Susanne Barth · Dan Milbourne *Editors*

Breeding Strategies for Sustainable Forage and Turf Grass Improvement

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Preface

From the 4–8th of September 2011, the Eucarpia Fodder Crops and Amenity Grasses Section held its 29th Meeting in the impressive surroundings of Dublin Castle in Ireland. Over one hundred and twenty scientists from 21 countries, all working in the area of the genetics and breeding of forage species, attended the meeting, which was themed '**Breeding strategies for sustainable forage and turf grass improvement**'. Why did we choose this theme?

Grasslands cover a significant proportion of the land mass of the world, and play a pivotal role in global food production. At the same time we are faced with several challenges that affect the way in which we think about this valuable set of resources. The population of the world is expected to exceed 9 billion by 2050, and increase of about one third relative to today's levels. This population increase will be focused in urban areas, and in what are currently viewed as "developing" countries, meaning that the buying power of this increased population will be greater—shifting the balance of demand from staple crops to high value items such as meat and dairy products. Overall this means that the world will have to approximately double agricultural output across all categories of food to meet the demands of this larger, urbanised population. This is occurring against a backdrop of equally large challenges in terms of global climate change. Agriculture is already a significant contributor to things such as greenhouse gas emissions, deforestation and soil erosion. The situation is made more complex by an increased emphasis on biofuels as a solution for our imminent oil shortage, resulting in increased competition between land utilised for food and fuel. In short, agriculture must continue to feed the world, whilst not contributing to damaging it further. It must be sustainable. Plant breeding plays a significant but frequently understated role in meeting the challenges presented by this complex and changing scenario. However, plant breeding and improvement is itself undergoing radical change, driven by technologies that, quite frankly, seem to have sprung from the pages of science fiction novels written decades ago.

Thus, it seemed to us, when given the opportunity to organise this meeting, that it was timely to explore how forage and turf breeding is changing and adapting to meet these challenges using the technological advances being experienced in plant breeding as a whole. Consequently, the meeting focused heavily on how next generation sequencing technologies are interacting with advanced phenotyping strategies for a variety of increasingly well defined traits. This type of analysis is powerful,

potentially telling us a lot about the genetic control of these traits, but also has the potential to revolutionise plant breeding via approaches such as genomic selection (GS).

A wonderful characteristic of the membership profile of Eucarpia is that the membership is composed of a mixture of plant scientists from multiple disciplines and practical breeders. While some of us wax lyrical about the potential of approaches such as GS, it's always useful to have breeders present who can ask pointed questions about how much this is going to cost them, and how it's better (i.e. more cost effective per unit of genetic gain) than what they currently do. This can sometimes be an uncomfortable experience, but it is through such a frank exchange of ideas that real progress is made.

As well as the focus on advanced technology, the meeting featured the usual interesting array of topics that attract the broad audience that attends the section meetings. Several contribution focused on the use of germplasm of grasses and legumes to improve the vegetation in different environmental conditions, particularly under conditions to be expected by climate change—these addressed the theme in a way in which we hadn't considered when we discussed it originally (again showing the advantage in a broad section membership). There were also regular topics such as the results of the EUCARPIA multi-site rust evaluation, showing that over a period of 11 years there is no evidence that crown rust resistance in individual *Lolium* cultivars was overcome by the pathogen), and the Festulolium satellite workshop.

This book contains papers based on many of the oral and poster presentations presented at the Dublin meeting. With some minor changes to represent the diversity of material presented, the papers are organised in sections fairly similar to the session topics, and for the purpose of this volume, are grouped into the following sections: European grasslands in the future; Breeding strategies; Novel emerging tools for the breeding of forage and turf crops; Breeding towards breeding objectives; Genetic variation and adaptation; and Agronomy and performance of forage and turf crops. We hope they present a good snapshot of a very stimulating meeting, and will be a useful resource for participants and those who couldn't attend.

We would like to acknowledge the enormous efforts of the local organising committee members (Connie Conway, Dermot Forristal, Dermot Grogan, Eleanor Butler, Patrick Conaghan), with a special mention for Connie Conway and Eleanor Butler, without whom the meeting would not have run so smoothly and efficiently. Finally, the work of the scientific committee and referee board for this book (Beat Boller, Bohumir Cagas, Christian Huyghe, Daniele Rosellini, Danny Thorogood, Dejan Sokolovic, Dermot Grogan, Dirk Reheul, Jan Nedelnik, Joost Baert, Michael Abberton, Michael Camlin, Niels Roulund, Paolo Annichiarico, Petter Marum, Roland Kölliker, Trevor Gilliland, Trevor Hodkinson, Ulf Feuerstein and Ulrich Posselt) must also be acknowledged, especially in providing their time so graciously and uncomplainingly to review the papers for this volume, and ensuring a high quality of presentation in these proceedings.

Carlow, Ireland

Susanne Barth
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Part I
Introduction: European Grasslands in the Future

Chapter 1
What Global and/or European Agriculture Will Need from Grasslands and Grassland Breeding over the Next 10–15 Years for a Sustainable Agriculture

D. Reheul, B. de Cauwer, M. Cougnon and J. Aper

Abstract The paper analyses actual trends in (European) ruminant agriculture and grassland based production systems. Consequences of reduced and/or zero grazing for grass breeding and grassland management are discussed. The impacts on eco-efficiency, recycling of minerals and ecosystem services are highlighted as well as the role of ley-arable farming. Special emphasis is on the potential use of tall fescue as a component of mixtures or as an interspecific cross. In grazed grassland, the role of white clover, the disease resistance and the nitrogen use efficiency of the grasses and the significance of biodiversity are considered. Based on an article published by Parsons et al. (2011) some reflections on the way ahead in grass and forage breeding are presented.

1.1 Introduction

At the start of the second decade of the twenty-first century, agriculture is changing faster than ever in most (European) countries. Attempts to realize some radical changes in the way we live, confront us with the tremendous complexity of societies. This results in important gaps between what should happen and what really is occurring. In theory, sustainable development aims at compromises between socio-economic and ecological imperatives. The transition from today's reality to this new world is a most difficult process passing along several stepping stones (Meerburg et al. 2009). It is occurring mostly within existing paradigms improving the eco-efficiency or eco-productivity ("producing more with less") of processes and making them cleaner and more rewarding. Next to this major development new paths are explored.

Agriculture is changing in line with the major drivers in society. Mainly driven by European policy, farming has become a very regulated business. To cope with

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this, the most striking development during the past years is the very fast expansion of agricultural enterprises both in terms of means of production, technology application, management and alliances. While this evolution is going on, scientists and policy makers already think way ahead of actual evolutions. A striking example is the newest report of the Standing Committee of Agricultural Research (SCAR) (Freibauer et al. 2011). The report clearly proposes to move away from the existing paradigm of productivity and replace it by the paradigm of sufficiency where consumer-driven, technology-driven and organizational innovation-driven pathways are building blocks of the transition. The report states "Scientific advance has the potential to bring forward agro-ecosystems that are both productive, respectful for ecosystems and resource saving. Demand increases need to be mitigated through behavioural changes, the internalization of environmental externalities and appropriate governance structures". If one has to reflect on what European agriculture will need from grassland, one cannot deny this report.

Actual trends form the main frame of this presentation. Grafted upon this main frame are scientific developments, potential implications of climate change and personal reflections.

Apart from the mentioned SCAR report, a number of recent high quality publications inspired the authors, e.g. Towards sustainable grassland and livestock management (Kemp and Michalk 2007), Genetic improvement of forage species to reduce environmental impact of temperate livestock grazing systems (Abberton et al. 2008), Proceedings of the international conference on grasses for the future (O'Donovan and Hennessy 2010), *Handbook of Plant Breeding*, fodder crops and amenity grasses (Boller et al. 2010), Producing milk from grazing to reconcile economic and environmental performances (Peyraud et al. 2010) and Past lessons and future prospects: plant breeding for yield and persistence in cool-temperate pastures (Parsons et al. 2011). The reader can find a lot of *quantified* data in these publications.

1.1.1 Very Intensively Used Grassland in the Lowlands

In the (lowland) areas of Europe with an intensive dairy industry, the number of dairy farms continues to decrease while numbers of cows in surviving farms are increasing rapidly. Economic scale effects and robot milking (improving the farmer's comfort) are important drivers for this evolution. As these drivers most probably will persist, this evolution is expected to continue. Although grazing may be the cheapest way to produce milk (O'Donovan and Hennesy 2010), grazing becomes difficult with very large herds particularly if the land around the milking parlour is restricted. Initially, the decision to work with large herds often goes along with restricted grazing but eventually grazing may disappear totally. The higher the numbers of dairy cows on a farm, the higher the probability that they stay in the barn year-round. In some parts of the world (e.g. New Zealand, The Netherlands), removable milking parlours may

sustain grazing even when herds become very large. The diet of cows held indoors is a combination of grass and other forages with conserved forages being far more important than fresh forage. Although grass remains an important component of the diet (mainly as a provider of nitrogen and of forage structure, the latter guaranteeing a good rumen fermentation), other forages (in many cases forage maize) become the main diet components very often supplemented with a source of concentrated protein. Hence, these large dairy farms need, next to the grassland area, a lot of arable land to produce their roughage and to recycle the nutrients in the slurry.

Zero grazing can comply well with a number of sustainability indicators.

1. Harvested dry matter is higher than under grazing. If silage losses are restricted the benefit remains. Uneven yearly distribution of grass yield becomes less important, since the animals are mainly fed with conserved forage.
2. A high nitrogen export going along with low (grass-clover) to very low (grass only) soil nitrate residues (Nevens and Reheul 2003a) makes zero grazing a good system for an optimal use of slurry. A high N-input combined with early cuttings (simulated grazing management) provides opportunities to restrict CH_4-emissions per produced milk quantum (Ellis et al. 2008, Bannink et al. 2010, van Zijderveld et al. 2011).
3. Nitrogen use efficiency (NUE) on the farm level can be substantially improved (a) by modern cowsheds and covered storage of slurry, (b) by uniform and emission-poor distribution of slurry reducing leaching and ammonia losses and (c) by the composition of animal diets balancing energy from non-grass feed and protein from the grass, improving the N utilization by the animal. The latter means that the chemical composition of the grass is less important than under grazing conditions, since excesses or shortages can be compensated by other forages.
4. Compared to continuous grazing, root depth of the grasses is on average deeper under a cutting regime offering opportunities for a better nutrient uptake efficiency and hence a better NUE by the grass plants (Crush et al. 2005; Abberton et al. 2008).
5. In large animal farms, most farmers live closer to their accountancy and their animals than to their crops. One can presume that grassland management will not (always) be the first priority of these industries. Mismanagement may deteriorate the grassland very quickly. On the other hand, farms may pay a lot of attention to good grassland management in order to cut feed costs.
6. According to the EU legislation on permanent grassland, farmers may avoid to keep all their grassland longer than 5 years in order not to lose degrees of freedom in their exploitation. Hence part of the grassland may be kept as temporary grassland. If managed under a high nitrogen input, it is difficult for legumes to maintain important abundances. On the other hand, farmers may cherish the legumes in order to save N-fertilizer costs.

What Are the Consequences of Reduced Grazing for Grassland Management, for Grassland Breeding and for the Ecosystem Services of Grassland? Given the intrinsic higher yield potential of early heading varieties, the attention for early varieties may increase in zero grazing systems, provided their persistence is high. According to Chaves et al. (2009) progress in the early varieties of *Lolium perenne* L. (perennial ryegrass) was lower than in the intermediate and late heading varieties offering opportunities for breeding, with a special emphasis on good quality. Good quality usually is very closely connected with leafiness. Hazard et al. (2006) showed that selection for longer leaves leads to earlier heading dates, indicating that selection for good quality may indirectly promote earliness (Barre et al. 2009). The trait "long leaves" has a high heritability (Cooper and Edwards 1961) and is mainly determined by leaf elongation rate, easily detectable as quick regrowth.

Since early varieties concentrate their production early in the season, the effect of summer droughts may be less detrimental than with intermediate or late varieties.

If zero grazing farms choose for temporary grassland, ley-arable farming offers a number of opportunities and threats (for a review see Vertès et al. 2007) but if well designed it may fit into a sustainable management.

In the short term, grassland sown into previous arable land significantly outyields grassland sown into ploughed down grassland (Reheul et al. 2007), particularly under dry conditions, most probably owing to the deeper rooting of the young grass plants. The establishment of white clover is better in grassland sown into former arable land and the clover tends to persist better (Reheul et al. 2007).

The rotation between grass and arable crops helps to manage weeds in the arable phase of the cropping system.

The opening crop in the arable phase can be grown without any nitrogen fertilizer (Nevens and Reheul 2002; Nevens and Reheul 2003b, Bommelé 2007; Reheul et al. 2007). Forage maize is an important component of ley-arable farming in large parts of Europe. In a sustainable system, forage maize is harvested early in the autumn offering the opportunity for a cover crop as *Lolium multiflorum* Lam. (Italian ryegrass) or *Secale cereale* L. (winter rye) to get established well before the winter. This way the cover crop prevents winter erosion, nutrient leaching and provides an early cut in the next spring. The Italian ryegrass may either be ploughed down, enhancing the soil organic matter or it may produce for the entire season, helping to overcome risks as the success of forage maize may be jeopardized by dry springs or very wet autumns. If the maize is harvested late, *Lolium multiflo*rum or even *Lolium perenne* may be undersown in the maize crop. Special machines are now available to sow or drill grasses into a forage maize crop. When this is done before the canopy is closing (maize height of approx. 40–50 cm), crop damage is minimal. Tetraploid varieties may offer advantages owing to their early vigour, good cold tolerance and presumed (has to be proven) deeper rooting.

Climate change is predicted to result in a higher frequency of extreme weather conditions as hot and dry summers and wetter winters in large parts of Europe. It is well known that ryegrasses and timothy may suffer from summer (or even spring) drought with low yields and low quality during the dry spells. Species with a better

drought tolerance as *Dactylis glomerata* L. or *Festuca arundinacea* Schreb. (tall fescue) can overcome poor performances during dry periods. The work of Pontes et al. (2007) showed that both species can deliver as much as or even more digestible dry matter and digestible crude protein than perennial ryegrass. Although these parameters may not be very relevant under grazing (animals graze -or reject- fresh grass and not digestible matter) they may be less irrelevant for conserved forage as is the case when ruminants stay indoors.

Quite a lot of work is currently done on fescue breeding. Mixing *Lolium* with *Festuca* may combine the advantages of both species (excellent forage quality of *Lolium spp.* and e.g. good drought resistance of *Festuca sp.*). The mixing can be done genetically in the form of *Festulolium* (see Eucarpia workshop of the Festulolium working group) or mechanically by sowing mixtures of *Lolium perenne* and/or *multiflorum* and *Festuca arundinacea*. While the abundance of *Festulolium* in a pure *Festulolium* sward is not expected to change dramatically over seasons and years this may be well the case with mixtures. Preliminary results in Belgian trials (both under grazing and cutting) do not show important shifts in species composition although the proportion of tall fescue in the harvested material is higher in early spring and during dry summer periods. A mechanical mixture may result in a transgressive over yielding driven by pairwise inter-specific interactions as indicated by Kirwan et al. (2007) who compared during 3 years mixtures with four components (two grass species and both white and red clover) in different locations across Mid and Northern Europe. Swards were managed under a cutting regime and dressed with maximum 200 kg N/ha/year. Preliminary results of our own cutting trials with mixtures of perennial ryegrass, tall fescue and white clover (dressed with about 160 kg/ha N) do not indicate a transgressive over yielding, probably because both perennial ryegrass and tall fescue belong to the same group of functional types[1] (Kemp and Michalk 2007) meaning that—according to the redundancy hypothesis—their mutual replacement has no significant impact on productivity.

The transgressive over yielding may extend into the animal, since the half life of fescue protein in the rumen is substantially higher than that of ryegrass protein, enhancing the probability of a better utilization of the protein by the animal (Abberton et al. 2008).

Different breeding programmes are currently providing new varieties of tall fescue with long and soft leaves resulting in improved palatability (Rognli et al. 2010). Many ecotypes have a high lignin concentration in the leaves lowering the digestibility, but owing to the high genetic variability of the species further progress is expected (De Santis and Chiaravalle 2001). Selection for a high leaf/stem ratio is a proper way to improve digestibility and measuring ADF and NDF are the best parameters to quantify the progress (De Santis and Chiaravalle 2001). In the mean time, the results of Mosimann et al. (2010), comparing mixtures in which either perennial ryegrass or tall fescue were the dominant component, indicated a similar digestibility throughout the year. Since tall fescue leaves have a longer life span than leaves of perennial

[1] Grime et al. (1988) described *Lolium perenne* as a CR/CSR type, while they categorized *Festuca arundinacea* as a CSR type (CSR: strategist, CR: ruderal competitor).

ryegrass (1.72 times longer, according to Lemaire et al. 2009), harvesting is quite flexible.

Tall fescue has a stronger and deeper rooting system than ryegrasses[2] (Abberton et al. 2008, Eickmeyer 2009; Bonos 2004). This results in a better water and nutrient use efficiency since tall fescue can retrieve water and nutrients from deeper soil layers. Its ability to protrude compacted soils makes it more resistant to mechanical soil compaction and allows a better water infiltration (Crush et al. 2005; Macleod et al. 2007). Simultaneously less nutrients are expected to be leached by heavy winter rains (Eickmeyer 2009). Compared to ryegrass, the deeper root system of fescue may stock a higher amount of organic carbon.

Although tall fescue and *Festulolium* may have promising traits, evidence is needed to show that these species perform well on the fragile sandy soils, where much of the intensive dairy is centralized. While tall fescue is growing in roadsides all over Europe, it is not abundant on sandy soils (were much of the animal production in the EU lowlands is concentrated) and on soils with a low pH. This may be an indication of poor performances/persistence on these soils. There is also a need to find out what the effects are of the lower digestibility of fescues when they are a component of a complex diet.

A warming up of the climate brings along new diseases and pests, advances their outbreaks and/or enhances their frequency (Kiritani 2007; FAO 2008; Ceccarelli et al. 2010). Therefore, breeding for disease (pest) resistance will become more important than ever, since in a sustainable agriculture the restriction of pesticide application is a prerequisite. This is particularly true for diseases striking the grass plants during seed production, since Mattner and Parbary (2007) showed a negative effect of a crown rust infection of a seed crop of *Lolium multiflorum* in (the non diseased) post-epidemic generation: the lower early vigour of the seedlings and poorer performances later on (registered in pot trials) were mainly due to the smaller seed size of the diseased seed crop.

A non-grazing management has consequences for the ecosystem quality and ecosystem services of grassland. According to Reidsma et al. (2006) the ecosystem quality of a region where grassland occupies a major part of the agricultural area, can be relatively high, even if the management is very intensive. They calculated a ecosystem quality of 20 % for intensive pastures as compared to 40 % for extensive pastures, while extensive crop production has 25 % and intensive crop produc-

[2] Breeding for a changing pattern of root distribution in *Lolium perenne* is reported by Crush et al. (2007). They reported a wide variation in genotypes for patterns of root distribution in a full-sib mapping population. They found no relationship between N-interception and patterns of distribution of DM weight of roots. Genotypes reacted on moisture stress either by increased or by inhibited root growth. Since root growth in artificial circumstances is very variable, hampering a reliable selection, they expect much of indirect marker-assisted selection of root traits in ryegrasses. This hope seems justified because of successes in rice (Steele et al. 2006) and maize (Ribaut and Ragot 2007). A high root/shoot ratio does not automatically reflect a good drought tolerance. In the experiments of Crush et al. (2005) timothy had a root/shoot ratio of 0.86 versus 0.63 for perennial ryegrass. Yet timothy is known to have a low drought tolerance.

tion 10 %. Among grassland systems, the species richness is substantially lower in cut than in grazed grassland (Smith and Rushton 1994).

Although temporary grassland is a better carbon sink than arable land, it stores about 50 % less carbon than permanent grassland and cut grassland stores about 50 % less carbon than grazed grassland (Mestdagh et al. 2004; Conijn 2007; Vertès et al. 2007), since a proper cutting management allows less senescent material to return to the soil.

There seems to be a trade-off between different sustainability indicators (emissons, carbon balances, ecosystem qualities). As a result it seems impossible to optimize all productivities, efficiencies and eco-efficiencies as already stated by Jansén (2000).

1.1.2 Grazed Grassland

In important parts of Europe, mainly hilly, mountainous land or land with shallow soils, grazing still is the best agricultural option for use of the land. This is reflected in large areas of permanent grazed grassland, with a relatively low frequency of reseeding. In order to be sustainable, grazing in the EU must comply with environmental prescriptions as expressed in the Nitrate Directive (91/676/EEC) and the Water Framework Directive (2000/60/EC). Hence, pasture management is pushed in different directions: lower stocking density (where the land is cheap), less external N inputs, restricted grazing, a combination of grazing and cutting where appropriate and a strong reliance on biologically fixed nitrogen. Also in these areas the trend of larger farms is striking and the evolutions toward larger farms is occurring remarkably fast.

