the material which we are breeding. Isoenzymes, as direct products of genes and reliable genetic markers can be used for this purpose. They are indicators of the level of homozygosity and genetic distance between inbred lines, which is important for determining the special combining ability. However, an integrated approach by using molecular markers and phenotypic features is recommended for a comprehensive analysis (Dudley et al. 1992; Hammer et al. 1999).

In Serbia, maize is grown without irrigation in most regions. Therefore, it is important to find lines that are productive under such conditions and at the same time yield a good quality grain. Eastern Serbia is the area with a specific climate that characteristically has long and cold winters and hot summers with very low precipitation. Since this part of the country is extensively agricultural, many of the small mountain farms still grow old corn varieties or populations rather than modern hybrids. One of the most important characteristics of inbred lines derived from local populations is excellent combining ability with geographically distant inbred lines. Also, those inbred lines have a broad adaptability to local growing conditions and good drought tolerance (Nikolić et al. 2004). Preliminary studies have determined that these populations have a high grain quality (Milenković et al. 2012).

Material and Methods

Plant Material

In this study 32 maize inbred lines in the fourth generation of inbreeding were examined. Seed for testing originated from the local maize populations collected from different locations in eastern Serbia (the areas surrounding Kalna, Pirot, and villages on the Stara Planina Mountain). Inbred lines were obtained with the standard method for developing maize inbred lines (Hallauer and Miranda 1988; Falconer 1989). The process of obtaining inbred lines began in 1999, and this study was performed during 2010 as part of a PhD thesis. The main objective was to select lines with a high content of lysine for further breeding. 32 inbred lines with high lysine content in grain (up to 2 %) were selected for this investigation.

Isoenzyme Analysis

Analysis of isoenzyme activity included five enzyme systems: acid phosphatase (Acp), malate dehydrogenase (Mdh), 6-phosphogluconate dehydrogenase (Pgd), phosphoglucomutase (Pgm) and phosphohexoisomerase (Phi). Sample preparation, gels, incubation mixtures for isoenzymes staining, as well as interpretation of the results, were performed by Stuber et al. (1988). Seeds of the inbred lines were germinated on moist filter paper; 5 days at 25 °C in the dark. The upper parts of the coleoptiles, approximately 1 cm, were used in the analysis. Polymorphism of enzyme systems was examined by horizontal electrophoresis on 12.9 % starch gel (24 × 21 × 1 cm). Electrophoresis was carried out for 4.5 h at 4 °C. After completion of electrophoresis, gel was sliced into four horizontal 1 mm thick layers (slices). Each layer was then immersed in a specific solution with the substrate and the color buffer and incubated in the thermostat at 37 °C. The process of staining gels was interrupted by rinsing in distilled water and then fixed in methanol, water and glacial acetic acid in the ratio 5:5:1. Isoenzyme analysis was performed in the Seed testing Laboratory, Biotechnology Department of the Institute for Field and Vegetable Crops in Novi Sad.

Statistical Analyses

Average polymorphism was calculated as a proportion of polymorphic loci and the total number of loci. A cluster analysis was performed to determine the genetic diversity and relationship between inbred lines using the software Statistica 5.0, Stat Soft Inc.

Results and Discussion

Different number and distribution of certain alleles in inbred lines of maize indicate their mutual distance and genetic divergence. Enzymes Pgm1 and Pgd2 were monomorphic; Mdh1 and Phi1 loci had two allelic variants; Mdh2, Pgm2 and Pgd1 had three allelic variants, while the locus Acp1 showed the highest variability with four alleles (Table 41.1).

Polymorphism is the proportion of polymorphic genes in the population. A gene is polymorphic if there are at least two variants of alleles. The difference between the investigated inbred lines can be seen based on the presence or absence of specific alleles in loci and their frequency. The highest number of alleles (14) was found in line 5. 14 inbred lines had 13 alleles, while line 9 had 12 alleles. Three lines each had 10 and 11 alleles, while line 30 had nine alleles. Eight lines had homozygous loci (Table 41.2).

Table 41.1 Number of alleles in investigated inbred lines

Locus	Alleles	Total
Acp1	2, 4, 2/4, 6	4
Mdh1	1, 6	2
Mdh2	6, 3/6, 6	3
Pgm1	9	1
Pgm2	1, 4, 8	3
Phi1	4, 5	2
Pgd1	2, 3.8, N	3
Pgd2	5	1
8 loci		19 alleles

Table 41.2 Allele number per inbred line, polymorphism (%)

Line	Number of alleles	Polymorphism (%)
1	12	62.5
2	13	62.5
3[a]	10	25.0
4	13	62.5
5	14	62.5
6	12	50.0
7[a]	10	25.0
8	13	62.5
9	12	62.5
10[a]	13	62.5
11	13	62.5
12	13	62.5
13	13	62.5
14	13	50.0
15	12	37.5
16	13	50.0
17	13	62.5
18	12	50.0
19	12	50.0
20	13	62.5
21[a]	11	37.5
22	12	50.0
23	11	37.5
24[a]	10	25.0
25	11	37.5
26[a]	12	50.0
27	13	62.5
28[a]	12	50.0
29	12	37.5
30[a]	9	12.5
31	13	62.5
32	13	62.5

[a] Lines with homozygous loci

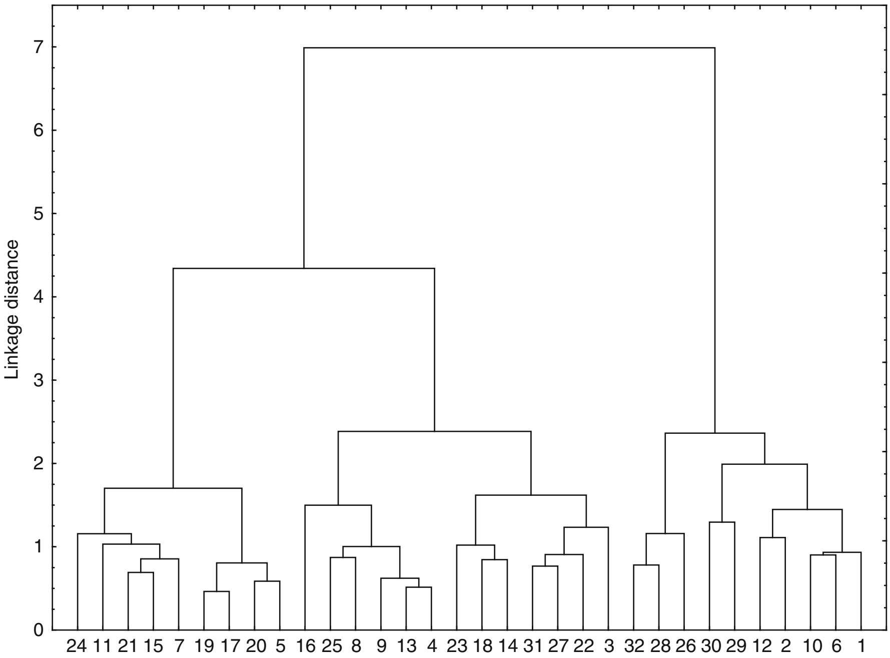

Fig. 41.1 The dendrogram of 32 inbred lines based on allele frequency

The cluster method was used for comparison of genetic diversity among the tested lines in order to obtain information about the origin of the breeding material. A dendrogram was formed based on the frequency of alleles in five studied enzymes and represents the mutual relations and genetic distance between 32 inbred lines (Fig. 41.1). There are three major groups of lines with different numbers of members. The first and second groups are joined together at a distance of 4.2, and form a new group of lines which is connected to the third group at the furthest distance of 7. The third group is clearly separated from the previous two. It consists of 10 lines of interconnected chain at distances from 0.75 to 2.25.

Conclusion

Given the common geographical area, all lines would be expected to have the same origin; however, the results based on allele frequency show that the lines originate from different populations. These results facilitate a clearer evaluation of the lines during the process of breeding and, together with other desirable features, a faster choice of material for further selection. The most interesting lines for combining in further work were lines 19, 17, 13 and 4 because of their genetic distance from the other investigated lines.

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Part VI
Breeding for New Roles of Multifunctional Forage Species

Chapter 42
Breeding for 'HealthyHay': Can We Optimise Plant Polyphenols in Legumes for Ruminant Nutrition, Animal Health and Environmental Sustainability?

Irene Mueller-Harvey

Abstract A small number of forage legumes are of great interest for sustainable livestock production because they contain various bioactive compounds, such as polyphenols, tannins and oxidases. The recently completed EU Marie Curie Research Training Network ('HealthyHay') reported large intra-species variation not only in agronomic characteristics but also in bioactive constituents during a detailed screening of a sainfoin (*Onobrychis viciifolia*) germplasm collection. Tannins, for example, showed considerable quantitative and qualitative differences: contents varied five-fold, the average polymer size varied seven-fold, prodelphinidins accounted for between 53 and 95 % and *trans*-flavanols for between 12 and 34 % of the tannins. Large variations were also observed amongst monomeric polyphenols and between enzymes involved in their biosynthesis. Total flavonol and flavan-3-ol contents were important variables for distinguishing between sainfoin accessions and dihydroflavonol 4-reductase and flavonol synthase proved particularly useful for screening purposes. Not surprisingly, considerable variation was also observed in terms of the effectiveness of the different accessions against parasitic worms, coccidia and generation of ruminal fermentation products.

This EU 'HealthyHay' project used existing and established several new techniques for screening legumes for their bioactive compounds, but also for their effects in terms of anti-parasitic, nutritional and environmental benefits. These included several different tannin methods, *in vitro* anthelmintic assays, an anti-coccidial assay, an automated pressure evaluation system (APES) for volatile fatty acid and methane production, a pepsin-cellulase digestibility assay and ensiling studies. Further work will be needed to identify the optimum concentrations and compositions of the bioactive compounds and to develop molecular markers for this under-exploited forage legume. Recent research on *Populus* indicated that the qualitative

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and quantitative tannin traits are under genetic control and thus amenable to plant breeding. It is likely that tannin traits are also under genetic control in sainfoin and that tannin composition can therefore be optimised to maximise specific benefits.

Keywords Sainfoin • Germplasm • Tannins • Beneficial effects

Introduction to the EU 'Healthyhay' Project

A recent EU Marie Curie 'HealthyHay' project trained 14 early stage and 6 experienced researchers in a multidisciplinary network, which included 13 partners from 10 countries (see: http://healthyhay.eu). The overall objective was to lay the foundations for a European breeding programme of sainfoin (*Onobrychis viciifolia*) by establishing a germplasm collection, screening programme and seed multiplication. This interesting forage legume used to be an important mainstay of ruminant feeding systems particularly on low fertility, drought prone, neutral to alkaline soils in Europe (Hayot Carbonero et al. 2011). Despite its numerous benefits, its cultivation has, however, declined since the wide-spread use of commercial nitrogen fertilisers.

Given that the literature contained some contradictory reports on the extent of the beneficial effects that can be derived from sainfoin, 'HealthyHay' explored this germplasm for variation in bioactive tannins, polyphenols, enzymes involved in flavonoid synthesis and oxidative reactions, nutritional properties (nitrogen use, volatile fatty acid production, digestibility, ensiling characteristics and environmental emissions) and anti-parasitic effects (anti-worming, *syn*. anthelmintic, and anti-coccidial properties). A major part of this project consisted of evaluating its agronomic performance in the UK and Spain, where work also started on developing molecular markers for a future breeding programme.

Beneficial Effects of Sainfoin

Ecosystem Benefits

Sainfoin builds soil fertility not only as a legume but also because of its large mass of fine roots (16,200 kg ha^{-1}), which compares very favourably with lucerne (*Medicago sativa*) (4,200 kg ha^{-1}) (Sergeeva 1955). It has a very long tap root, which allows cultivation on drought-prone land. Not surprisingly, therefore, nineteenth century covenants stipulated that tenant farmers in parts of the UK had to include sainfoin in their crop rotations (Hill R, personal communication 2008).

Sainfoin is also prized for its fine honey (up to 400 kg/ha) and is visited not only by honey bees but also bumble and other types of bees. Its flowers provide a rich source of pollen and nectar over many months and lasts well beyond oilseed rape. Compared to white clover, they attract ten times more bees (Taki et al. 2009; Howes 2007; McGregor 1976).

Effects on Animal Nutrition and Health Plus Environmental Benefits

The term 'sain(t) foin' tells us that its importance has been recognised since ancient times as either 'healthy' or 'holy' hay (Mueller-Harvey 2009). Unlike lucerne and some clovers, it is a non-bloating legume and animals can graze it safely (Wang et al. 2012). Sainfoin is the only perennial forage legume that contains a high water-soluble carbohydrate content; i.e. between 48 and 143 g total water-soluble carbohydrates per kg of freeze-dried sainfoin were measured in the 'HealthyHay' collection (Mueller-Harvey et al. unpublished results). Sucrose was a major metabolite and accounted for approximately 35 % of the acetone/water extract (Marais et al. 2000). Such high levels could explain the very high intakes and excellent growth rates (Mueller-Harvey 2009). They are also responsible for the fact that sainfoin can be relatively easily ensiled in contrast to most other forage legumes (Lorenz et al. 2010).

Some reports also suggest that the nutritional benefits are due to the 'rumen-escape protein' phenomenon. Sainfoin is one of a select group of forage legumes, which contain bioactive tannins; these tannins complex and thus protect dietary protein from microbial attack in the rumen. The 'escaped' protein can lead to enhanced amino acid absorption in the hindgut (Mueller-Harvey 2006).

A slight shift from ruminal protein degradation to more amino acid absorption in the hindgut also generates another benefit: it reduces urinary nitrogen and slightly increases faecal nitrogen emissions. This is important for two reasons: (i) it can lead to better nitrogen use by ruminants and (ii) urinary nitrogen is quickly converted by urease and other enzymes into nitrous oxide, which is a 296 times more potent greenhouse gas than carbon dioxide (Kingston-Smith et al. 2010). This combination of better nutrient use efficiency and lower environmental emissions has the potential for improving the sustainability of ruminant production systems. Interestingly, evidence also exists that bioactive tannins can reduce the amount of methane that is emitted by ruminants (Jayanegara et al. 2012).

In addition to preventing animal deaths from bloat, sainfoin and a few other forage legumes (*Dorycnium rectum*, *L. pedunculatus*, *L. corniculatus*, *Hedysarum coronarium*, *Lespedeza cuneata*) can also be used to reduce parasitic worm burdens due to their bioactive tannins (Hoste et al. 2005, 2012; Heckendorn et al. 2007; Terrill et al. 2012). This is an important feature as parasitic worms are becoming increasingly resistant to veterinary drugs (Molento 2009). Such anthelmintic resistance is causing distress to animals and large financial losses to farmers across the world. Several studies have demonstrated that tannins can interfere at each stage of the parasitic life cycle and cause significant reductions (Hoste et al. 2006). It has been suggested that helminth control is more likely to succeed if animals are allowed to develop immunity in the presence of low worm burdens and veterinary drugs should only be used as a last resort, as they tend to eliminate (non-resistant) worms almost completely.

Table 42.1 Concentrations of condensed tannins in anthelmintic forage legumes (Min and Hart 2003; Ramirez-Restrepo and Barry 2005; Mueller-Harvey 2006)

Legume	Tannin content (g/100 DM)
Dorycnium rectum	15–20
Big trefoil (*Lotus pedunculatus*)	5–10
Birdsfoot trefoil (*Lotus corniculatus*)	2–5
Sulla (*Hedysarum coronarium*)	2–12
Sainfoin (*Onobrychis viciifolia*)	1–8
White clover flowers (*Trifolium repens*)	5–8
Sericea lespedeza (*Lespedeza cuneata*)	5–18

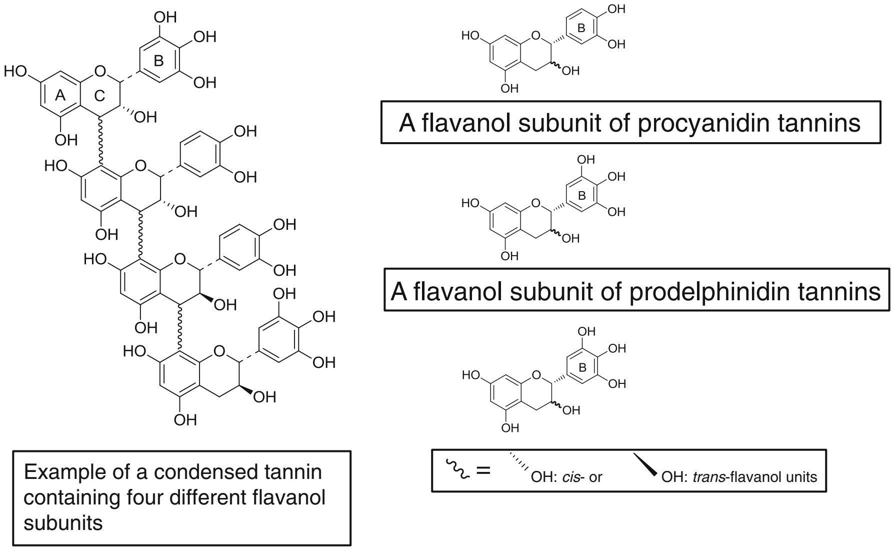

Fig. 42.1 Example of a condensed tannin tetramer and its flavanol subunits, which give rise either to procyanidins or prodelphinidins

Tannins as Active Ingredients

This section will briefly summarise the main features of tannins; for more detailed information the reader can consult reviews by Moilanen et al. (2013), Mueller-Harvey (2006) and Khanbabae and van Ree (2001). Tannins are a sub-group of plant polyphenols. Land-based plants synthesise three tannin groups: condensed tannins, gallotannins and ellagitannins. This paper will focus on the condensed tannins, as much more is known about the potential benefits that can be derived from these tannins when present in forage legumes.

Condensed tannins from forage legumes shown in Table 42.1 consist of repeating flavanol subunits (Fig. 42.1). Procyanidin tannins are characterised by the presence of two OH-groups on the B-ring and prodelphinidin tannins have three OH-groups on the B-ring.

Table 42.2 Tannin composition of the five most contrasting sainfoin accessions (Stringano et al. 2012)

NIAB accession number	Description and origin	Tannin content (g/100 DM)	Average number of flavanol sub-units per tannin	Prodelphinidin tannins (%)	*Trans* flavanol units (%)
1071	Hampshire Common, UK	1.1	12	68	20
1256	Wkt 10, Turkey	2.8	18	95	32
1220	247, Morocco	0.6	25	83	27
1127	63767, USA	0.9	84	83	30
1179	CPI 63820, Spain	1.1	18	53	17

Research in New Zealand has shown that procyanidins from *L. corniculatus* generate the useful 'rumen-escape' protein but the prodelphinidins from *L. pedunculatus*did not (Mueller-Harvey 2006). However, these prodelphinidins appeared to be better at reducing parasitic worm burdens (Burke et al. 2012; Min and Hart 2003).

'HealthyHay' research probed in more detail into the nature of sainfoin tannins because of contradictions in the literature. Some – but not all – reports suggested that sainfoin, like *L. corniculatus*, generated 'rumen-escape protein'. However, it had also been reported that sainfoin contains mostly prodelphinidin rather than procyanidin tannins, and therefore it is puzzling that sainfoin generated 'rumen-escape protein' (Waghorn 2008).

Germplasm Variation

Table 42.2 reveals the extent of variation in tannin content and structural parameters amongst 37 diverse sainfoin accessions (Stringano et al. 2012). Tannin contents varied five-fold from 0.9 to 2.8 g/100 dry matter, the average polymer size varied seven-fold with mean degree of polymerisation (mDP) ranging from 12 to 84, prodelphinidins accounted for between 53 and 95 % and *trans*-units for between 12 and 34 % of the flavanol subunits.

Leaves contained not only more tannins than stems, butleaf tannins were also larger and had a higher proportion of prodelphinidins (Theodoridou et al. 2011). Tannin composition in plants from three sainfoin accessions grown in France changed during the first growth cycle as follows: tannin contents increased slightly from an average of 4–5 g kg^{-1} DM, mDP-values from ca. 25 to 45, prodelphinidins from 63 to 73 %, and *cis*-flavanols from ca 75 to 80 %. Changes during the second growth cycle were less pronounced: tannin contents were higher and averaged ca. 7 g kg^{-1} DM, mDP values ca. 45, prodelphinidins ca. 70 % and *cis*-flavanols ca. 80 % (Theodoridou et al. 2011). Azuhnwi et al. (2013a) noticed that the tannin polymer profiles were relatively stable over two harvests in multiple-flowering (Bifera) accessions but were more variable in single-flowering (Communis) accessions.

Taken together, these results may provide a possible explanation for the above mentioned contradictory results. However, most previous sainfoin feeding trials had provided little information on the sainfoin accession and included no tannin analysis of the feeds. Therefore, it is not possible to conclude with any certainty whether the positive nutritional effects (rumen-escape protein) had been obtained with accessions having high procyanidin contents and the positive anthelmintic effects were due to accessions having high prodelphinidin contents. This sort of problem demonstrates how important interdisciplinary research is in order to unravel the scientific puzzle posed by bioactive legumes.

Screening Tools for Bioactive Legumes

Polyphenol and Tannin Analysis Methods

Polyphenols, including tannins, are often analysed by relatively non-specific colorimetric methods, which provide little new information (Lorenz et al. 2010). LC-MS methods are capable of yielding much more useful information and one such method was specifically developed for screening flavonoids, cinnamic acid derivatives and other polyphenols in this sainfoin germplasm (Regos et al. 2009).

The HCl-butanol reagent is widely used for analysing condensed tannins but the reaction conditions were only recently optimised for the direct screening of plant material by adding acetone, which proved essential to improve the reproducibility and colour yield of the reaction in the case of *Lotus* tannins (Grabber et al. 2013). Unlike the acetone-HCl-butanol assay, thiolytic degradation provides information not only on tannin content but also on composition and was adapted for the direct screening of plant material from the sainfoin germplasm collection (Gea et al. 2011). Information on tannin structures, e.g. polymer size, procyanidin/prodelphinidin and *cis*/*trans* ratios, is of particular interest as previous surveys of plant tannin contents have not yet enabled us to establish why some, but not all, tannin-containing plants exert beneficial nutritional or veterinary effects.

In our quest for analytical tools that are suitable for screening large numbers of tannin-containing germplasm material, we also evaluated near-infrared spectroscopy (NIRS) and used the results from the above thiolysis method for the calibration. Initial results indicated that NIRS is suitable not only for predicting tannin contents whether determined by HCl-butanol or by thiolysis assays but interestingly it also showed promise for predicting polymer size, procyanidin/prodelphinidin and *cis*/*trans* ratios (Mueller-Harvey et al. 2011). Professor J-P. Salminen's laboratory at the University of Turku, Finland, is currently developing a novel and very fast mass spectrometric method, UPLC-MS/MS, as a tannin screening tool.