Low external N inputs allows legumes to persist in the grassland. The quantity of biologically fixed nitrogen (BNF) in the grass-clover herbage can be estimated by multiplying the white clover DM yield (expressed in ton/ha) in the herbage by 35 (BNF35) and corrected for applied mineral N (kg/ha). The total quantity of biologically fixed nitrogen *in the herbage*, $\mathrm{BNF} = \mathrm{BNF35} * 1 - (0.282 * \mathrm{N})/100$ (Humphreys et al. 2008). *Total white clover BNF* (including the non harvested clover DM (stubble, stolons, roots) is estimated by multiplying BNF by 1.27, which brings the total fixation at approx. 50 kg/ha per ton DM of white clover. The correction factor was calculated by Hansen (1995), based on the work and data of Nesheim et al. (1990). The latter applied no more than 80 kg/ha mineral N either as fertilizer N or as cattle slurry to Swiss swards dominated by perennial ryegrass, meadow fescue and white clover. Later publications (e.g. Humphreys et al. 2008) use the same correction factor for much higher mineral N dressings, asssuming that the linear relationship holds beyond the originally tested low mineral N applications. Anyway, the formula quantifies common knowledge: to take maximum advantage of the biological fixation, external mineral N-input should be low. There is ample scientific evidence that grass-clover pastures produce almost as much DM as pastures consisting of pure grasses dressed with 200–250 kg/ha mineral nitrogen (e.g. Peyreaud et al. 2010) provided soils are deep and water supply in summer is sufficient. Experience on organic farms,

with no fertilizer N input, demonstrates that such grass-clover swards comply very well with the environmental regulations.

In these circumstances, grasses with high nutrient use efficiency (NUE) are requested. The trait NUE can be disentangled into a number of physiological more precise components: NUE can be expressed as the product of the uptake efficiency (NUptE) with the utilization efficiency (NUtE) (e.g. Gallais and Hirel 2004). NUpt refers to the efficiency with which roots absorb nitrogen (absorbed versus supplied nitrogen) while NUtE refers to the quantity of dry matter produced per unit N present in the dry matter. The latter depends on the retention time and the remobilization possibilities, again depending on the leaf longevity. Experimental breeding research in grasses has mainly focused on the NUtE at a given (mostly low) N supply (e.g. Baert et al. 1999, 2003). The strategy to focus on NUtE gets support from other species, since Gallais and Hirel (2004) and Schmidt (maize breeder at KWS in Germany; personal communication) indicated that under low N, NUtE was the driver for a better NUE in (grain) maize. Despite at lot of research (e.g. the past NIMGRASS EU-project, in which several EU grass breeders participated) and experimental breeding work, to our knowledge only few varieties are advertised strongly to have a better N use efficiency. However, grass breeders always have indirectly selected for a better NUE, since at a given N supply, the most productive varieties have the best nitrogen use efficiency. Eventually it is the (N)UE at farm level that is the most important driver for a production system with low emissions. Hence NUE in the grass plants should be integrated with NUE in the ruminants. If not, too many nutrients left unused by the animals, return to the soil or are lost in the atmosphere. In case the animals are fed with grass exclusively (or dominantly), the balance between N and WSC may improve the NUE in the animal and hence at farm level.

From a theoretical point of view genotypes or species with an extensive root system and a longer life span of leaves offer the best opportunities to improve NUE. However, genotypes with longer life spans may be more prone to leaf diseases, hence a good resistance is a prerequisite. The same applies to varieties with long leaves. Long leaves are advantageous in grazing since they guarantee a high supply of good quality herbage, the high supply being necessary for a high intake as demonstrated e.g. by the studies of Delagarde et al. (2001, 2006) and Delagarde and O'Donovan (2005). If long leaves are the result of a high leaf elongation rate, a quick regrowth after defoliation offers steady high herbage mass (Barre et al. 2009). As the rate of development of foliar diseases often is refrained by high N concentrations in the leaves, a low nitrogen use demands varieties with an excellent resistance to leaf diseases and this trait may become more important when the climate gets warmer. Although there is often a negative correlation between leaf length and number of tillers and a positive correlation between numbers of tillers and persistence, grass breeders have bred persistent varieties with long leaves.

Extensive root systems are able to restore soil structure in cases of trampling due to adverse weather conditions. Several studies demonstrate the deeper and stronger roots of fescues as already stated here-above and the positive effects of the association of grasses and clovers to guarantee a good soil porosity and water infiltration (e.g. Van Eekeren 2010).

Peyraud et al. (2010) give an overview of reasons why multi-species swards under mild fertilization are the way ahead for a sustainable animal production based on grazed grassland. The reader is referred to follow the MULTISWARD EU-project (http://www.multisward.eu/multisward_eng/) to get an idea of existing knowledge and ongoing research.

A recently highlighted function of (grazed) grassland is its value as a sink for soil organic carbon (SOC). Sonneveld and Van Den Akker (2011) report values of 9–21, 7 kg/m^2 in the upper 20 cm of sandy and peat soils respectively in the north of the Netherlands. When ploughed down, the rate with which the SOC is initially lost is approximately twice the rate of its accumulation as reported by Johnston (1986). Indirectly this is a plea for persistent grassland and for breeding of varieties with an excellent persistence.

There is currently a lot of work going on studying the carbon footprint of animal production systems. Although today there is no standardized methodology, it is clear that the carbon footprint heavily depends on the farming system. There seems to be a link with productivity: in many cases low input systems are also low output systems with a high carbon footprint expressed per kg produce. Eco-efficient production systems seem to comply best with low carbon footprints. Indeed, according to Edwards-Jones et al. (2009), emissions by on-farm activities were by far dominated by fertilizer-N and concentrates. Not surprisingly, these are also the most important drivers of N-surpluses. This is again a strong argument for a limited input of fertilizer-N and concentrates and for grassland systems heavily leaning on biologically fixed nitrogen.

The relation between carbon sequestration by (grazed) grassland and climate change is a much studied topic (for a review see e.g. Bartlett et al. 2008 and De Deyn et al. 2008). Provided there is no water shortage and no shortage of minerals, a rising temperature and a rising atmospheric CO_2 concentration are expected to stimulate the growth of the C_3 forbs and grasses, both above and under the ground. More roots, more dead material and more root exudations are expected to stimulate the microbial web in the soil. An enhanced heterotrophic respiration may be responsible for an initial carbon loss from the system. Enhanced mineralization of recent and old SOC may provide more nitrogen, stimulating again growth, strengthening the circle of accumulation and mineralization and carbon fluxes. Eventually the growing microbial biomass may immobilize N, refraining plant growth and carbon fluxes to the soil, except when legumes are providing extra N input. So it remains to be seen if the net result of climate change will increase or decrease the carbon sink in grassland. Whatever the outcome will be, the room to manipulate here is quite small, although Dijkstra et al. (2006) and De Deyn et al. (2011) showed that species richness (with an important role for legumes) continues to be the best guarantee for carbon sequestration.

Grazed grassland has a high potential for biodiversity both above and under the ground (Smith and Rushton 1994; Kemp and Michalk 2007; Van Eekeren et al. 2008, 2010 and Van Eekeren 2010). In a number of regions a reasonable yield and an acceptable biodiversity can go hand in hand, but in quite large parts of Europe, biodiverse grassland systems need to be financially supported, e.g. by agro-environmental

schemes. In the absence of this support, these grasslands risk to be quickly abandoned, with the loss of a number of ecosystems and ecosystem services in the short term. It remains to be seen how the economic crisis in the world will influence the protection of these areas and their ecosystem functions.

To conclude: the semi-intensive grazed grassland of the future will have multifunctional roles (Reheul et al. 2010). The swards are multi-species swards, comprising persistent grasses and legumes with a dominant role for white clover in temperate regions: grasses have a long growing season, long leaves, a quick regrowth, good disease resistances, an extensive rooting system and a high NUE. The grassland and the animal production system is managed in a way to be as eco-efficient as possible, by applying best practices and common sense.

1.1.3 Reflections on the Paper of Parsons et al. (2011)

The paper of Parsons et al. (2011) should be compulsory reading for any (grass) breeder. Based on a thorough analysis of past breeding work, successes and failures, the paper partly questions if the (experimental) breeding—as it is actually conducted-is the right way to quickly move forward, given—according to the authors—the moderate (compared to other crops) breeding advances. The authors propose a more academic approach of the breeding work based on a quantified definition of breeding goals in clearly defined environments. They suggest focusing on specific traits, starting from—or referring to—physiological processes in the plant, unraveling how they are genetically regulated and interact with the environment and they propose to find out how traits eventually may be locked into varieties. They question the value of some experimental variety (field) trials—as they are actually designed and conducted—and would like to focus more on a tiered approach, with an important emphasis on the "proof of the concept", i.e. an early testing of trait performance rather than on variety performance. The latter is deduced from the finding that traits may be diluted (or eventually lost) during the development of synthetic varieties and that permanent grazed grassland can be such a complicated plant community with interactions above and under the ground, that genetic progress may be difficult to prove in experimental trials or in farm situations with different settings. Indirectly they ask for more fundamental research.

Essentially they rake up an old dilemma, very nicely defined as two models by Coors (2006). In model 1 "form follows function", while in model 2 "function follows form". Model 1 means that by selection of phenotypes the breeders' goal eventually is to change genotypes, while in model 2 one first changes genotypes in order to create new phenotypes. Model 1 is the model that breeders are applying for over a century now with proven success. Model 2 results from developments in molecular biology and genetics. The transition from model 1 to model 2 seems to be happening—as quoted by Coors (2006)—"by default, without any discussion and challenge". Put it in another way: it refers more or less to the confrontation between an academic view and the view of people working in the real world, between laboratory breeding and

plant breeders who work "with mud and dirt and drought and wind" (quote of Prof. T. DeJong, tree crop physiologist, UC Davis). Or to conclude with Coors (2006), "at the end, it is the phenotype that matters".

Some reflections on the article

1. I have once read a scientific report (but unfortunately have lost it) stating that "there has not been a single (commercially viable) success booked in plant breeding programmes that were driven by deliberately creating genotypes with altered plant physiological characters". Parsons et al. (2011) show an example of such a failure (decreasing respiration), but they do try to explain the failure.
2. Tiered approaches are common in risk assessment (e.g. of genetically modified plants). Some scholars argue that standardized lab tests are necessary to "prove concepts", while others are urgently demanding "in planta" experiments. The reasons for the dispute are analogous to those given by Parsons: the farther away from standardized *ex situ* experiments and the closer to the real complex *in situ* world, the more difficult it becomes to prove anything. But in the end of the day, it is the reactions of organisms in the real world that matters.
3. Parsons et al. (2011) emphasize the importance of "fitness of traits", meaning that changed traits have to sustain during the process of variety building and in the complex communities of (grazed) grassland and management settings of animal farms. They show by smart analysis how varieties with a higher concentration of water soluble carbohydrates (WSC) do have a positive effect on NUE at low nitrogen inputs but that the effects fade away at high nitrogen input. Could (F1-) hybrids—e.g. based on CMS as proposed by Gaue and Baudis (2002)—bring more stability? At least no loss of traits is expected during the variety construction and we do know both from maize and cereal hybrids as well as from hybrids grown for their vegetative parts (sugar, forage beet, a series of vegetables) that they perform remarkably well in different environments, with in many cases, the best yield bonuses in rather poor environments. Moreover, the idea of fitness of traits, fits exactly in the "breeding model 1" as cited above.
4. Questioning if we really can increase the growth of perennial grasses, in particular by altering their growth strategy, Parsons et al. (2011) "speculate with credible evidence that our perennial grasses are holding back and not growing to the limits of their resource supply" since they have to combine good annual growth with the storage of reserves to guarantee persistence. Yet Chaves et al. (2009) demonstrated that progress over the last 40 years in dry matter yield and persistence (sic) in the short living *Lolium multiflorum* was very comparable to the perennial *Lolium perenne*. For crown rust resistance, progress was even better in *Lolium perenne* than in *Lolium mulitiflorum.*
5. As in many cases, the truth probably will lie in the middle. As Coors (2006) says: we do know that the model 1 is working as proven by more than a century of breeding; we do not know how successful model 2 will or can be, particularly for traits regulated by QTL's. It would be unwise to throw away a century of experience, as it would be unwise to neglect new developments. However, in line with the vision of Parsons et al. (2011) it is my opinion that there should be

more clarity and continuity and perseverance into the focus of the application of new techniques or tools: this will enhance the probability to be successful or will quickly create clarity on the (non) feasibility and the (non) practicality. I have seen many projects focusing on molecular techniques passing by. All held bright initial promises that became less and less brilliant the closer the project came to an end. Once a project finishes, new projects are proposed with new and often completely divergent promises and new focuses. There is no clear link between the series of continuously emerging new techniques (or improvements of their performance) and achieved results in plant breeding.

1.1.4 Conclusions

Forage grasses are expected to excel in vegetative growth with good forage qualities during several harvests per year, to persist in these characteristics over many years and in many different settings and yet to have a good generative growth in order to produce enough seeds. Unlike a crop as e.g. forage maize, there is no possibility for compensation between generative and vegetative characteristics, and unlike some vegetables, grown for their vegetative parts, grasses can not yet take advantage of heterosis offered by hybrids. No surprise that genetic gain is slower than in many other crops.

There will always be funded or hyped arguments to select for extra traits. Plants breeders are well aware that the probability to create excellent all-round varieties is decreasing, the more traits are involved[3]. I do think that it is wise to focus on the core: producing good forage in an eco-efficient way with the application of best practices. Furthermore, I do think that there is no need to become nervous owing to induced hypes and/or alarming messages about dramatic evolutions in food production and climate change. The current breeding strategies and techniques have proven to create a steady progress and they should be continued. The introduction of new breeding tools into the existing programmes applying recurrent selection to create improved populations—as a base for variety development—may accelerate the selection response provided they are well focused. Hybridization may change the whole breeding progress, provided the created heterosis justifies higher seed costs.

As the era of plenty seems to have gone and in line with the recent SCAR-report (Freibauer et al. 2011), the transdisciplinarity between scientific disciplines as grass breeding, grassland management, forage use and animal sciences may be key for speeding up the transition to sustainable grassland based production systems. Reflecting on adjusted production systems followed by proper actions and applying best practices in every element of the production chain can make the whole process more sustainable. As, according to the presumed developments presented in the SCAR report, among other things, consumer behavior is expected to change (how

[3] The number of genotypes in an F_2 population equals 3^n with n being the number of different loci. The greater n, the smaller the probability to find the ideal genotype.

difficult this will be?) and environmental externalities are expected (who knows when?) to be internalized in markets, the demand for animal products may (locally?) decrease[4] and/or their price may rise. It is unclear today how the effect of this evolution will affect grassland: will animal production with ruminants become even more concentrated in the very intensive areas and take advantage of both the economy and ecology of scale, or will we see the opposite?

If there is one particular worrying evolution, much more cumbersome than the conceptualized rather slow progress in grass breeding, it is the growing shortage of skilful agronomists, grassland scientists and plant breeders. We are losing a valuable expertise and a valuable professional genetic diversity which are all sources of vital creativity. Without these people it will be difficult to achieve any necessary transition and we will not be able to convince society that some actual hypes drain away a lot of energy and efforts that would be much more rewarding if they were focused on the core business instead of circling around it. Science can change the world, but science has to be honest. I think it is unwise to transform science into an advertising agency, concentrating on the regular emission of new promises. It is in a series of old values, methods and perceptions that lay many foundations of sustainability.

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[4] In university cities as Gent and Leuven in Belgium (and most probably in other major European cities), action groups promote one "veggie day" per week as a starter to decline meat consumption and the consumption of animal proteins. If a large part of the population goes along with this evolution a substantial decrease in the demand of animal products is expected, with inevitable consequences for animal production systems and their orientation.

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Part II
Breeding Strategies

Chapter 2
Marker Assisted Selection Made Cheap and Easy

H. Riday

Abstract Molecular markers in perennial forages are becoming ubiquitous for marker assisted selection (MAS). Nevertheless, widespread implementation of MAS in perennial forage breeding programs for highly quantitative traits has not yet occurred. A primary reason is likely the cost associated with genotyping plants using molecular markers. Presented are cost-effective MAS strategies that are immediately implementable in most diploid or polyploidy perennial forage breeding programs. Breeding methods developed during the pre-MAS era maximize the ratio between additive variance and the square root of the phenotypic variance (i.e. $h\sigma_A$). With the advent of molecular markers, many new breeding methods based primarily on correlated selection responses between molecular markers and quantitative traits have been proposed (i.e. $r\sigma_A$). MAS strategies should be considered using molecular markers to improve $h\sigma_A$ by enhancing traditional breeding methods. A selection strategy pyramid is envisioned with traditional maternal halfsib selection serving as the base followed by marker assisted paternity selection. With no additional cost within maternal and paternal maximum linkage disequilibrium based MAS strategies can be added. Finally, as the total cost to genotype one individual decreases, residual linkage disequilibrium based MAS strategies can be added to the above mentioned strategies or at some price point supplant these strategies.

2.1 Introduction

In most cases forage varieties available today perform better than varieties developed in the past; much of this increased performance can be attributed to plant breeding. Plant breeding has been part of the human experience since the dawn of agriculture. Starting in the twentieth century, modern plant breeding began exploiting the theory and knowledge of the mode of inheritance to accelerate selection gains. Breeding methods developed during this era typically seek to maximize the ratio between additive variance and the square root of the phenotypic variance (i.e. $h\sigma_A$). With the advent of molecular markers, many new breeding methods based primarily on correlated selection responses between molecular markers and quantitative traits have been

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proposed (i.e. $r\sigma_A$). The advantage of breeding systems built upon correlated selection responses based on molecular markers are that theoretically selection can occur in a non-target environment due to elimination of the phenotypic variation (i.e. σ_P) from the denominator of the selection gain equation and correlation values potentially being up to one (if enough molecular markers are used).

So why have marker assisted selection (MAS) schemes as of yet not been widely incorporated into forage breeding programs, resulting in new varieties? A primary reason is likely the cost associated with genotyping plants using molecular markers. Compared to animal breeding, as well as plant breeding systems involving inbred line development, phenotyping costs in outbred forage species are comparatively much lower. Therefore, MAS schemes are usually not cost competitive, especially in cases where many molecular markers are required. Furthermore, due to the slow pace of genetic gain in highly quantitative traits, such as biomass yield and persistence, it is often difficult to distinguish between elite varieties. This may make it difficult for seed companies to justify increasing costs to accelerate improvement of these traits, as it may be more strategic to invest only enough resources in these traits to remain competitive in the marketplace rather than to excel. Additionally, competitors will almost always be only one selection cycle behind the breeder, making extra cost recuperation for such core quantitative traits difficult, further depressing the incentive to excel. For value-added traits in forages, which are often more expensive to phenotype, MAS schemes become more feasible, especially if premiums for value-added traits can be obtained during seed sales. However, in markets allowing transgenic traits, it is often more beneficial to pursue transgenic approaches, which may have more dramatic phenotypic changes and which usually afford better intellectual property protection and associated ability to recapture costs through premiums.

End users of forages still have an immense interest in core quantitative trait improvement, such as for biomass yield and persistence. In the interest of end users of forage varieties, as well as for the greater good of agriculture, efforts should be made to improve the rate of plant breeding progress. The challenge is to make these improvements in a cost neutral way (i.e., increased efficiency at the same price) so that market-driven plant breeders have an incentive to adopt new methods in order to retain competitive plant breeding programs.

A further challenge and opportunity is the reality of the diseconomy of scale in plant breeding that is a result of the nature of selection intensity (k) (Fig. 2.1).

There is a disincentive to increase selection fractions beyond 1 in 1000 because around this selection fraction, exponential increases in selection fractions increase k-values at sub-linear rates. Therefore, increasing program size is not a cost effective option; rather, selection gains at lower selection intensities need to be increased. Traditionally, molecular markers have been incorporated in schemes to increase the r-values in correlated selection responses in order to eliminate the need for extensive phenotyping. Less attention has been given to the use of molecular markers in increasing selection efficiency, especially in schemes that would necessitate phenotyping. Since forage breeding phenotyping is relatively inexpensive, it makes sense to explore such schemes.

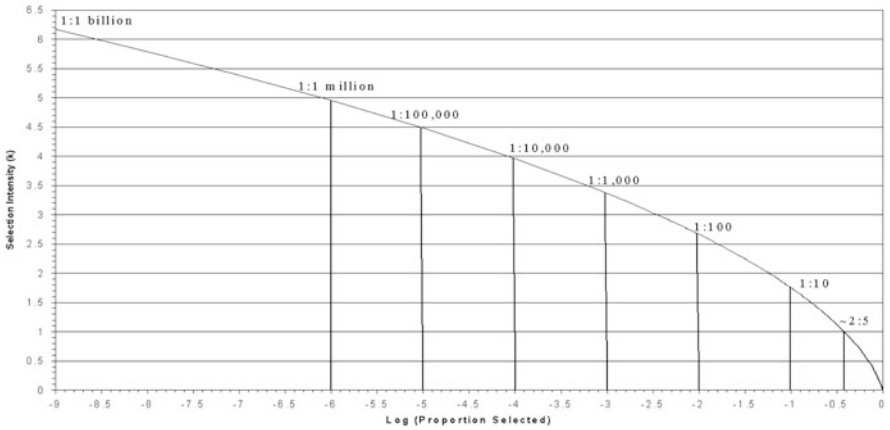

Fig. 2.1 Selection intensity (k) value at various selection proportions (log scale)

2.2 Family Based Selection

In outbred forage species, maternal identity of plants is often known. Paternity, however, is usually unknown. Riday (2011) describes the use of paternity testing to increase selection gains in red clover, a diploid forage legume species. Since the completion of this work, a pilot study was implemented to conduct paternity testing in an autotetraploid alfalfa breeding program. Software was developed and paternal identity of progeny of a 16-parent polycross were ascertained using an exclusion analysis and 16 SSR markers (Riday 2012). These two studies establish that paternity testing is now feasible in any forage species, no matter the ploidy configuration. In most cases, paternity testing should be accomplishable using less than 30 polymorphic molecular markers. Increased selection gain is made by increasing parental control and it contributes to increased efficiency in selection schemes based on $kh\sigma_A$. Riday (2011) describes various ways such information could be incorporated into a breeding program. He suggests that the risk-reward consideration of using this scheme is low while confidence in determining paternity with a set of SSR is very high and genetic gains based on paternal halfsib selection are not affected by any type of marker-quantitative trait linkage considerations.

An extension of this strategy could be applied to the less informative situation where potential number and genotypes of parents of a polycross are unknown. Progeny genotypes from a polycross would be clustered into groups based on molecular markers; these groups would then be defined as pseudo-families. Breeding values would then be assigned to pseudo-families and selection would be conducted among the pseudo-families. Computer programs, such as 'Structure' (Pritchard et al. 2000), for example, could be used to cluster genotypes into groups, with genotypes being assigned to each potential cluster at a given probability and multiple runs of 'Structure' used to determine the most probable number of clusters. (It should be

noted that the instructions for using 'Structure' say the program should not be used on genotypes with known family structure; however since in this case *some* form of clustering is desired and "true" population structure is not relevant, this violation may be acceptable). This procedure was run on 550 progeny of a 19-mother by 96-father polycross. 'Structure' determined that 19 clusters were the most probable number of clusters. There was some correlation between genotype assignment to the 19 clusters and the actual 19 maternal halfsib families of the genotypes. Between pseudo-halfsib family variance in this case was approximately 70 % of the actual known maternal halfsib variance. Due to violations of halfsib family inheritance of additive genetic variance, it is unclear how much, if any, of the 70 % of the variance determined among the pseudo-halfsib families would be predictive of progeny performance in the next generation. If further study shows that some of the between pseudo-halfsib variance is heritable, this would be another potentially quick and easy use of molecular markers to increase selection response.

2.3 Correlated Selection Response Based Selection

With known maternal and paternal identity of plants and with molecular marker information collected to accomplish the paternity test, it becomes a risk free proposition to pursue molecular marker-based correlated selection responses (i.e. even if no linkage is evident between molecular markers and the quantitative traits the molecular markers used for paternity testing). It is currently popular to investigate and describe whole genome selection strategies (Jannink et al. 2010). This is indeed a good strategy, but becomes riskier at lower molecular-marker-genome coverage depending on residual linkage disequilibrium between markers and traits. Such linkage disequilibrium has been reported to be very low in outbred forage species (Isobe et al. 2009). Presented is a potentially less informative correlated selection response strategy that can be used at lower molecular marker density. Rather than selecting on residual linkage disequilibrium (i.e. whole genome selection), maximum linkage disequilibrium is targeted instead, theoretically allowing molecular markers to be used at much lower molecular marker density. The traditional problem with maximum linkage disequilibrium approaches is that trait marker linkage rapidly breaks down at greater recombination distances, making breeding values assigned to specific markers unreliable. However, if the correlated selection response is restricted to distinguishing between the two possible homologues inherited from the same halfsib parent, targeted selection can be conducted at lower risk. Essentially this is a molecular marker-correlated selection response on within halfsib family additive variance. The limitation of this method is that some accompanying phenotypic data is necessary to assign breeding values to heterozygous markers associated with common halfsib parent-derived homologues. Furthermore, the maximum amount of additive genetic variance that can be captured per halfsib parent is one-quarter (i.e. $r1/4\sigma_A$ for the maternally derived marker and $r1/4\sigma_A$ for the paternally derived marker).

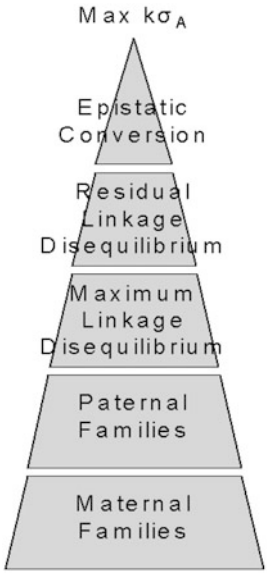

Fig. 2.2 Selection strategy pyramid with *maternal halfsib family* selection at the base followed by various molecular marker assisted selection strategies including: *paternal halfsib selection*, *maximum linkage disequilibrium* based within halfsib family selection, *residual linkage disequilibrium* based selection, and finally *epistatic conversion*

2.4 Combined Methodologies

Halfsib seed-derived maternal family information, molecular marker-derived paternal halfsib information, and maximum linkage disequilibrium based within halfsib family correlated selection response information can all be combined into a common strategy using the four orthogonal or independent partitions of the additive genetic variance. Strategically, the four terms can be viewed as building blocks with maternal identity and accompanying breeding values forming the foundation. Additional resources can then be used to determine paternal identity and within halfsib family maximum linkage disequilibrium correlated selection responses (Fig. 2.2).

The advantage of this strategy is that, depending on the availability of breeding resources and the perceived value of a particular polycross, the resources commitment can be increased or decreased with some additional selection gains realized at each increased level of resource commitment.

If a few molecular markers are known *a priori* to be very close to quantitative trait loci or specific genes such that residual linkage disequilibrium-correlated selection response strategies can be pursued (i.e. $r\sigma_A$), then these could be added to a combined selection strategy in combination with maternal halfsib selection, paternal halfsib selection, and within maternal and paternal molecular marker-based halfsib selection. Based on the cost of each strategy and amount of additive variance described for each selection strategy, optimization equations could be written to pursue the most efficient selection strategy on a cost and selection gain basis. Obviously as genotyping costs decrease and molecular marker densities increase the breeding strategy would move more and more towards whole genome selection. A critical point to consider is that

before committing from the start to a potentially high cost whole genome selection strategy one should consider that there are many molecular marker assisted selection strategies in conjunction with traditional family based selection strategies that can be used in stead of or in conjunction with whole genome selection strategies that may be more cost effective or strategic to achieve improved selection gains (Fig. 2.2).

2.5 Conclusions

A final caution needs to be discussed in relation to the use of molecular marker assisted breeding strategies. Molecular markers used to describe family structure or used to identify correlations with traits under selection are *always* descriptive of genotypic potential; molecular markers *never* determine a plant's genotypic potential. A plant's genotypic potential is always determined during the random genome reshuffling of parental genomes during reproduction (unless transgenic approaches are taken). This means that the perfect genotype will only be observed rarely, irrespective of how many molecular markers are used to identify it (i.e., even if the perfect theoretical genotype is known *a priori*, it still has to be created via random mating and physically identified from among all products of random mating). The core process of extracting DNA from a genotype is not drastically decreasing in cost. Therefore, from a practical perspective, forage breeders will still be evaluating a limited number of selection units in their programs (i.e. 100–100,000). It is therefore unnecessary to have an unlimited number of molecular markers for selection among a smaller number of genotypes since only a certain amount of molecular marker information will be necessary to rank selection units, with less information needed for accurate ranking among fewer genotypes.

It should be apparent that in the combined molecular marker strategy described above, the greatest amount of information per molecular marker will be obtained from an initial set of markers. However, as the number of markers utilized is increased, the amount of information per marker at some point will decrease. The number of markers utilized in a selection strategy should be optimized so the maximum amount of resources can be applied to increase the number of selection units under evaluation. Even under the perfect correlated selection response scheme (i.e. $k\sigma_A$) selection intensity and the amount of additive variance present are still paramount drivers of selection gain.

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Chapter 3
Genome-wide SNP Marker Development and QTL Identification for Genomic Selection in Red Clover

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Abstract Genomic selection (GS) has experienced remarkable advances in genome technologies over the past few years. However, employing GS for forage breeding has been considered difficult because forage species generally show short linkage disequilibrium (LD) across the genome. To elongate the LD, an Advanced Intercross Line (AIL) population was generated from crosses between six individuals originating from Switzerland, Russia and Japan. The suitability of this population was demonstrated for GS or association analysis. For high throughput genotyping, single nucleotide polymorphism (SNP) markers were developed by comparing transcriptome sequences obtained from two red clover individuals. An Illumina Golden Gate platform for 1,536 candidate SNPs was used for polymorphic analysis in the AIL population. A total of 784 SNP markers were identified as polymorphic. In addition, 75 polymorphic SSR markers were used for genotyping the AIL population. Approximately 200 plants each were established in 2010 in the fields of Palampur, Moscow region, Zurich and Chiba. Seed yields, flowering characteristics and morphological traits were evaluated in each region. Significant QTLs and QTL interactions were identified for the traits investigated by GMM analysis. The results suggest that the AIL population can be used for GS and association analysis.

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3.1 Introduction

Genomic selection (GS) has experienced remarkable advances in genome technologies during the past few years. Because of its ability to identify all significant QTLs (Quantitative Trait Loci), including those having small effects, as well as not requiring phenotype validation of breeding populations, GS was expected to become a major technique for molecular breeding (Heffner et al. 2009). Whenever GS is performed, it is essential to consider the relationship between the genetic distance of the markers and the LD (linkage disequilibrium). This is because the exact breeding values are estimated by using DNA markers that have a strong LD with the targeted genes (Meuwissen et al. 2001). The genetic distance showing a significant LD depends upon the targeted species and the type of population. Employing GS for forage breeding has been considered difficult because forage species generally show short LD across the genome.

Establishing populations with family relations is nearly the only approach to elongate the genetic distance showing significant LD. Darvashi and Spoller (1995) proposed using AIL (Advanced Intercrossed Line) populations for more accurate estimations with QTL mapping. AIL populations are produced by randomly and sequentially intercrossing a population that initially originated from a cross between two inbred lines or some variant thereof. Sequential intercrossing is often employed in forage breeding. Therefore, AIL populations may be appropriate for the association mapping and GS of forage species.

In this study, an AIL population was established from crosses between six individuals originating from Switzerland, Russia and Japan, and this AIL population was demonstrated to be suitable for association analysis and future GS. In addition, for high throughput genotyping, single nucleotide polymorphism (SNP) markers were developed to establish a high throughput genotyping system in red clover.

3.2 Materials and Methods

3.2.1 Plant Materials

Three F_1 populations were generated from crosses between 'HR × R130', 'M137 × Violetta6' and 'M366 × M372'. The former two crosses were reported as parental crosses for linkage map construction (Sato et al. 2005, Herrmann et al. 2008). 'M366' and 'M372' were derived from varieties bred in Switzerland. Ten F_1 progeny of each of the three crosses were intercrossed in a field in Sapporo (National Agricultural Research Center for Hokkaido Region) and Zurich. The produced seeds from both countries were used for a second intercross in a greenhouse at Kazusa DNA Research Institute (KDRI). The second polycrossed seeds were designated the 'RC-AIL' population and were used as the mapping population for this study.

3.2.2 Phenotype Investigation

Approximately 200 seeds of RC-AIL were sown in Chiba, Zurich, Shimla and the Moscow Region in the autumn of 2009. The seedlings were transplanted in the field with spaced planting. Morphological traits, flowering time and seed yield were analysed in 2010 according to UPOV guidelines.

3.2.3 SNP Discovery

Transcriptome sequencing of the leaves of two red clover individuals, 'HR' and 'R130', were performed using a Roche 454 GS-FLX Titanium according to the standard protocol provided by Roche Diagnostics. The transcript sequences obtained in this study and the 26,394 ESTs (Expressed Sequence Tags) of red clover published previously (Sato et al. 2005) were assembled using the MIRA 3.0.5 program. Candidate SNPs were identified as base mismatches in the alignment of the transcript sequences. Of the candidate SNPs, 1,536 SNPs were screened based on QV (Quality value) scores and alignment coverage.

3.2.4 Genotype Investigation and Data Analysis

DNA of each plant was isolated from fresh or dried leaves. SNP marker genotyping for each of the 65 plants grown in the four countries was performed using the Illumina Golden Gate® assay according to the provided protocol. In addition to SNP markers, a total of 75 SSR (single sequence repeat) markers, mapped on the red clover consensus linkage map (Isobe et al. 2009) at intervals of 10 cM, were used for genotype analysis using a fragment analyser (ABI3730, Applied Biosystems). GGT 2.0 software was employed to determine the LD between two markers (van Berloo 2008). QTL identification was performed using GMM (Genotype Matrix Mapping) version 2.1 (Isobe et al. 2007).

3.3 Results and Discussion

3.3.1 Phenotype Investigation

The RC-AIL population grew well in Chiba, Zurich and Shimla. However, plants suffered cold temperatures in the Moscow Region, and 108 of the 200 plants died after the first winter. A total of 28 traits were investigated in the four regions (Table 3.1). Of the traits, three traits were investigated in all four regions, while seven,

Table 3.1 List of the investigated traits in four different regions

Traits	Number of investigated regions	Traits	Number of investigated regions
Flowering date	4	Intensity of green colour	1
Seed yield	4	Powdery mildew resistance	1
Plant length	4	Dry plant weight	1
Number of flowerheads	3	Colour of stems	1
Number of stems	3	Hairness	1
Number of internodes	3	Colour of flowers	1
Stem thickness	3	Genestin	1
Length of leaflets	3	3-Hydroxy Biochanin A	1
Width of leaflets	3	Formo Numbernetin	1
Colour of stipules	3	Biochanin A	1
Number of florets	2	Seedling vigour	1
Plant height	2	Vigour after winter	1
Leaf mark	2	Vigour in august	1
Growth habit	2	Vigour in June	1

four and 14 traits were evaluated in three, two and one region, respectively. Large variations were observed in each investigated trait. Examples of trait distributions are shown in Fig. 3.1.

3.3.2 SNP Discovery and Polymorphic Analysis of the RC-AIL Population

The number of transcript sequences read by Roche 454 pyrosequencing were 499,241 and 447,259 in R130 and HR, respectively. Along with the 26,394 published ESTs, the transcript sequences were assembled into 78,835 contigs and 535,128 singlets using MIRA 3.0.5. The total length of the consensus sequences was 45,990,294 bp. The average, longest and N50 sequence were 583, 7,865 and 564 bp, respectively. By comparing the base mismatches of the aligned transcript sequences, a total of 129,019 candidate SNPs were identified between HR and R130. Of the candidate SNPs, those with high quality were screened based on the following criteria: QV of the consensus sequence > 50, QV of the candidate SNPs with two or greater base types > 30, absence of SNPs on 60 bp up and downstream of the candidate SNP. As a result, a total of 2,227 candidate SNPs were screened. Of the screened SNPs, 1,536 were validated as SNP markers in the RC-AIL population and the F_1 progeny of HR × R130. The polymorphic ratios of the tested markers were approximately the same in the two populations: 51.0 % (784/1,536) in RC-AIL and 51.4 % (789/1,536) in HR × R130. Because the number of overlapped polymorphic markers was 412, a total of 1,161 SNP markers confirmed their availability. The majority of SNP markers that showed polymorphisms were mapped onto a red clover linkage map (data not shown).

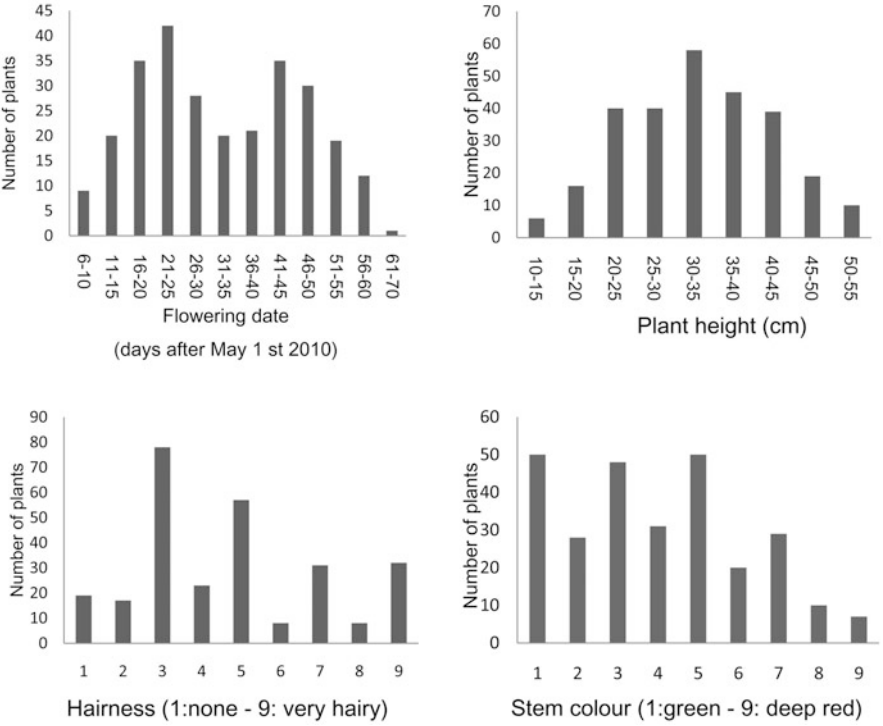

Fig. 3.1 Distribution of the investigated traits in Chiba

Meanwhile, the 75 SSR markers used for polymorphic analysis identified one to 12 alleles per marker. The mean number of alleles identified for a marker was 6.5. The genotype data derived from the 75 SSR and 784 SNP markers was used for the LD investigation and QTL identification. The average genetic distance between two markers was 1.01 cM.

3.3.3 Relationship Between the LD and the Genetic Distance

The relationship between the LD (D′) and the genetic distances of three different types of red clover were investigated. The unrelated population consisted of 1,156 plants derived from 24 varieties. Details of the unrelated population were described by Isobe et al. (2009). Linear regression was observed in a F_1 mapping population ($r = -0.929$) while no significant relationship was observed in an unrelated population ($r = -0.039$). The intensity of the relationship between the LD and the genetic distance in the RC-AIL population was in between that of the F_1 and unrelated populations ($r = -0.24$). This result suggested the possibility of QTL identification in the RC-AIL mapping population using 1 cM interval markers (Fig. 3.2).

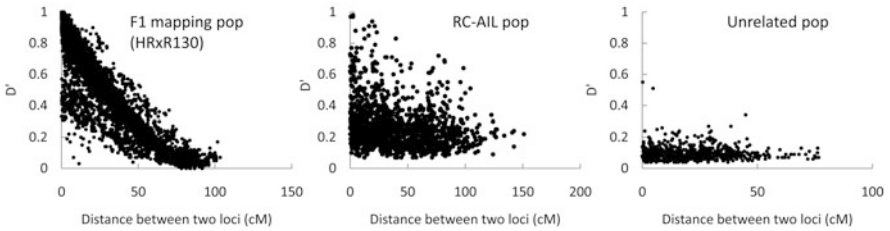

Fig. 3.2 Relationship between the LD (D′) and the genetic distance of two marker loci

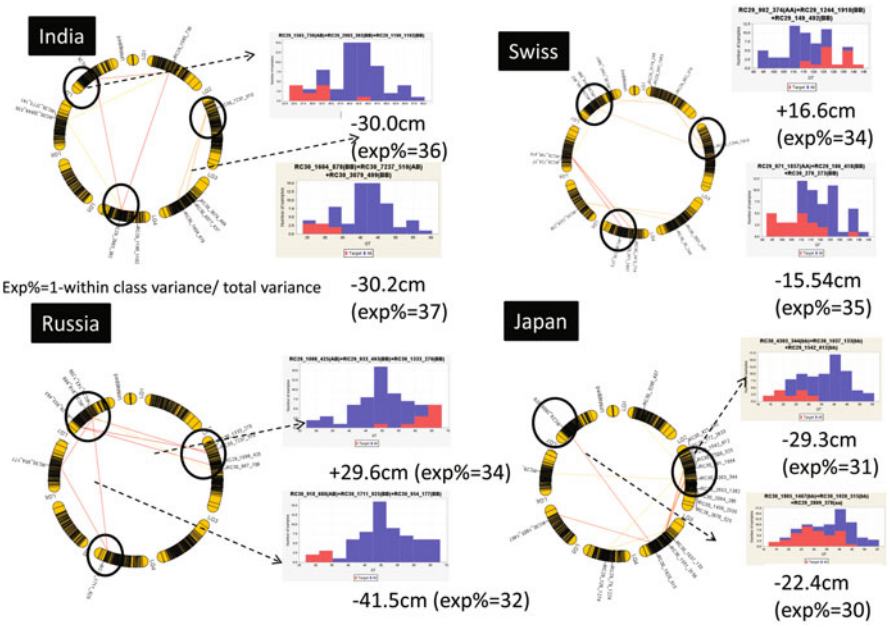

Fig. 3.3 Significant QTLs and QTL interactions for plant length in the RC-AIL population investigated in the four regions. *Large circles* indicate the linkage map of red clover and *small circles* show the common QTLs across four regions. Letters outside of the *large circles* represent marker name identified QTLs and lines inside the *large circles* show significant QTL interactions under three QTL combinations. *Bar graphs* indicate the distribution of phenotype; *red* and *blue bars* show the distribution of selected QTL-allele combinations and others, respectively

3.3.4 QTL Identification

QTL identification was performed for plant length and flowering date (Fig. 3.3 and 3.4). Significant QTLs and QTL interactions were identified for the two traits. QTLs commonly identified across the four regions were for plant length and flowering date. However, the interactions were often region-specific. With regard to plant length, three common QTLs were identified on LG2, LG4 and LG7. The phenotypic

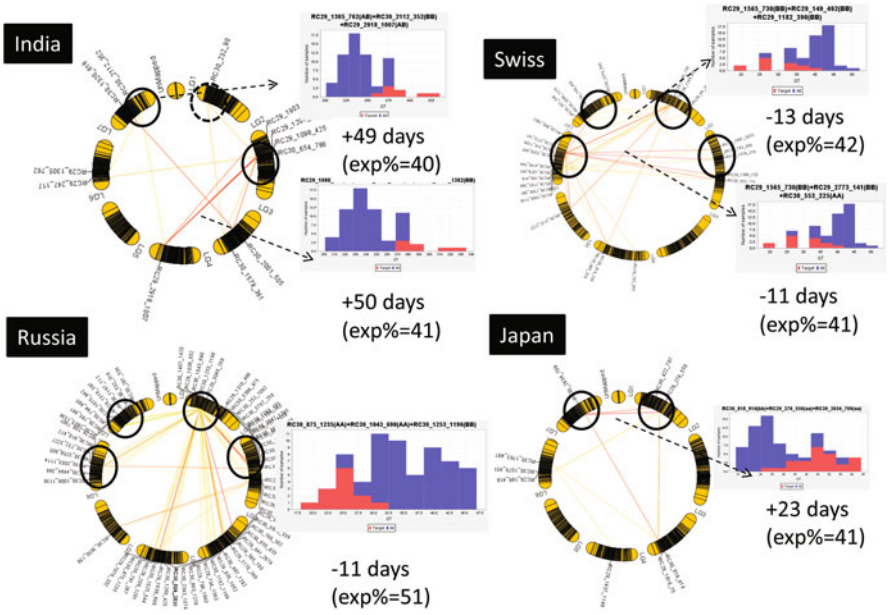

Fig. 3.4 Significant QTLs and QTL interactions for flowering date in the RC-AIL population investigated in the four regions. The details of this figure are the same as that of Fig. 3.3

variance explained percentage of the identified QTL interactions (three QTL combinations) varied from 30–37 %. Most of the significant QTLs showed negative effects, while interactions between LG2 and LG7 showed positive effects. With regard to flowering time, four common QTLs were identified on LG1, LG2, LG6, and LG7. The phenotypic variance explained percentage of the identified QTL interactions (three QTL combinations) was estimated between 40–42 % in Shimla, Zurich and Chiba, while the maximum value in the Moscow region was 51 %. The QTL interactions identified in Shimla and Chiba showed positive effects, and those identified in Zurich and the Moscow region showed negative effects. The large magnitude of the percentage of phenotypic variance suggests that the RC-AIL mapping population is appropriate for genome wide association analysis and GS with fewer than 1,000 DNA markers.