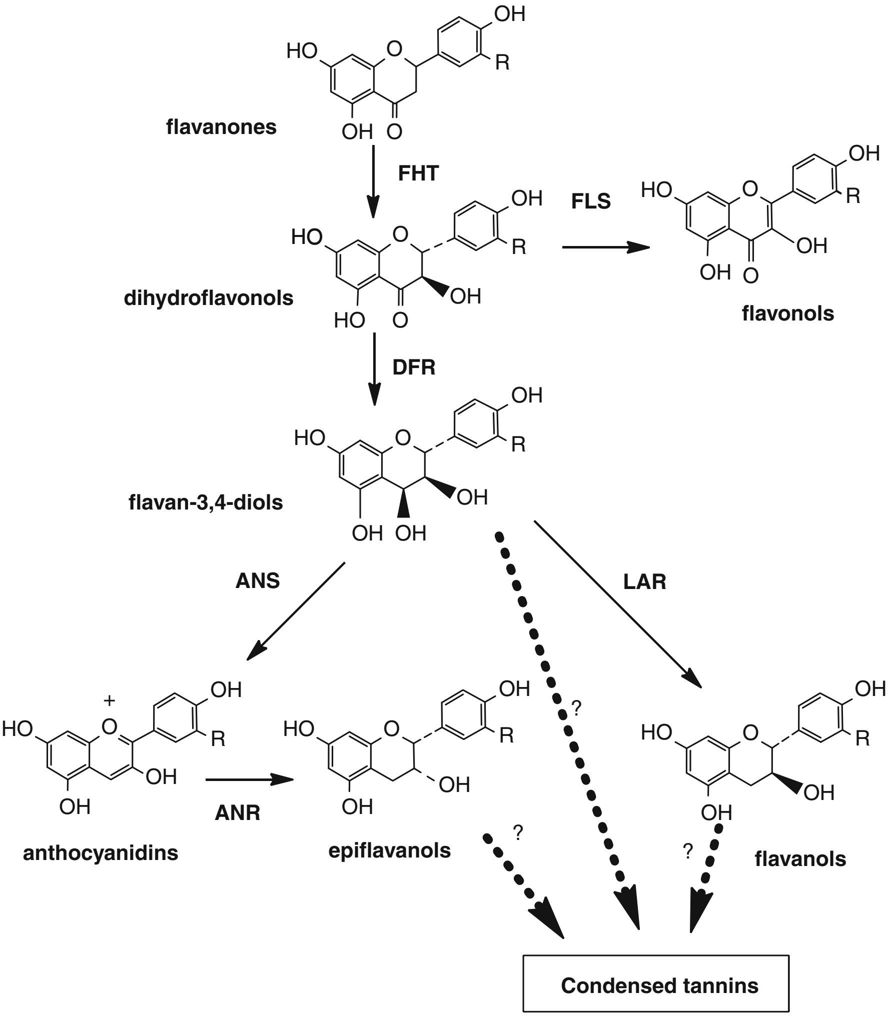

Fig. 42.2 Brief summary of flavonoid biosynthesis (Thill et al. 2012). *ANR* = anthocyanidin reductase, *ANS* = anthocyanidin synthase, *DFR* = dihydroflavonol 4-reductase, *FHT* = flavanone 3-hydroxylase, *FLS* = flavonol synthase, *LAR* = leucoanthocyanidin reductase

Enzyme Assays

The 'HealthyHay' project also investigated whether measurements of enzyme activities, which are involved in the flavonoid pathway (Fig. 42.2), could serve as screening tools for predicting polyphenol and tannin composition in sainfoin. Given the elusive nature of thetannin-condensing enzyme, the ratio of dihydroflavonol 4-reductase and flavonol synthase (DFR/FLS) activities proved promising for selecting accessions that differ in flavonol/tannin ratios (Thill et al. 2012).

Of particular interest was the finding that several sainfoin accessions had a high peroxidase (POD) activity, which was still active after 48 h in the rumen of sheep (Ahmad et al. 2010). Somewhat surprisingly, POD activities in hay samples were higher than in fresh samples. The authors concluded that POD activities may modify sainfoin polyphenol composition during processing into hay or silage. Therefore, there is a need to ascertain if and how polyphenol oxidation may impact on the nutritional and veterinary effects of different sainfoin accessions.

In Vitro *Anthelmintic and Anticoccidialassays*

Several *in vitro* screens have been developed to assess the impact of plant extracts or purified compounds on the different life cycle stages of parasitic worms (Jackson and Hoste 2010). Assays exist for measuring the inhibition of egg hatching, feeding, exsheathment or penetration into mucosa; *in vitro* fecundity cannot yet be measured with parasitic worms, as these require a host, and therefore *in vitro* assays are being developed instead with the free-living nematode, *Caenorhabditis elegans*. Once a potentially interesting plant extract or compound has been identified, feeding trials are necessary to test *in vivo* efficacy.

As expected the different sainfoin accessions differed in their ability to generate *in vitro* anthelmintic effects (Novobilský et al. 2013; Manolaraki et al. 2011). These studies found that not only tannin content, but also polymer size and percentage of prodelphinidins influenced anthelmintic efficacy. *In vivo* feeding trials have also shown promise for sainfoin and *Lotus* species, to name just two of the tannin-containing legumes, in the form of fresh forage, hay or silage (Azuhnwi et al. 2013b; Heckendorn et al. 2006, 2007; Paolini et al. 2005; Min and Hart 2003; Min et al. 2003), but more research will be needed to ensure consistent results.

Preliminary research on *in vitro* anticoccidial assays showed that more work will be needed to refine these assays. However, the feeding of sainfoin to lambs appears to be a promising strategy for reducing Coccidiosis caused by *Eimeria* species (Saratsis et al. 2012).

In Vitro *Digestibility Assays*

Screening tools for *in vitro* digestibility are now well established and consist of measuring organic matter disappearance and gas production or protein degradation in the presence of enzymes such as pepsin-cellulase (Aufrère et al. 2007) or a microbial rumen inoculum (Pellikaan et al. 2011; Lorenz et al. 2012). The HealthyHay germplasm material exhibited significant differences between sainfoin accessions in terms of organic matter digestibility, production of volatile fatty acids and methane gas (Theodoridou et al. 2011; Hatew et al. 2011).

Exploring the Genetic Diversity of Sainfoin

As there was a dearth of information on the genetic diversity of the *Onobrychis* species, a cytological characterisation of the HealthyHay germplasm was undertaken at NIAB, Cambridge, UK, and this resolved some contradictory reports. Hayot Carbonero et al. (2013) found that most of the *O. viciifolia* accessions and some other *Onobrychis* species were tetraploid. These authors studied the phylogeny of sainfoin and identified DNA sequences, which enabled an assessment of the robustness of the *Onobrychis* taxonomy (Hayot Carbonero et al. 2012). Substantial genetic diversity among *Onobrychis* species was found and separated accessions into two clusters, i.e. a western European and an eastern European plus Asia group. Interestingly, these clusters also reflected differences in agronomic and tannin characteristics (Hayot Carbonero et al. 2012; Stringano et al. 2012). Researchers from CITA in Zaragoza, Spain, identified six transferrable microsatellite EST-SSR markers from *Medicago truncatula* (Demdoum et al. 2012), which facilitated fingerprinting and studies into the genetic similarity of 23 *O. viciifolia* accessions, all of which clustered into one group that was well separated from two wild species, *O. argentea* and *O. pyrenaica*. Furthermore, this analysis showed that the British accessions were distant from the Eastern European accessions; whilst the French and Italian accessions fell between these two groups (Demdoum et al. 2012).

Research in a current EU Marie Curie Initial Training Network (www.legumeplus.eu) is investigating marker-trait associations in sainfoin in order to develop marker assisted selection tools using high-throughput cDNA sequencing and/or quantitative real-time PCR.

Breeding for Tannins as a Novel Trait

Given that the 'HealthyHay' project found large variations in the bioactive traits of these sainfoin accessions, a concerted breeding programme is now warranted to enhance not only agronomic performance but also bioactive traits for the different regions in Europe. Although some 'mixing' of local with introduced sainfoin germplasm has occurred (Demdoum et al. 2012), there are still many landraces and other accessions that are worth developing because they are already well adapted to local soils and climate (Pecetti et al. 2009).

Kelman (2006) concluded that selection for optimal levels of condensed tannins and herbage productivity were feasible breeding objectives for *Lotus* species. However, definitive evidence that both structural traits, i.e. tannin size and composition in terms of flavanols, are heritable has emerged only recently in experiments that examined *Populus* and its hybrids (Scioneaux et al. 2011). Therefore, it should be possible for sainfoin breeding to optimise the various tannin traits, including tannin polymers, as these appear particularly relevant to anthelmintic properties. We noted that tannin polymer distribution profiles differed between sainfoin Communis and Bifera accessions and were more stable for the Bifera accessions (Azuhnwi et al. 2013a).

Research will be needed to establish optimum levels of tannin content and composition for improved ruminant nutrition, lower greenhouse gas emissions and anthelmintic effectiveness. In addition harvesting and preservation procedures will need to be optimised in order to maintain the beneficial effects of sainfoin and other bioactive forage legumes (Lorenz et al. 2010). This will help to inform breeding targets for novel traits in sainfoin and other tannin-containing legumes (e.g. Table 42.1) and will complement important agronomic targets such as seed germination, establishment, competitiveness against weeds and persistence under different grazing regimes (Hayot Carbonero et al. 2011; Pecetti et al. 2009). Farmers will need lines that possess relatively stable compositions of the desired features and it is interesting that the polyphenolic composition of a few sainfoin and sorghum accessions was indeed more stable than most others (Azuhnwi et al. 2013a; Mueller-Harvey and Dhanoa 1991). Breeding for sustainable and resilient animal feeding regimes in a changing climate will continue to remain an important objective and tannin-containing legumes hold much promise.

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Chapter 43
High-Throughput Genome-Wide Genotyping to Revive the Use of Natural Diversity in Forage and Turf Breeding

Jean Paul Sampoux, Philippe Barre, and Isabelle Litrico

Abstract European genebanks maintain collections of natural accessions of forage and turf species that represent a wide sampling of the adaptive diversity existing in these species. The increasing availability of technologies for the screening of genome-wide polymorphisms offers challenging opportunities to search for genomic diversity involved in the adaptive response of natural populations. New high-throughput genotyping technologies can be combined with information from environmental databases covering large spatial scales at fine resolution to search for associations between genomic polymorphisms and environmental variation at sites of origin of the natural diversity. Furthermore, methods to detect signature of selection using dense genome-wide genotyping data have been developed from population genetics theory. This methodological framework is well suited for studies at the macro-environmental level, which is the relevant level to investigate adaption to climatic constraints. We suggest it can be applied to genebank accessions collected throughout the natural distribution area of forage and turf species, in order to provide a new kind of information to guide the use of natural diversity in breeding programs.

Keywords Adaptive diversity • Association genetics • Environmental variations • Genetic resources • Genome-wide genotyping • Signature of selection

Introduction

A large part of the adaptive variability existing in the natural diversity of cultivated species and their wild relatives has likely remained under-exploited, and may provide useful genetic resources for modern plant breeding. Genebanks maintain collections of natural accessions that all together may represent comprehensive

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sampling of the genetic variability available across the environmental distribution of species. The increasing availability of technologies for the efficient screening of genome-wide polymorphisms offers new opportunities to investigate genomic diversity and more specifically to search for genomic diversity involved in the response of populations to natural selection (Luikart et al. 2003; Storz 2005). Environmental databases covering large spatial scales at fine resolution are now available, especially regarding climatic information. High-throughput genotyping technologies and detailed environmental information may hence be combined to search for associations between genomic polymorphism and environmental variation at sites of presence of the natural diversity (Holderegger et al. 2008). Furthermore, methods to detect signature of selection using dense genome-wide genotyping data have been developed from population genetics theory (Beaumont 2005). Combining the results of association studies between molecular polymorphisms and environmental variations with results of signature of selection studies is expected to be an efficient way to reveal molecular polymorphisms linked to environmental adaptation. This methodological framework is well suited for studies at the macro-environmental level, which is in particular the relevant scale to investigate adaption to climatic constraints. We propose that it should be applied to genebank collections of natural diversity of forage and turf species. Markers of adaptive diversity revealed by such investigations could contribute to optimise the sampling of natural diversity of forage and turf species for conservation in genebanks, to set up breeding pools of these species aiming to recombine allelic diversity at adaptive loci and to investigate the determinism of the revealed adaptive diversity.

Detecting Adaptive Diversity in Genome Polymorphisms: Background

During the evolution of the diversity of a species, selection events are accompanied by selective sweeps on the genome (i.e. a reduction of genetic diversity) in the vicinity of the loci responding to selection. Linkage disequilibrium (LD) is thus created in the genome zone subjected to selective sweep. The conservation of LD created by selective sweep over subsequent generations depends on mutation and recombination rates and on selection intensity at the locus under selection. Significant associations between the mostly neutral diversity revealed by genome-wide genotyping methods (AFLP, DarT, RAD Sequencing) and the variability of environmental parameters can thus be due to physical linkage between some of these markers and the loci under selection. However, such associations may also be caused by LD due to genetic drift in genetic pools isolated from each other. Associations between genomic polymorphisms and environmental variations are thus expected to reveal putative adaptive diversity, provided that they are assessed within a group of populations from the same phylogeographic lineage, in which alleles conferring an adaptive advantage have experienced a spatial expansion without marked genetic structure outside the selective sweep zone. This approach has been developed for several years in ecological genetics and is part of an emerging disciplinary field called “landscape genomics” (Manel et al. 2010).

Monestiez et al. (1994) searched for spatial genetic structure in the natural diversity of perennial ryegrass using multivariate geo-statistics models applied to some phenotypic traits. By this mean, they estimated that the range of gene flow and the range of environmental selection for a phenotypic trait (summer growth) reached 120 and 300 km, respectively. These results suggest that the distance between collection sites of neighbouring populations could be set between 120 and 300 km when searching for associations between genomic diversity and environmental variation at large spatial scale in perennial ryegrass. This spatial resolution should avoid bias in association models due to autocorrelation patterns caused by gene flow. On the other hand, it should permit to find more or less similar selection pressures beyond the range of gene flow. Spatial patterns of genetic structure and phylogeographic trends have been documented in the case of perennial ryegrass. Balfourier et al. (1998a) assessed polymorphism at isozyme loci in this species over Europe and notably found that diversity decreased with increasing latitudes. Furthermore, Balfourier et al. (2000) evidenced the existence of several routes of expansion of natural perennial ryegrass in Europe by using phylogeographic analyses of chloroplastic DNA diversity. These results stress out the necessity to take into account spatial genetic structure and phylogeography in the search for associations between genomic polymorphisms and environmental variations.

An alternative way to identify adaptive diversity in natural populations is to use methods that detect signatures of selection based on population genetics theory which do not explicitly incorporate environmental data. Demography and history of populations have an impact on neutral diversity at the whole-genome level whereas selection only affects specific loci and associated flanking regions. It is thus anticipated that dense genome-wide genotyping will reveal a majority of loci showing similar patterns of variation, in agreement with neutral model expectations, whereas some outlier loci will deviate from these expectations. These outlier loci can be considered as having possibly been under selection or as being physically linked to loci under selection (Gebremedhin et al. 2009; Luikart et al. 2003; Nielsen 2005; Nosil et al. 2009; Storz 2005). The status of ‘outliers’ conferred to some markers by tests of signature of selection may confirm significant associations of these markers with some environmental parameters revealed in a landscape genomics study (Holderegger et al. 2008).

Recent reports of landscape genomics studies, combined or not with outlier detection tests, addressed various plant and animal species and involved various sampling scales and genotyping methods. All these studies revealed a small proportion of genomic markers (from 1 % to more 10 %) that were significantly associated to environmental parameters (Holderegger et al. 2008; Jump et al. 2006; Joost et al. 2007; Manel et al. 2009; Nunes et al. 2011; Poncet et al. 2010).

Genebank Collections of Forage and Turf Species: A Relevant Material to Search for Adaptive Diversity

Genebank accessions collected in the natural diversity of forage and turf species are likely to provide a valuable material to search for molecular polymorphisms associated with environmental variations at large environmental scales. For most forage and turf species, the addition of collections from the different European genebanks covers pretty well their primary area of expansion across Eurasia and North-Africa. For example, the EURISCO Catalogue gathers 2,361, 2,163, 1,392 and 590 different natural accessions of *Dactylis glomerata*, *Lolium perenne*, *Festuca pratensis* and *Festuca arundinacea*, respectively, that were collected throughout the distribution area of these species. European genebank databases, such as EURISCO, ECPGR and AEGIS are indeed straightforward sources of information to draw sets of accessions of forage and turf species that could be suitable to investigate adaptive diversity at large environmental scale. Genebank databases furthermore provide spatial coordinates of collection sites. These spatial coordinates can be used to extract environmental data from environmental databases. More particularly, it is possible to extract climatic data and land-cover data from databases covering wide areas at fine resolution scale (Sampoux and Badeau 2009). Satellite views may also be processed to obtain data for various parameters like global solar radiation and land-cover. In addition, genebank databases may provide environmental information recorded by collectors, such as land-use, soil texture, slope, aspect.

Genome-Wide Genotyping Methods to Detect Adaptive Diversity

Linkage disequilibrium (LD) is shaped in plant genomes by many factors, namely the recombination and mutation rates, the genome regions, the mating system, the selection pressures, the population structure and the effective population size (Flint-Garcia et al. 2003; Gupta et al. 2005). In the major temperate forage and turf species, LD is expected to decrease fairly rapidly due to their outbreeding mating system and their high level of diversity. A good knowledge of LD in the material under investigation is essential in order to define the number of markers needed to cover the whole genome. For example, in natural perennial ryegrass, LD decays to $r^2=0.2$ over 500–3,000 bp (Auzanneau et al. 2007; Ponting et al. 2007). In elite cultivars of perennial ryegrass, LD decay varies according to cultivars, from 150 bp in cv. Herbie to 6.6 cM (about 23.1 Mbp) in cv. Aberdart (Auzanneau et al. 2011; Brazauskas et al. 2011). If LD decay to $r^2=0.2$ is assumed at 1,000 bp for a collection of natural populations of perennial ryegrass with a haploid genome size of 2.7 Gb (Wang et al. 2009), 2.7 million markers should be required to cover the whole genome. Genotyping a great number of individuals with so many markers is now feasible with new technologies but remains expensive. Nevertheless, a lower density of markers could be expected as sufficient to reveal a part of genomic polymorphisms linked to adaptive loci. Many genome regions are expected to have been submitted to adaptive selection pressures in the natural diversity of a species and LD should be stronger in such regions because of selective sweep. To date, experimental results are lacking to assess the level of LD existing among natural populations of forage and turf species. However, some studies undertaken on various plant and animal species have been able to reveal genome zones linked to adaptive diversity with relatively small number of markers (Jump et al. 2006; Joost et al. 2007; Manel et al. 2009; Nunes et al. 2011; Poncet et al. 2010).

In order to genotype a large set of markers on many individuals, AFLP markers have been extensively used on different species because their use does not require sequence information. Nevertheless, scoring could be laborious and it could be difficult to obtain the DNA sequence of a band of interest. DarT markers circumvent these drawbacks and have been used to reveal DNA polymorphisms in different species (Jaccoud et al. 2001). However, the number of markers revealed by AFLP or DarT markers remains in the range from several hundreds to one or two thousands. Genotyping strategies based on single nucleotide polymorphisms (SNPs) are able to provide much more genomic markers (Deschamps et al. 2012). SNP micro-arrays technology involve three steps: i/detecting SNPs by sequencing a subset of individuals from the genetic diversity of interest, ii/designing SNP markers and iii/genotyping all individuals by using techniques based on the recognition of the polymorphic locus by hybridization. The interest of this technology is that the analysis of raw data provides directly the genotype of each individual at each SNP marker, and it is furthermore possible to genotype many individuals for a large number of SNPs at affordable cost. However, polymorphisms can only be detected for SNPs discovered in the initial subset of individuals. An alternative strategy uses a direct sequencing of a fraction of each individual genome, and it is therefore called genotyping-by-sequencing (GBS) (Davey et al. 2011; Elshire et al. 2011). This alternative strategy has the ability to discover new SNPs that are specific of populations under study but it requires a heavy workload in raw data analysis (short sequences).

Genetic Analysis Methods to Detect Adaptive Diversity

Association Between Molecular Polymorphisms and Environmental Variations

The detection of associations between molecular polymorphisms and environmental variations is based on statistical models. These models are set up to predict the presence or frequency of the allelic forms of molecular markers as a function of environmental parameters used as explanatory or independent variables. Note that, in such models, the allelic forms of the molecular markers are the dependant variables to be predicted, whereas they are the independent variables, or predictors, in association models aiming to predict phenotypic traits from molecular polymorphisms.

Statistical modelling techniques convenient to model binary distributions (presence – absence of alleles) or proportions (frequencies of alleles) can be used. Several kinds of models have already been used to predict presence – absence of alleles: GLM (Generalized Linear Models, Joost et al. 2008), GEE (Generalized Estimating Equations, Poncet et al. 2010) and MAXENT (Maximum Entropy Distribution, Banta et al. 2012). The approach can be population-based. Several individuals can be genotyped in a number of populations and allele frequencies in populations can be modelled. However, a great number of individuals may need to be genotyped if wide environmental gradients are to be investigated. The alternative is to adopt an individual-based approach in which only one random individual is genotyped in each of numerous populations and presence/absence or frequency of alleles are modelled at individual level. The drawback of tests of associations between molecular polymorphism and environmental variations is that false positive associations can be detected if it happens that spatial neutral genetic structure matches to some extent with some environmental variations. One possibility to deal with this issue is to first investigate spatial genetic structure and then to fit models within each unstructured subset. A better alternative will be offered in next future by modelling methods including structure patterns in the modelling process.

Tests of Signature of Selection

Different kinds of tests of signature of selection have been devised. Single-population tests, like the Tajima's D and the Ewens-Watterson test, aim to detect genome regions with lower diversity than expected under neutral model expectations (Nielsen 2005; Storz 2005). Pairwise-population tests intend to detect genome regions with contrasted within-population allelic diversity at some loci (Schlotterer 2002). Multiple-population tests, based on a principle initially proposed by Lewontin and Krakauer (1973), are the most convenient tests to detect signatures of selection across numerous populations. They are based on the assumption that similar patterns of differentiation among populations should be observed for all neutral loci in the genome, which are expected to make to the great majority of marked loci. On the other hand, loci under selection, or physically linked to adaptive polymorphisms, are expected to exhibit outlier patterns of differentiation departing from the patterns of the great majority of neutral loci. Several tests have been proposed to detect such outlier loci. Beaumont and Nichols (1996) proposed a method to detect outlier loci based on the identification of loci whose inter-population F_{ST} significantly departs from the neutral model hypotheses, whereas Beaumont and Balding (2004) used a Bayesian method based on posterior allelic distribution. However, the fact that these tests are population-based may be a difficulty when using accessions from genebanks, as it could be that allele frequencies in genebank seed lots deviated from frequencies in the original populations due to different causes (flawed collection procedures, drift during ex situ seed regenerations).

Tests of signature of selection may confirm results of tests of associations between molecular polymorphisms and environmental variations when same molecular polymorphisms are identified as linked to adaptive diversity by the two approaches. Conversely, molecular polymorphisms detected by an association approach that are not recovered by tests of signature of selection can be suspected as being false positives caused by an incidental distribution of structural groups along some environmental gradients. On the other hand, tests of signature of selection may reveal diversity for adaptation to environmental variations that are not taken into account in association tests.

Which Benefits from Detecting Adaptive Diversity in Natural Populations of Forage and Turf Species?

An Alternative to the Phenotypic Assessment of Adaptive Diversity

Environmental adaptation in the natural diversity of a plant species can be investigated at the phenotype level. With this aim, a set of diverse individuals can be assessed for phenotypic performances in several experimental conditions. However, assessing a large range of diversity, and consequently a large number of individuals, necessarily restricts the number of phenotypic observations to a limited number of simple traits and a few environmental conditions. On the other hand, new genome-wide high-throughput genotyping techniques offer opportunities to directly detect adaptive diversity at the molecular level for a large number of individuals originating from a wide range of environmental conditions. However, one should keep in mind that fitness is the only feature that is consistently selected for natural adaptation. Conversely, agronomic performance involves multiple traits that might in some cases be negatively correlated with fitness. For this reason, the detection of adaptive diversity at the molecular level must remained supplemented with an assessment of traits related to agronomic value. Furthermore, it can be considered to perform tests of association between phenotypic variations and molecular polymorphisms in parallel with the search of adaptive molecular diversity.

An Enhanced Insight in Genetic Diversity for an Optimised Sampling of Ex Situ Collections

Ex situ collections with numerous accessions are difficult to handle and maintain in the long term. Frankel and Brown (1984) proposed the concept of core-collection which consists of identifying a sub-collection representing as best as possible the genetic diversity of a larger collection with a limited number of accessions. To set up a core-collection, Brown (1989a) suggested a simple random sampling of 10 % of accessions in the base collection. From this basic method onwards, several refinements have been proposed in order to maximise the genetic diversity captured in the core-collection. Some indirect approaches have been suggested that consist in diversifying spatial origins of selected accessions (Brown 1989b). The choice of a core-collection may also be based on phenotypic data. Several methods have been suggested to maximise the capture of phenotypic variability (Noirot et al. 1996; Balfourier et al. 1998b). However, maximising phenotypic variability for a limited number of traits recorded in a few environments does not ensure that the capture of adaptive diversity is maximised. Other strategies have been proposed based on the maximisation of molecular marker diversity. However, until recently, only a limited number of neutral markers were available. In these conditions, maximising the molecular diversity was efficient only if the neutral structure was matching by chance with some contrasted adaptive responses. The possibility to dispose of markers of adaptive diversity revealed by dense genome-wide genotyping would provide means for a better sampling of adaptive diversity in core-collections. More specifically, adaptive diversity could be sampled along the main environmental gradients determining the presence of a species (Sampoux and Badeau 2009). Furthermore, dense genome-wide genotyping, releasing a great number of neutral markers, would provide a better insight into neutral structure patterns and phylogeographic trends which remain important features to take into account in the choice of a core-collection.

Models to Predict Present and Future in Situ Distribution of Natural Diversity

The statistical methods that can be used to model associations between genomic polymorphisms and environmental variations are quite similar to the ones used to model the environmental distribution (or ecological niche) of species, as done for example in Sampoux and Huyghe (2009) or in Cheaib et al. (2012). Association models between genomic polymorphisms and environmental variations could be considered as multi-dimensional models of environmental distribution of alleles, in a conceptual framework similar to the one of the realized niche of a species (Hutchinson 1957). Such models could be used to predict the probability of presence of alleles at nodes of spatial grids of environmental databases, hence enabling to draw maps of probability of presence of alleles over territories in the same way as maps of probability of presence of species described in Sampoux and Badeau (2009). Models of environmental distribution of alleles could be used to compare the environmental tolerance of different alleles at loci involved in adaptive diversity, in terms of mean position and amplitude along environmental gradients (Banta et al. 2012). Furthermore, models of environmental distribution of alleles could be projected in climate change scenarios, hence enabling to foresee the possible spatial shifts of areas suitable for these alleles.

New Tools for the Use of Adaptive Diversity in Breeding Forage and Turf Species

High-throughput genome-wide genotyping in natural populations of forage and turf species could provide valuable molecular information to breed for improved resilience against environmental variations. Markers of adaptive diversity could be used to identify natural populations harbouring genes useful to introgress in elite materials. At least in the first steps of the breeding process, markers of adaptive diversity identified by association methods and/or signature selection tests could be used in marker-assisted selection. Furthermore, anonymous markers of adaptive diversity could be the starting point to the discovery of neighbouring genes involved in adaptive diversity. Another interesting perspective would be to apply methods of detection of adaptive diversity to allelic polymorphisms revealed by resequencing known candidate genes in natural populations, as done for example by Banta et al. (2012). The finding of outlier loci in the sequence polymorphism of candidate genes and/or significant associations between their allelic polymorphism and environmental variations would contribute to validate their adaptive function and would help to guide the use of their polymorphism in breeding programmes.

Conclusion

Methods to detect adaptive diversity in genomes from genome-wide genotyping data have been initially devised in the frame of population and ecological genetics. The relevance and expected efficiency of these methods are now boosted by new high-throughput genotyping methods based on SNP discovery. Applying such methods to detect adaptive diversity in natural populations of cultivated species opens unprecedented capacities to capture wild adaptive alleles for use in plant breeding. More specifically, introgressing such alleles in elite germplasms could be expected as a mean to improve resilience of cultivars against environmental variations. The extensive collections of natural populations existing in European genebanks of forage and turf species form an especially promising material for applying such methods to bridge the gap between genetic resources and breeding in these species.

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Chapter 44
Monitoring, Analysis and Modeling of Yield and Quality Dynamics of *Lolium perenne* Varieties for Biogas Production

Anita Swieter, Sylvia Moenickes, Bernhard Ohnmacht, Jörg-Michael Greef, and Ulf Feuerstein

Abstract The cutting date is an important determinant for biogas production from forage grass, as yield and digestibility of grass vary greatly during one growing season. Hence, the objective of this study was to increase the biogas yield of perennial ryegrass (*Lolium perenne*) by model-based determination of the optimal cutting date for biogas production. This cutting date was defined as date of the maximum digestible yield, calculated from dry matter yield and crude fiber. For modeling the digestible yield, a model for yield and crude fiber development of *L. perenne* varieties was set up, which includes the most important environmental conditions, such as temperature, global radiation and soil moisture. For model parameterization, eight *L. perenne* varieties with different maturing times were cultivated at four sites in Germany and harvested in time series around the usual first cut. Yield and quality of *L. perenne* and environmental conditions were monitored. The model gave a good fit to both, yield and quality data of the varieties at all sites. Variety-specific model parameters reflected differences in yield and quality development of the varieties. For each variety, the digestible yield at model-based optimized cutting date was increased compared to the digestible yield at the date of ear emergence. By predicting the variety-specific digestible yield for one initial value per variety, differences especially between the early and late varieties became obvious. We conclude, that this model allows us to characterize *L. perenne* varieties and predict the optimal cutting date for achieving an increased biogas yield.

Keywords Perennial ryegrass • Model • Biogas • Varieties • Cutting date

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Introduction

Biogas is promoted in the EU as a renewable energy source, like wind and solar energy. It is environmentally friendly and CO_2 neutral. It comprises primarily methane (CH_4) and carbon dioxide (CO_2) and is produced by fermentation of biodegradable materials, in Germany mainly by silage maize, about which there are some environmental issues (Sieling et al. 2013). Forage grasses are also suitable crops for biogas production (Prochnow and Heiermann 2009; McEniry and O'Kiely 2013).

Yield and quality depend on the growth stage and vary during one growing season, so the date of cutting directly influences the yield and quality achieved.

The optimal cutting date for forage grass is considered to be the time of ear emergence (Gilliland et al. 1995). The optimal cutting date for biogas production is so far unknown.

Perennial ryegrass (*Lolium perenne*) is the most productive forage grass under maritim mid-european conditions (Kornher and Taube 2008). Maturing time of *L. perenne* varieties in the descriptive variety list of Germany differs by up to 43 days (Bundessortenamt 2009). As a consequence, the optimal cutting date is variety-specific as well.

The objective of this study was to increase the biogas yield achieved for perennial ryegrass by determining the optimal cutting date for biogas production by modeling the variety-specific yield and quality development. As the first cut generates the largest percentage of the total yield, we concentrated on the first cut.

Materials and Methods

Model Description

The optimal variety-specific cutting date for biogas production was defined as the date of the maximum difference between dry matter (DM) yield and crude fiber (XF) or the date of the maximum digestible yield. Therefore, a model for DM yield and crude fiber development was set up. Experiments were conducted on one hand to verify the determination of the optimal variety-specific cutting date and on the other hand for model parameterization.

The model comprises one constitutive equation for yield and one for quality development. DM yield y (kg m^{-2}) is modeled based on logistic growth with the growth rate r_{max} (d^{-1}) and the maximum yield y_{max} (kg m^{-2}). Additionally, the yield model includes the environmental response ϕ, which can be a temperature response, a soil moisture response or a global radiation response:

$$\frac{dy}{dt} = r_{\max} \cdot \phi \cdot y\left(1 - \frac{y}{y_{\max}}\right) \qquad (44.1)$$

The temperature response is an optimum function according to O'Neill (Krenek et al. 2011), which includes the optimum temperature T_{opt} and the temperature coefficient Q_{10}. The soil moisture response is a Weibull distribution with a value for a critical soil moisture. The global radiation response is a saturation function with a value for the maximum radiation β (Herrmann et al. 2005).

The quality model is coupled to the yield model. The development of crude fiber y_F (kg m^{-2}) is defined as:

$$\frac{dy_F}{dt} = \theta_F \cdot \left(y - y_F\right) \tag{44.2}$$

with the rate of crude fiber recharge θ_{F}.

Experiments

A field experiment was conducted at four sites in Germany. At all sites, eight *L. perenne* varieties with different maturing times were cultivated in a randomized block design with two replications. The cultivated *L. perenne* varieties were harvested in time series around the usual first cut. Harvests were carried out with ten cutting dates in 5 days intervals. At each cutting date, growth stage, DM yield, crude fiber (XF), crude ash (XA) and digestible organic matter (DOM) were determined. Concentrations of XF, XA and DOM were estimated by a near-infrared reflectance spectroscopy (NIRS) on dried ground samples with a Foss 5000 spectrometer (Foss GmbH, Hamburg, Germany). In addition to yield and quality data, the daily mean temperature, global radiation and calculated soil moisture were recorded for every location.

Verification of the Optimal Cutting Date

In order to validate the determined optimal cutting date for biogas production, methane yield was calculated independently with DOM and XA (Weißbach 2008) and compared to the difference between DM yield and XF.

Model Parameterization

The variety-specific parameters r_{max}, Q_{10}, β and θ_F were fitted by the local optimization algorithm of Matlab 2011b (Mathworks®, Natick, Massachusetts) to experimental yield and crude fiber. The optimum temperature T_{opt} was estimated from the field experiments. The remaining parameters were coincident for each variety.

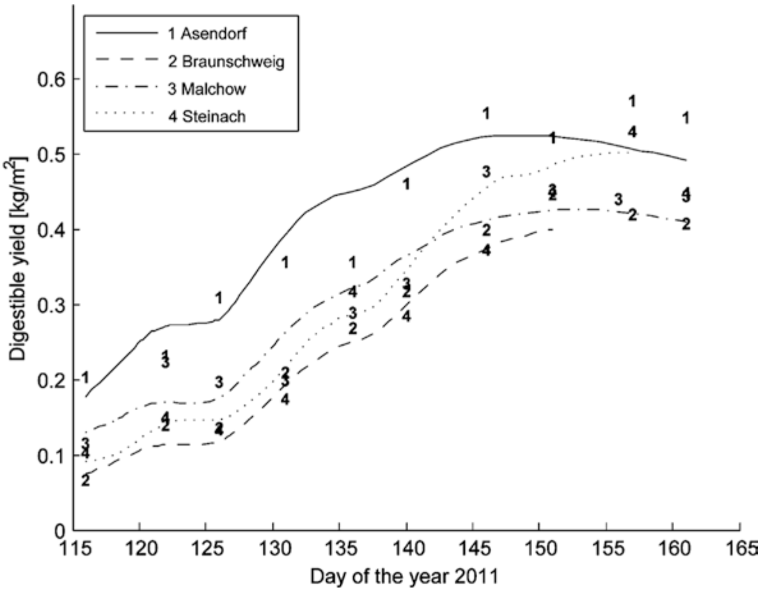

Fig. 44.1 Measured (*numbers*) and modeled (*lines*) digestible yield of variety Aubisque during the first growth at sites Asendorf (*1*), Braunschweig (*2*), Malchow (*3*) and Steinach (*4*); digestible yield was modeled until the maximum yield

Results and Discussion

The model for variety-specific development of the digestible yield, calculated as the difference between DM yield and crude fiber, gave a good fit to the measured yield and crude fiber data of all sites (see Fig. 44.1 for variety Aubisque as an example). The coefficient of determination R^2, averaged over all sites, was 0.99.

Differences in yield and quality development of the investigated varieties were reflected by the model through differences in rates of growth and temperature coefficients (Table 44.1). Early varieties tended to have lower rates of growth and temperature coefficients than later varieties.

For each variety, the digestible yield at model-based optimized cutting date or the date of maximum digestible yield was increased compared to the digestible yield at usual cutting date (date of ear emergence). This is shown by the example of site Braunschweig (Table 44.2). For the earliest variety Lipresso, the digestible yield at optimized cutting date was nearly twice the digestible yield at date of ear emergence.

Table 44.1 Investigated *L. perenne*-varieties, their maturity time and the variety-specific parameters of the yield model T_{opt} (optimal growth temperature), r_{max} (growth rate) and Q_{10} (temperature coefficient)

Variety	Time of ear emergence[a] (days after April 1st)	T_{opt} (°C)	r_{max} (d^{-1})	Q_{10} (°C^{-1})
Lipresso	41 (early)	16	0.11 ± 0.01	2.15 ± 0.52
Abersilo	46 (early)	16	0.11 ± 0.01	1.84 ± 0.42
Aubisque	50 (intermediate)	17	0.14 ± 0.00	2.88 ± 0.18
Respect	51 (intermediate)	17	0.15 ± 0.01	3.00 ± 0.21
Fennema	52 (intermediate)	17	0.14 ± 0.01	2.54 ± 0.27
Bargala	52 (intermediate)	17	0.13 ± 0.01	2.91 ± 0.23
Aberavon	59 (late)	18	0.15 ± 0.01	2.72 ± 0.18
Sponsor	63 (late)	18	0.14 ± 0.01	2.77 ± 0.15

[a] Federal Plant Variety Office, 2009

Table 44.2 Modeled digestible yield of the *L. perenne* varieties at site Braunschweig at usual cutting date (time of ear emergence) and at model-based optimized cutting date (time of maximum digestible yield)

Variety	Digestible yield at usual cutting date (kg m^{-2})	Digestible yield at model-based optimized cutting date (kg m^{-2})	Days between usual cutting date and optimized cutting date
Lipresso	0.26	0.43	20
Abersilo	0.30	0.40	17
Aubisque	0.34	0.43	10
Respect	0.38	0.50	11
Fennema	0.38	0.48	11
Bargala	0.38	0.40	5
Aberavon	0.30	0.40	11
Sponsor	0.34	0.46	15

Predicting the development of the digestible yield for each of the varieties by means of the variety-specific model parameters, the environmental conditions and the initial value per variety at day 140, made differences especially between the early and the late varieties easy to observe: The digestible yield of the early varieties decreases approximately 5 days earlier than the digestible yield of the late varieties (Fig. 44.2).

We can conclude, that the presented model allows us to characterize *L. perenne* varieties, on the basis of yield, quality and environmental response. Furthermore, it can help to predict the optimal cutting date to achieve an increased biogas yield.

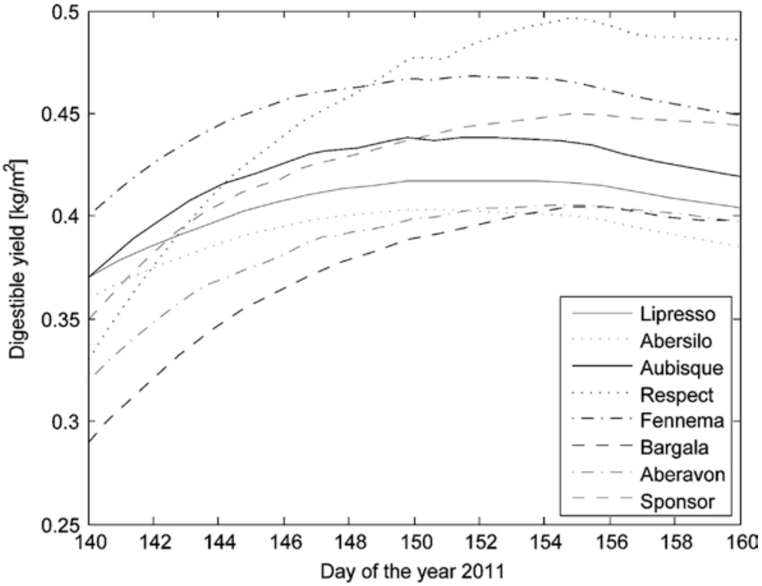

Fig. 44.2 Predicted digestible yield for the early varieties Lipresso and Abersilo, the intermediate varieties Aubisque, Respect, Fennema and Bargala and the late varieties Aberavon and Sponsor at site Braunschweig with variety-specific initial values at day 140

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Chapter 45
Effects of Rhizobacterial Inoculation of a Preceding Oat Crop on Alfalfa (*Medicago sativa* L.) Yield

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Abstract The aim of this study was to test if oats (*Avena sativa* L.) inoculated with plant growth promoting rhizobacteria (PGPR) could promote growth of alfalfa, *Medicago sativa* L. as a subsequent crop. A pot experiment was carried out with unsterilized soil under greenhouse conditions. Oat plants were inoculated with strains belonging to *Sinorhizobium meliloti*, *Azotobacter* spp., *Pseudomonas* sp. as well as *Enterobacter* sp. and *Bacillu*s *megaterium*. The experiment was conducted as a completely randomized design with eight inoculated treatments and three replicates in each treatment. Inoculated treatments were compared with an uninoculated control treatment. Results indicated that the growth of alfalfa plants was improved by oat inoculation with *B. megaterium* strain SNj and *Azotobacter* sp. A1, as well as with a mixture of all strains included in this study. Shoot dry weight of alfalfa was increased by up to 20 % in inoculated treatments compared to the uninoculated control, but the increase varied between strains. The nitrogen content were higher in alfalfa plants exposed to strains *B. megaterium* SNj (18 %) and *Azotobacter* sp. A1 (15 %). Results showed that the beneficial effects of inoculation of oat plants on yield and N-assimilation in alfalfa were specific to certain rhizobacteria.

Keywords Alfalfa • Plant growth promotion • PGPR • Inoculation • Preceding crop • Oats

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Introduction

To maintain a high, profitable yield of good quality in alfalfa (*Medicago sativa* L.) large quantities of nutrients from the soil are required. The concentrations of plant nutrients in soils are variable and nutrients are often present in a form unavailable to the plants. Therefore, the application of mineral fertilizers can significantly increase the quality and quantity of yield. However, in recent decades, economic and ecological problems associated with the excessive use of mineral fertilizers and other synthetic chemicals have emerged, brought on by the increase in the world's human population. The principal goal of agriculture is the production of high quality, safe and affordable food for human population. Nutrient management is the practice of using nutrients wisely for optimum economic benefit, while minimizing the impact on the environment. Consequently, there has been an ever-increasing interest in the use of autochthonous and allochthonous beneficial microorganisms to improve plant health and productivity, while ensuring safety for human consumption and protection of the environment (Avis et al. 2008; Figueiredo et al. 2010). Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonize the rhizosphere and plant roots and have ability to enhance plant growth through direct action or via biological control of plant diseases. The principal mechanisms of growth promotion include increasing nitrogen (N) uptake (biological N-fixation, BNF), solubilization of mineral nutrients, stimulation of root growth (phytohormone production) and suppression of root diseases (antibiosis), as well as bioremediation of polluted soils, which was more recently discovered (Martínez-Viveros et al. 2010; Hayat and Ali 2010; Bhattacharyya and Jha 2012). PGPR are associated with many plant species and are commonly present in several types of environments (Kumar et al. 2011). Strains with reported PGPR activity mainly belong to the phylum Proteobacteria (genera *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas, Serratia*), but other rhizobial bacteria have also been reported (Abbas-Zadeh et al. 2010; Antoun and Prevost 2005; Bhattacharyya and Jha 2012). Due to their ability to promote plant growth, PGPR have been used as bacterial inoculants – a promising complement to mineral and organic fertilizers, pesticides and other supplements (Bhattacharyya and Jha 2012).

Oats (*Avena sativa* L.) are one of the major cereal crops and are followed by legumes in crop rotation (Lassiter et al. 2011). Rotation is important for disease management, reducing the potential weed burden, lowering risk of herbicide residues and for increasing soil health. Good cover crops, like oats should be considered an integral part of any farming system that wants to efficiently utilize nutrients, improve soil quality and increase farm profitability by reducing herbicide costs. An important advantage of using oats as a cover crop is its ability to suppress weeds through physical and chemical (allelopathic) mechanisms (Nagabhushana et al. 2001). The use of an oat crop has the potential to increase productivity of subsequent crops and to reduce herbicide inputs for subsequent crops (Reeves et al. 2005). Based on these characteristics, oats are suitable as the preceding crop to alfalfa. Inoculation of preceding crops with PGPR could help the establishment of a population of beneficial bacteria in the soil and in the rhizosphere of the next crop (Goos et al. 2001).

In recent years there has been a considerable interest in the use of bacterial inoculants as biofertilizers. The selection of effective strains with PGPR properties is one of the most important steps in the evaluation of bacterial inoculants potential. The experiment presented here was designed to determine the possibility of promoting alfalfa growth by inoculating of a preceding oat crop with rhizobacteria, in order to select effective strains that could to be applied as biofertilizer in crop rotation.