3.4 Conclusion

A total of 1,161 polymorphic SNP markers were developed in this study. The markers will be useful tools for genetic analysis and molecular breeding in red clover. The large phenotype variation of the RC-AIL population suggests its efficiency as a mapping population for association analysis and as a training population for GS. QTL identification was performed for plant height and flowering date, and signifi-

cant QTLs were identified. Of the identified QTLs, common QTLs expressed in four different regions were observed. However, significant QTL interactions were rather region-specific. These results suggest a possible estimation of the breeding value in GS with genotype data of 1-cM intervals using an AIL population.

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Chapter 4
Breeding for Resistance to Bacterial Wilt in Ryegrass: Insights into the Genetic Control of Plant Resistance and Pathogen Virulence

F. Wichmann, F. Widmer, F.-J. Vorhölter, B. Boller and R. Kölliker

Abstract Bacterial wilt caused by *Xanthomonas translucens* pv. *graminis* (*Xtg*) is a severe disease of forage grasses leading to drastic losses in pure and mixed stands. Italian ryegrass (*Lolium multiflorum*) is particularly susceptible to bacterial wilt and breeding for resistance is the only practicable means of disease control. A detailed understanding of the genetic control of this complex host-pathogen interaction is indispensible for the further development of *L. multiflorum* cultivars with increased resistance to bacterial wilt and to refine and optimise breeding procedures. While several recent studies have revealed novel insights on plant resistance, little is known about the processes involved in host-colonization and disease development. Therefore, factors influencing pathogen virulence were investigated in *Xtg* using conserved primer approaches and whole genome sequencing. Knock-out mutation of components of the type three secretion system (T3SS), a major virulence factor in many *Xanthomonas* spp., showed that the T3SS is important for *Xtg* virulence but not for *in planta* multiplication. Analysis of the draft genome sequences revealed a substantial number of effectors apparently characteristic for *Xtg*. In conlusion, our investigations on the *Xtg* x *L. multiflorum* interactions provide fundamental insights for the development of innovative resistance breeding approaches.

4.1 Introduction

Bacterial wilt caused by *Xanthomonas translucens* pathovars is estimated to account for annual forage yield losses of up to 20 % in swards (Schmidt and Nuesch 1980), but yield losses of up to 80 % have been reported from experiments involving artificial leaf inoculation (Wang and Sletten 1995). Of the different pathovars infecting forage grasses, *X. t. pv. graminis* (*Xtg*) is the most abundant (Egli and Schmidt 1982) and has a relatively broad host range including several major forage grass species such as *Lolium multiflorum*, *L. perenne*, *Festuca pratensis*, *Phleum pratense* and

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Poa pratensis. Italian ryegrass (*L. multiflorum*) is particularly susceptible to bacterial wilt caused by *Xtg*. Breeding for resistance to bacterial wilt in ryegrasses is usually based on artificial seedling inoculation and subsequent recurrent phenotypic selection. Although some progress has been achieved (Boller et al. 2009), further advances in breeding programs have often stagnated after several cycles of recurrent selection and susceptible individuals are still observed in advanced breeding populations. Therefore, breeding methods have to be refined and optimised to develop *L. multiflorum* cultivars with increased resistance to bacterial wilt, thereby ensuring productive and sustainable grassland agriculture. Molecular genetic tools for discovering and targeting genes and alleles in germplasm collections and/or breeding lines have the potential to enhance the efficiency of breeding programs through marker assisted selection (MAS). Although a number of quantitative trait loci (QTL) associated with resistance to diseases such as crown rust and bacterial wilt have been identified in *L. perenne* and *L. multiflorum* (Dracatos et al. 2010; Studer et al. 2006), so far no example of successful MAS has been reported for these species. This may arise from the narrow genetic base of mapping families when compared to the range of variation available in the breeding germplasm or the often unknown genetic composition of pathogen populations (Dracatos et al. 2006; Kölliker et al. 2006). Therefore, a detailed characterization of the *Xtg* × *L. multiflorum* interaction has to take into account factors influencing plant resistance as well as factors influencing pathogen virulence. This contribution briefly reviews recent advances towards a better understanding of plant resistance to *Xtg* and then reports on the investigation of potential virulence factors by means of virulence-gene mutagenesis and whole genome sequencing.

4.2 Genetic Control of Bacterial Wilt Resistance in *L. multiflorum*

QTL analyses in a mapping population of *L. multiflorum* have demonstrated that bacterial wilt resistance is controlled by one major QTL on linkage group (LG) 4 explaining between 43 and 84 % of the total phenotypic variance for resistance (Studer et al. 2006). Phenotypic traits that result in the identification of one single QTL explaining such a high percentage of the total observed phenotypic variance have often been shown to be controlled by one or only a few major *R*-genes (Mutlu et al. 2006). In order to investigate whether *Xtg* resistance was based on a major, race-specific resistance gene, 62 selected plant genotypes were artificially inoculated in the greenhouse with six different bacterial isolates (Wichmann et al. 2011b). Significant differences in resistance among *L. multiflorum* genotypes ($p < 0.001$) and in virulence among *Xtg* isolates ($p < 0.001$) were observed using the area under the disease progress curve (AUDPC). The ranking of virulence of the six bacterial isolates in this study was for the most part congruent with the differences in virulence observed by Kölliker et al. (2006), which had been based on three different *L. multiflorum* cultivars. This demonstrates that the bacterial isolates used are reproducibly virulent on different plant material and during different experiments. No significant genotype–

isolate interaction ($p > 0.05$) using linear regression modelling was observed. Simple sequence repeat (SSR) markers were used to identify marker-resistance associations in the same plant genotypes and using the same bacterial isolates. The SSR marker NFA027 located on LG 5 was significantly associated with bacterial wilt resistance across all six bacterial isolates and explained up to 37.4 % of the total variance of AUDPC values. The major QTL on LG 4 (Studer et al. 2006) could not be confirmed in the F_2 progeny derived from the initial mapping population. This may be due to insufficient linkage between the SSR markers used and the QTL and the small number of parental alleles observed in the investigated individuals (Wichmann et al. 2011b). Neither the inoculation experiment nor the SSR analyses revealed major host genotype–pathogen isolate interactions, thus suggesting that *Xtg* resistance, so far observed, is effective across a broad range of different bacterial isolates and plant genotypes.

The partial transcriptomes of two Italian ryegrass genotypes, one resistant and one susceptible to bacterial wilt, were compared at four time points after *Xtg* infection (Wichmann et al. 2011a). The transcriptome analysis of the resistant genotype revealed in total 158 genes differentially expressed after *Xtg* inoculation. Twenty up-regulated genes were observed at 48 hours post infection (hpi), 52 genes were differentially expressed 192 hpi (42 up- and 10 down-regulated), and 124 genes were differentially expressed 288 hpi (76 up- and 48 down-regulated). No significant differential expression was detected 8 hpi (Wichmann et al. 2011a). Recognition of bacterial effector proteins and the induction of HR usually occur within 24 hpi (Scheideler et al. 2002). Thus, transcriptional changes leading to a HR were either absent or below the detection threshold of the microarray analysis. The transcriptome analyses revealed a number of promising candidate genes for bacterial wilt resistance. For example, a gene strongly up-regulated after *Xtg* infection was highly similar to a gene encoding the low silicon transporter Lsi1, a protein which belongs to a Nodulin26-like major intrinsic protein sub-family of aquaporins. Lsi1 has been reported to be involved in silicon (Si) uptake in many plant species and Si is thought to be essential for resistance against biotic and abiotic stress (reviewed in: Ma and Yamaji 2008). Another promising candidate gene found to be up regulated in the resistant genotype after *Xtg* inoculation was the germin-like protein GLP6 (Wichmann et al. 2011a). GLP6 belongs to the oxalate oxidase (OXO)-like GLPs (Carrillo et al. 2009), which have been associated with QTL for disease resistance in rice and barley (Ramalingam et al. 2003). Among others, these genes present promising candidates for further characterization of *Xtg* resistance in *L. multiflorum*.

4.3 Genetic Control of Virulence in *X. translucens* pv. *graminis*

Virulence factors are produced by the pathogen to enable host colonization, to evade or inhibit the host's immune response and to acquire nutrients from the host. They include secretion systems as well as secreted effector proteins. The most extensively studied secretion system of *Xanthomonas* spp. is the type III secretion system (T3SS) which is responsible for the secretion of a broad range of effectors (Cornelis 2006).

The genes encoding the T3SS are typically localised in large gene clusters termed *hrp* (hypersensitivity response and pathogenicity) genes (Arnold et al. 2003). Expression of the *hrp* gene cluster results in the formation of a membrane-spanning secretion apparatus, the Hrp-pilus or type III injectisome, which mediates the delivery of bacterial effectors into the host cell (Cornelis 2006). In *X. campestris* pv. *vesicatoria*, expression of the T3SS gene cluster is induced *in planta* by the HrpG/HrpX two-component regulatory system (Büttner and Bonas 2010). Approximately 20–30 effectors with overlapping activities and diverging composition are typically secreted by one single *Xanthomonas* strain (reviewed in: Büttner and Bonas 2010). These effectors fulfill multiple functions and often are involved in effector triggered susceptibility or the induction of HR on resistant plants (White and Yang 2009). Although some striking similarities in virulence factors exist within the genus *Xanthomonas*, secretion mechanisms and effector repertoires vary greatly among species, pathovars and strains (White et al. 2009). Phylogenetic analyses have shown that *Xtg* is quite distantly related to well characterised xanthomonads such as *X. campestris* pv. *vesicatoria* (*Xca*), *X. oryzae* pv. *oryzae* (*Xoo*) and *X. axonopodis* pv. *citri* (Parkinson et al. 2009). It is therefore expected, that specific and/or novel effectors and secretion pathways are involved in the *Xtg* × *L. multiflorum* interaction. The aim of this study was to clarify the role of the T3SS for virulence of *Xtg* by means of site directed mutagenesis of the regulatory *hrpG* gene and to characterise T3SS components and encoded effector proteins by means of whole genome sequencing and comparative genomics.

4.3.1 Materials and Methods

4.3.1.1 Mutagenesis of *hrpG*

The *hrpG* gene of *Xtg*29 (Kölliker et al. 2006) was sequenced using conserved primers designed on the consensus sequence of publicly available sequences of the *hrpG* gene of other *Xanthomonas* spp. Subsequent primer walking on genomic DNA resulted in a contig of 2,045 bp length. Two DNA fragments of approximately 500 bp of each of the two *hrpG* flanking regions were connected using Soeing PCR (Horton 1995). The resulting fragment referred to as Δ*hrpG* fragment was cloned into the suicide vector pKNG101 (Kaniga et al. 1991) resulting in the plasmid pCC101 (Fig. 4.1). Electrocompetent *Xtg*29 were transformed with the suicide vector pCC101 and the transformants were selected with streptomycin. Double homologous recombination leading to a *hrpG* deletion was induced after culturing in media devoid of streptomycin and plating on sucrose plates. The resulting Δ*hrpG* mutant was verified by PCR.

A highly susceptible *L. multiflorum* genotype was used for virulence screening of the *Xtg29* wildtype and the Δ*hrpG* mutant. A negative control treatment consisted of cutting the plants without inoculum. Assessment of bacterial wilt symptoms was performed using four replications per genotype x treatment combination in a completely randomized block design.

In planta multiplication was assessed by re-isolation of wildtype and mutant strains from inoculated *L. multiflorum* plants grown in replicates under controlled

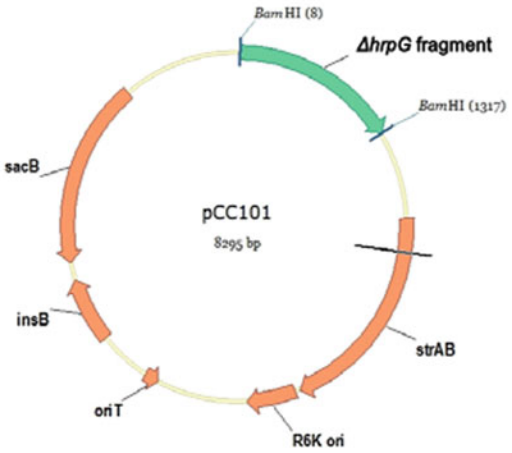

Fig. 4.1 Suicide vector pCC101 derived from pKNG191 containing the Δ*hrpG* fragment

conditions. Leaves and tillers were harvested at four different time points after infection: 6 hours post infection (hpi), 4, 7 and 14 days post infection (dpi). Surface sterilization was performed by incubating the plant material in 1 % Chloramine-T solution (Honeywell Riedel de-Haën, Seelze, Germany) for 10 min. Serial dilutions of the homogenized plant material were prepared on CircleGrow medium (MP Biomedicals) and bacterial cell counts per g of fresh plant material were determined after incubation of the plates at 28 °C for 7 days.

4.3.1.2 Genome sequencing

A draft sequence of the *Xtg*29 (Kölliker et al. 2006) genome was obtained by employing a whole genome shotgun approach by means of 454 sequencing (Roche). For this purpose, a library of paired-end (PE) shotgun fragments was prepared from the genomic DNA. The fragment library was sequenced using the 454 Titanium technology. Genome data were annotated using the GenDB software (Meyer et al. 2003). DNA and protein sequences were compared with other *Xanthomonas* spp. using BLASTn, BLASTx or BLASTp (Altschul et al. 1990).

4.3.2 Results and Discussion

4.3.2.1 Mutagenesis of *hrpG*

A strain of *Xtg*29 deficient of the *hrpG* gene was generated using double homologous recombination after transformation with the pCC101 plasmid (Fig. 4.1) and selection on media containing 5 % sucrose. The different recombination events were verified

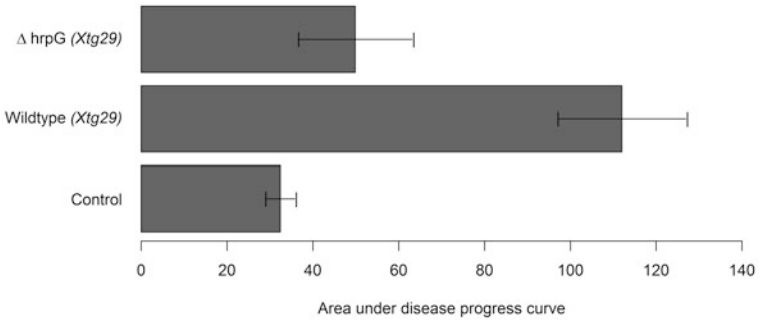

Fig. 4.2 Area under the disease progress curve (AUDPC) values of a highly susceptible *L. multiflorum* genotype inoculated with the *Xtg*29 wildtype and the Δ*hrpG* mutant. Data were collected at 7, 14, 21 and 28 days after inoculation. Standard deviation is indicated by horizontal lines

using PCR and hrpG specific primers. *In vitro* growth of the Δ*hrpG* mutant was not affected by the *hrpG* mutation or the presence of an extrachromosmal plasmid on GYC plates, and the colonies could not be distinguished from the wildtype strain (data not shown).

The symptoms caused by the Δ*hrpG* mutant were significantly weaker than the symptoms caused by the wildtype *Xtg*29 isolate. Average disease scores ranging from 1–9 for the plants infected with the wildtype isolate of *Xanthomonas translucens* pv. *graminis Xtg*29 increased from 2.00 ± 0.82 at 7 days post inoculation (dpi), to 5.00 ± 0.00 at 14 dpi, to 6.00 ± 1.15 at 21 dpi and 6.00 ± 0.82 resulting in an AUDPC value of 112.00 ± 14.85 (Fig. 4.2). On the other hand, disease scores of the plants infected with the Δ*hrpG* mutant ranged from 1.75 ± 0.50 at 7 dpi, to 1.75 ± 0.96 at 14 dpi, 2.25 ± 0.96 at 21 dpi, and 2.75 ± 0.50 at 28 dpi, respectively, resulting in an average AUDPC value of 49.88 ± 13.21. Control-treated plants were described with disease scores 1.00 ± 0.00 at 7 dpi, 1.00 ± 0.00 at 14 dpi, 1.5 ± 0.58, at 21 dpi and 2.25 ± 0.50 at 28 dpi, respectively, resulting in an average AUDPC value of 32.28 ± 3.35.

The fact that symptom development is impaired by site-directed mutagenesis of the *hrpG* gene in *Xtg* indicated that *Xtg* depends on a T3SS for pathogenicity. In order to characterize the role of the *hrpG* gene for *in planta* multiplication of *Xtg* in *L. multiflorum*, both the wildtype and the Δ*hrpG* mutant were re-isolated from infected plant material and population densities were determined. Population densities of the Δ*hrpG* mutant quantified in leaves at 6 h post infection were about 0.9-fold those of the wildtype isolate *Xtg*29 (Fig. 4.3). At 4 days post infection (dpi), both isolates multiplied and reached average $\log_{10}$ population densities per gram of fresh weight of 8.72 for the wildtype and 8.07 for the Δ*hrpG* mutant and a further increase in population densities followed until 14 dpi. These results indicated that the *hrpG* gene is not primarily responsible for survival during the early infection stages. However, when infecting *L. multiflorum* plants with *Xtg*, the leaf tissue is wounded by cutting with scissors. This procedure enables direct contact of the bacterial cells with the site or tissue of multiplication i.e. the xylem. Therefore, we hypothesize that due to

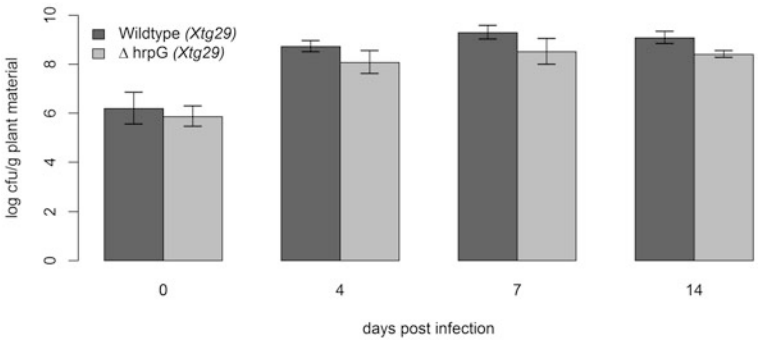

Fig. 4.3 Colonization of *L. multiflorum* by *Xtg29* wildtype and Δ*hrpG* mutant 0–14 days post infection. Population densities at 4 and 14 dpi were significantly different based on a two sided t-test ($p < 0.05$)

the fact that *Xtg* cells gain direct access to the xylem, *Xtg* can make use of nutrients available inside the host throughout the infection process.

4.3.2.2 Genome sequencing

Preliminary analysis of the data obtained for *Xtg29* resulted in a draft sequence consisting of 908 contigs with gaps between the contigs and a G + C content of 68.6 % was calculated. This G + C content is higher than in all the previously determined complete genomes of *Xanthomonas* spp., where the G + C content was well conserved at about 63–65 %. The number of insertion sequence (IS) elements or IS element fragments in the genome of *Xtg*29 is estimated to develop into several hundred copies. High numbers of both complete and fragmentary IS elements have been observed for all *Xoo* genomes so far, as well as for *X. o.* pv. *oryzicola*. Hence, this finding relates *Xtg* to the rice pathogenic *Xanthomonas* strains, where the number of IS elements ranges from 251 complete to 714 fragments of IS elements. Predicted genome size of *Xtg29* is 5,057,066 bp consisting of one single, circular chromosome and no plasmids.

Despite a number of similarities to the other sequenced *Xanthomonas* spp. (e.g. the *gum* gene cluster, EPS production and conserved *hrp* genes), the isolate *Xtg*29 was found to be different from the other sequenced *Xanthomonas* spp. in terms of T3SS architecture and protein sequence identities. The two genes *hrpG* and *hrpX* encoding the two response regulators of the T3SS were found to be localized within the *hrp* gene cluster in *Xtg*29 which is in contrast to the other sequenced *Xanthomonas* spp., where *hrpG* and *hrpX* are situated together and outside of the *hrp* gene cluster. A similar architecture with the genes encoding *hrpG* and *hrpB* (which is homologous to *hrpX* of *Xanthomonas* spp.) within the T3SS has been reported for the *Ralstonia solanacearum* isolate GMI1000 for which the T3SS has been shown to be located on the megaplasmid pGMI1000MP (Salanoubat et al. 2002). A set of homologous genes

encoding T3SS secreted effectors that certainly is important for the interaction of *Xtg* with *L. multiflorum* was found. These effectors may be specific for this host-pathogen interaction and may also determine the host range of *Xtg*. Nevertheless, whether these homologs are functional and/or race-specific for the isolate *Xtg*29 or are also found in other *Xtg* isolates will require further experimental data and comparative analysis with other *Xtg* strains and *X.t.* pathovars. From the data obtained so far, it can be speculated that not only virulence factors and symptoms may be distinct for the *L. multiflorum*-*Xtg* interaction, but also that resistance mechanisms may differ from well characterised systems such as rice-*Xoo* and pepper-*Xcv*.

4.4 Conclusions

The genomic and transcriptomic approaches undertaken so far have revealed valuable insights into bacterial wilt resistance of *L. multiflorum* and *Xtg* virulence. Taken together, phenotypic and molecular genetic characterization as well as the transcriptome analyses indicated that *Xtg* resistance is conferred by various resistance mechanisms and genes that contribute to resistance quantitatively. Two independent analyses revealed two different genomic regions to contribute to a significant degree to *Xtg* resistance. In addition, the transcriptome analyses revealed two interesting candidate genes for *Xtg* resistance such as *Lsi1* and *GLP6*. To shed light on the *L. multiflorum*-*Xtg* interaction from a different perspective, virulence factors of *Xtg* were investigated. Interactions between *R. solanacearum* and solanaceous crops may serve as more suitable models due to comparative analyses that revealed a T3SS with a genetic organization different from other sequenced *Xanthomonas* spp., but very similar to the β-proteobacterial plant pathogen *Ralstonia solanacearum*. In contrast to other *hrpG* deficient *Xanthomonas* strains of other species, the *hrpG* gene of *Xtg*29 is not crucial for *in planta* multiplication and survival, highlighting the distinctness of the *Xtg*-*L. multiflorum* interaction. Combined, the approaches of *L. multiflorum* resistance mechanisms and *Xtg* virulence factors have allowed to gain a more profound understanding of this host-pathogen system and will enable the identification of further candidate genes and the development of MAS tools in the future.

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Chapter 5
Mechanisms Utilised Within the IBERS Diploid *Lolium perenne* L. Forage Grass Breeding Programmes to Improve Rumen Nitrogen Use Efficiency

R. C. Hayes, J. A. Lovatt and M. T. Abberton

Abstract Researchers at Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, have completed 3 years of a 5 year LINK project, sponsored by Defra through the Sustainable Livestock Production LINK programme (www.greener-grasslands.ibers.aber.ac.uk) which aims to breed new forages that will reduce the environmental footprint of livestock production. One of the key objectives is to improve nitrogen use efficiency (NUE) in the rumen through the breeding of new forage grasses to improve protein utilisation. Previously it has been shown that feeding ryegrasses with higher water soluble carbohydrate content leads to improved rumen efficiency and evidence suggests that this results in increased meat and milk production and reduced nitrogen losses with lower ammonia and nitrous oxide emissions through improved protein utilisation. IBERS diploid perennial ryegrass breeding programmes involve a combination of spaced plant assessment and half-sibling plot performance as a basis for recurrent selection over many generations, the current focus of which is to combine increased NUE along with other desirable agronomic traits including improved yields, increased persistency and higher dry matter digestibility.

5.1 Introduction

Researchers at Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, have completed 3 years of a 5 year LINK project, sponsored by Defra and industry partners through the Sustainable Livestock Production (SLP) LINK programme (www.greener-grasslands.ibers.aber.ac.uk). One of the key objectives is to enhance nitrogen use efficiency in the rumen through the breeding of new forage grasses to improve protein utilisation. Feeding ryegrasses with higher water soluble carbohydrate (WSC) content has been shown to lead to improved rumen

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efficiency (allowing increased microbial protein synthesis) and evidence suggests that this results in reduced nitrogen (N) losses through lower ammonia and nitrous oxide emissions via improved protein utilisation (Lee et al. 2003; Moorby et al. 2006)

Ruminant agriculture plays a significant role in contributing to greenhouse gas emissions, estimated at 18 % of anthropogenic emissions worldwide (FAO 2006), and environmental damage through N losses from excreta, both in terms of losses to air as ammonia and nitrous oxide, and as nitrates leaching into groundwater. Previous breeding targets at IBERS have focused on the genetic improvement of ryegrass for traits to improve NUE in the rumen, for example ryegrass varieties that incorporate increased dry matter digestibility (DMD) and WSC content have already been developed and marketed. Previous studies and models have demonstrated that these grasses supply energy to rumen microbes at the time required for microbial protein synthesis which increases rumen NUE (Lee et al. 2002, Ellis et al. 2011). There is a need to build on this proof of concept and breeding progress to develop grasses that realise the full potential of increased WSC levels through an optimised energy to protein ratio.

IBERS' diploid perennial ryegrass breeding programmes involve comprehensive trialling and assessment of spaced plants and half-sibling plot performance as a basis for recurrent selection over many generations. The diploid intermediate heading date breeding programme is now in the 14th generation of selection; some of the successful varieties from past generations include 'AberDart' (F7), 'AberStar' (F9), 'AberMagic' (F10) and the most recent commercial variety, 'AberGreen' (F11). Current diploid IBERS varieties are recognised via Recommended List trials as some of the best in their category throughout the UK. In this paper the variety progress achieved during the 12th generation will be discussed, in addition, predictions of performance for the newest varieties synthesised from the 13th generation of selection in 2011 will be reviewed.

5.2 Materials and Methods

A breeding trial (13th generation, intermediate diploid *Lolium perenne* L.) was undertaken at IBERS, Gogerddan, Aberystwyth, Wales. The soil type was a Rheidol series. The breeding trial was undertaken over two cutting seasons (March to November in 2009 and 2010). A seed rate of 30 kg/ha was used with broadcast sowing during autumn 2008 to prepare four randomised complete block replicated plots per half-sibling family or control variety measuring 1×1.2 m. Fertiliser rates of 190 kg/ha/year N, 35 kg/ha/year P, 120 kg/ha/year K and 25 kg/ha/year sulphur were applied as a split dressing after each cut. A seven cut management was used as an intermediary treatment between conservation and grazing managements and cut using a Haldrup plot harvester. Assessment of forage yield over 2 years was made, additionally, quality data including N, WSC, dry matter digestibility (DMD) and fibre fraction, were collected in year one from cuts three, four and five and analysed using

laboratory based NIRS, 10 % of samples underwent chemical analysis to confirm the NIRS calibrations. The breeding trial included five control varieties (cv. 'AberDart', 'AberMagic', 'Premium', 'Ba14074 Syn1' and 'Ba14074 Syn2') and 55, 13th generation, intermediate heading half-sibling progeny sets, generated from crossing 240 selected 12th generation half-sibling progeny, with equal representation of 60 progeny from each of four 12th generation families.

New varieties were synthesised by selecting four mother plants based upon the above range of desirable traits that had been identified via half-sibling progeny plot trials. Most traits were quantitatively assessed to determine heritability scores and significant differences. Predictions of performance for new synthetic varieties were obtained by calculating the mean score for the four selected mother plants for each trait from the half-sibling progeny breeding trial results. Each chosen mother plant was cloned and an equal number of each polycrossed in pollen proof isolation chambers. Seed was harvested, threshed and cleaned and the Syn1 variety seed stock generated using equal quantities from each mother plant.

The synthetic variety 'Ba14074' was selected and produced during the 12th generation of selection using the same breeding method described above for the 13th generation. Syn 1 and Syn 2 generations of 'Ba14074' were assessed in evaluation trials sown in 2007 and 2008 respectively, at IBERS using an experimental design of four randomised complete blocks against a range of commercial varieties. The cutting regime used was five cuts in years 1 and 3 to simulate conservation management and nine cuts in year 2 for simulated grazing. Quality measurements were taken from cuts one and two in year 1 and obtained using laboratory based NIRS, 10 % of samples underwent chemical analysis to confirm the NIRS calibrations. Fertiliser rates of 165 kg/ha/year N, 30 kg/ha/year P, 105 kg/ha/year K and 20 kg/ha/year sulphur were applied as a split dressing after each cut.

The synthetic varieties, Ba14150–152, were selected and produced from the 13th generation of selection from the intermediate heading breeding programme using the same methodology described above. Predictions of performance for the new varieties were obtained as previously described.

5.3 Results

The variety, 'Ba14074', demonstrates the accuracy of predictions obtained from the results of 12th generation half-sibling recurrent selection breeding plot trials as a basis for varietal construction when compared to actual performance of the finished variety (results for Syn 1 and Syn 2 generations did not significantly differ) in IBERS evaluation plot trials (Fig. 5.1). The results presented in Fig. 5.1 also show the gains in overall performance of 'Ba14074' (F12) compared to 'AberDart' (F7) for a range of traits from IBERS evaluation trials; dry matter yield, WSC and ground cover. For the above traits the values obtained from IBERS evaluation trials were at least as good as or better than those previously predicted for 'Ba14074', predictions of

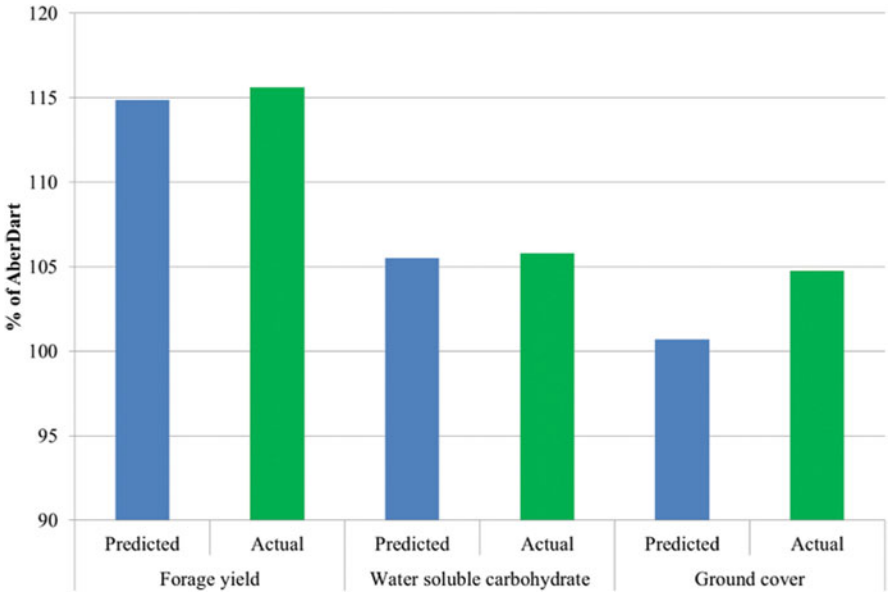

Fig. 5.1 Predicted and actual agronomic performance of 'Ba14074' in IBERS trials, performance is presented for 'Ba14074' as a percentage of that achieved by 'AberDart' in the same breeding and evaluation trials ('AberDart' = 100 %)

performance for 'Ba14074' were obtained as described in the materials and methods section.

WSC and DMD measurements of the 13th generation half-sibling progeny plot breeding trials exhibited a wide range of variation (Fig. 5.2), with a number of populations showing improvements over 'AberMagic', thus enabling the selection of mother plants to synthesise varieties which are predicted to have an energy content better optimised for ruminant digestion. Many of the mother plants observed with the desired WSC content were also seen to exhibit many additional desirable agronomic traits as well as variation in protein content, thus enabling the selection of three sets of four elite mother plant clones to synthesise three new 13th generation intermediate heading varieties (Ba14150–152).

Performance predictions were calculated for three new 13th generation synthetic varieties as presented in Fig. 5.3, in comparison with the performance of 'Ba14074' (Syn 1 and Syn 2 generations) from the 13th generation breeding trial. Calculated performance predictions were used to determine the best combination of mother plants to produce new varieties, in this instance the varieties were all targeted at slightly different markets and end use. 'Ba14150' was selected principally for very high yield (5.2 % above 'Ba14074') and WSC (10.2 % above 'Ba14074') to be used as a biofuel source as well as determining how far the WSC trait can be increased. 'Ba14151' was selected to provide excellent all round performance, with very high ground cover (4.2 % above 'Ba14074'), an optimised energy to protein ratio and superior seed production potential. 'Ba14152' was designed to extend the grazing

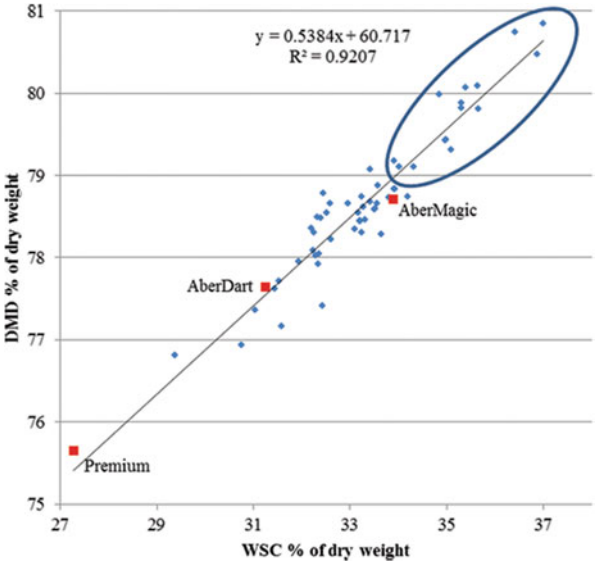

Fig. 5.2 Variation in WSC and DMD within the 13th generation of selection of IBERS intermediate diploid *Lolium perenne* L. breeding population compared to three commercial control varieties. The *oval* highlights populations' superior to 'AberMagic', the *blue dots* represent the scores assigned to each of 55 mother plants from the 13th generation half-sibling progeny breeding plot trial

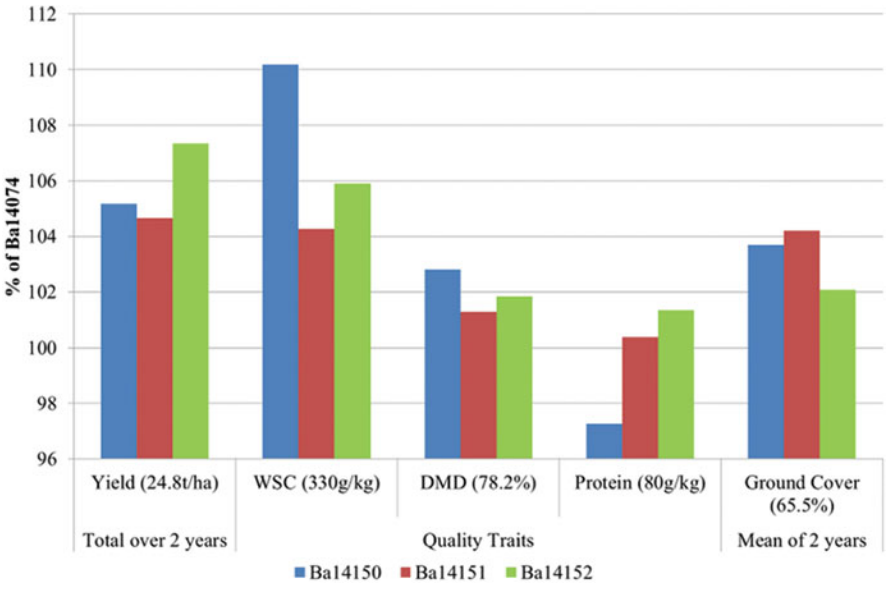

Fig. 5.3 Predicted agronomic performance of three new intermediate diploid varieties synthesised from the 13th generation, all were designed to optimise the ratio of energy to protein. Values in parenthesis were used as the 100 % baseline and were obtained as a mean of Syn 1 and Syn 2 'Ba14074' from the breeding trial

season with enhanced spring and autumn growth, giving a total yield 7.3 % greater than seen for 'Ba14074', in conjunction with superb all round performance and optimal WSC to protein content.

5.4 Discussion

During the project a number of new varieties of ryegrass were synthesised from the intermediate heading diploid breeding programme that seek to optimise the energy to protein ratio in forage, thus enhancing rumen efficiency; this optimisation is in conjunction with gains to yield and superior all round agronomic performance. The three predictions of varietal performance derived from the intermediate population illustrate the value of half-sibling recurrent selection as a breeding tool. This methodology provides access to a genetic base that has been improved over many generations to select from, and enables the identification of multiple desirable traits in selected genotypes through phenotypic selection for mother plant selection and variety synthesis.

Future breeding objectives at IBERS for forage grasses include improvements to N and P utilisation and recycling, increasing polyunsaturated fatty acid content, specifically that of alpha linolenic acid, improving seed yield and enhancing the digestible to non-digestible fibre fraction. These traits will provide greater added value to farmers and reduce the environmental impact of ruminant farming in addition to providing all-round improved forage performance and quality.

In addition to ryegrass breeding, complementary research at IBERS has investigated the variation in leaf protein content of elite genotypes of white clover. This has led to the development of a lower protein variety. Leys of new ryegrass varieties and the reduced protein clover are predicted to enhance rumen NUE beyond levels currently obtainable with existing grazing options. Animal trials will explore more definitively the relationship between the ratio of WSC and protein in the diet (at different WSC and protein levels), excretion of urinary N, milk yield and quality using mixtures of high WSC grass varieties and white clover varieties selected for lower protein content.

5.5 Conclusions

Half-sibling recurrent selection has been shown to be a powerful breeder's tool, without which improvements seen in modern IBERS varieties would not have been possible. IBERS' ryegrass breeding programmes have been seen to advance important agronomic traits and add additional benefits both to the farmer, in the form of improved meat and milk production from grazed grass, and also to the environment by reducing the proportion of nitrogen lost as ammonia and nitrous oxide during rumen digestion. The commercialisation of new IBERS' varieties is expected to assist farmers with maximising profits from grazed forage.

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Chapter 6
Population Genetics of the Grass Self-incompatibility System—Practical Implications for Grass Breeding Programmes

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Abstract Self-incompatibility (SI) is a mechanism that prevents plants from self-pollination through molecular recognition between pollen and pistil; but neither the genes nor the proteins involved are known. Determining haplotype diversity of the SI locus region is an indirect way of predicting incompatibility genotype which would enable plant breeders to develop strategies to ensure precision crossing for exploiting heterotic combinations. In the case of ryegrass (*Lolium perenne*), SI is known to be a gametophytic reaction involving two loci, *S* and *Z*. This paper reports on an assay of marker allele diversity around the *Z* locus for a population produced from twelve generations of mixed and half-sib family selection. Using conventional genotyping of marker length polymorphisms, as well as high resolution melt (HRM) curve analysis, the 55 plants of the population were classified into thirteen groups according to their genotypes for each marker. HRM genotyping proved to be more discriminating than STS markers and could enhance the precision of *Z* genotype prediction. Although half-sib family selection is expected to lead to allele fixing, this initial analysis was encouraging in that it indicated high haplotype diversity around the *Z*-locus suggesting maintenance of a high degree of cross-compatibility in the breeding population. This would be expected as SI alleles are subjected to frequency dependent selection.

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Keywords Haplotype diversity · High resolution melting · Frequency dependent selection · Plant breeding · *Lolium perenne*

6.1 Introduction

Many plants possess self-incompatibility (SI) mechanisms that prevent inbreeding by blocking fertilisation of ovules by like-pollen. These mechanisms have evolved several times and a number of different mechanisms have been described (Franklin-Tong 2008). Related species often possess the same mechanism, and in the grass family, the SI system is controlled gametophytically by two complementary loci, *S* and *Z*, which both consist of a highly diverse series of alleles. Incompatible pollen (determined by the possession of an *S*-*Z* allele pair from the individual pollen gametophyte matching that of any of the allele pairs of the receptive stigma) is arrested very quickly after alighting on the stigmatic surface and is characterised by pollen-tube tip arrest and massive callose occlusion of the pollen grain.

Genes controlling SI are subject to negative frequency dependent selection (FDS) where rare alleles are at a selective advantage and are maintained in plant populations. This has led to the evolution of a highly polymorphic series of alleles. According to theory (Wright 1939; Castric and Vekemans 2004) *S*-alleles in single gene systems are expected to be subject to balanced selection, where, over time, new alleles accumulate until their frequency is equal to all other alleles in the population.

In the grasses there are only two published examples of estimates of the number and frequency of *S*- and *Z*-alleles in populations, both having been documented in perennial ryegrass (*Lolium perenne* L.). One of these is of a natural hay meadow population (Fearon et al. 1994) and the other is of a synthetic cultivar (Devey et al. 1994). Both cases were characterised by having large numbers of both *S* and *Z* alleles but with markedly different allele frequencies.

It is an extremely laborious process to genotype SI loci in plants possessing multi-locus SI systems by intra-population pollination tests. But with the advent of technology to easily and cheaply generate polymorphic molecular marker data, and the development of physical marker maps for both *S* and *Z* loci, screening for allelic variation at grass SI loci is made easier.