Material and Methods

The influence of inoculation of oat with potential PGPR on the yield of a subsequent alfalfa cultivar K-28 crop was examined in a pot experiment under greenhouse conditions. *Sinorhizobium meliloti* strain L3Si, *Bacillus megaterium* SNji, *Enterobacter* sp. strain E1, *Azotobacter* sp. strains A1 and A2, as well as *Pseudomonas* sp. strains P1 and P2 from the Collection of the Institute of Soil Science were used for oat inoculation. Bacterial inoculums were prepared in specific growth media. *Bacillus* sp. and *Enterobacter* sp. strains were cultivated in nutrient broth medium for 24 h, whereas *Pseudomonas* sp. strains were cultivated in King's B medium, also for 24 h (King et al. 1954; Holt 1984–1989; Sarić 1989). The *S. meliloti* strain was cultivated in yeast mannitol broth (YMB) for 48 h, while *Azotobacter* sp. strains were cultivated in N-free mannitol broth with Vinogradsky solution for 72 h (Vincent 1970; Tchan and New 1984).

The pot experiment was designed to imitate the effects of inoculation of a preceding oat crop on a subsequent alfalfa crop, by planting alfalfa plants in pots after a first crop of inoculated oats had been cut down. The oat plants were inoculated with single strains, as well as with a mixture of all seven strains, giving a total of eight inoculation treatments. Treatments were compared with a control, where the preceding oats had not been inoculated. The experiment was carried out in triplicate, using a completely randomized block design (RCB).

Each pot was filled with 1.9 kg of non-sterile soil with the following properties: pH (in H_2O) 7.4, 0.09 % N, 1.48 % C, 21 mg kg^{-1} available P (P_2O_5), 400 mg kg^{-1} K_2O. Surfaced-sterilized oat seeds (0.1 % $HgCl_2$) were inoculated with 2 ml plant^{-1} of liquid culture of either a single strain or an equal mixture of all seven strains (Vincent 1970). Liquid cultures of single strains contained >10^9 cells ml^{-1}. Ten seeds per pot were planted and after 2 weeks seedlings were thinned to 5 plants pot^{-1}. Oat plants were removed at the grain filleting stage and uninoculated alfalfa was sown in the same pots. Alfalfa seeds were surfaced-sterilized, planted and thinned in the same way as the oat seeds. The alfalfa plants were let to grow for 6 weeks before harvesting. Plant shoots were separated from roots and dried in an oven at 70 °C to constant weight and the average dry weight per plant was calculated. The percentage of shoot-N was determined from dried and ground plant samples using

Table 45.1 Alfalfa properties after inoculation of a preceding oat crop with PGPR

	Alfalfa parameters				
PGPR[a] strain	Height (cm)	SDW (mg $plant^{-1}$)	Total-N content (mg $plant^{-1}$)	SDW index	Total-N content index
Ø[b]	30.4 a	479 c	17.0 c	100	100
A1	28.8 a	527 b	19.6 a	110	115
A2	32.3 a	457 cd	15.7 d	95	92
P1	28.8 a	480 c	16.7 c	100	98
P2	30.5 a	420 e	14.2 e	88	83
L3Si	29.8 a	445 de	15.0 de	93	88
SNj	32.1 a	583 a	20.0 a	122	118
E1	33.1 a	442 de	15.5 d	92	91
Mix[c]	35.1 a	578 a	18.1 b	121	106
LSD 0.01;0.05	/	23.1	0.77		
	5.62	30.7	1.04		

[a]Plant grow promoting rhizobacteria, [b]uninoculated control treatment, [c]mixture of all strains, *a–e* homogeneous subgroups according to Duncan's multiple range test (at the significance level of $\alpha = 0.05$)

a CNS analyzer and used to calculate total-N content (mg $plant^{-1}$). The data were statistically analyzed using the LSD and Duncan tests in the statistical program SPSS 10.0. Correlation coefficients were calculated to study the associative relations between measured traits. All references to significance in the text imply statistical significance at $p < 0.05$, unless otherwise stated.

Results

The height of the alfalfa plants ranged from 28.8 to 35.1 cm, with no significant differences between treatments. The highest mean shoot dry weights (SDW) of alfalfa plants were obtained after inoculation with *B. megaterium* strain SNj and the strain mixture, followed by *Azotobacter* sp. strain A1, (583, 578 and 527 mg $plant^{-1}$, respectively) (Table 45.1). These results indicated that alfalfa SDW was significantly higher after inoculation of the preceding oat crop with SNj, A1 or the strain mixture, then after inoculation with any other strain or in the control. However, the effect of strain A1 on SDW was significantly lower than that of the SNj strain or the strain mixture. The increase in SDW, compared to the control, was 22 % for SNj and 10.00 % for A1. Application of a mixture of strains resulted in an increase in SDW by 21 %, which was comparable to that obtained by application of strain SNj. There was also a highly significant, positive correlation between SDW and shoot total-N content ($r = 0.927$). The results highlight significant plant growth promotion abilities of these rhizobacterial strains.

The highest mean total-N content was also detected in the SNj, A1 and strain mixture treatments. However, the two single strain treatments had significantly higher total-N content than the strain mixture. The treatment with *B. megaterium* SNj and with *Azotobacter* sp. A1 resulted in a total-N content of 20.0 and 19.6 mg plant^{-1}, respectively. The strains increased the total-N content of alfalfa plants by up to 18 %, in comparison to the control. In contrast, the influence of the strain mixture on total-N increase was much lower.

None of the other strains included in this study significantly improved alfalfa properties when compared to the control.

Discussion

In the era of sustainable crop production, the plant–microbe interactions in the rhizosphere play a pivotal role both in the transformation, mobilization and solubilization of nutrients from the limited nutrient pool and in the subsequent uptake of essential nutrients by plants. Thus, plant–microbe interactions enable plants to realize their full genetic potential. Consequently, soil bacteria have been used as inoculants (biofertilizers) in crop production, including legumes and cereals, for decades (Figueiredo et al. 2008; Hayat and Ali 2010; Figueiredo et al. 2010). In addition, there is an interest in inoculation of preceding crops with PGPR, with the intention of establish PGPR populations in the soil and in the rhizosphere of the next crop (Domit et al. 1990; Goos et al. 2001).

Our results showed that out of seven strains, only two individual strains (*B. megaterium* strain SNj and *Azotobacter* sp. A1), and a mixture of all seven strains, had the ability to significantly promote plant growth. Increased growth and yield of agronomically important crops (soybean, common bean, peanut, alfalfa, oat, barley, corn, potato etc.) in response to inoculation with PGPR have been extensively reported (Zhang et al. 1996; Gupta et al. 2002; Vessey 2003; Gray and Smith 2005; Figueiredo et al. 2008).

Diverse populations of aerobic, endospore-forming *Bacillus* species are present in arable soil and contribute to crop productivity by various mechanisms of growth promotion. It is very likely that plant growth promotion by rhizosphere bacilli may be a result of the combined action of two or more of these mechanisms (Minorsky 2008; Richardson et al. 2009; Hayat and Ali 2010; Kumar et al. 2011). These bacteria competitively colonize the roots of plants and can act as biofertilizers or antagonists (biopesticides). Some bacteria can even act as biofertilizer and biopesticide simultaneously. In the present study, *B. megaterium* SNj displayed significant growth promoting abilities, with alfalfa yield SDW and total-N content increasing by 22 and 18 %, respectively.

Our results suggest that the plant growth promotion abilities of *Azotobacter* sp. strains A1 and A2 differ from those in *B. megaterium* SNj, which indicates that the growth-promoting ability of a bacterium may be highly specific to a plant species, cultivar or genotype (Lucy et al. 2004). Based on these results and a previous study (Delić et al. 2013), we conclude that strain A1 had significantly greater plant growth promoting potential, when used as an inoculum. It is likely that the N-fixing activity of this strain caused the observed increase in total-N content in alfalfa (15 % increase compared to the control). It has been reported that certain species of *Azotobacter*, in association with some cereals, can fix up to 90 kg N ha^{-1}, which indicates that members of the *Azotobacter* genus are indeed good diazotrophs and can be of use in both cereal and legume production (Franche et al. 2009).

In this study, *S. meliloti* strain L3Si was ineffective when applied as an inoculant of preceding oats, despite of its highly effective role in BNF in alfalfa in a previous study (Delić et al. 2012). Significantly lower values of measured alfalfa properties compared to the control (Ø) indicated an ineffective relationship between the symbionts.

In addition, the other rhizobacteria included in this study (*Azotobacter* sp. A1, *Enterobacter* sp. E and the two *Pseudomonas* sp. strains) did not produce any positive effects on alfalfa yield. This is inconsistent with previous research, where *Enterobacter* and *Pseudomonas* genera were identified as PGPR for various plants (Minorsky 2008; Franche et al. 2009; Zabihi et al. 2010). The lack of positive effects in our experiment could be due to natural variations in the environment, the soil and the indigenous microflora between different areas, as well as due to variations in host specificity and the associated rhizosphere effect. This heterogeneity represents a major challenge in the use of bio-inoculants (Niranjan Raj et al. 2005; Mehboob et al. 2008; Martínez-Viveros et al. 2010).

Conclusion

Improved yield and N-accumulation in alfalfa were obtained through inoculation of a preceding oat crop with certain strains of *Azotobacter* sp. and *B. megaterium*, highlighting their significant alfalfa growth promotion abilities. Our results showed that the application of PGPR have the potential to contribute to the development of sustainable agricultural systems and should be evaluated under field conditions.

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Chapter 46

Phalaris arundinacea L.: Variations in Seed Shattering and Related Traits in a Breeding Collection

Karin Förster, S. Pentschew, J. Braune, and Ulf Feuerstein

Abstract Reed canary grass is a perennial forage grass species with a high biomass yield potential and a wide adaptability to both, dry and excessive wet growing conditions. Its seed yield depends on number of plants per m^2, number of panicles per plant, number of spikelets per panicle as well as flowering time, seed setting, seed development and seed retention at the time of maturity. Unfortunately, in many years seed shattering causes seed yield losses up to 90 %. Because of the limited variation of seed retention in the available germplasm of reed canary grass, a mutation experiment was carried out in 2011 using the alky-lating agent ethyl methanesulfonate. Results of seed shattering and seed yield of single plants, growing in 2011/12 after mutagenic seed treatment are presented for the first time.

Keywords Seed shattering • *Phalaris arundinacea* L • Seed yield • Chemical mutagenesis

Introduction

In grasses, quantity, quality and economics of seed production influence the growing area of a species. Reed canary grass has a high biomass yield potential and is adapted to dry and wet growing conditions. Otherwise, its seed shattering behaviour is similar to wild plants. Although Wrobel et al. (2009) have shown the potential for improvement in seed retention, in Europe no shattering resistant variety is cultivated.

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A project to improve seed shattering resistance and seed yield by chemical mutagenesis was started. Results reported here are from the first selection and testing year.

Material and Methods

Plant Material

Well characterized uniform seed of five reed canary grass populations were provided by Euro Grass Breeding.

Methods

- Ethyl methanesulfonate treatment of seed includes: preparation, pre-soaking, mutagen treatment (short-term: 3 h, long-term: 5 h; untreated control) and post-treatment steps following a standardized procedure (unpublished).
- 685 selected, vernalized plants were cultivated in a space-planted nursery (100 × 90 cm) at the experimental station Etzdorf.
- During the vegetation period growth, development and flowering behaviour of each single plant were evaluated. At the end of open bloom on each single plant panicles in identical stage of development were isolated to prevent seed loss. The isolated ripe panicles were harvested. Mass of seeds per panicle was measured from each plant. Three parameters were estimated: m1: mass of natural shattered seed, m2: mass of seeds after seed shattering treatment under controlled provocation conditions, and m3: mass of non-shattered seed.
- Seed shattering resistance percentage was calculated using the equation $m3 * 100/(m1 + m2 + m3)$.
- Statistical analysis: ANOVA was applied on populations and treatments using procedures of the Statistical Analysis System, Version 9.2 (SAS Institute, Cary, NC, USA). Paired comparisons of means with Tukey-Kramer method, $\alpha = 0.05$, were performed for yield per panicle.

Results and Discussion

Seed shattering resistance of the tested single plants varies between 5 and 98 % (Fig. 46.1). On average the initial population means (UC) are lower than those of the respective long-term mutagenic seed treatment. Beyond it, differences in response to mutagenic treatments exist among populations. The average shattering resistance

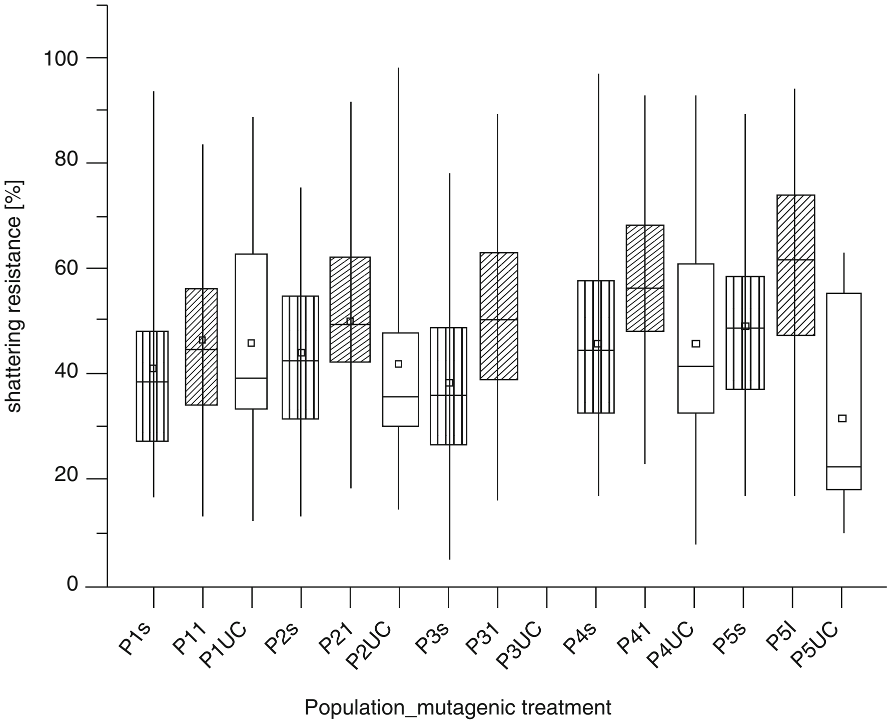

Fig. 46.1 Seed shattering resistance of open-pollinated progenies of five populations after mutagenic treatment. *P1…5* number of original population, *s* short-term, *l* long-term mutagenic treatment, *UC* untreated control, N = 685, P3UC not measured

of population 5 is higher than that of all other populations. Shattering resistance after short-term mutagenic treatment increases especially in population 5. However, in both treatments as well as in the untreated control outliers with a very high shattering resistance are found.

Inter-population specific differences are also found for the trait seed yield per panicle (Fig. 46.2). On average the total seed yield (m1 + m2 + m3) of P2UC, P4UC and P5UC is lower than that of P1UC. In contrast to seed shattering resistance yield increases after the short mutagenic treatment. The average yield per panicle of P1, P2 and P5 after the short mutagenesis is significant higher than that of the untreated control. In comparison with the untreated control the long-term mutagenesis reaches same yields (exception: P4).

The results of the first year show the potential of the breeding material in spite of seed shattering and seed yield. Seed shattering and yield both depend on environmental conditions (Hermann 1975; Sahramaa and Hömmö 2000). It is necessary, therefore, to continue the evaluation of the material and to check the characteristics of their progenies.

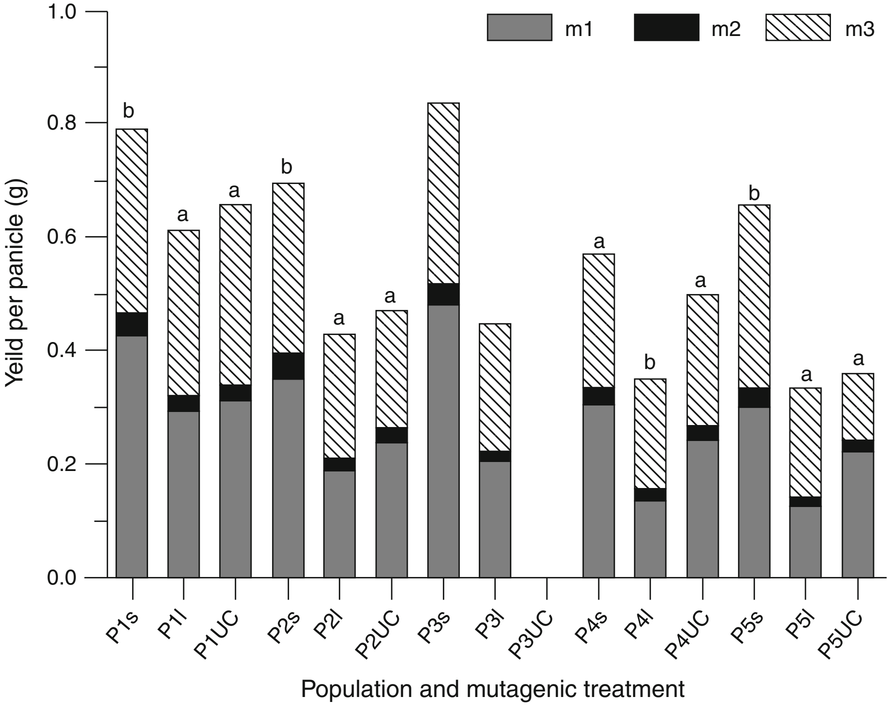

Fig. 46.2 Seed yield components. Values are means; m1 mass of shattered seed, m2 mass of shattered seed after seed shattered treatment under controlled conditions, m3 mass of non-shattered seed; N = 685; pairwise comparison of short-term (*s*) or long-term (*l*) mutagenic treatment against untreated control (*UC*)/population; different letters: means are significantly different; P3UC not measured

Conclusions

Mutagenic treatment has a high potential for increasing the variation of agronomic traits in reed canary grass.

Differences in response to mutagenic treatments exist among populations.

The long-term mutagenic treatment causes an increase of seed shattering resistance.

Plants with higher yield and good seed shattering resistance can be selected.

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Chapter 47
Effect of Alfalfa Cultivar on Pollinator Visitation, Seed Yield and Yield Components

Goran Jevtić, Bojan Anđelković, Jasmina Radović, Bora Dinić, and Snežana Babić

Abstract Seed production is a critically important trait in alfalfa breeding, and understanding the relationship between pollinator visitation and seed yield could help the development of high seed-yielding cultivars. The effect of three alfalfa cultivars on pollinator visit, seed yield and seed yield components was researched. To determine visitation rates, pollinators within a 1 m^2 area were counted during a 30 min period in the morning, at noon and in the afternoon. Most pollinators were attracted to cv. K-28 (13.2). Cultivar NS Medijana was visited by 9.81 pollinators and cv. K-22 by 9.06. Cultivar K-28 attracted the highest number of honey bees (40.1 %), followed by cv. NS Medijana (31.3 %) and cv. K-22 (28.6 %). The ratio of honeybees to other observed pollinators was approximately 3:1. Cultivar K-28 achieved a seed yield of 553.3 kg ha^{-1}, cv. Medijana 472.8 kg ha^{-1} and the seed yield of cv. K-22 was 469.4 kg ha^{-1}. Cultivar K-22 had the most flowers and fertilized pods in the middle and highest inflorescences as well as seeds per pod in the highest inflorescences. Cultivar Medijana had the most flowers on the lowest inflorescences. Cultivar K-28 showed the highest values for other observed traits. Cultivar K-28 showed very significant differences from the other varieties in percentage of fertilized pods at the highest inflorescences. In relation to seed yield, numbers of flowers, pods and seeds per pod at the lowest inflorescences, cv. K-28 was significantly better than other cultivars, which positively correlated with the number of visiting pollinators. For other yield components, differences between cultivars were not statistically significant.

Keywords Alfalfa • Cultivar • Pollinators • Seed yield • Seed yield components

Introduction

Due to its large number of flowers, alfalfa has a very high potential for seed production. Different authors suggest that alfalfa could potentially produce more than 2 t ha^{-1} of seed (Pedersen et al. 1956). Yields this high are unfeasible in practice due

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to several limiting factors in alfalfa seed production in relation to the production of alfalfa forage (livestock feed). Agro-meteorological factors, the level of agricultural technology, row spacing, seed rate, cultivar and other factors have a great influence on alfalfa seed production. Failure to meet one of these conditions can be a limiting factor in alfalfa seed production.

Alfalfa, being an almost entirely cross-pollinated plant species (xenogamy), for normal fertilization requires the presence of a sufficient number of appropriate pollinators whose activity is closely related to the agro-meteorological conditions at the time of flowering. To enhance the effectiveness of pollinators, the following have been undertaken: selection programs for honey bee based on alfalfa pollen collection, (Nye and Mackensen 1970), the domestication of certain species of solitary bees (*Megachille rotundata* L., *Nomia melanderi* L., etc.), their utilization in alfalfa seed production (Richards 1984; Cane 2002), and even the introduction of bumblebees (*Bombus terrestris* L.), (Cecen et al. 2008). In addition to the selection of honey bees, there has been a comparative selection of alfalfa for more efficient pollination, and, as a result, lines of alfalfa have been developed with increased nectar secretion (Tauber and Green 1996), and with easier flower access (Knapp et al. 1996). The rate of honey bee visits to an alfalfa seed crop, besides climatic conditions, is affected by: the presence of competitive pastures at the time of alfalfa flowering, pollination facilitation, time of day, distance of hives from seed crops and the alfalfa cultivars (Кырну et al. 1977; Lukić 2000; Jevtić et al. 2001, 2004, 2006). Most of these factors affect alfalfa seed yield and seed yield components.

The aim of this study was to determine the influence of local cultivars on various pollinator visits, as well as the response of alfalfa seed yield and seed yield components.

Material and Methods

Three local cultivars of alfalfa were used for this experiment. K-22 and K 28 are local cultivars, created at the Institute for Forage Crops in Kruševac, and cv. NS Medijana was created at the Institute for Field and Vegetable Crops in Novi Sad.

The number of honey bees and other pollinators was determined by counting all honey bees and pollinators that flew to within 1 m^2 of alfalfa in full bloom during a 30 min period. Counting was carried out on the 5 days of the experiment at the following times: 0900–0930, 1300–1330, and 1700–1730 at second cut. During the count, the species of other pollinators was not taken into account, only their abundance on alfalfa.