We are endeavouring to develop polymorphic molecular markers, closely linked to *S* and *Z* that can be used to predict *S* and *Z* genotypes and enable us to study *S* and *Z* allele diversity in *Lolium perenne* breeding populations. This will enable us to gather and analyse more empirical data to help us to understand the population genetics of SI in multi-locus systems and compare it to theoretical expectations based on FDS. We are developing a marker screening strategy using a *Lolium perenne* population produced at the Institute of Biological, Environmental and Rural Sciences, Aberystwyth University from nine base plants over five cycles of mixed selection followed by seven cycles of multiple half-sib family selection. Such a selection procedure is likely to lead to significant fixation of neutral alleles. However, SI allele diversity might be expected to be maintained as it is influenced by negative FDS.

The aim of our study, using a 'test' population was to be able to assess *Z* region haplotype or haplotype-combination diversity and ultimately predict *Z* genotypes of individuals and allele numbers and frequencies in our test population. We were particularly intrigued by the possibility of using HRM curves to differentiate PCR products to enhance our ability to distinguish polymorphic regions of our DNA region of interest, both simply and cheaply.

6.2 Materials and Methods

We studied 55 inter-related plants which were the half-sib progeny of four mother plants that were derived from twelve previous generations of mixed (first five generations) and half-sib family (next seven generations) selection. A single genotype progenitor of a top-cross between a competitor's commercial variety and AberDart was added to the mother plants at the 9th and another at the 10th generation. In total, the breeding population was derived from nine genotypes. Plant genotyping was done using twelve markers covering a physical genomic distance of approximately 200 Kb flanking the *Z* locus. Two of these markers were STS markers, that showed length polymorphisms during capillary electrophoresis and ten were PCR-amplified markers that revealed contrasting high resolution DNA melting (HRM) curves. The twelve markers covered a physical genomic region of 200 kb around the *Z*-locus of *Lolium perenne*.

For the capillarity electrophoresis, PCR reactions were made on fluorescent primers, the fluorescent products of which were detected using the ABI3130, Applied Biosystems, Foster City, CA, USA system. For HRM analysis the PCR products are heated causing the dissociation of the double strand DNA, accompanied by a decrease in fluorescence. The degree of fluorescence is recorded continuously over time and relates to the rates of double strand dissociation. Fluorescence curves are produced that vary depending on the molecular structure of the DNA fragments, including the number of polymorphisms present in the PCR amplicon, fragment length and base-pair constitution. Such melt curves will therefore be able to resolve any type of polymorphism due to genotypic differences. For the HRM, PCRs were performed according to Studer et al. (2009). In short, master mix containing the fluorescent dye LCgreen (1X LightScanner Master Mix, Idaho Technology Inc., Salt Lake City, UT, USA) was used.

6.3 Results

The two STS markers were genotyped based on fragment size differences revealed using Genemapper software (example illustrated in Fig. 6.1a). With three exceptions, genotypes could be allocated to each plant for the STS2 marker. In contrast, HRM markers revealed a complete data set, as illustrated for the marker HRM8 in Fig. 6.1b.

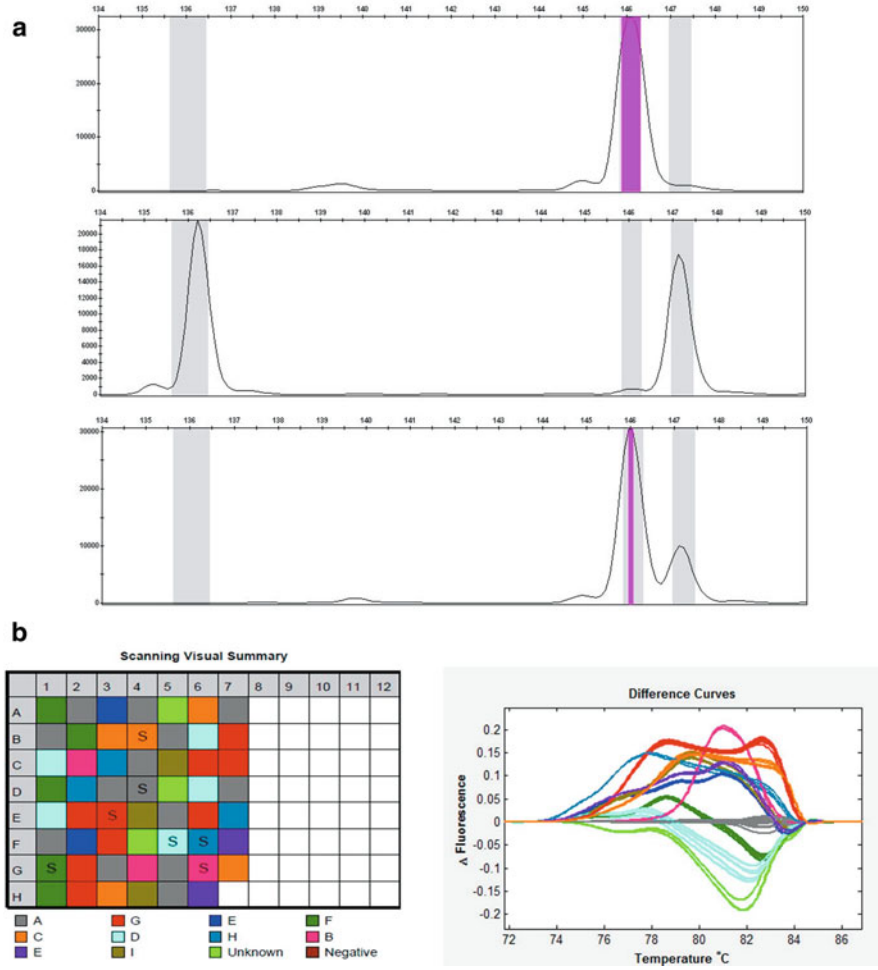

Fig. 6.1 Comparison of the result formats between STS marker (**a**) and HRM markers (**b**). **a** represents the allele size and intensity of the marker STS 1 for 3 different plants of the population using Genemapper 3.7. **b** represents the results given by the LightScanner® System software for the marker HRM8. Each coloured curve represents one genotype (haplotype combination)

In order to estimate the total number of genotypes at Z, genotyping data for each HRM and STS marker were grouped according to their melting curve profiles and STS genotypes, respectively, and we identified a total of 13 haplotype combinations (genotypes) (Table 6.1).

Some markers were more discriminatory than others. The most discriminatory markers were STS2, HRM8 and HRM9 where, in combination, all the 13 genotypes could be distinguished (Table 6.1). Moreover, one recombination event within the 200 Kb flanking the *Z* locus was detected in the breeding population.

Table 6.1 Classification of the 55 plants into different groups according to HRM and STS markers. We have represented the groupings based on HRM markers 8 and 9 and the STS2 marker. Other markers followed similar patterns but, in almost all cases, were less discriminatory. The colours in the table indicate the different genotype for each marker but there is no relation between colours of different markers. The capital letters differentiate genotypes, lower case letters indicate alleles

	HRM8	STS2	HRM9
1	A	aa	A
	A	aa	A
	A	aa	A
	A	aa	A
2	I	aa	A
	I	aa	A
	I	aa	A
	I	aa	A
	I		A
3	F	aa	E
	F	aa	E
	F	aa	E
4	H	ab	A
	H	ab	A
	H	ab	A
	H	ab	A
5	G	ab	F
	G		F
	G	ab	F
	G	ab	F
	G	ab	F
	G	ab	F
	G	ab	F
	G	ab	F
	G	ab	F
6	A	ac	A
	A	ac	A
7	C	ad	A
	C	ad	A
	C	ad	A
	C	ad	A
	C	ad	A
8	D	ad	B
	D	ad	B
	D		B
	D	ad	B
	D	ad	B
9	A	bb	A
	A	bb	A
10	E	bb	D
	E	bb	D
	E	bb	D
	E	bb	D
1	B	bd	A
	B	bd	A
	B	bd	A
12	A	dd	B
	A	dd	B
	A	dd	B
	A	dd	B
	A	dd	B
	A	dd	B
13	A	dd	C
	A	dd	C
	A	dd	C

6.4 Discussion

In a pilot attempt to genotype the *Z* SI locus we investigated twelve genetic markers covering a 200 Kb stretch of DNA around the Z locus. We estimate that, in generating our population of 55 plants, there have been around 4,000 opportunities for recombination events to take place over the thirteen generations of selection. In independent mapping studies with an unrelated mapping population we have screened over 5,000 plants for markers in the same region and have rarely found recombinants between the most distant markers. Therefore we speculate that the genotypes we have identified are unlikely to have been created subsequent to a recombination event. These genotypes therefore represent either the genotypes of the original parents of the first cross, or genotypes that have been ingressed either deliberately (one genotype at F9 and one at F10) or by accident.

We used two STS markers that discriminated alleles based on DNA fragment lengths. However we are aware that such markers will not reveal polymorphisms that are based on base pair constitution (e.g. SNP variation) rather than base pair number. The analysis of PCR products by HRM technology has a far greater discriminatory power as fluorescence curves are influenced by base-pair constitution and even epigenetic factors such as DNA methylation.

Our HRM marker genotypes matched with our STS markers in complete linkage disequilibrium. But further, they were more discriminatory: we were able to separate a group of plants into three genotype groupings where the two STS markers could only distinguish two. We therefore suggest that markers based on HRM can provide a useful tool for genotyping plants, maximising allelic diversity revealed.

With thirteen different *Z* genotypes revealed, we suggest that a minimum of five different *Z* alleles are present. The frequencies of individual *Z* genotypes (and alleles) are significantly different, most likely due to founder effects and genetic drift, yet *Z* allele diversity alone (regardless of what the situation for the *S* locus is, and we do not expect it to be very different!) in this population is sufficient to allay fears of a limitation imposed on intra-population-compatibility and seed production potential.

We are in a position to predict the *Z* genotypes of our 55 plants but we need to be able to empirically test these predictions by carrying out semi-*in-vivo* test pollinations. However, as the SI reaction is dependent on complementary action of the *S* locus, this needs to be done in combination with knowledge of the *S* genotypes of our plants. We plan to use a similar screening approach to analyse *S* locus genotype variation.

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Chapter 7
Use of Molecular Marker Information in the Construction of Polycrosses to Enhance Yield in a *Lolium perenne* Breeding Programme

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Abstract The aim of this study is to evaluate the use of molecular marker information in the construction of polycrosses to enhance yield in a diploid *Lolium perenne* breeding programme. The starting material used for this study consisted of 30 diploid late perennial ryegrass plants selected from advanced breeding material. Pairwise Jaccard similarities among these 30 genotypes were determined on the basis of 559 AFLP markers. Jaccard similarities varied between 0.334 and 0.686, with a mean value of 0.467. Nine polycrosses (PC) of four parental plants with different levels of genetic similarity were composed in 2007: three wide PC with low Jaccard similarities between parental plants (0.334–0.447), three medium PC with intermediate Jaccard similarities (0.405–0.540) and three narrow PC with high Jaccard similarities (0.523–0.686). Some parental plants were used in more than one polycross. The seed was harvested in 2008 and a progeny evaluation trial was sown in April 2009. The dry matter yield was measured in the growing season of 2010 and will be measured in 2011. Progenies from medium and wide polycrosses were on average significantly higher yielding than the progenies from the narrow polycrosses (yield increase of 4.2 % and 6.1 % respectively) in 2010. There was a trend but not significant that the yield of the progenies of the same parental plant from wider polycrosses was higher compared to progenies from narrow polycrosses. We may conclude that the application of molecular markers to select genetically diverse polycross parents can result in an average yield increase.

7.1 Introduction

Perennial ryegrass is a wind-pollinated species and cultivars are usually produced through random mating of selected parental plants resulting in population-based synthetic cultivars. A high level of heterozygosity should be obtained to maximize the agronomic performance. The parental plants are usually selected based on phenotypic characteristics determined by visual scoring of desirable traits. Molecular markers offer powerful tools for the analysis of genetic diversity independent of the

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Table 7.1 Parental component number and pairwise Jaccard similarity coefficients between the 4 parental clones used in nine polycrosses (PC)

PC number	PC type	Comp. Number				Jacc. sim. coeff.	
		1	2	3	4	Mean	Range
1	Wide	2	3	12	15	0.386	0.343–0.435
2	Wide	3	20	26	28	0.389	0.334–0.429
3	Wide	2	11	19	28	0.410	0.388–0.447
4	Medium	10	11	29	30	0.463	0.422–0.534
5	Medium	5	8	9	12	0.463	0.405–0.502
6	Medium	13	19	24	25	0.463	0.412–0.540
7	Narrow	9	11	17	27	0.570	0.523–0.651
8	Narrow	10	14	15	16	0.581	0.549–0.686
9	Narrow	9	10	13	14	0.637	0.566–0.686

environment. The use of molecular marker information in the parental selection may optimise the parental combinations to maximize heterosis. According to Kölliker et al. (2005) application of molecular markers to select genetically diverse polycross parents can result in an average yield increase. In this study molecular markers were used to compose polycrosses with different levels of genetic diversity. The progenies are being evaluated in a yield trial.

7.2 Material and Methods

The starting material used for this study consisted of 30 genotypes of late heading diploid perennial ryegrass selected from advanced breeding material of the ILVO breeding programme. Leaf material of all 30 parental plants was harvested for DNA extraction in March 2007. The AFLP marker information was generated according to Vandepitte et al. (2009). Each individual was profiled with six primer combinations to generate polymorphic bands. For comparing pairs of individual plants, the Jaccard similarity was calculated using information of 559 polymorphic AFLP markers. Twenty-two genotypes were retained on the basis of molecular marker diversity, to compose nine polycrosses (PC) of four parental plants with different levels of genetic similarity: three wide PC with low Jaccard similarities, three medium with intermediate similarities and three narrow with a high similarity. Some parental plants were clonally divided and used in more than one polycross. In none of the nine polycrosses half-sibs were represented. Table 7.1 shows the parental clone numbers and Jaccard similarities between the clones.

Halfsib seeds were harvested on each parental genotype in July 2008. For yield determination a progeny evaluation trial was established in April 2009 in a randomized block design with three replicates. Each of the halfsib families was sown.

The standard varieties in the trial were Aberavon, Cancan and Melways. The trial was cut five times in 2010 and the DMY was measured. The DMY will be determined again in 2011.

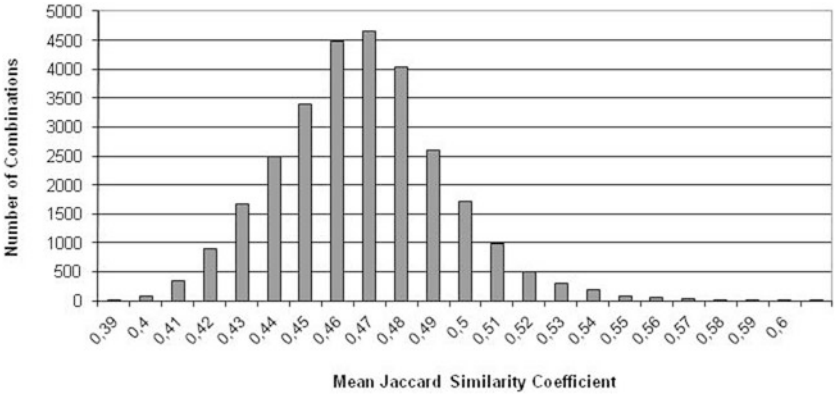

Fig. 7.1 Frequency distribution of the mean Jaccard similarity coefficient of all possible polycrosses of four parental clones selected from the 30 original genotypes

7.3 Results and Discussion

7.3.1 Genetic Similarity

Pairwise Jaccard similarities among the 30 genotypes were determined on the basis of 559 AFLP markers. Jaccard similarities varied between 0.334 and 0.686, with a mean value of 0.467. The mean Jaccard similarity for all possible combinations of 4 parental clones was calculated and varied between 0.386 and 0.637. This molecular marker data was used to construct the nine polycrosses described in Table 7.1. Figure 7.1 shows the frequency distribution of the mean Jaccard similarity coefficient of all possible polycrosses of four parental clones.

7.3.2 Dry Matter Yield

On average, progenies from medium and wide polycrosses were significantly ($P > 0.05$) higher yielding than the progenies of the narrow polycrosses in 2010. There was a yield increase of 4.2 % and 6.1 % respectively. Figure 7.2 shows the total DMY in 2010 of the nine polycrosses. The wide PC2 and PC3 and the medium PC4 were the best performing polycrosses in the trial and were significantly higher yielding than the narrow polycrosses. The narrow PC 9 had the lowest DMY of all polycrosses.

Some parental clones were used in more than one polycross (Table 7.1). Seven of these clones were included in polycrosses with a different type of molecular diversity (wide, medium, narrow). There was a trend but not significant that the yield of the progenies of the same parental plant from wider polycrosses was higher compared

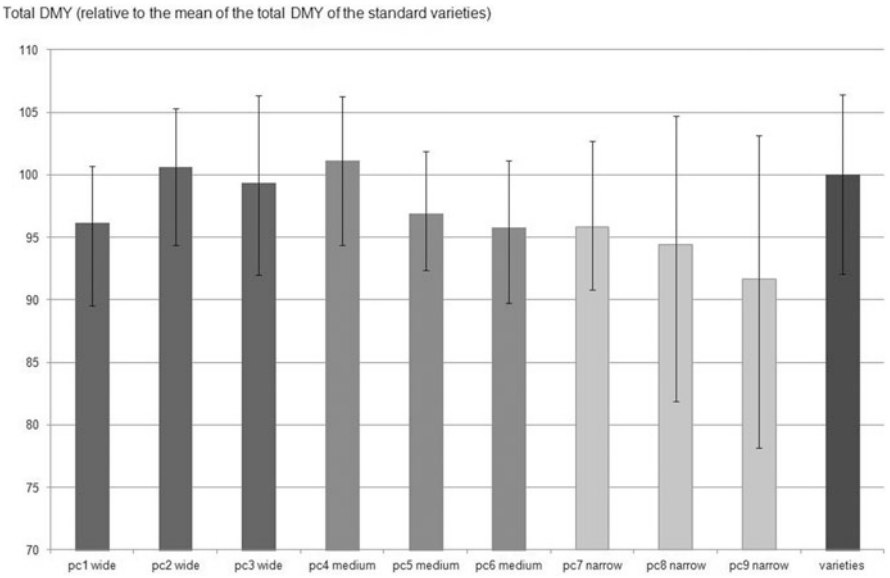

Fig. 7.2 Total dry matter yield (relative to the mean of the total DMY of the standard varieties) of the nine polycrosses composed with different levels of genetic similarity: wide, medium and narrow and of the standard varieties. Error *bars* indicate the highest and the lowest DMY of the four halfsibs of the PC and the highest and the lowest DMY of the three standard varieties

Table 7.2 Differences of the total dry matter yield (relative to the mean of the total dry matter yield of the standard varieties) of the progenies of the same parental plant used in different types of polycrosses

Clone number	Wide PC	Medium PC	Narrow PC
9		−6.8	−8.9
10		+3.8	−0.2
11	+0.9	+1.0	−2.3
12	−1.6	−2.8	
13		−6.4	−10.7
15	−3.3		−5.6
19	−2.4	−4.8	
Average	−1.6	−2.7	−5.5

to progenies from narrow polycrosses. Table 7.2 shows the differences of the DMY of the progenies of the seven clones that were used in different types of polycrosses.

On average the DMY of progenies of the parental clones used in wider polycrosses was 3.9 % higher than when used in narrow polycrosses. Clone 10 is performing better than most of the other clones. Clone 10 is being used in PC 4, 8 and 9. The high variation in total DMY between the half sibs in PC 8 and 9 (Fig. 7.2) can be explained by the good general combining ability of clone 10. In PC 9 the progenies of clone 10 are significantly higher yielding than the other half sibs in the PC. Clones

with a very good general combining ability can result in high performing progenies even when they are used in narrow polycrosses.

7.4 Conclusion

The total dry matter yield of the first production year was higher for the wide and medium polycrosses when compared to those of the narrow polycrosses. If the results of 2011 confirm the first year results, we may conclude that molecular markers can be used for the selection of genetically diverse polycross parents in the breeding programme of perennial ryegrass. This can result in an increase of dry matter yield of the synthetic cultivar without compromising phenotypic uniformity.

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Chapter 8
An Analysis of Chromosome Pairing Behaviour in Newly Synthesized Alfalfa Tetraploids by Means of SSR Markers

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Abstract A duplication of a species' chromosomes results in the formation of a polyploid with polysomic inheritance, or autopolyploid, while the union of the genomes of different species results in the formation of a polyploid with disomic inheritance, or allopolyploid. Cultivated alfalfa shows polysomic (tetrasomic) inheritance; however, no information of chromosome pairing behaviour is available for newly tetraploidized *M. sativa.* We are studying two tetraploid plants obtained by bilateral sexual polyploidization, that is, by crossing a diploid *Medicago sativa* subsp. *falcata* plant that produces 2n eggs (PG-F9) with a 2x *Medicago sativa*. subsp. *coerulea* x *falcata* plant that produces 2n pollen (12P). We are employing SSR markers to investigate the chromosome pairing behaviour of these two plants. They were crossed with an unrelated tetraploid pollen donor, and parental SSR allele segregation patterns are examined in the two progenies. Our results so far, indicate that random pairing, and consequently tetrasomic inheritance, is the rule in newly tetraploidized *M. sativa*.

8.1 Introduction

Polyploidization is an increase in genome number and occurs in nature as the consequence of the union of 2n gametes (sexual polyploidization), or as the consequence of somatic genome duplications (somatic polyploidization). A duplication of a species' chromosomes results in the formation of a polyploid with polysomic inheritance, or autopolyploid, while the union of the genomes of different species results in the formation of a polyploid with disomic inheritance, or allopolyploid. Some polyploids have both modes of inheritance (segmental allopolyploids). Cultivated alfalfa (*Medicago sativa* L. 2n = 4x = 32) shows polysomic (tetrasomic) inheritance (Quiros 1982; Julier et al. 2003).

Cultivated tetraploid (4x) alfalfa likely derives from wild diploid (2x) *M. sativa* through sexual polyploidization. Whether a newly formed polyploid behaves like a polysomic or a disomic polyploid depends on the genotypes involved. When the union of 2n gametes occurs between genetically distant types, the two homologous

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Table 8.1 Informative SSR markers

Marker	Chromosome	Primer sequences		Annealing
FMT13	1	GATGAGAAAATGAAAAGAAC	CAAAAACTCACTCTAACACAC	50
MTIC451	2	GGACAAAATTGGAAGAAAAA	AATTACGTTTGTTTGGATGC	55
MTIC332	4	CCCTGGGTTTTTGATCCAG	GGTCATACGAGCTCCTCCAT	60

chromosome from one parent may pair preferentially (disomic behaviour). On the contrary, if the 2n gametes come from similar genotypes the four 'homologs' tend to pair at random (polysomic behaviour); pairing behaviour may also vary among chromosomes.

We are studying two tetraploid (4x) plants obtained by crossing a diploid (2x) *M. sativa* subsp. *falcata* plant that produces 2n eggs (PG-F9) with a 2x *M. sativa*. subsp. *coerulea* x *falcata* plant that produces 2n pollen (12P). We are employing previously mapped SSR markers to investigate the chromosome pairing behaviour of these two plants.

8.2 Materials and Methods

PG-F9 and 12P were hand crossed without emasculation and the progeny obtained screened for the 4x state by root tip chromosome count. Two 4x plants, named S29 and S48, derived by bilateral sexual polyploidization, were randomly selected and crossed without emasculation with a plant from the Italian variety Classe (4x) used as pollen donor. Progeny seeds were germinated on Petri plates and the plants reared in a greenhouse. The genomic DNA was extracted (GenElute Plant Genomic DNA Miniprep Kit—SIGMA) from 50 plants of the S29 x Classe and S48 x Classe progenies, and from the three parental plants (PG-F9, 12P and Classe).

Eighteen SSR markers were selected from the published literature (Diwan et al. 2000; Julier et al. 2003; Sledge et al. 2005; Mun et al. 2006), on the basis of chromosome location. SSR amplification was performed as follows: buffer 1X, $MgCl_2$ 1,5 mM, dNTP 0,2 mM, primer FOR/REV 0,5 µM, Taq (SIGMA) 1U, genomic DNA 30 ng, in 20 µl final volume. PCR cycling was 94 °C 3 min, 40 cycles at 94 °C 30 s, Ta °C 30 s, 72 °C 30 s (See Table 8.1 for the temperatures of annealing, Ta). After screening in agarose, fluorescinated primers were used for amplification of the three parental plants and the two progenies (43 plants each) with 8 selected primer pairs, and capillary electrophoresis was performed (3130x Genetic Analyzer, Applied Biosystem). Electrophoretic data were analyzed using the software Gene Mapper 4 (Applied Biosystem). SSR allele segregation was compared with those expected with tetrasomic or disomic inheritance and X^2 values calculated. Bivalent pairing is assumed in this work (Ma et al. 2002), so the occurrence of double reduction was not considered; in any case, it was found to be limited (Julier et al. 2003).

Table 8.2 Allelic patterns of three SSR markers in the 2x and 4x plants studied. Numbers are allele sizes in bp

SSR Markers	Alleles	Plants				
		PG-F9 (2x)	12P (2x)	Classe (4x)	S29 (4x BSP)	S48 (4x BSP)
FMT13	1		144	144	144	144
	2		147	147	147	147
	3	152			152	152
	4			156		
	5			166		
MTIC332	1			110		
	2			123		
	3		127	127	127	127
	4	128			128	128
	5		129			129
	6			136		
	7	138			138	138
MTIC451	1	124				124
	2		130	130	130	
	3			134		
	4	136			136	136
	5		138	138	138	138

8.3 Results and Discussion

Five to eight alleles per marker were counted for the 8 selected markers; the two diploid parents were found to be genetically different, as demonstrated by the low number of common alleles (3 in 25, data not sown). Informative polymorphisms were found so far for only 3 of the 8 selected markers, distributed on 3 chromosomes. Two were useful for S29 and three for S48 (Table 8.2).

Considering, for example, the PG-F9 alleles 1 and 4 of marker MTIC451 in the plant S48 (Table 8.2), a gametic segregation 1/6(M1 M4):2/6(M1 -):2/6(- M4):1/6(- -) is expected with complete random pairing, whereas a segregation 1/2(M1 -):1/2(M4 -) is expected with complete preferential pairing (Fig. 8.1). Two allelic configurations of the S29 and S48 4x plants were useful for estimating chromosome pairing behaviour: (1) two PG-F9-specific alleles, or (2) one homozygous PG-F9-specific allele (Table 8.2). Alleles of 12P were not informative, because they were shared with Classe, with only three exceptions. This was not completely unexpected, because 2x *M. coerulea* (parent of 12P) is the prevalent wild ancestor of cultivated *M. sativa*.

Three PG-F9 and two 12P alleles were not transmitted to S29; two PG-F9 and two 12P alleles were not transmitted to S48 (examples in Table 8.2, markers MTIC332 and MTIC 451). This is likely due to homozygous gamete formation, that occurs when the mechanism of 2n gamete formation is second division restitution (SDR) without crossing-over between the centromere and the locus, or when, in case of first division restitution, a crossing-over occurs (Bingham 1980). In PG-F9, the SDR mechanism of 2n gamete formation has been observed (Tavoletti 1994).

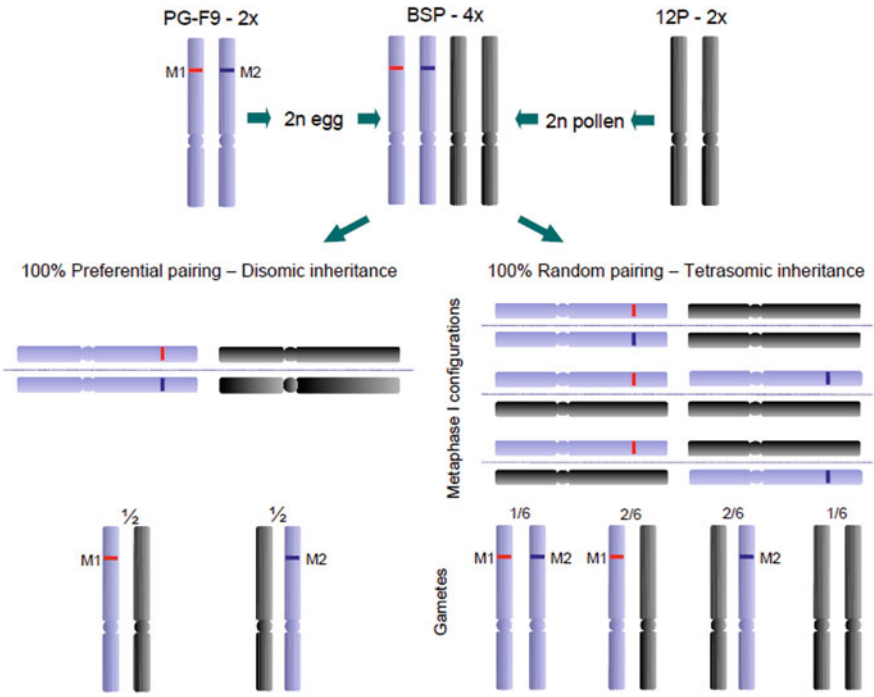

Fig. 8.1 Scheme of the formation of a tetraploid through sexual polyploidization, and of segregation of two alleles from one parent (disregarding those from the other parent) in the tetraploid meiosis under complete preferential or random pairing models

Complete preferential pairing did not occur, as demonstrated by the presence of all 4 gamete types for markers MTIC332 and MTIC451 (Table 8.3). X^2 calculations indicate tetrasomic inheritance in 4 out of 5 cases; only chromosome 4 in the S48 progeny appeared to deviate from complete random pairing, due to lower than expected 'M4M7' and higher than expected '- -' gamete types (marker MTIC332, Table 8.3). However, this result derives from segregation distortion, not from preferential pairing. The disomic inheritance hypothesis could only be tested by X^2 for the FMT13 marker, because two gamete types are expected, but at different frequencies between the two pairing behaviours. The X^2 values were highly significant, thus rejecting the disomic hypothesis.

Significant preferential chromosome pairing was evidenced in a mapping study performed using a population obtained by crossing *M. falcata* × *M. sativa* tetraploid genotypes (Ma et al. 2002). Our results involve less distant, but still different genotypes; however, maybe surprisingly, our data are more in line with those of Julier et al. (2003) that did not support preferential pairing in cultivated *M. sativa*.

Table 8.3 X^2 analysis of disomic vs tetrasomic segregation in the progenies of two newly tetraploidized plants

Plant, Chromosome, Marker	Gamete types	Numbers of progenies			X^{2a}	
		Observed	Expected disomic	Expected tetrasomic	Disomic	Tetrasomic
S29, Chromosome 1, FMT13	M3M3, M3 -	38	21	35	27.52 **	1,54
	- -	4	21	7		
S29, Chromosome 4, MTIC332	M4M7	4	0	7		
	M4 -	17	21	14	NT	1.93
	- M7	14	21	14		
	- -	7	0	7		
S48, Chromosome 1, FMT13	M3M3, M3 -	34	21	35	16.10**	0,17
	- -	8	21	7		
S48, Chromosome 2, MTIC451	M1M4	4	0	7	NT	1,57
	M1 -	15	21	14		
	- M4	15	21	14		
	- -	8	0	7		
S48, Chromosome 4, MTIC332	M4M7	2	0	7.2	NT	8,49*
	M4 -	14	21.5	14.3		
	- M7	14	21.5	14.3		
	- -	13	0	7.2		

NT non testable by X^2 because of 0 expectation for some gamete types
*significant at $P \leq 0.05$; **significant at $P \leq 0.01$
[a]X^2 for 1 df and $P = 0.05$ is 3.84; X^2 for 3 df and $P = 0.05$ is 7.81

8.4 Conclusions

Considerable genetic differences between the two diploid parental genomes of the two newly tetraploidized plants studied here can be assumed based on genealogy and were confirmed by SSR allelic diversity. Despite this, we obtained evidence that, once merged in a new tetraploid individual, the four homologous chromosomes pair at random, and consequently the new tetraploids originated by sexual polyploidization have tetrasomic inheritance. This indicates that, in alfalfa, chromosome pairing is not influenced significantly by genetic differences between subspecies.

Alfalfa belongs to the so-called *M. sativa* complex that includes 2x *M.coerulea*, 2x and 4x *M.sativa* ssp. *falcata*, and 4x *M. sativa* ssp. g*lutinosa*. Our data suggest that, even at the very moment of tetraploidy acquisition by sexual polyploidization, genomic divergence within this complex does not result in significant levels of preferential chromosome pairing and disomic inheritance. We are completing this analysis with markers for all the chromosomes.

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Chapter 9
Genome Constitution in Selected and Unselected Plants of F_2–F_4 Generations Derived from an Allotetraploid *Festuca pratensis* × *Lolium perenne* Hybrid

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Abstract The objective of this work was to assess the genomic constitution and intergeneric recombination in three successive unselected generations, (F_2–F_4), derived from an intergeneric hybrid between *Festuca pratensis* Huds. ($2n = 4x = 28$) and *Lolium perenne* L. ($2n = 4x = 28$). Examination based on genomic *in situ* hybridization analyses of randomly chosen plants in each generation indicated progressive changes in genome balance towards that of *Lolium*. The dominance of *Lolium* chromatin over *Festuca* likely resulted from extensive recombination between chromosomes of the parental genomes, together with substitutions of whole *Festuca* chromosomes by whole *Lolium* chromosomes. The total number of *Lolium* chromosomes increased from generation to generation. The number of recombinant chromosomes, and recombination breakpoints per genotype, also increased in successive generations, but their number was higher for *Festuca* than for *Lolium*. The patterns of genome constitutions and recombination were similar to those we observed in selected generations (F_2–F_4 breeding populations) developed from the same F_1 hybrid plants.

Keywords Allotetraploid · Genome constitution · GISH · *Festulolium* · *Festuca pratensis* × *Lolium perenne* · Recombination

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9.1 Introduction

Festulolium hybrid cultivars that combine high yield and quality of ryegrasses (*Lolium multiflorum* and *L. perenne*) with the persistence, and abiotic and biotic stress resistance of fescues (*Festuca pratensis* and *F. arundinacea*), are considered ideal components of agricultural and turf-grass systems (Ghesquière et al. 2010). The development of cultivars derived from synthetic amphiploids (alloploids) has been limited in the past because homoeologous chromosome pairing can lead to aneuploidy and genetic instability in advanced generations. Relatively low seed fertility, and its high level of variation, observed in advanced breeding populations of *Festulolium,* are reasons why such amphiploids cannot be included successfully in breeding programmes. Recently, cytogenetic investigations of genome architecture of *Festulolium* cultivars, using genomic *in situ* hybridization (GISH), have demonstrated extensive homeologous recombination in the following populations developed from two allotetraploid ($2n = 4x = 28$) hybrids—*F. pratensis* × *L. multiflorum* and *F. pratensis* × *L. perenne* (Zwierzykowski et al. 1998, 2006, 2011; Canter et al. 1999; Kopecký et al. 2006). In our previous research, we studied the genome constitutions and dynamics of intergeneric recombination in breeding populations of F_2–F_8 generations developed from *F. pratensis* (4x) × *L. perenne* (4x) hybrids (Zwierzykowski et al. 2006, 2011). Extensive recombination between chromosomes of the two parental genomes, as well as substitution of whole *Festuca* chromosomes by whole *Lolium* chromosomes, was observed in these progeny. In this work, we present preliminary results on genome constitution and recombination in unselected materials of F_2–F_4 generations derived from the tetraploid *F. pratensis* (4x) × *L. perenne* (4x) hybrid, and compare the results with those obtained in breeding materials of F_2–F_4 generations (developed from the same F_1 hybrid plants), selected for agronomic traits, including vigor, winter hardiness, drought tolerance and fertility (Zwierzykowski et al. 2006).

9.2 Materials and Methods

9.2.1 Plant Materials

Tetraploid hybrids of *F. pratensis* (Fp) × *L. perenne* (Lp) ($2n = 4x = 28$) were generated by intercrossing autotetraploid forms of both species (Zwierzykowski et al. 2006). Five F_1 partially male and female fertile hybrids were intercrossed under controlled conditions, and F_2 progeny were produced. In each of the F_2–F_4 generations studied, 150 randomly collected genotypes were grown in control conditions in a glasshouse. Thirty randomly chosen plants, taken from each generation, were used for cytogenetic analysis.

9.2.2 *Genomic in Situ Hybridization (GISH)*

To determine the number and genomic structure of chromosomes, root-tip 'spreads' were prepared according to Zwierzykowski et al. (1998), and GISH was performed according to Zwierzykowski et al. (2006). To discriminate between Lp and Fp chromosomes, total genomic DNA of Lp cv. Solen was used as a probe and labeled with digoxigenin-11-dUTP by a standard commercially available 'nick translation' protocol. Total genomic DNA of Fp cv. Westa was used as a blocker. The cytogenetic analyses were performed to characterize: (i) the total number of chromosomes, (ii) the number of parental chromosomes, (iii) the number of recombinant chromosomes, and (iv) the number of recombination breakpoints.

9.3 Results and Discussion

In unselected plants of three successive generations of open pollination, progressive changes in genome balance in favour of the 'dominant' the *Lolium* genome were observed (Table 9.1). This genomic dominance of the *Lolium* chromatin resulted from extensive recombination between chromosomes of the parental genomes, and from a substitution of whole *Festuca* chromosomes by whole *Lolium* chromosomes. The total number of *Lolium* chromosomes increased from a mean 14.17 in the F_2 to 15.27 in the F_4, and the total number of *Festuca* chromosomes decreased correspondingly from a mean of 13.66 to a value of 12.70. The number of recombinant chromosomes and recombination breakpoints per genotype also increased from generation to generation, although the respective values of both characters were higher for *Festuca* (1.20–4.47 and 2.60–6.77) than for *Lolium* (0.90–2.10 and 0.90–2.41).

The patterns of genome constitutions and intergeneric recombination were similar to those we observed in F_2–F_4 breeding populations, for example, the total number of *Lolium* chromosomes increased from 14.36 in the F_2 to 15.81 in the F_4, and the number of recombination breakpoints per genotype also increased from generation to generation, although the respective values of this character were higher for *Festuca* (1.14–5.19) than for *Lolium* (0.68–4.04) (Table 9.1; Zwierzykowski et al. 2006).

The reasons for the dominance of *Lolium* over *Festuca* are not understood, although it appears to operate in the same way in both the selected and unselected populations. There is no evidence that gametic competition, pollination effects or selection for vigour in the early stages of seedling growth can be the causative agents. We are therefore left with a single line of conjecture that the process might involve differences in centromere organization and competitiveness (Jones and Hegerty 2009). Does centromere drive operate here, possibly at female meiosis, in a way we have hitherto not even suspected; and are there any differences in the *CENH3* genes, or loading of the centromere-specific CENH3 histone variant which could underlie such a process? Loading differences are known to be at the basis of uniparental chromosome elimination in hybrids of *Hordeum vulgare* × *H. bulbosum*, rather than silencing of *CENH3* genes (Sanie et al. 2011). We have no answers to such questions

Table 9.1 GISH analysis in selected and unselected plants of the F_2-F_4 generations obtained from *F. pratensis* (4x) × *L. perenne* (4x) hybrids. Data of selected plants from the F_2-F_4 generations were published previously. (Zwierzykowski et al. 2006)

Generation		*Lolium* chromosomes						*Festuca* chromosomes					
		Total no. of chromosomes		No. of recombinant chromosomes		No. of breakpoints		Total no. of chromosomes		No. of recombinant chromosomes		No. of breakpoints	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
F_2	Selected	14.36	14–16	0.68	0–4	0.68	0.4	13.57	12–14	0.86	0–4	1.14	0–6
	Unselected	14.17	12–17	0.90	0–2	0.90	0–2	13.66	11–15	1.20	0–3	2.60	1–6
F_3	Selected	14.77	13–17	2.00	0–5	2.23	0–6	13.27	11–15	2.85	1–5	3.77	1–7
	Unselected	14.60	13–17	1.80	0–4	2.00	0–5	13.33	10–15	3.33	1–5	4.80	1–11
F_4	Selected	15.81	13–20	3.46	1–5	4.04	2–6	12.12	8–15	3.89	2–6	5.19	2–10
	Unselected	15.27	12–18	2.10	1–5	2.41	1–6	12.70	10–16	4.47	2–6	6.77	4–11

in *Festulolium*, neither do we know if the drift involves *Lolium* chromosomes in a non-specific way. In any event the mechanism appears to be progressive by small incremental shifts at each generation.

9.4 Conclusions

In each of the F_2-F_4 generations of tetraploid hybrids of *F. pratensis* × *L. perenne* ($2n = 4x = 28$) GISH analysis showed that the balance of chromatin changed. There was a progressive shift in the balance of chromatin in favour of *Lolium*, and a corresponding decline in that of *Festuca*. This dominance of *Lolium* chromatin over *Festuca* appears to result from extensive recombination between chromosomes of the parental genomes, together with substitutions of whole *Festuca* chromosomes by whole *Lolium* chromosomes. The outcome of the experiments mirror that found in previous work based on selected breeding materials of F_2-F_4 generations. No mechanism for this process of chromatin drift has yet been identified, but it is hypothesized that it may be based on the differential activity of the centromeres of the two genera concerned.

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Chapter 10
Estimation of Temporal Allele Frequency Changes in Ryegrass Populations Selected for Axillary Tiller Development

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Abstract The effects of selection on allele frequency at five genes with putative effect on shoot morphology were examined in two perennial ryegrass (*Lolium perenne* L.) populations undergoing recurrent selection. A synthetic C_0 population was created by intercrossing five unrelated ryegrass genotypes within the EU FP5 project 'GRASP'. Two rounds of selection (both positive and negative) for axillary tiller formation were performed, leading to selected populations C_1^+ and C_1^-, respectively. The mean number of axillary tillers per plant was 2.18, 3.90 and 0.22 for C_0, C_1^+ and C_1^-, respectively. Five ryegrass genes putatively involved in the control of plant architecture and hormone response were SNP genotyped in all three populations. A test of selective neutrality (Waples' test), which tests the hypothesis of genetic drift versus selection, was applied. This test indicated selection for the gene *LpIAA1* in C_1^-, where allele frequency changes could not be explained by genetic drift alone ($p < 0.05$). *LpIAA1* belongs to a large family of genes, called *Aux/IAA*, which comprises genes that are auxin-regulated and were shown to control shoot morphology in Arabidopsis and rice.

10.1 Introduction

Shoot morphology as one of the main components of plant architecture plays an important role in fodder grasses. The architecture of the shoot system affects the light harvesting potential of plants, the synchrony of flowering and seed set, and

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ultimately the reproductive success of a plant. Growth and development in grasses has been described by Langer (1979). Briefly, perennial ryegrass plants consist of numerous basal shoots or tillers. During vegetative growth, each tiller is made up of several leaves on a short stem. An axillary shoot meristem is present in the axil of each leaf, and its outgrowth results in formation of a new daughter tiller. Daughter tillers develop their own root systems and become independent from their mother tiller allowing clonal growth as a means of vegetative reproduction. Leaf and tiller production continues until plants switch to reproductive development in response to specific environmental cues. Perennial forages have opposing selective pressures placed upon them during plant breeding. Seed production practices favour genotypes with high numbers of reproductive tillers, and good seed yields. However, nutritional quality is reduced during flowering (Jung et al. 1996), so the end-user, the farmer, requires varieties with fewer reproductive tillers. Effective regulation of resource partitioning between vegetative and reproductive growth is important in perennial ryegrass and other perennial forage grasses.