Thirty alfalfa stems per repetition were selected for seed yield component determination. Three inflorescences per stem were selected (low, middle and peak) and the flowers were counted. Seed yield components were number of flowers per stem, number of flowers per inflorescence, number of inflorescences with fertilized pods per stem, number of pods per inflorescence and the number of seeds per pod. In addition, fertilization of inflorescences (number of fertilized inflorescences/total number of inflorescences per stem × 100) and fertilization of pods (number of pods/number of flowers × 100) were counted.

Seed yield was determined after plant maturation. Plants were harvested and seed was threshed and measured. The yield was expressed in kg ha^{-1}.

Monofactorial variance analysis was done by a completely random plan for alfalfa seed yield and seed yield components. Any differences between cultivars were tested using LSD test.

Results and Discussion

Most honey bees were attracted to cv. K-28, followed by cv. NS Medijana, and the fewest honey bees visited cv. K-22 (Table 47.1). Cultivar K-28 attracted 9 % more honey bees than cv. NS Medijana and 11.5 % more than cv. K-22. Besides being more attractive to honey bees, cv. K-28 was also more attractive to other pollinators, meaning that, of the overall number of other pollinators, 42.1 % visited this cultivar. It is interesting to note that cv. K-28 was most visited by honey bees in the morning period (0900–0930), and the other two cultivars at 1300–1330.

Pollinator visitation was greatly influenced by weather conditions during alfalfa flowering. In years with more precipitation, an increase in the number of other pollinators was recorded in the alfalfa fields, while in dry and warm years, honey bees were the dominant pollinators (Jevtić et al. 2010). Significantly higher seed yield was achieved from cv. K-28 (553.3 kg ha^{-1}) while the other two cultivars had a similar yield (Table 47.2), which is in positive correlation with the number of visiting pollinators. Nevertheless, cv. K-28 had the highest percentage of fertilized inflorescences and fertilized pods at low, middle and peak inflorescences. This cultivar had the most fertile inflorescences per stem, the most pods on the lowest inflorescences and the most seeds per pod at the lowest and middle inflorescences. The seed yield of ten alfalfa genotypes had a very high correlation with the number of pods and the number of seeds per inflorescence, ranging from 426 kg ha^{-1} in the genotype Le-6 to 651 kg ha^{-1} in cv. Evropa, and the resulting differences were highly significant (Ilic and Đukic 2006).

Some authors report significantly greater variation between alfalfa cultivars when determining seed yield: for example, the differences in research carried out by Ecalle et al. (2001) range from 421 to 1,021 kg ha^{-1}. Cultivar K-22 had the most flowers on the middle and peak inflorescences, but since it failed in seed yield, it can be concluded that there was inadequate flower pollination. This cultivar was also the least attractive to honey bees and other pollinators. Cultivar NS Medijana had the most flowers on the lowest inflorescences, while the other components were somewhat lower or on a par with other observed cultivars. However, although this cultivar had lower values for almost all the observed components, it had a slightly better yield than cv. K-22. This fact is certainly related to pollinator visitation because this cultivar had many more pollinators compared with cv. K-22., especially in the morning and mid-day periods.

Table 47.1 Number of honey bees and other pollinators on different alfalfa cultivars

Cultivar	Pollinator	0900–0930	1300–1330	1700–1730	Total pollinators	Total	Average	Honey bees (%)	Other (%)
K-22	Honey bee	5.0	8.8	5.8	27.2	19.6	6.53	28.6	
	Other	1.2	3.6	2.8		7.6	2.53		26.2
NS Mediana	Honey bee	8.6	10.0	2.8	30.6	21.4	7.13	31.3	
	Other	2.2	3.6	3.4		9.2	3.07		31.7
K- 28	Honey bee	11.0	9.4	7.0	39.6	27.4	9.13	40.1	
	Other	3.8	4.2	4.2		12.2	4.07		42.1
Honey bee					97.4	68.4	7.60	100	
Other						29.0	3.22		100

Table 47.2 Seed yield and seed yield components in different cultivars of alfalfa

	Cultivar						
						Lsd	
Trait	K-22	NS Med.	K-28	Average	P	0.05	0.01
Seed yield (kg ha^{-1})	469.36^{b}	472.85^{b}	553.34^{a}	498.43	0268**	58.73	88.99
No. of inflorescences	23.60	19.83	25.37	22.93	2185^{ns}	6.96	10.54
No. of fertilized inflorescences	19.87	15.67	21.8	19.11	2693^{ns}	6.14	9.30
Fertilization of inflorescences (%)	84.19	79.02	85.93	83.05	7060^{ns}	11.04	16.72
No. of flowers on low inflor.	9.37^{ab}	10.57^{a}	8.47^{b}	9.47	0250*	1.39	2.10
No. of pods on low inflor.	4.38	4.83	5.02	4.74	0972^{ns}	1.10	1.67
Fertilization of pods on low inflor. (%)	46.74	45.69	59.27	50.57	9198^{ns}	22.02	33.36
No. of flowers on middle inflor.	16.03	14.93	13.4	14.79	1392^{ns}	2.91	4.40
No. of pods on middle inflor.	8.52	7.28	7.84	7.88	1370^{ns}	1.24	1.88
Fertilization of pods on middle inflor. (%)	53.15	48.76	58.51	53.47	1273^{ns}	15.74	23.85
No. of flowers on peak inflor.	22.00	21.33	17.97	20.42	1912^{ns}	5.22	7.91
No. of pods on peak inflor.	7.45	6.43	7.04	6.97	3010^{ns}	1.59	2.40
Fertilization of pods on peak inflor. (%)	33.86^{b}	30.14^{b}	39.18^{a}	34.39	0020**	3.77	5.71
No. of seeds on low inflor.	4.82^{b}	4.55^{b}	5.59^{a}	4.99	0297*	0.77	1.16
No. of seeds on middle inflor.	4.22	3.95	4.78	4.32	1310^{ns}	0.93	1.41
No. of seeds on pick inflor.	3.84	3.71	3.65	3.73	6361^{ns}	0.76	1.15

ns not significant; *statistically significant at $p<0.05$; **statistically highly significant at $p<0.01$

a, *b*, *ab* indicate high homology, meaning absence of statistically significant differences (Fisher's LSD test, $p<0.05$)

Differences in seed yield and pod fertilization on the highest inflorescences between cultivars were statistically highly significant ($p < 0.01$). Differences in the number of flowers and pods at the lowest inflorescences and number of seeds per pod on the lowest inflorescences were significant ($p < 0.05$).

Morphology of plant, inflorescences and flowers could affect pollinator visitation and seed yield. Cultivar K-28 had the highest number of inflorescences per plant but had the fewest flowers per inflorescence, so pollinators visited it more often. A reason for the more frequent visits could be that cultivar K-28 may have had a greater quantity of nectar per flower as a result of the smaller number of flowers per plant.

Conclusion

Based on the presented results on the impact of different alfalfa cultivars on pollinator visitation, seed yield and seed yield components, the following conclusions can be made:

- Most pollinators were attracted to cv. K-28 (average 13.2), while cvs NS Medijana and K-22 were visited by fewer pollinators (10.2–9.06).
- During pollinator monitoring, it was determined that there were 2.5 times more honey bees than other pollinators in the alfalfa field. There was a variation in pollinator visits according to the time of day, and the greatest number of pollinators was recorded at 1300–1330.
- Cultivar affected seed yield and seed yield components. Cultivar K-28 achieved the highest seed yield and the best results for most of the observed yield components. Differences in seed yield and pod fertilization in the highest inflorescences were statistically highly significant ($p < 0.01$). Differences in the number of flowers, number of pods and number of seeds per pod at the lower inflorescences were statistically significant ($p < 0.05$).

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Chapter 48
The Influence of Individual and Combined Inoculants on Development of Alfalfa on Acidic Soil

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Abstract In this study, the influence of pre-sowing inoculation with individual and combined cultures of rhizobium (*Rhizobium meliloti*), azotobacter (*Azotobacter chroococcum*) and actinomycetes (*Streptomyces* spp.) on growth parameters of alfalfa plants (height, number of stems and plant weight) was investigated. The mass of plant root was also determined. Analyses were carried out at the onset of flowering. The experiment was a two-factorial design, where the growth of plants influenced by seven variants of microbial inoculation was compared with the growth of non-inoculated controls for two alfalfa cultivars (K-28 and Syntéza 1). The experiment was carried out in pots in semi-controlled conditions. For this study, plants were sown on acid soil with pH 4.7. For tested parameters, interaction between cultivar and inoculation resulted in a positive effect compared to the control. Highest plant height was achieved in cv. Syntéza 1 using an inoculum that contained all of the species of microorganisms. By using this microbial variant, the highest value for green mass and number of stems per plant was achieved in cv. K-28. In this cultivar, using the combined cultures of rhizobium and actinomycetes, the highest value for root mass was achieved.

Keywords Alfalfa • Inoculation • Rhizobium • Azotobacter • Actinomycetes

Introduction

Alfalfa is one of the oldest and most important forage crops that, in addition to high yield potential and biomass quality, is characterized by the ability to fix nitrogen. This plant species can fix 100–400 kg N ha^{-1} per year in association with *Rhizobium*

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meliloti (Peoples et al. 1995). Soils with high acidity have negative effects on the abundance and activity of *Rhizobium* and on the intensity of nitrogen fixation (Soto et al. 2004). To ensure the successful production of alfalfa on soils of high acidity, pre-sowing inoculation using *R. meliloti* strains results in satisfactory nitorgen-fixing activity in such soils. Rhizobia provide macrosymbionts with nitrogen and also synthesize polysaccharides, vitamins B_{12}, B_1, B_2 (Denison and Kiers 2004). In addition to the symbiotic nitrogen-fixers, the increase of nitrogen balance is achieved by the application of free nitrogen-fixers, primarily *Azotobacter* spp., which synthesize gibberellins, auxins, pyridoxine and nicotinic acid. Actinomycetes produce antibiotics and vitamins that act favourably on physiological processes in plants (Kumar et al. 2010). These microorganisms are present, in higher or lesser abundance, in the soil, and after harvest they can end up on the seed and plant roots. However, the application of selected microorganisms for seed inoculation leads to better results in crop production. The effect of inoculation depends on host plant activity, species, strain and cell concentration of microorganisms in the inoculum, the structure of indigenous populations, chemical and physical properties of soils, and agricultural practices (Walker et al. 2003).

The aim of this study was to examine the influence of rhizobium, actinomycetes and azotobacter (especially in combination) on the development of alfalfa.

Material and Methods

For the purposes of research, soil with acidity pH 4.7 (KCl) was used. The soil is of the eutritic brown type. This soil has a medium to high nitrogen content (0.204 %), is medium rich in humus (4.30 %) and easily accessible phosphorus (10.20 mg/100 g), and is very rich in potassium (51.00 mg/100 g) (Table 48.1).

The experiment was a two-factorial, random block design with five replicates, where the first factor was alfalfa cultivar and the second was the variant of microbial inoculation. Research was conducted on two alfalfa cultivars: K-28 (bred in the Institute for Forage Crops in Kruševac) and Syntéza 1 (bred in the Plant Production Research Center (PPRC) in Piešťany).

The inoculation was done using the following inocula:

1. *Rhizobium meliloti* (10.8×10^{11} per ml);
2. *Azotobacter chroococcum* (28×10^{11} per ml)*;*
3. *Streptomyces* spp. (actinomycetes), (7.6×10^{10} per ml);
4. *R.meliloti* + *A.chroococcum;*
5. *R. meliloti* + *Streptomyces* spp.;
6. *R. meliloti* + *Streptomyces* spp.;
7. *R. meliloti* + *A.chroococcum* + *Streptomyces* spp.;
8. Control – no inoculation.

The *R. meliloti* cultures were grown on YM substrate by Vincent (1970), *A. Chroococcum* cultures were grown on the liquid substrate by Feodorov (1949),

Table 48.1 The chemical composition of the soil

Parameter	pH/H_2O	pH/KCl	Total nitrogen (%)	Humus (%)	P_2O_5 (mg/100 g)	K_2O (mg/100 g)
Value	5.64	4.77	0.204	4.30	10.20	51.00

and *Streptomyces* spp. were grown on the substrate by Krasiljnikov (1965). These microorganisms were obtained from the collections of the Department of Microbiology of the Faculty of Agriculture in Novi Sad. Alfalfa seed was sterilized with 0.2 % solution of $HgCl_2$ and 70 % ethanol, rinsed several times with sterile tap water and then immersed in the corresponding inocula. Ten seeds from each inoculum variant were planted in pots filled with soil. Ten milliliter of the appropriate inoculum was added to each pot.

The analysis of plant and root traits were done in the second cut at the beginning of flowering. The following traits were measured: plant height (cm), number of stems per plant, green mass per plant (g) and root weight per plant (g).

The results were processed using the statistical package STATISTICA 8.0. The significance of the difference between the investigated treatments was determined by analysis of variance and LSD tests.

Results and Discussion

The results obtained showed that the application of microorganisms and combinations thereof can affect the parameters of plant mass and root mass yield.

For most treatments, inoculation positively influenced plant height. The greatest effect on height was noted in the treatment with combined cultures of *R. meliloti*, *A. chroococcum* and *Streptomyces* spp. (7). By using a mixed inoculum of these microorganisms, Jarak et al. (2007) reported the fastest germination and maximum height and weight of alfalfa. However, in our experiments, in treatments with pure A. *chroococcum* (2) and *Streptomyces* spp. (3), the height of the plants of both cultivars was not statistically different from the control. Increases in height were achieved by applying combined cultures of these microorganisms (inoculum 6) to cv. Syntéza 1.

Applied inocula increased green mass. Only the application of individual cultures of *Streptomyces* spp. (3) to cv. K-28 did not result in a statistically significant increase in green mass per plant compared to the control.

Positive effects of microbial inoculation on number of stems were recorded in almost all the treatments on cv. K-28. For inoculation treatment with combined cultures of *A. chroococcum* and *Streptomyces* spp. (6), the number of stems was not statistically different to the control. However, in cv. Syntéza 1, the number of stems was significantly increased, but only by inoculation with combined cultures of *R. meliloti* and *A. chroococcum* (4) and with *Streptomyces* spp. (5). Research results obtained by Stavarache et al. (2010) showed that inoculation had no significant influence on the number of stems in alfalfa.

Table 48.2 The effect of inoculation and cultivar on the parameters of alfalfa

Cultivar	Variant	Plant height (cm)	Green mass per plant (g)	Number of stems per plant	Root mass per plant (g)
K-28	1	$41.00^{d,e}$	5.61^{h}	$5.10^{a,b,c}$	8.53^{b}
	2	$40.60^{d,e,f}$	5.40^{i}	$5.30^{a,b}$	7.66^{d}
	3	$40.40^{e,f,g}$	5.28^{j}	$4.80^{b,c,d}$	7.56^{e}
	4	$42.80^{b,c}$	6.30^{d}	$5.30^{a,b}$	8.74^{a}
	5	42.30^{c}	6.49^{b}	5.40^{a}	8.68^{a}
	6	41.30^{d}	5.56^{h}	$4.70^{c,d,e}$	8.19^{c}
	7	$43.00^{a,b,c}$	6.58^{a}	5.50^{a}	8.76^{a}
	8	$40.10^{f,g}$	5.24^{j}	$4.20^{e,f,g}$	7.68^{d}
Syntéza 1	1	$42.90^{a,b,c}$	$6.00^{f,g}$	$3.60^{h,i}$	$7.16^{f,g}$
	2	$39.90^{f,g}$	6.02^{f}	3.40^{i}	$6.70^{h,i}$
	3	39.60^{g}	5.94^{g}	$3.60^{h,i}$	6.57^{j}
	4	$43.20^{a,b}$	6.40^{c}	$4.30^{d,e,f}$	7.25^{f}
	5	$43.50^{a,b}$	6.32^{d}	$4.30^{d,e,f}$	7.13^{g}
	6	$40.20^{e,f,g}$	6.22^{e}	$4.00^{f,g,h}$	6.77^{h}
	7	43.70^{a}	6.47^{b}	$4.20^{e,f,g}$	$7.20^{f,g}$
	8	39.70^{g}	5.17^{k}	$3.70^{g,h,i}$	6.67^{i}

Note: Same letters in superscript indicate high homology, meaning absence of statistically significant differences (Fisher's LSD test, $p<0.05$)

The application of microbial inoculants, especially those that contained *R. meliloti*, had a positive effect on root mass (Table 48.2). Compared to the control the mass was not statistically different only in the treatment with pure inoculum of *A. chroococcum* (2) in both cultivars. Similar results were obtained by Heydar (2008). In soils of high acidity, the concentration of indigenous microbial populations is small, so the use of inoculation with effective strains of microorganisms can intensify microbiological processes which, in turn, can increase available nutrients for plants. The results obtained in our research indicate that plant response to the application of appropriate microorganisms and their combination was generally positive. Better effects were achieved using a combined culture of microorganisms, especially when combining *R. meliloti*, *A. chroococcum* and *Streptomyces* spp. inoculations simultaneously, since more microbiological processes are activated.

Considering the cultivar influence on the tested parameters of alfalfa, there was no clear trend, except in root mass, which was better in cv. K-28 than cv. Syntéza 1 in all treatments.

The results suggest that artificial inoculation with a mixture of inoculants could be ecologically feasible for improving the production of alfalfa in areas with high soil acidity.

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Chapter 49
Alfalfa Genetic Variation for Biomass Production in Pure and Mixed Stands

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Abstract New interest is given to alfalfa, an important forage legume crop, because it can contribute to agriculture sustainability. Mixtures of alfalfa with grass species combine, in a single sward, the advantages of the two species: seasonal growth pattern, biochemical composition, low nitrogen input. Forage production, but also the survival of each species, result from the ability of plants to get the resources needed for their growth. The present study aimed at describing alfalfa genetic variation for biomass production in different treatments: spaced plants, dense monocultures and alfalfa-tall fescue mixtures. Four harvests were taken in 2011 and 2012. In analyses of variance, the effects of alfalfa genotype and treatment on alfalfa biomass were highly significant. The interaction between genotype and treatment effects was significant, showing that genotype ranking for biomass depended on the treatment. These results will be complete with data on fescue biomass, plant height and nitrogen content to propose an alfalfa ideotype suitable for both monoculture and mixture.

Keywords *Medicago sativa* • *Festuca arundinacea* • Legume • Genetic variation

Introduction

In the context of increasing agricultural sustainability, swards based on alfalfa-grass mixtures regain importance in forage production systems (Capitaine et al. 2008). The benefits ascribed to mixtures are economy of mineral nitrogen (less energy consumption, less costs, less pollution), improved distribution of crop production, high feeding value, less susceptibility to diseases, less weeds (Malézieux et al. 2009). The survival of each species results on the ability of the plants to get the resources needed for its growth among which light radiation and nitrogen are of major importance. The introduction of alfalfa into tall fescue swards has been demonstrated to provide the equivalent in dry matter production of that provided by the

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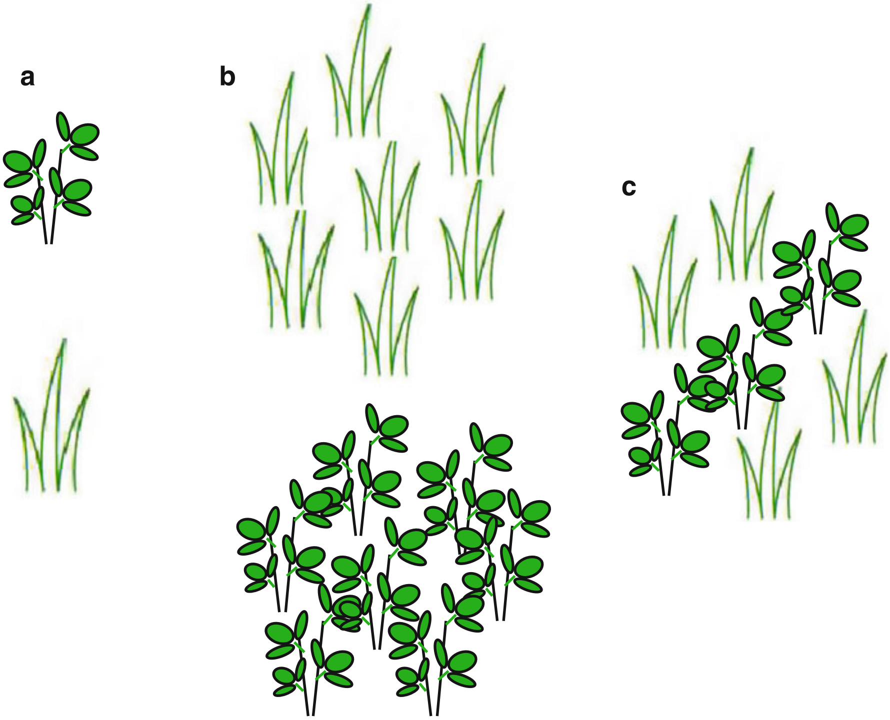

Fig. 49.1 The experimental design: (**a**) spaced plant, (**b**) dense monoculture, (**c**) mixture with fescue

application of about 200–300 kg of nitrogen to pure stands of tall fescue (Chamblee and Collins 1988). The balance between the legume and grass components should range between 30 and 70 %. At the community level, biomass production has generally been considered an ideal measure of abundance (Chiarucci et al. 1999). The aim of this study was to compare biomass production of alfalfa genotypes in several treatments: isolated plants (nursery), monocultures and mixtures with tall fescue.

Materials and Methods

Forty six contrasting genotypes from ten alfalfa varieties and two genotypes of tall fescue (a forage type and a turf type) were planted on September 2010 at INRA, Lusignan. The experimental design was a randomized completed block design with three repetitions, and four treatments: “Spaced plants” of alfalfa and fescue, “dense monocultures” (plots of seven plants, the target plant being surrounded by six plants, 7 cm apart), “mixtures with fescue forage type” and “mixtures with fescue turf type” (plots of three alfalfa and four fescue plants, 7 cm apart) (Fig. 49.1). Each genotype of alfalfa and the two genotypes of fescue were clonally propagated except for the dense monoculture in which the six surrounding alfalfa plants were

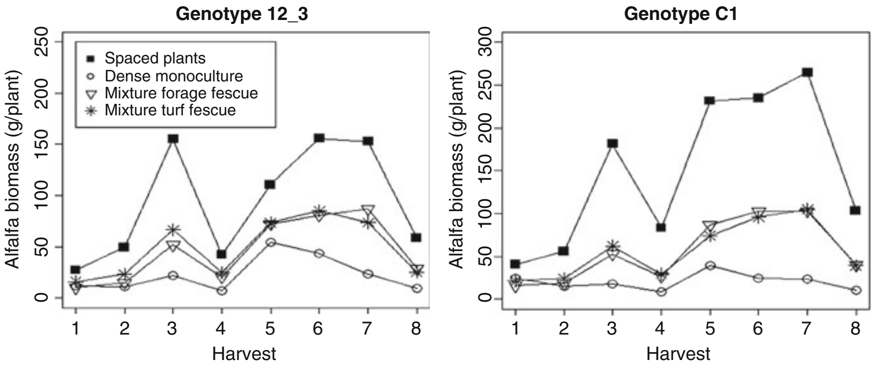

Fig. 49.2 Biomass production of alfalfa genotypes: 12_3 and C1 in different treatments during two years of harvest

seedlings from the variety “Orca”. Four harvests per year were taken in 2011 and 2012. Whole plant samples were dried at 60 °C and dry matter biomass was recorded for the target plant and the surrounding plants.

Analyses of variance were carried out to test the effect of treatment, genotype, block and treatment x genotype on the biomass of alfalfa plants. To better understand the interaction genotype x treatment, Wricke's ecovalence (1962) was calculated for each treatment *i* and genotype *j*: $W_{ij} = (Y_{ij} - Y_{i.} - Y_{.j} + Y_{..})^2$, where Y_{ij}: observed biomass (averaged across experiment replicates), $Y_{i.}$ and $Y_{.j}$: biomass means over genotypes and treatments, respectively, $Y_{..}$: grand mean.

Results and Discussion

Alfalfa biomass, expressed in g per plant was the largest for the spaced plants, the lowest for the dense monoculture and intermediate for the mixtures (Fig. 49.2). Variation among alfalfa genotypes for biomass production was large in each treatment. As expected, both alfalfa genotype and treatment had significant effects on biomass. A significant genotype x treatment interaction was also evidenced in each harvest except for the second one in 2011. We concluded that ranking of genotypes for biomass is thus influenced by treatment.

The analysis of ecovalence (Table 49.1) showed that the treatment “dense monocultures” mainly contributed to treatment x genotype interaction. Some alfalfa genotypes contributed a lot to ecovalence (ex: genotype C1) and other contributed little (ex: genotype 12_3).

Alfalfa biomass in mixture was positively correlated ($R = 0.79$, $P < 0.001$) to alfalfa biomass in dense monoculture (Fig. 49.3a). Some genotypes were highly productive in mixture and moderately productive in dense monoculture. Similarly, alfalfa biomass in mixture was correlated to biomass in spaced plants ($R = 0.88$, $P < 0.001$, Fig. 49.3b).

Table 49.1 Ecovalence (%) for dry matter biomass of alfalfa calculated for each treatment in each harvest

	Dense monoculture	Spaced plants	Mixture with forage fescue	Mixture with turf fescue
Harvest 1 (May 2011)	25.50	24.94	24.00	25.56
Harvest 3 (August 2011)	37.79	22.95	18.71	20.56
Harvest 4 (October 2011)	28.29	25.49	26.34	19.88
Harvest 5 (May 2012)	32.38	24.97	21.52	21.13
Harvest 6 (July 2012)	35.19	24.42	19.82	20.57
Harvest 7 (August 2012)	27.37	28.75	20.04	23.84
Harvest 8 (October 2012)	31.43	24.86	20.45	23.25

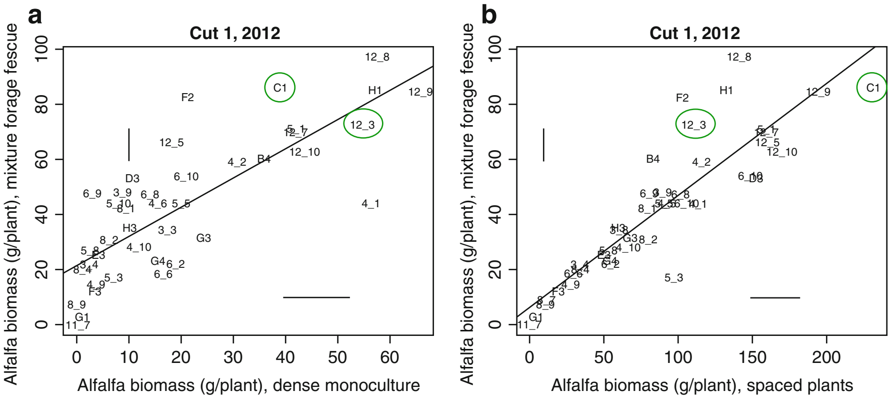

Fig. 49.3 Biomass of alfalfa genotypes in mixtures with forage fescue in comparison with dense monocultures (**a**) and spaced plants (**b**). Bars are for residual standard errors

Conclusions

Large genetic variation for alfalfa biomass production in mixture was observed. Positive correlations among treatments were noticed, but some genotypes were more productive in dense monoculture and others were more productive in alfalfa – fescue mixture. In addition to biomass production, traits related to mixture performance (alfalfa/fescue biomass proportion, nitrogen status of fescue) will be analyzed. A definition of alfalfa ideotypes for both monoculture and mixture use is expected. In addition, alfalfa plant evaluation in mixed conditions would help to identify the most promising genotypes.

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Part VII
Forage and Seed Production

Chapter 50
Drought Effect on Yield of Perennial Ryegrass (*Lolium perenne* L.)

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Abstract Drought tolerance forms a major challenge for plant breeding and gains now more interest in Europe, because drought periods are expected to increase in the twenty first century as a result of climate change. During August 2003, August 2009, spring 2010 and spring 2011, four different drought periods in Belgium caused severe reductions in dry matter production of the corresponding cuts in our fodder grass field plot trials. Dry matter production was clearly inhibited for perennial ryegrass genotypes, while tall fescue performed relatively better during the drought periods. Nevertheless, a high yield variation among perennial ryegrass families was found during and after the drought periods. In addition, the dry matter yield of different perennial ryegrass families after a drought period was positively correlated between three drought periods. Both the considerable variation and important correlations in response to drought within these genotypes show potential to improve drought tolerance in perennial ryegrass.

Keywords Drought tolerance • Perennial ryegrass • Tall fescue

Introduction

Blum (2011) stated that "Global warming is a serious engine of plant stress which requires specific expertise towards breeding solutions". Nowadays, breeders in North-western Europe face the choice between breeding for drought tolerant varieties of the current dominant fodder grass species or choosing more drought tolerant species and improving their digestibility and palatability. This study wanted to explore

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the drought periods in the past decennium in Belgium and their impact on yield. Furthermore, the drought response of tall fescue and the available genetic variation for drought tolerance in our perennial ryegrass breeding pool was evaluated.

Material and Methods

The standardized precipitation index (SPI, McKee et al. 1993) was calculated for the period 1981–2012 based on daily precipitation data measured at the meteorological station of Merelbeke (Belgium) using the program SPI_SL_6 (National Drought Mitigation Center 2013). When the SPI exceeded −1.5, the period was considered a severe drought period. To see whether these drought periods had an important influence on the yield of perennial ryegrass, we analysed yield data of 'Roy', a tetraploid perennial ryegrass (*Lolium perenne* L.) variety which was included as one of the reference varieties in 13 yield trials during the period 2002–2011. The trials were sown in spring in Merelbeke (Belgium) in plots of 8.10 m^2 (2002–2007) or 7.83 m^2 (2007–2011) in two or three replications and mown three to five times in the following 2 years. Fertilization in the first mowing year decreased from 400 (2002), 350 (2003, 2004) to 300 (2006–2011) kg N ha^{-1}. The dry matter production of the first mowing year was analysed. The data from the 'non-drought' mowing years were used to fit a regression curve using the drc package in R (Ritz and Streibig 2005).

The dry matter production of a tall fescue variety (*Festuca arundinacea* Schreb.) together with a diploid and tetraploid perennial ryegrass variety was evaluated in an experiment sown in 2009 and mown five times in 2010 and five times in 2011 (fertilization: 300 kg N ha^{-1}).

The coefficient of variation among families and varieties was calculated for six trials: a0801 – a0802 (sown in 2008), a0902 – a0903 (sown in 2009) and a1004 – a1006 (sown in 2010) consisting of 36, 24, 42, 36, 30 and 48 perennial ryegrass (half-sib) families and varieties respectively. Correlations were determined for the trials sown in 2008 and 2009 between the cuts after each identified drought period.

Results and Discussion

Based on precipitation data, four agronomic important drought periods were identified in the past 15 years using the standardized precipitation index ($SPI < -1.5$) (Fig. 50.1): two summer droughts: August-September 2003 and August-September 2009 and two spring droughts: April-May-June 2010 and March-April-May 2011.

The accumulated dry matter production of 'Roy' was plotted for the four different 'drought years' against a fitted curve based on the data of the 'non-drought years' (Fig. 50.2). The regression equation of the fitted curve (Dry matter yield = 19

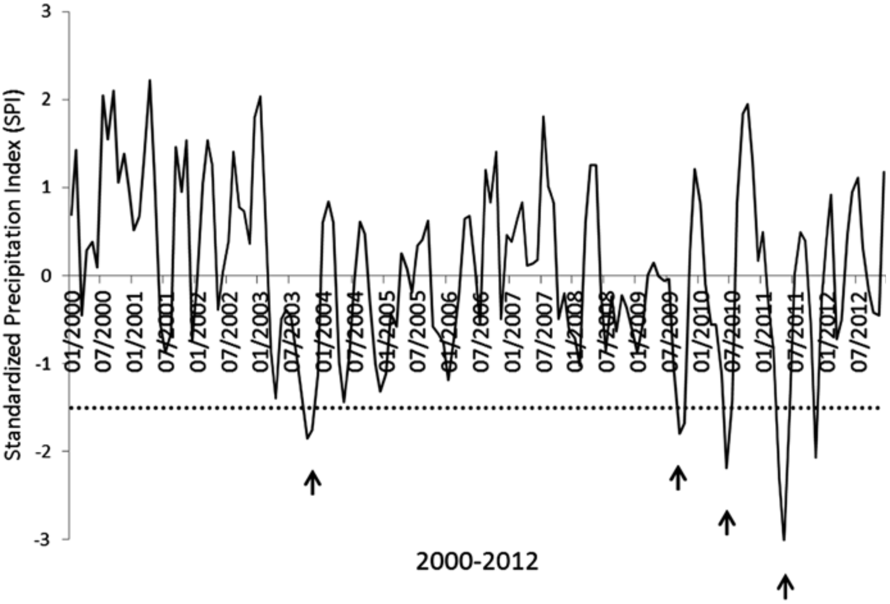

Fig. 50.1 Standardized Precipitation Index time series calculated for Merelbeke, Belgium, 1981–2012, using a time scale of 3 months. ↑ drought periods

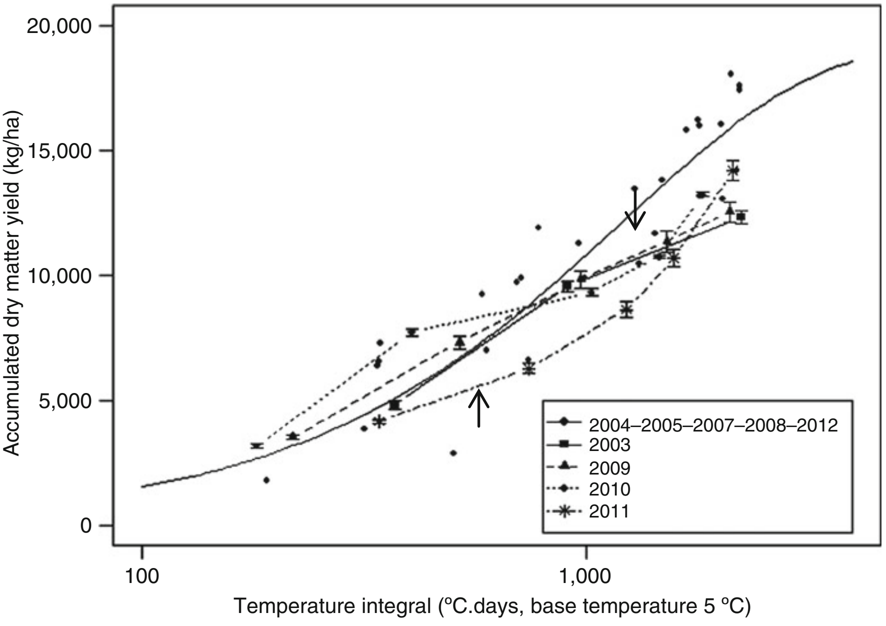

Fig. 50.2 Dry matter yield of 'Roy' in the first year after sowing for the period 2003–2012. ↑: May 2011, ↓ August 2003 and August 2009

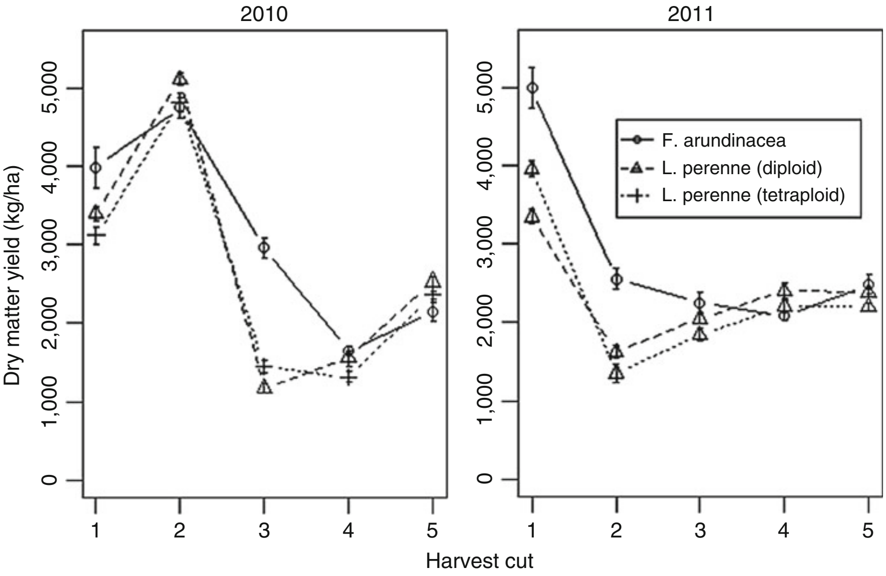

Fig. 50.3 Dry matter yield of varieties of tall fescue, diploid and tetraploid perennial ryegrass in the *first* (2010) and *second* (2011) year after sowing. ↑ cut after the drought period

,272) * (1 – $e^{-0.000824*\text{Temperature integral}}$) enabled us to compare the relative growth rate during a drought period and the relative growth rate of the fitted curve. The final dry matter production in the 'drought years' was clearly lower compared to the fitted curve of the non-drought years. Moreover, the average relative growth rate of 'Roy' was in May 2011 only 66 % of the average relative growth rate of the fitted curve and it even decreased to 54 and 56 % in August 2003 and August 2009 respectively.

Yield trials in 2010 and 2011 clearly demonstrated an inhibition of dry matter production of the diploid and tetraploid perennial ryegrass varieties, while tall fescue performed better (approximately 1,000 kg/ha extra) during the cuts after the drought periods (Fig. 50.3: third cut in 2010 and second cut in 2011).

The coefficient of variation among families (averaged for different trials in 1 year) showed, next to the peaks associated with the first cut, three peaks corresponding with the drought periods of 2009, 2010 and 2011 (Fig. 50.4). Significant positive correlations were found between dry matter production after two drought periods of perennial ryegrass families in one trial (Table 50.1). The considerable variation in response to drought among perennial ryegrass families and the good correlation in dry matter production of families after drought periods show potential to improve drought tolerance in perennial ryegrass.

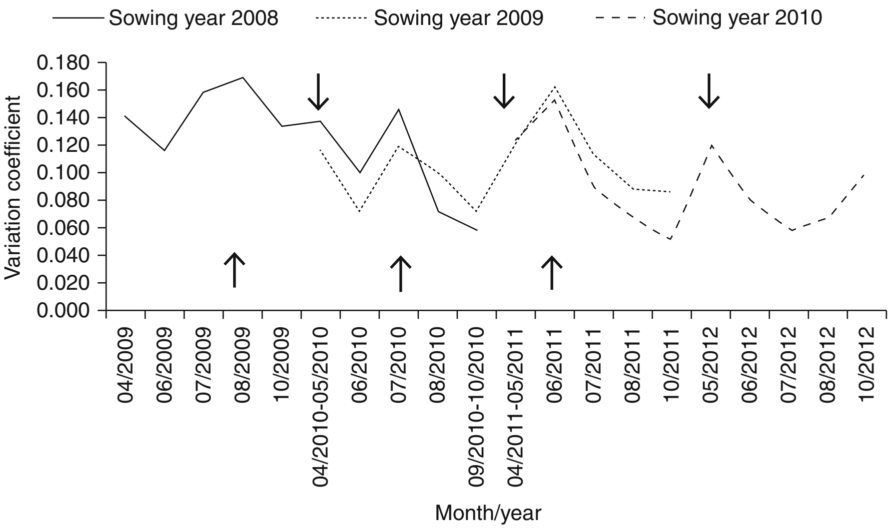

Fig. 50.4 Coefficient of variation of dry matter production per cut from six yield trials with diploid and tetraploid perennial ryegrass families sown in 2008, 2009 and 2010. ↓ variation peaks associated with the first cut, ↑ variation peaks associated with a drought period

Table 50.1 Correlation coefficients[a] between dry matter production after a drought period in the first and second mowing year from four yield trials with perennial ryegrass families

Drought periods	Trial a0801	Trial a0802	Trial a0902	Trial a0903
08/2009–07/2010	0.70***	0.59***	–	–
07/2010–06/2011	–	–	0.67***	0.46*

[a] * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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Chapter 51
Optimising Stand Density and Nitrogen Fertiliser Rates for Seed Production in the Tetraploid Italian Ryegrass Cultivar K29T

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Snežana Babić, and Dušica Delić

Abstract Tetraploid cultivars of Italian ryegrass have been developed and used as an alternative to diploid cultivars in an attempt to improve forage yield and quality. But, very often, production of tetraploid cultivars is reduced by unfavourable weather conditions. The objective of this research was to determine seed production stability of the tetraploid Italian ryegrass cultivar K29T in variable and contrasting environmental and meteorological conditions in Serbia. The study was conducted in four consecutive years and generations of seed multiplication. Every year, seed was harvested from the primary growth in the first production year after the year of establishment. Data of investigated traits (heading and harvest dates, tiller length, number of spikelets per spike, seed yield, dry herbage yield and 1,000 seed weight) were recorded for different management practices (stand densities and nitrogen applications). It was determined that seed yield, dry matter production and tiller length were presumably most affected by different environmental factors in different production years, and different management practice, especially different sowing densities. In contrast, number of spikelets per spike and 1,000 seed weight were less affected by management practice, except in the extremely arid 2003 year. Variation in seed yield was more related to variation in seed numbers than to variation in seed weight. In the first seed production year, the best density in this experiment was 15–20 kg ha^{-1} of seed sown at an interrow spacing of 60 cm without fertilizing or with only 50 kg ha^{-1} of N. Therefore, seed of tetraploid Italian ryegrass cv. K29T can be produced in diverse environmental conditions in Serbia, but lower stand densities allow for better plant tillering and the production of a greater volume of seed and, at the same time, good dry matter yield of the residual biomass.

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Keywords Italian ryegrass • Seed production • Stand density • Fertilizing • Environmental conditions • Stability

Introduction

With one exception, perennial forage grasses are not domesticated; 'wild' collections are generally phenotypically indistinct from cultivated forms (Casler and Duncan 2003). The single exception is Italian ryegrass (*Lolium multiflorum* Lam.), which was developed by unconscious selection prior to the twelfth century in the Lombardy and Piedmont plains of Italy (Beddows 1953). Italian ryegrass is valued for its high-yield potential, fast establishment, potential applications in reduced tillage systems, and high degree of tolerance to climatic variations in temperate climates (Moore 2003). Also, it is well adapted to high rainfall, but can be grown where a minimum of about 500 mm of rainfall occurs during the growing season (Evers et al. 1997).

The production of grass seed has often been organized in areas characterized by seasonally variable climates, such as Western Serbia (Simić et al. 2012a). Seed yield is significantly reduced in the temperate continental climate of Serbia compared with those observed in Western Europe, mostly due to the summer drought period and uneven rainfall distribution. Chastain (2000) suggested that rainfall events and short-term rainfall patterns have a much greater influence on seed yield than do temperature events or patterns.

Optimum stand density for maximum seed yield in Italian ryegrass has not yet been determined (Young et al. 1996). A very dense crop stand, inadequate nitrogen nutrition and/or variable environmental conditions can play a negative role in seed productivity. The spatial arrangement of plants at seeding was reported to have a strong influence on the yield and longevity of seed stands of Italian ryegrass (Young et al. 1996; Kunelius et al. 2004; Venuto et al. 2004), but such differences tend to disappear during the later stages of development. Maximising first-year seed yield is much more important than increasing cumulative productivity over several consecutive years, due to disorganization of established spatial conditions (tillering, seed shattering) and an inability to predict the next stand density. As the amount of seed shattered can easily be 10 % of the total harvested seed, it can develop into a very dense stand in subsequent seasons, thus increasing the seeding rate more than ten-fold. As a consequence, the effect of dense stands is a reduction in seed yield (Young et al. 1996). Therefore, Italian ryegrass is usually treated as an annual seed crop.

Excess nitrogen can increase vegetative growth, leading to swathing difficulties (Griffith 2000). On the other hand, increasing N fertilizer levels applied (100 kg ha^{-1} N), may improve competition of ryegrass with weeds and increase overall seed yield (Simić et al. 2012b).

Watson et al. (1983) observed differences among cultivars of Italian ryegrass in yield stability, although diploid cultivars showed a much wider range than tetraploids.

Genetic correlations of individual seed yield components with overall seed yield in Italian ryegrass are generally low (Elgersma et al. 1989) but some components may vary between production years depending on the prevailing growing conditions, and thus affect seed yield in certain production years (Simić et al. 2010).

The objective of this study was to improve the stability of Italian ryegrass tetraploid cultivar seed production traits in the first production year, by determining optimum stand density and N fertilizer application and to identify genetic correlations among key seed production traits in Italian ryegrass.

Material and Methods

The field plot experiment was conducted for four consecutive years, between 2002 and 2006, on a field located in a semi-humid region (with very variable years) (44°47'N, 19°35'E, 80 m a.s.l.) in western Serbia. The main characteristics of the soil (depth: 0–30 cm) were as follows: soil texture: loam; pH_{KCl}: 5.25; K: 12.5 mg kg^{-1} and P: 1.31 mg kg^{-1}.

Seeds from the primary growth of tetraploid Italian ryegrass cv. K29T were harvested in the first production year after the year of establishment. Italian ryegrass was sown each autumn with seeding rates equivalent to 5, 10, 15 and 20 kg ha^{-1} providing 12 plant spatial treatments in combination with interrow spacings of 20, 40 and 60 cm. The fertilizer treatments (lime ammonium-nitrate, 27 % N) were applied to the plots in the spring of each year (N_1 – 0 kg ha^{-1}; N_2 – 50 kg ha^{-1}; N_3 – 100 kg ha^{-1} and N_4 – 150 kg ha^{-1}). The plot size was 10 m^2 (2.5 × 4 m) in four replications in a complete randomized block design. Prior to the seed harvest, measurements of generative tiller length and the number of spikelets per spike were made based on ten randomly sampled tillers from each plot.

The harvest was started at the stage of full seed ripening when a gentle hand rubbing of spikes would result in evident seed shattering. After air-drying, the seed was threshed, cleaned, dried to a seed moisture content of 150 g kg^{-1} and weighed (ISTA, 2004). After seed harvest, the remaining biomass was cut, collected together with threshed straw and presented as dry herbage yield. To evaluate seed quality, 1,000-seed weight (TSW) was measured. TSW was determined from samples dried at room temperature for 2 months.

Data were analyzed by ANOVA and significant differences determined by LSD test, both with Statistica 8.0 software package.

Meteorological data were collected at the Mitrovica weather station near the experimental site. Accumulated growing degree days (GDD) was calculated by the formula $\{[(T_{max} - T_{min})/2] - T_{base}\}$, where T represents daily maximum and minimum temperatures. The T_{base} for Italian ryegrass was 0 °C, and values were summed from the first of January (Griffith et al. 1997) (Fig. 51.1). Accumulated precipitation during the same period was also calculated and is presented in Fig. 51.2.

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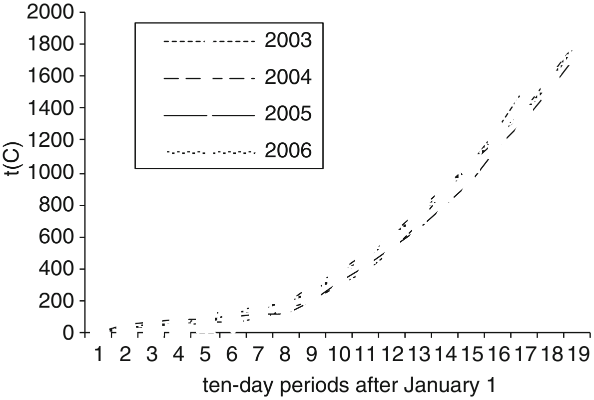

Fig. 51.1 Growing degree days during 4 consecutive years in Italian ryegrass grown for seed

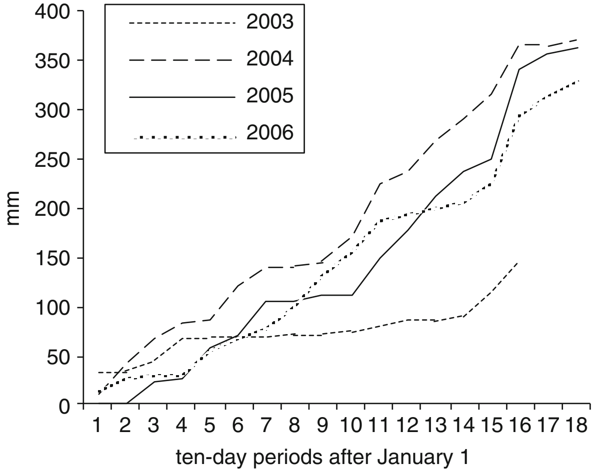

Fig. 51.2 Accumulated precipitation during 4 consecutive years in Italian ryegrass grown for seed

Results and Discussion

The main seed yield components and seed yield *per se* varied significantly among investigated years, but also among different treatments used in the experiment. Average tiller length was very variable (CV from 7–17 %), and it was almost doubled in the humid conditions of 2004 and 2005 compared to the extremely arid year, 2003 (Table 51.1). Higher precipitation during the consecutive years (2004 and 2005) resulted in great biomass accumulation. As a result, lodging and photomorphogenic plant responses were observed. Thus, tillers in a dense sward (treatment A_1 – 20 cm interrow spacing) with low red/far-red ratios developed longer leaves and longer shoots, as was also reported by Casal et al. (1985), achieving the greatest tiller length in 2004 and 2005 and in total (105.5 cm). Excessively tall plants and long tillers, resulting from high rainfall and N fertilization, may result in lodging, which is an ubiquitous problem in Italian ryegrass grown for seed (Griffith 2000;

Table 51.1 Effect of density and nitrogen rate on seed yield components and seed yield (average values for period 2003–2006 and coefficients of variation, CV %)

Traits	Tiller length (cm)	Spikelets per spike	Seed yield (kg ha^{-1})	Dry herbage yield (t ha^{-1})	1,000 seed weight (g)
Densities					
A_1B_1	101ab[a]	24.9ab	904bc	3.73de	4.26ab
A_1B_2	108a	26.4a	1,035abc	3.92bcde	4.50a
A_1B_3	105ab	25.9a	1,116ab	4.83ab	4.29ab
A_1B_4	108a	25.2ab	1,151ab	5.22a	4.29ab
A_2B_1	94c	23.8b	795c	3.15e	4.32ab
A_2B_2	106a	24.7b	1,021abc	3.80cde	4.36ab
A_2B_3	103ab	24.9ab	1,189ab	4.70abcd	4.23b
A_2B_4	102ab	24.8b	1,146ab	4.48abcd	4.24ab
A_3B_1	98bc	24.0b	978abc	3.26e	4.27ab
A_3B_2	104ab	25.4a	1,094ab	3.84cde	4.41ab
A_3B_3	106a	25.0ab	1,284a	4.60abcd	4.31ab
A_3B_4	107a	25.6a	1,228ab	4.78abc	4.39ab
A1 average	105.5a	25.6a	1,051.5a	4.42a	4.34a
A2 average	101.3a	24.6a	1,037.8a	4.03a	4.29a
A3 average	103.8a	25.0a	1,146a	4.12a	4.35a
B1 average	97.7b	24.2a	892b	3.38b	4.28a
B2 average	106.0a	25,5a	1,050ab	3.85b	4.42a
B3 average	104.7a	25.3a	1,196a	4.71a	4.28a
B4 average	105.7a	25.2a	1,175ab	4.82a	4.31a
Nitrogen rate					
N_1	103.6a	25.1a	1,086a	4.21b	4.32a
N_2	104.2a	24.5b	1,040b	4.61a	4.33a
N_3	103.7a	24.8ab	1,007b	4.60a	4.34a
N_4	104.6a	24.7ab	1,005b	4.59a	4.36a
Average 2003	72	24.7	785	1.81	3.15
CV 2003	16.9	10.5	54.7	50.4	7.54
Average 2004	131	27.2	955	6.75	4.77
CV 2004	8.5	7.5	22.7	18.0	4.90
Average 2005	115	21.6	1,559	5.94	5.12
CV 2005	7.3	7.6	22.5	23.3	3.74
Average 2006	98	25.6	839	3.51	4.31
CV 2006	13.9	6.9	20.8	40.5	6.80

A_1B_1 20 cm interrow spacing, 5 kg ha^{-1} sowing rate, A_1B_2 20 cm, 10 kg ha^{-1}, A_1B_3 20 cm, 15 kg ha^{-1}, A_1B_4 20 cm, 20 kg ha^{-1}, A_2B_1 40 cm, 5 kg ha^{-1}, A_2B_2 40 cm, 10 kg ha^{-1}, A_2B_3 40 cm, 15 kgha^{-1}, A_2B_4 40 cm, 20 kg ha^{-1}, A_3B_1 60 cm, 5 kg ha^{-1}, A_3B_2 60 cm, 10 kg ha^{-1}, A_3B_3 60 cm, 15 kg ha^{-1}, A_3B_4 60 cm, 20 kg ha^{-1}, N_1 0 kg ha^{-1}, N_2 50 kg ha^{-1}, N_3 100 kg ha^{-1}, N_4 150 kg ha^{-1}

[a] Average values with the same letters are not significantly different at $P<005$

Simić et al. 2012a). Tiller lengths in treatments with sowing rates of 10, 15 and 20 kg ha^{-1} did not differ significantly, while those in treatments with 5 kg ha^{-1} were statistically significantly shorter (97.7 cm). Differences of this trait among densities were statistically significant, with particularly shorter tillers in densities A_2B_1 and A_3B_1. Different rates of N application had no influence on this trait (Table 51.1).

CV of number of spikelets per spike varied from 7 to 10 % in different years, ie. the average number was from 21.6 to 27.2. The impact of different interrow spacing and sowing rate on this trait was not significant, but there were some stand densities which showed significantly higher numbers of spikelets per spike. The value of this trait decreased with an increase in the rate of N fertilizer applied (Table 51.1).

Seed and dry herbage yield, commercially the most important traits for Italian ryegrass seed production, showed the greatest differences among treatments. Generally, the highest seed and dry herbage yield were determined for the highest sowing rates (B_3 and B_4). With regards to interrow spacing, the best seed yield resulted from plots with 60 cm between rows, while the best dry herbage yield was achieved in plots with 20 cm between rows. The highest seed yield was determined in lowest densities (combinations A_3B_3 and A_3B_4) where vegetation space per plant was greatest. On the contrary, the best dry herbage yield was established in the combinations of interrow spacing 20 cm and sowing rates 15 and 20 kg ha^{-1}. Statistically, the highest seed yield was determined in treatments without added N, while all treatments with added N showed good dry herbage yield of about 4.6 t ha^{-1} (Table 51.1).

No statistically significant effect of any factor was detected on 1,000 seed weight.

According to Mueller-Warrant and Caprice Rosato (2002), drought damage had more effect on ryegrass vegetative growth than on reproductive allocation.

The one goal in this trial was to determine ways of controlling stand density in the first production year because all of the shattered seed from the previous crop could be mixed into the soil during seed bed preparation; thus, many Italian ryegrass stands are too dense to achieve maximum seed yield in the second production year. Seed and dry herbage yield varied in the arid conditions of 2003 (CVs more than 50 %), while in favourable years CVs were around 20 %. Ryegrass may achieve a good seed yield on heavy, temporarily waterlogged, soils if well established (Valenzuela and Smith 2002), but heavy rains and flooded conditions during spring 2006 caused a high level of seedling mortality in all sown stands. However, the stand re-grew but with a reduced plant population, and dry herbage yield had a high coefficient of variation of 40.5 %. In contrast with the previous year where higher tiller density had a deleterious effect on seed yield, environmental conditions (flooding) forced stands with greatly varied tiller populations to reach a similar stand density and productivity.

Average seed yield in favourable years 2004–2006 was twice that of the dry year 2003, while dry herbage yield was four-fold larger. Thousand seed weight showed a CV of only 8 % in all experimental years. For optimum seed yields, Italian ryegrass has to be harvested as soon as the majority of the heads have begun to senesce and the seeds have reached approximately 40–45 % moisture content (Simić et al. 2010).

Correlation coefficients developed during this study show a low correlation between tiller characteristics and seed yield (Table 51.2), but with a significant correlation in unfavourable years 2003 and 2006. According to Nelson et al. (1997), genetic correlations of seed yield components with seed yield in Italian ryegrass are

Table 51.2 Correlation coefficients between harvest characteristics and morphological components

	Seed yield		Dry herbage yield			1,000 seed weight	
Year	r TL	r SPS	r TL	r SPS	r SY	r SY	r DHY
2003	0.67[*]	0.36[*]	0.66[*]	0.34[*]	0.96[**]	0.24[*]	0.23[*]
2004	–0.28[*]	–0.13	–0.09	–0.13	0.11	0.01	–0.09
2005	–0.13	0.06	0.11	0.14	0.37[*]	–0.11	–0.21[*]
2006	0.48[*]	0.31[*]	0.55[*]	0.18[*]	0.65[*]	0.24[*]	0.27[*]

TL tiller length, *SPS* spikelets per spike, *SY* seed yield, *DHY* dry herbage yield

[*] P value <0.05

[**] P value <0.01

generally low. But, in all years, an increase in dry herbage yield resulted in a statistically significant seed yield improvement. Similar correlations were noticed between 1,000 seed weight and harvest parameters, with most significant correlations in unfavourable years 2003 and 2006 (Table 51.2). It is noticeable that, almost generally, a higher seed yield was positively interrelated with good seed quality.

Conclusions

Italian ryegrass seed production represents a viable alternative to traditional crops in Serbia. As seed is economically the most important output of this kind of Italian ryegrass production, it can be concluded that for the first seed production year, the best density in this experiment was 15–20 kg ha^{-1} of seed sown at interrow spacings of 60 cm without fertilizing or with only 50 kg ha^{-1} of N. Variation in seed yield was more related to variation in seed number than to variation in seed weight.

It was determined that levels of seed yield, dry matter production and tiller length were most affected by environmental factors in different production years, but also in different management practice, especially by different sowing methods. In contrast, the number of spikelets per spike and 1,000 seed weight were less affected by different management, except in extremely unfavourable years.

Considering the factors of stand establishment and nitrogen application, it can be concluded that the former significantly affected all the observed parameters during the experiment. Nitrogen application had a significant effect on almost all parameters excluding tiller length and 1,000 seed weight.

Based on the results obtained, it can be concluded that seed of tetraploid Italian ryegrass can be produced in diverse environmental conditions in Serbia. Lower stand densities allow for better tillering of the plants and a higher seed yield, together with a good dry matter yield of the remaining biomass. This makes Italian ryegrass seed production sustainable and profitable in the context of changeable weather conditions.

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Chapter 52
The Influence of Climatic Conditions on Forage Yield and Quality of Certain Types of Grass

Tihomir Čupić, I. Varnica, G. Jukić, S. Popović, and M. Tucak

Abstract The aim of this study was to examine the potential cultivation and yields of perennial grasses of foreign origin in western and eastern Croatia. Accessions within a grass species were minimally distinguishable by yields of green mass and hay. In contrast, the differences in crude protein yield were significant. The characteristic with the highest variation was yield of protein per unit area (coefficient variability CV 8.16 %). The influence of location was significant only for yields of hay and crude protein, while the impact of the year by location was not significant for all characteristics. Impact of year as a separate variable had a significant influence on all the yield properties. Italian ryegrass (TLJ2) produced the highest yields of green mass and hay, while cocksfoot (KO2) achieved the highest protein yield.

Keywords Grass • Forage yield • Quality • Environment

Introduction

Milk producers in southeastern Europe are becoming more dependent on hay, which is necessary for feed during the winter period. Hay from perennial grasses and legumes is the primary source of valuable forage for the dairy cows. Unlike legumes, perennial grasses are less sensitive to soil acidity and more tolerant to diseases and insects. Natural grasses in the Republic of Croatia account for approximately 49 % of total agricultural land (Knežević et al. 2004). Most new grasslands are from foreign cultivars because very little has been done to develop new grass cultivars since the 1990s in Croatia. A systematic gathering of information about adaptability to climatic zones within a region is therefore needed (Leto et al. 2006). Current hay production in the dry conditions of southeastern Europe does not meet the needs of

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Table 52.1 Information about the origin of the investigated species of grasses and number of cuts

		No. cuts/site			
Species	Country of origin	OS2007	OS2008	ZG2007	ZG2008
L. multiflorum/TLJ1	Germany	3	3	4	3
L. multiflorum/TLJ2	Denmark	3	3	4	3
L. perenne/ELJ1	Germany	3	3	4	3
L. perenne/ELJ2	Denmark	3	3	4	3
L. perenne/ELJ3	Denmark	3	3	4	3
L. boucheanu/OLJ1	Germany	3	3	4	3
L. boucheanu/OLJ2	Denmark	3	3	4	3
L. boucheanu/OLJ3	Denmark	3	3	4	3
D. glomerata/MR1	Denmark	3	3	4	3
D. glomerata/MR2	Denmark	3	3	4	3
P. pratense/KO1	Denmark	3	4	4	3
P. pratense/KO2	Denmark	3	4	4	3

milk producers in terms of quality and quantity (Arcioni et al. 1980, 1985). Therefore, it is very important to know how different grasses react to agroecological conditions and to know nutritive hay values when choosing species and planning production. The dry matter yield being achieved was very low and quality was very poor.

Climatic conditions such as drought and high summer temperatures significantly increase risks to hay production by reducing the number of grass cuts (Leto et al. 2006). The optimal cutting stage of grass is when dry matter content is 15–20 % (Vranić et al. 2010). The dry matter content in plant mass increases as the plant grows. At the earing up stage, grasses comprise 20–30 % DM and at the flowering stage 30–50 % DM, while during the wax stage of seeds DM content can be more than 50 % (Chamberlain and Wilkinson 2002). The main prerequisite for growing quality forage is timely cutting during the earing up stage. The best leaf/stem ratio is achieved during this period.

The aim of this study is to compare yield and quality of hay grown from different introduced perennial grass species and examine their productivity potential in the agro-ecological conditions of Croatia.

Material and Methods

Data Source

The yield and nutritive values of the hay of several perennial grass species were compared in this study. Several varieties of the most important grass species, *Lolium multiflorum* Lem., *Lolium perenne* L., *Lolium boucheanum* Kvnth., *Dactylis glomerata* L. *Phleum pratense* L., which are of foreign origin, were analyzed (Table 52.1).

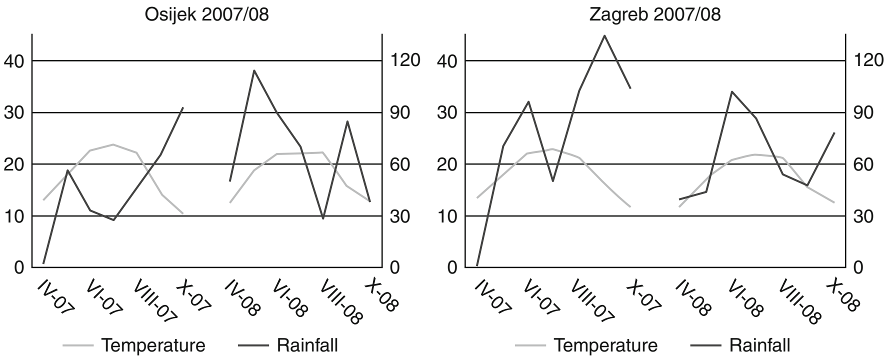

Fig. 52.1 The Walter's climate diagram in a ratio 1:3 for Osijek and Zagreb in 2007 and 2008

The analyzed grass accessions at the Osijek and Zagreb sites were sown on 08. 09. 2006, and were cut during 2007 and 2008. Experimental fields were located in the northeast (Osijek) (45° 52 'N, 18° 68' E), and the northwest (Zagreb) of Croatia (45° 82 'N, 16° 03' E). Experimental fields at both locations were fertilized with 500 kg ha^{-1} of NPK 7:20:30 and 136 kg ha^{-1} of urea (46 % N) and again every year in spring with 150 kg ha^{-1} CAN (27 % N). Italian ryegrass, perennial ryegrass, hybrid ryegrass and cocksfoot were sown in quantities of 25 kg ha^{-1}, while timothy was sown at 11 kg ha^{-1}. The experiment was a complete randomized block design with four replications and the basic plot size was 8×1.25 m, while the plot size for calculation was 5×1 m. Cutting was done at the stem elongation/heading stage. If that stage had not been reached plants were still cut, but at 7 weeks after the preceding harvest. Green mass yield (GMY) and hay yield (HY) are expressed in t ha^{-1}, while crude protein yield (CPY) is expressed in kg ha^{-1}. The chemical analysis was performed on the middle row of each plot following the AOAC (1990) procedure.

Statistical Analyses and Weather Conditions

The data was analyzed by CROPSTAT 7.2 (2007) (International Rice Research Institute Inc., Cary, NC, USA, 2002–2003.) statistical program, by GLM procedure, and differences in the mean values were tested by Duncan test at the level of $p < 0.01$. Climate diagrams by Walter show significantly less rainfall during the vegetation period in 2007, especially at the Osijek site (Fig. 52.1).

Results and Discussion

Analysis of variance (Table 52.2) showed that the varieties of grasses and interactions with year and site locations have a highly significant effect on the expression

Table 52.2 Combined analysis of variance for investigated traits

Source	DF	GMY	HY	CPY
Loc	1	21.4ns	7.87**	63.7**
R (Loc)	6	31.5n.s	2.1n.s	8.6n.s
Year	1	19,258**	763**	3,416**
Locx Year	1	910.4**	193**	1,139**
Rx Year (Loc)	6	6.6n.s	0.45n.s	1.9n.s
Spec	11	835.2**	55.6**	330**
Spec x Year	11	797.7**	37.8**	3,307**
Spec x Loc	11	84.6**	4.3**	150.9
Year x Loc x Spec	11	304.4**	16.1**	31.2**
Error	132	12.24	0.81	4.7
C.V.%		7.74	7.73	8.16

n.s. not significant, * (P=0.05), ** (P=0.001)

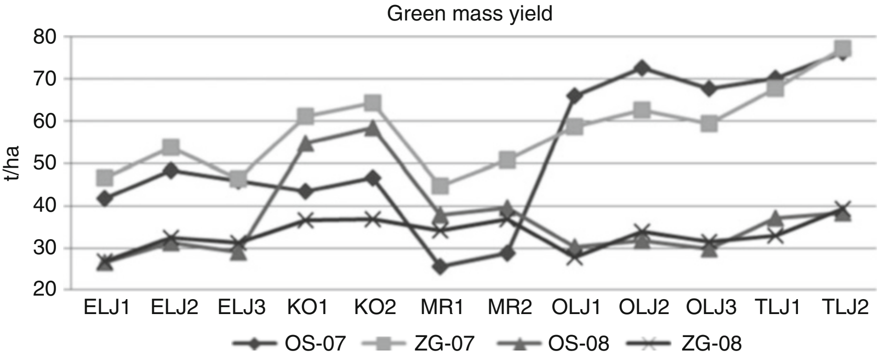

Fig. 52.2 The green mass yield per accession and site location

of all traits. The study determined the significant influence of year on all yield properties. The site location variable had a significant effect on grain yield and protein content of hay. The impact of the year variable per location had no significant influence on the studied properties.

Green mass yields varied the most between years per grass species. Italian ryegrass varieties achieved the highest yields of green mass at both locations in 2007, while the cocksfoot variety KO2 achieved the highest yield in 2008. Cocksfoot varieties had the highest level of variance in both years and locations, and achieved the highest yields at Zagreb in 2007 and Osijek in 2008. Timothy grass varieties had the lowest yield of green mass at all locations in both years (Fig. 52.2). The TLJ2 variety, originating from Denmark, achieved the highest significant yield in a 2 year average when compared with other varieties. This result is probably due to the faster growth and development of plants in the first cut.

The yield of hay followed a similar trend to green mass yield by year and location with the exception of the hybrid ryegrass which achieved significantly lower

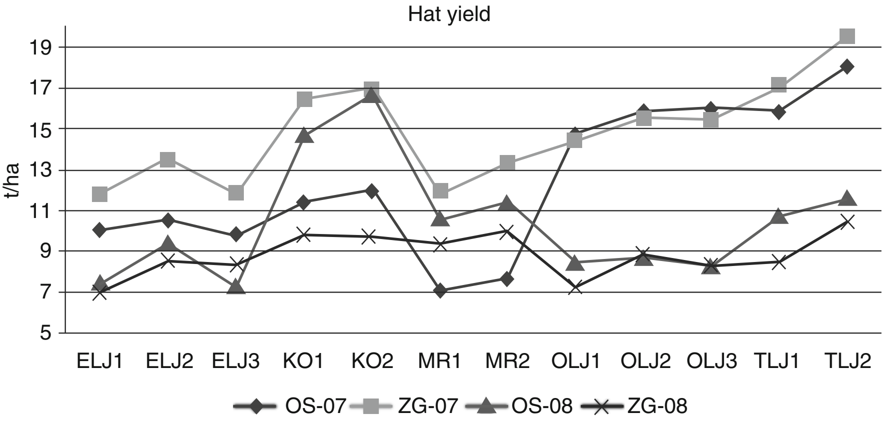

Fig. 52.3 Hay yield per accession and site location

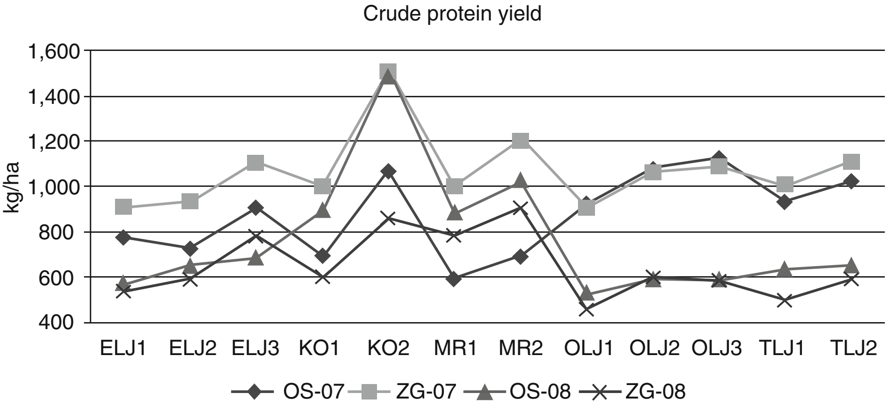

Fig. 52.4 Crude protein yield per accession and site location

yields of hay than green mass. This reduction in hay yield compared with green mass resulted from a lower dry matter content at the time of harvest due to a slower rate of development compared with other species. It is important to point out that varieties KO1 and KO2 achieved high yields of hay, although statistically significantly less than the TLJ2 variety (Fig. 52.3). Similar results were found by Kohoutek et al. (2010) among other species but with much higher yields. The differences between results were caused by lower rainfall at the test site locations.

The yield of crude protein per unit area is the trait most commonly used to indicate the quality of a fodder crop. This trait had the highest coefficient of variation at 8.16 %. Crude protein yield displayed smaller differences between years than green mass and hay yields. The cocksfoot variety KO2 achieved the highest protein yield. It had the highest yield in all the site locations except in Zagreb 2008, where the variety MR2 achieved the highest yield (Fig. 52.4).

The poor leaf/stem ratio influenced the low protein yield in the KO1 variety. The lowest variability was determined among the species of perennial ryegrass, whereas varieties of Italian ryegrass and hybrid ryegrasses varied the most between sites. The achieved results of protein yield of Italian ryegrass and hybrid ryegrass do not agree with those of Wilman et al. (1996) who pointed to the superior quality of perennial and Italian ryegrass. The poor quality of the Italian ryegrass was mostly caused by the rapid rate of development. At the time of the heading stage cut, the Italian ryegrass was overdeveloped compared with other grass species which were cut at the stem elongation stage. Similar results were obtained by Bruno-Soares et al. (1998), Leto et al. (2006) and Sokolović et al. (2007) in their ryegrass trials in similar climatic and soil conditions. Their results of achieved yields of protein from 619 to 815 kg ha^{-1} were in accordance with our results.

Based on the results of green mass, hay and crude protein yield we can conclude that the varieties tested could be successfully used in the agroecological conditions of Croatia. In particular, the very favorable balance of yield and quality of cocksfoot, a species that is well adapted to high temperatures and drought, should certainly not be ignored when developing grass mixtures.

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Chapter 53
Indicating Soil Quality Using Urease and Saccharase Activity in Abandoned Grassland and Differently Managed Crop Fields

Ligita Baležentienė

Abstract In order to ascertain and make a comparison of bioactivity variation during vegetation period, soil samples were collected in fields of different fertilizing and farming systems: extensive (ExF), conventional (CF) and organic (OF) in order to determine the saccharase and urease activity responses to different land management systems and main soil quality indices: soil organic carbon (SOC), total nitrogen (N), and C/N ratio. Assessed hydrolases were observed as sensitivity indicators suitable to evaluate parameters of soil quality. The highest decrease and the lowest rates of urease (1.13 mg NH_4^+–N g^{-1}) and saccharase (8.40 mg CG g^{-1} 24 h^{-1}) activities were observed in abandoned grassland soil where mineral fertilizers were not applied. Enzyme activity correlated stronger with SOC ($r=0.7$) or C/N ratio ($r=0.6$) than that with total nitrogen content ($r=0.5-0.6$). A significantly higher 3 year mean value of saccharase (27.00 and 12.6 mg CG g^{-1}) and urease (5.78 and 4.16 mg NH_4^+–Ng^{-1} 24 h^{-1}) activities were observed in soil under conventional and organic management compared with abandoned grassland. Thereafter enzyme activities might be related to the different levels of soil fertility.

Keywords Agroecology • Indicators • Enzyme activity

Introduction

Hydrolytic enzymes make nutrients available for plants and soil microorganisms from a wide range of complex substrates and are influenced by a wide range of soil conditions such as pH, organic matter and texture, and also by farming management and anthropogenic impacts (Li et al. 2008). Hydrolytic enzymes, namely saccharase and urease are related to the C and N cycles, which are the fundamental factors in forming soil fertility (Dilly et al. 2007). Among other indices,

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enzyme activity is proposed as an index of soil fertility or contamination. These enzymes are non-cellular and persistent in the soil matrix though they are sensitive to abiotic factors, especially to inorganic fertilizers. Some literature has pointed out an increase in the abundance of microorganisms as well as some enzyme activity when organic and mineral fertilizers have been applied (Sang et al. 2009). Past studies have shown that high doses (>120 kg ha^{-1}) of mineral fertilizers as well as the species of cultivated crop will change microorganism and ferment composition and abundance (Lagomarsino et al. 2009). Therefore evaluation of enzyme activity is bio-indicator specific to biochemical alteration of soil organic matter.

The aim of this study was to evaluate the impact of farming management systems (conventional, organic, and extensive) and different crops (legumes and grasses) on the activity of urease and saccharase related to C and N cycles in 15 years period on different soils. In addition, this study addressed the question, which of the main biochemical indicators was most suitable for soil fertility evaluation and rating?

Materials and Methods

Soil Sampling

Soil samples were collected in accordance with ISO 10381-2:2002 from different farming fields (Aleksandras Stulginskis University): (i) extensive -abandoned barley field (ExF b-cg), grassland (ExF g) and fodder galega (*Galega orientalis* Lam.) stand (ExFl ga); (ii) conventional – winter wheat for grain, legumes mixtures, and (iii) organic – winter wheat w for grain and legumes mixtures for fodder (Table 53.1).

This study relies on chemical analysis data, obtained in a previous project (Sabiene et al. 2010). SOC was determined through a dry combustion method (ISO 10694:1995) and N was measured as Kjeldahl nitrogen (ISO 10694).

Enzyme Bioassay

Saccharase (EC 3.2.1.26) activity was measured according to the modified Hofmann and Seegerer method and expressed in conventional glucose (CG, mg g^{-1} of air dried soil) released. Urease (EC 3.5.1.5) activity was assayed by employing the modified Hofmann and Schmidt spectrometric method. Urease activity was expressed in mg NH_4^+–N g^{-1} 24 h^{-1} and saccharase activity – in mg conventional glucose (CG) g^{-1} of air dried soil.

Variation of annual temperatures and precipitations were observed during the study period of 2007–2009. Average temperatures ranged between −6.2 °C (2007)

Table 53.1 Trial design during 2007–2009

Management type	Crop rotation	Treatment acronym	Fertilizing	Year	Soil classification	Location
Conventional farming	Barley;	CF b	$N_{120}P_{50}K_{60}$	2007	*Hapli–EpihypogleyicLuvisol (LVg-p-w-ha)*	54°52′40″N 23°50′99″E
Extensive farming (abandoned crop)	Couch-grass Couch-grass	ExF b-cg	0 0	2008 2009		
Extensive farming (abandoned grassland)	Grassland	ExF g	0 0 0	2007 2008 2009	*Hapli-AlbicLuvisol (LVe-ha)*	54°53′83″N 23°51′61″E
Extensive farming (abandoned crop)	Legumes (galega)	ExFlga	0 0 0	2007 2008 2009	*Hapli-AlbicLuvisol (LVe-ha)*	54°53′32″N 23°40′85″E
Conventional farming (synthetic fertilizers; weed controlled by tillage and herbicide)	Oat-vetch; Winter wheat; Barley-clover	CF ov-w-bcl	$N_{60}P_{50}K_{60}$ $N_{120}P_{50}K_{60}$ $N_{60}P_{50}K_{60}$	2007 2008 2009	*Hapli-EpihypogleyicLuvisol (LVg-p-w-ha)*	54°52′32″N 23°51′48″E
Conventional farming (synthetic fertilizers; weed controlled by tillage and herbicide)	Oat-vetch; Barley	CF ov-b	$N_{120}P_{50}K_{60}$ $N_{120}P_{50}K_{60}$	2008 2009	*Albi-EpihypogleyicLuvisol (LVg-p-w-ab)*	54°51′66″N 23°48′40″E
Conventional farming (synthetic fertilizers; weed controlled by tillage and herbicide)	Winter wheat; Oilseed rape; Bare fallow	CF w-r-bf	$N_{120}P_{50}K_{60}$ 0 0	2007 2008 2009	*Albi-EpihypogleyicLuvisol (LVg-p-w-ab)*	54°52′92″N 23°51′42″E
Organic farming ([a]certificated 15 years)	Oat-pea; Barley; Barley-clover	OF op-b-bc	Manure, 80 t ha^{-1}	2007 2008 2009	*Hapli-EpihypogleyicLuvisol (LVg-p-w-ha)*	54°52′44″N 23°51′39″E
Organic farming	Winter wheat; Oat-pea; Barley	OF w-op-b	Manure, 80 t ha^{-1}	2007 2008 2009	*Hapli-EpihypogleyicLuvisol (LVg-p-w-ha)*	54°52′30″N 23°51′40″E

[a] Organic certification by the EKOAGROS (Lithuanian Committee for Organic Agriculture)

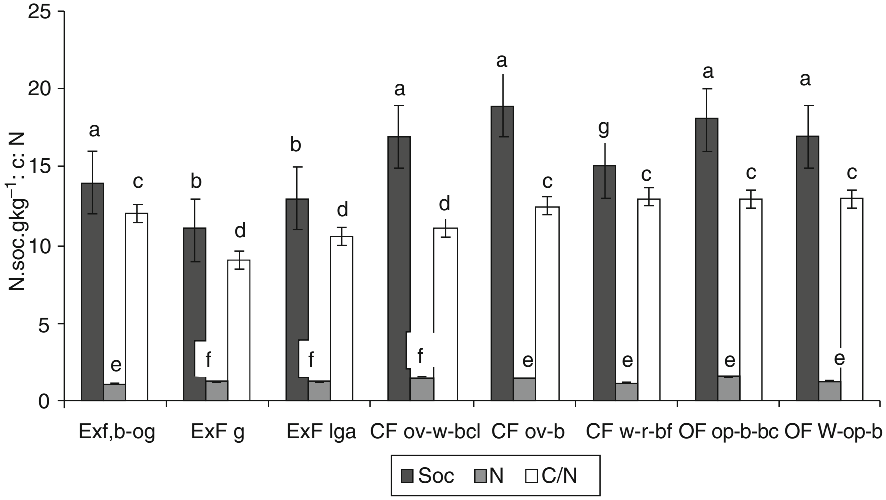

Fig. 53.1 Soil carbon, nitrogen and their ratio in different field management (mean ± SE, $p = 0.05$)

and 18.5 °C (2005); an average precipitation ranged between 8.6 mm (2009) and 148.7 mm (2007).

Statistical Analyses

The confidence limits of the data were based on one–way analysis of variance by *ANOVA* (in case of significant interactions) followed by post hoc Tukey theoretical criterion. The least significant differences between treatment means were determined using Fisher's least significant differences (LSD_{05}). LSD, standard error (SE), correlation coefficient (r) has been calculated at level of statistical significance $p < 0.05$. Results of urease and saccharase activity are presented as a mean of four independent analyses at the $P = 0.05$ probability level.

Results and Discussion

According to the literature (van Diepeningen et al. 2006; Nannipieri et al. 2002), farming management type and crop rotation indicated significant impact on soil fertility indices, namely on SOC ($r = 0.7$), N ($r = 0.4$) and C/N ($r = 0.7$) (Fig. 53.1).

As Singh and Kumar (2008) stated, saccharase is used as a reliable index of soil bioactivity and fertility due to association with humus, K_2O and P_2O_5 content. Therefore saccharase activity with the exception of CF crop stands was observed to

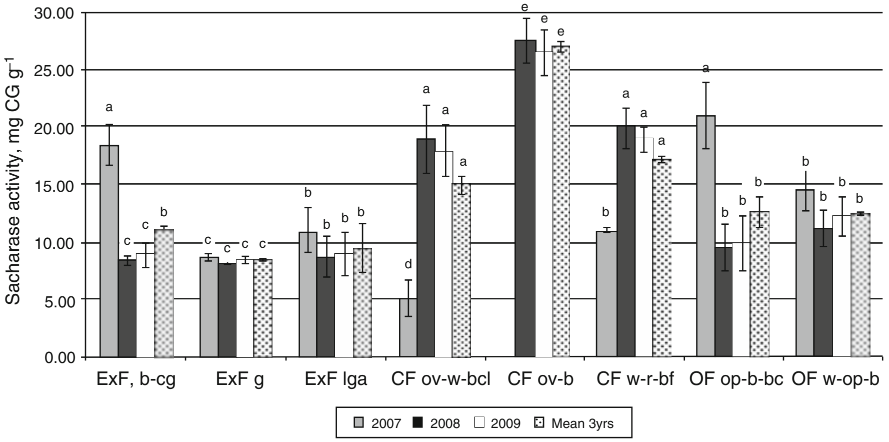

Fig. 53.2 Saccharase activity responses to farming type and different crop stands (mean ± SE, $p < 0.05$).

be significantly different and dependent on farming type as well as crop type during the three study years (r = 0.3; Fig. 53.2). During 3 year conventional farming, the winter wheat stand (27.00 mg CG g^{-1}) significantly induced the highest saccharase activity, supposedly due to appropriate aeration conditions (Sang et al. 2009) and sufficient C containing substrate supply (Li et al. 2008). The lower saccharase activity was observed in CF fallow (17.18 mg CG g^{-1}) and CF mixtures with legumes (15.87 and 14.95 mg CG g^{-1} respectively), as compared with that of CF w. wheat treatment (Fig. 53.2).

Relatively high saccharase activity in stands with legumes could be explained by formation of nodules on leguminous plant roots. As Cooper (2004) found, nodulated plant roots produce glycosides containing flavonoids, which is decomposed by saccharase.

Saccharase activity was observed to be significantly the lowest in cereal (8.40 mg CG g^{-1}) and legume (9.38 mg CG g^{-1}) crops as compared with CF, OF and ExF. Response of saccharase activity to different farming types could be explained by its strong correlation with SOC content (r = 0.7), which varied across different farming systems and ranged between 4.86 in ExF meadow and 12.4 % in IF w. wheat stands. The highest mean values of soil fertility indices were observed in fields using conventional (17 g kg^{-1} SOC, 1.4 g kg^{-1} N and 12 C/N ratio) and organic (17.5 g kg^{-1} SOC, 1.36 g kg^{-1} N and 13 C/N ratio) management systems. In accordance with the chemical parameters of soil fertility, the highest mean activity of saccharase was observed in CF (19.71 mg CG g^{-1}) and OF (12.57 mg CG g^{-1}) respectively. Therefore our study demonstrated that conventionally and organically managed soils exhibited greater saccharase activity due to beneficial conditions for accumulation of SOC ($r = 0.7$, Fig. 53.3) and forming favourable C/N ratio ($r = 0.7$, Fig. 53.1), which, in turn, guarantee sufficient amount of C containing substrate (García-Ruiz et al. 2008).

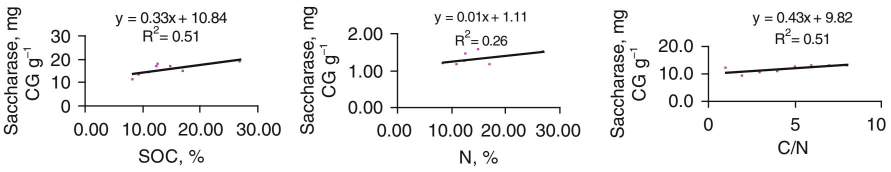

Fig. 53.3 Correlation between saccharase activity and soil fertility indices (SOC, N and C/N) ($p < 0.05$)

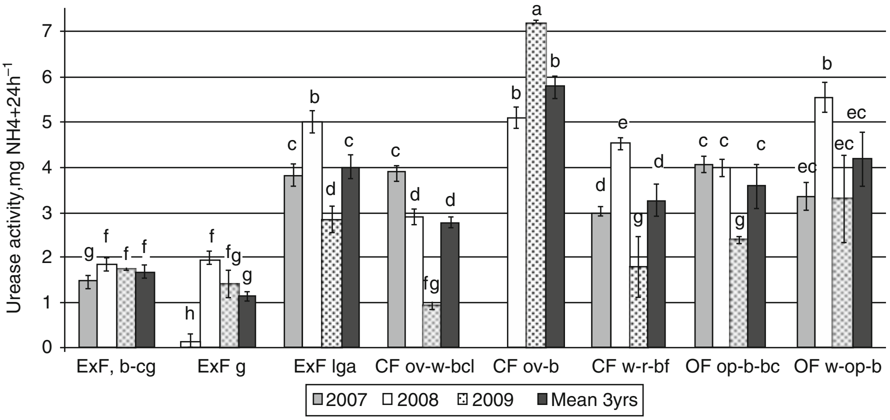

Fig. 53.4 Potential ammonification rate responses ($r = 0.6$) to farming type and different crop stands (mean ± SE, $p < 0.05$)

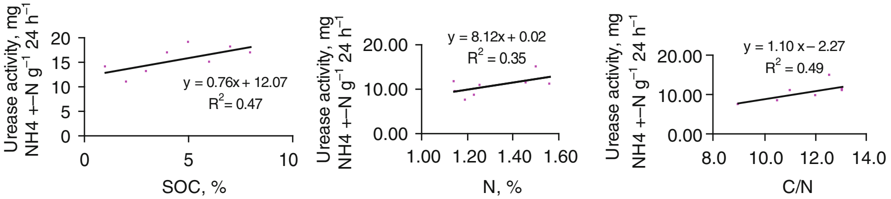

Fig. 53.5 Correlation between urease activity and soil fertility indices ($p < 0.05$)

The most active SOC decomposition was observed in CF and OF, where the highest SOC content was determined. Uncultivated and unfertilized soils of ExF did not accumulate organic matter and other elements important when forming soil fertility, therefore lowest SOC content (5.67–4.86 g kg^{-1}) and saccharase activity (8.6–11 mg CG g^{-1}) were observed.

Urease activity was related to nitrogen–containing substrate dynamics in response to land management intensity and crop type (r = 0.6) (Fig. 53.4). The highest urease mean activity has been observed in CF and OF wheat stands (5.79 and 4.16 mg) possibly due to application of heavy nitrogen rates (N$_{100–120}$). This trend also found by other researchers (García-Ruiz et al. 2008), of significantly higher potential ammonification in the conventional farms is consistent with the long-term application of ammonium or urea.

Urease activity correlated (Fig. 53.5) stronger with SOC ($r=0.7$) or ratio C/N ($r=0.7$) than with total nitrogen content ($r=0.6$). Thus urease is related to total soil fertility (Renella et al. 2007). Decrease of urease activity was observed in soil of EF abandoned grassland (1.13 mg NH_4^+–N g^{-1} 24 h^{-1}) and barley-coach grass (1.67 mg NH_4^+–N g^{-1} 24 h^{-1}) due to the lowest total N (1.2 g kg^{-1}) and SOC (11 g kg^{-1}) content. Due to lower rates of soil quality indices (12.7 g kg^{-1} SOC, 1.19 g kg^{-1} N and 10.5 C/N ratio), the lowest urease mean activity (2.27 NH_4^+–N g^{-1} 24 h^{-1}) was observed in fields of extensive farming. Increase of soil fertility indices in conventional (17 g kg^{-1} SOC, 1.40 g kg^{-1} N and 12.7 C/N ratio) and organic (17.5 g kg^{-1} SOC, 1.36 g kg^{-1} N and 13 C/N ratio) farming indicated better agronomic management there. Land management stimulated the increase of mean urease activity in conventional (3.93 mg NH_4^+–Ng^{-1} 24 h^{-1}) and organic (3.88 mg NH_4^+–N g^{-1} 24 h^{-1}) farming.

Summarising, soil chemical indices (N and SOC) and assessed hydrolase activity indicated positive impacts and successful agricultural practices of conventional farming in studied sites. Nonetheless, C/N ratio (13.0) was significantly higher and more crop-favourable in organic farming, than in conventional (12.2). Agro-chemical properties alone were not sensitive enough to track relatively subtle soil quality improvements in farms over the past 15 years since the shift from conventional to organic farming in the investigated sites. Hydrolytic enzymes suggest that soil functionality was also enhanced under conventional management. Indeed, 15 years practices undertaken routinely were not sufficient for improving soil quality indices in the organic farms covered in this study. Observed variation of the assayed soil hydrolase activity might have occurred due to different site properties (i.e. soil type, landscape characteristics, or environmental conditions) in accordance with van Diepeningen et al. (2006).We found that site is a much stronger determinant of the soil agro-chemical composition than management type.

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