Selection mapping (SM) refers to a range of approaches that identify alleles, loci, and epistatic interactions using populations that have been subjected to iterative cycles of recombination and selection (Wisser et al. 2008). The effects of selection can be seen as differences in allele frequency, diversity, and/or patterns of recombination, through comparisons of temporally or spatially defined subpopulations. A fundamental challenge in SM, however, is to differentiate the effects of selection from those of genetic drift. Selection increases the frequencies of favourable alleles while genetic drift is a random change in allele frequencies due to small population size. A loss of favourable alleles due to random genetic drift leads to reduction in genetic variance and, thus, limits future selection response (Guzman and Lamkey 1999). The assessment of the effects of random genetic drift and selection is important for designing efficient recurrent selection (RS) programs. Standard statistical tests (e.g. χ^2, G tests) for assessing significant changes in allele frequencies neglect the effects of random genetic drift and are, therefore, not appropriate for the analysis of changes in allele frequencies in RS with finite population size. In contrast, Waples provided a test statistic for monitoring allele frequency changes, which takes into account the increased variance in allele frequencies between generations caused by random genetic drift. Up to now, only a limited number of studies utilized Waples' neutrality test (Waples 1989) in plant breeding with all instances coming from studies on maize (Labate et al. 1999; Pinto et al. 2003; Coque and Gallais 2006; Falke et al. 2007; Wisser et al. 2008). Hence, a detailed evaluation of allele frequency changes in populations undergoing RS in other plant species is still lacking.

In the present study, we evaluated the selection response of two perennial ryegrass populations after one cycle of recurrent selection on the basis of SNP markers in five candidate gene regions. Our objective was to investigate allele frequency changes in candidate regions due to the effects of random genetic drift and selection.

10.2 Materials and Methods

Five of the 20 perennial ryegrass (LTS) genotypes, studied in the EU FP5 project 'GRASP' (Posselt et al. 2006), were crossed in all possible pairwise combinations including reciprocal crosses. Two of the five genotypes, LTS15 and LTS16, are ecotypes, two, LTS03 and LTS04, are parent genotypes of VrnA mapping population (Jensen et al. 2005) and one, LTS11, is a ryegrass mutant expressing enhanced axillary tillering (Pašakinskienė 2005). Equal amounts of seed from each combination were sown in the greenhouse (single plant per pot) to form the synthetic C_0 population. 340 C_0 plants were evaluated for axillary tiller development by counting the average number of axillary tillers per 20 primary tillers at the beginning of flowering. The 34 plants with the highest and the lowest average number of axillary tillers per plant were selected to produce selected subpopulations C_0S^+ and C_0S^-, respectively. These selected plants were intercrossed in isolated greenhouses by open pollination to produce populations C_1^+ and C_1^-, respectively. In order to synchronize flowering all plants were vernalized at 6 °C with 8 h photoperiod per day for 100 days. Following the same selection procedure as in C_0 34 plants were selected in each C_1^+ and C_1^-. The selected plants from C_1^+ and C_1^- along with 34 random plants from C_0R and five LTS genotypes, were used for DNA extractions from young leaf tissue following the CTAB protocol.

Five ryegrass genes with putative effects on shoot morphology and hormone response were chosen as candidate genes for genotyping. Details on primer design and SNP detection for these genes in 20 LTS genotpes were published earlier (Brazauskas et al. 2010). The SNP selection procedure consisted of several steps. First, a minimum number of SNP subsets was determined for each candidate gene to distinguish all haplotypes present among the parental genotypes using software PolyMin (Frei et al. 2009). Second, the best combination of SNPs was determined to meet Sequenom MassARRAY sequence requirements, namely sufficient sequence conservation next to each SNP to enable diagnostic primer annealing. Third, duplicate SNPs were selected, where available, for genotyping error control and rigorous haplotype calling. SNP genotyping was performed using the Sequenom MassARRAY (Maldi-TOF mass spectrometry) system at the Centre for Integrative Genomics (CIGENE, Aas, Norway). SNP genotyping results were used to calculate haplotype frequencies in each of the four samples, LTS, C_0R, C_1S^+ and C_1S^-. A test of selective neutrality (Waples' test) was applied. It tests, whether the observed variation in allele frequency between two samples taken at different times can be explained as a sample drawn from a population of size N_e (effective population size) that has undergone t generations of genetic drift. The test statistic is distributed as a chi-square (Waples 1989) and is calculated as $\chi^2 = (x - y)^2/\mathrm{var}(x - y)$, where x equals the estimated allele frequency in an initial sample, y equals the estimated allele frequency in a subsequent sample and $\mathrm{var}(x - y)$ equals the variance of this difference. The derivation of the variance in $(x - y)$ is explained by Waples (1989) in detail. We assumed that the effective population size is equal to twice the number of selected plants ($2\,N = 68$) in a population of allogamous diploid genotypes, where migration was controlled by

Table 10.1 Average number of axillary tillers per plant in C_0, C_1^+, C_1^- and respective selected subpopulations

Cycle	Total population		Subpopulation S^+		Subpopulation S^-	
	N[a]	Ax. tiller number[b]	N	Ax. tiller number	N	Ax. tiller number
C_0	340	2.18 (0.10–4.35)	34	3.41 (3.00–4.35)	34	1.83 (0.10–2.42)
C_1^+	340	2.46 (1.25–6.20)	34	3.90 (3.00–6.20)	–	–
C_1^-	340	1.98 (0.00–3.85)	–	–	34	0.22 (0.00–0.45)

[a]N, number of genotypes
[b]Average axillary tiller number per plant and it's range in parenthesis

isolation and mutation could be ignored (Labate et al. 1999). Bonferroni correction was applied to account for multiple testing.

10.3 Results and Discussion

Plants in C_0 expressed substantial variation for axillary tiller formation with the mean number of axillary tillers per plant ranging from 0.10 to 4.35, and an overall average of 2.18 (Table 10.1). A reciprocal selection at 10 % intensity (34 selected genotypes from a total of 340) resulted in selected subpopulations C_0S^+ and C_0S^- with an average number of 3.41 and 1.83 axillary tillers per plant, respectively. Substantially higher selection differential ($D = 1.23$) was observed for positive selection as opposed to that for negative selection ($D = -0.35$). The selected genotypes were intercrossed to produce C_1^+ and C_1^- of 340 plants each. Despite substantially higher selection differential the resulting selection response was ony slightly higher for positive selection ($R = 0.28$) in comparison to negative selection ($R = -0.20$) and the average number of axillary tillers reached 2.46 and 1.98 in C_1^+ and C_1^-, respectively. A second round of reciprocal selection was performed with the same intensity as during the first cycle (10 %, 34 selected genotypes), and resulted in two selected subpopulations C_1S^+ and C_1S^-. Here, a higher selection differential was observed for negative selection ($D = -1.76$) in comparison to positive selection ($D = 1.44$). The average number of axillary tillers per plant was 3.90 and 0.22 in C_1S^+ and C_1S^-, respectively.

SNP genotyping with 25 SNPs in five genes (Table 10.2) was performed on a random subsample of 34 C_0 genotypes (C_0R), two selected subpopulations C_1S^+ and C_1S^-, and the five parental genotypes (LTS). Nineteen haplotypes were identified in five genes in total. Five haplotypes per gene were detected in *LpRUB1* and *LpBRI1* while *LpIAA1*, *LpSHOOT1* and *LpTB1* had only three haplotypes per gene among the five parental genotypes. The loci under investigation were dispersed over linkage groups 2, 3, 4 and 5 (Table 10.2).

All 19 haplotypes detected in parental genotypes were also present in C_0R and no novel haplotypes were detected. Furthermore, a minor haplotype frequency change

Table 10.2 GeneBank accession numbers, putative functions, linkage groups (LG), number of SNPs genotyped and haplotypes detected for five perennial ryegrass genes

Gene	GeneBank	Putative function	LG	No of SNPs	No. of haplotypes
LpIAA1	GU987119	Auxin signaling	4	5	3
LpRUB1	GU987120	Auxin signaling	5	5	5
LpBRI1	GU987121	Brassinosteroid signaling	3	6	5
LpSHOOT1	GU987122	Outgrowth of axillary buds	4	7	3
LpTB1	GU987123	Outgrowth of axillary buds	2	2	3

Table 10.3 Waples' test[a] for temporal changes in allele frequency between Cycle 0 and Cycle 1 for both positive and negative selection

Gene	$N(C_0R)$[b]	$N(C_1S^-)$	$\chi^2(C_1S^-)$	$N(C_1S^+)$	$\chi^2(C_1S^+)$	df[c]
LpIAA1	34	34	10.30*	34	1.25	2
LpRUB1	34	34	8.34	34	6.64	4
LpBRI1	34	34	8.39	34	2.68	4
LpSHOOT1	34	34	0.42	34	0.03	2
LpTB1	34	34	1.84	34	0.13	2

*Significant at $p < 0.05$
[a]Based on $N_e = 68$
[b]N equals sample size at the respective cycle
[c]df, degrees of freedom

was observed from LTS to C_0. This change was expected and could be attributed to both genetic drift and sampling variance in C_0 due to the low number ($N = 34$) of genotypes in C_0R. After two cycles of selection, two haplotypes (10.5 %) were lost in C_1S^+ and one haplotype (5.3 %) was eliminated in C_1S^-. All three lost haplotypes had low frequencies (0.02–0.10) in both LTS and C_0R. An increase in the number of haplotypes belonging to the extreme classes of frequencies were earlier reported (Pinto et al. 2003). This is a feature of a dispersive process in which the intermediate allele frequencies tend towards the limits of zero (lost) or 1 (fixation). No fixed haplotypes were detected in our data set while the highest haplotype frequency reached 0.74 (*LpTB1*).

Waples' test (1989) was applied to each locus to detect if any of the observed changes in allele (haplotype) frequency between C_0 and C_1 within populations were greater than those expected by drift alone. Loci were tested for statistically significant changes in allele frequency assuming an effective population size of $N_e = 2\ N = 68$, where N is the number of selected lines ($N = 34$). No additional significant tests would be obtained using any smaller value of N_e because such tests would be more conservative. The Waples' test indicated selection for the gene *LpIAA1* in C_1^-, where allele frequency changes could not be explained by genetic drift alone ($p < 0.05$) (Table 10.3).

The recurrent selection process is aimed at the enrichment of rare alleles. This selection response was earlier observed in a complex maize population selected for quantitative disease resistance for four cycles (Wisser et al. 2008). Authors speculate that loci under selection are more likely to be detected through the increases

in frequency of rare alleles at linked marker loci than through frequency shifts of more common alleles. The more frequent alleles at marker loci are more likely to be associated with multiple alleles at the genes under selection, including favourable and unfavourable alleles, thus the higher-frequency alleles would change in both directions under selection. However, in our study, allele frequency changes were estimated within candidate genes with putative effects on axillary tiller formation. This implies that a tight linkage with alleles under selection should be expected and both higher and lower frequency alleles should be under direct selection response. This was observed in our data set where low-frequency alleles showed only small fluctuations in their frequencies while all three haplotypes at non-neutral locus *LpIAA1* had intermediate frequencies ($p = 0.21 - 0.43$) in C_0.

The effective size of a population, N_e, determines the rate of change in the composition of a population caused by genetic drift and is crucial in determining the level of variability in a population, and the effectiveness of selection relative to drift. The probability of obtaining a significant Waples' test statistic strongly depends on the size of the effective population. Smaller N_e leads to larger variance in allele frequencies and a higher percentage of statistically significant tests. However, this effect is of lower magnitude in the early generations and the ratio of S/N_e, where S is the number of sampled individuals, becomes more important (Waples 1989). We assumed that effective population size in our data set is equal twice the number of selected lines ($N_e = 2\ N = 68$) as the bottleneck generation of 34 selected lines will create the strongest effect on genetic drift. Other factors, such as migration, non-random mating or variation in offspring number were controlled by the experimental design. Several methods exist for empirical N_e estimation from marker data, including inbreeding and variance effective population sizes (Charlesworth 2009). Labate et al. (1999) estimated effective population sizes based on temporal changes in allele frequencies between C_0 and C_{12} for two maize populations undergoing reciprocal recurrent selection. Authors indicate that directional selection deflates effective population size where loci under selection yield N_e estimates closer to $N_e = 0.5\ N$ in contrast to that of neutral loci with $N_e = 2\ N$.

LpIAA1 is orthologous to *AUXIN RESISTANT 5* (*AXR5*), a member of a large family of genes, called *Aux/IAA*, which comprises genes that are auxin-regulated (Reed 2001). *AXR5* was isolated from a mutant of Arabidopsis called *axr5-1* (Yang et al. 2004). Mutant plants are resistant to auxin and display a variety of auxin-related growth defects including defects in root and shoot tropisms. More specifically, there are fewer lateral branches on the primary inflorescence of arx5-1 plants, but more inflorescence branches growing from rosette. More recently an ortholog of *AXR5*, called *OsIAA1*, was also characterized in rice (Song et al. 2009). The *OsIAA1*-overexpression transgenic plants showed distinctive morphological changes such as decreased plant hight and loose plant architecture. Results from our study as well as mutant studies from Arabidopsis and rice indicate that *LpIAA1* is a promising candidate for shoot morphology control and could be used for the development of functional markers for marker assisted breding in perennial ryegrass.

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Chapter 11
Understanding the Genetic Basis for Slow Plant-Mediated Proteolysis in *Festulolium* Hybrids

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Abstract Inefficiencies in the rumen associated with excessive rates of degradation of forage protein have meant that resource capture is poor compared to the potential gains achievable from use of new grass cultivars. Increased rumen efficiency through improved utilization of feed protein will decrease emissions of environmentally damaging wastes and greenhouse gas emissions. New interspecific forage grass hybrids (*Festulolium*) derived from hybridizing ryegrass species *Lolium perenne* or *Lolium multiflorum* with the related fescue species *Festuca arundinacea var glaucescens*, native to mountain pastures in Mediterranean regions provide an opportunity to enhance ruminant nutrition and provide an environmental safeguard. Under rumen conditions, the fescue protein is degraded at a significantly slower rate than that of ryegrass which should lead to increased efficiency of feed N-utilization and aid livestock gain whilst also reducing N losses that contribute to greenhouse gas emissions. Procedures are underway to determine the necessary genome composition required to optimize expression of the fescue trait in a ryegrass genetic background and to determine the most effective plant breeding strategy to ensure that this is achieved. It is hypothesized that mechanisms evolved in the fescue species necessary for adaptations to high temperatures in Mediterranean locations are also functional and relevant for protein protection in stress conditions in the rumen. Combining these with ryegrass-derived traits for forage yield and quality should assist strategies for more sustainable livestock agriculture at a time of climate change.

11.1 Introduction

Food security for an increasing world population is threatened by increased summer droughts and high temperatures. Providing food security is not only a problem in developing countries but is also re-emerging as a problem for developed countries and is creating an increasing pressure on the planet's resources (Oweis and Peden 2008). Livestock agriculture is a major provider of food security in the UK, but due to its large land area usage and the inefficiencies of ruminant nutrition, contributes significantly to emissions of environmentally harmful wastes including greenhouse

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gasses (methane and nitrous oxide) and ammonia. Grassland accounts for over 70 % of crude protein (CP) consumed by ruminants in the UK compared with alternative sources that include cereals (14 %), oilseeds (10 %) and legumes (peas and beans < 5 %) (Wilkins and Jones 2000). Ruminant digestion can successfully convert fibrous biomass such as grass, which is in abundance around the world, to produce high quality protein sources fit for human consumption and thereby circumvent use of alternative CP sources that have in the past incurred health scares such as BSE. However, the advantages of grassland agriculture must be weighed against the environmentally damaging concomitant greenhouse production (Moss et al. 2000).

Poor Protein Capture Poor protein capture by livestock from the large amounts of plant biomass that is consumed has serious implications for livestock agriculture's sustainability. There is a poor return with only 30 % of nitrogen ingested being converted into animal protein products milk and meat. Ruminants are inefficient utilisers of grass protein due in part to the impacts of rumen stresses that induce plant–mediated proteolysis (Kingston-Smith et al. 2010). Plant biomass eaten as forage is exposed in the rumen to hostile conditions including darkness, no oxygen, high temperatures (39 °C) and microbes that in unison cause the plant cells to initiate an early regulated cell death (senescence). This results in poor utilization of grass-based nutrients and the creation of N waste in the forms of urea and ammonia (Kingston-Smith et al. 2008).

Improvement of Ruminant Dietary Intake for Future Agricultural Sustainability The cause of poor utilization of dietary protein and loss of N from the rumen has been attributed to the 'Asynchrony Hypothesis' with regard to the imbalance between energy and protein supply and demand by the rumen microbial populations. Asynchrony occurs because ruminants grazing on fresh forages supply the rumen microbial populations with excess RDP (Rumen Degradable Protein) relative to fermentable energy; this is because of rapid availability of plant proteins due to their degradation by plant and microbial enzymes (Nolan and Dobos 2005; Moorby et al. 2008). This loss of non-protein nitrogen (NPN) limits ruminal microbial growth. It is the post-ruminal flow of microbial protein that contributes directly to amino acid absorption and host animal tissue production.

New Models for the Reduction of Environmentally Damaging Wastes Caused by Livestock Agriculture and Improving Ruminant Nutrition Nitrogen and energy use efficiency in ruminant digestion has been addressed using two approaches involving alternative plant breeding techniques: Matching the availability of energy to protein intake, and reducing protein degradability to synchronize with energy availability (Moorby et al. 2008).

Energy-use-efficiency and reduction of waste N is achieved by increasing the fermentable energy available to the rumen microbial populations from freshly ingested forage (Humphreys 1989; Miller et al. 2001) and in conserved forages such as silage (Merry et al. 2006). Practically, this matching of energy to protein intake has been addressed by using improved perennial ryegrasses (*Lolium perenne*) known as high-sugar grasses (HSG's). These have been bred for increased water soluble carbohydrate (WSC) content for improved forage nutritional value for grazing animals.

In the second approach, the synchronization of reduced protein degradability to energy availability was addressed by (Kingston-Smith and Theodorou 2000) who challenged the traditional view that breakdown of ingested forage in the rumen was a process mediated by rumen micro-organism communities. The authors have shown that plant-mediated proteolysis plays a major role in the rapid break down of freshly ingested forage protein and that fresh living forage grasses ingested by grazing ruminant animals are still mostly intact when they enter the rumen (Kingston-Smith and Theodorou 2000). The hostile rumen environment of high temperatures and anaerobic conditions (39 °C, no $O_{2,}$), has been shown to trigger living cells in freshly ingested forage to release enzymes which could be part of a defense mechanism referred to as programmed cell death (PCD) (Kingston-Smith et al. 2005).

Plant Breeding Solutions to Achieve more Efficient Forage for Livestock Agriculture
In vitro comparisons of protein stability from grass accessions demonstrated differential sensitivity of the forage protein to rumen conditions. Ryegrasses are particularly susceptible to proteolysis under rumen conditions (Beha et al. 2002). However, protein from the fescue species *Festuca arundinacea var. glaucescens* (Boiss.) was found to be more resilient given identical stress conditions (Shaw 2006) so that the rate of plant-mediated proteolysis was considerably slower for *F. arundinacea var glaucescens* compared to *Lolium*. Specifically, the mean protein half-life of *L. perenne* was 4.33 h, in *L. multiflorum* this was 2.06 h, whereas the mean protein half-life under rumen-simulated conditions for *F. arundinacea var glaucescens* was 15.83 h. The enhanced rumen-stress tolerance of the fescue species may have arisen from its natural adaptations to high temperatures and water limiting conditions which it encounters regularly in its natural habitat in Mediterranean grasslands (Shaw 2006). Therefore, combining the desirable and complementary characters of ryegrass (high digestibility and yield) and *Festuca arundinacea var glaucescens* provides opportunities to both improve ruminant nutrition and increase resilience of the crop to the onset of increased summer stresses thereby helping sustain forage production and achieving yield potential.

Shaw (2006), attempted to transfer the protein-protective mechanism of *F. arundinacea var glaucescens* into *L. multiflorum* through the use of a backcross breeding programme and introgression-mapping technologies, returning over two backcross generations to a diploid *L. multiflorum* genome with some genotypes having one or more trans-located *Festuca* chromosome segments.

It is likely that the mechanisms for protein protection in the fescue species are complex and governed by a suite of interacting genes (quantitative trait loci; QTL) of greater or lesser effect. To monitor transfers of *F. arundinacea var glaucescens* genes into *Lolium*, Shaw (2006) employed fescue-specific amplified fragment length polymorphism (AFLP) markers. The same and other marker technologies and a near-identical breeding programme was employed successfully at the same time to transfer a QTL for drought and heat-tolerance from *Festuca* into *Lolium*, thereby confirming the efficacy of the technique used, at least for the traits studied (Humphreys et al. 2005). However, Shaw (2006) during her marker-assisted backcrossing programme whilst although able to demonstrate the successful transfer of a number of

F. arundinacea var glaucescens chromosome segments onto *L. multiflorum* chromosomes, the heritability of the *Festuca* trait for protein protection over generations was inconclusive. The significant reduction in the expression of the *Festuca* trait over generations led to questioning of the suitability of the introgression-mapping approach for transfers of a functional protein protection mechanism from *Festuca* into *Lolium*.

However, as well as the successful use of *Festuca* gene introgression for improved drought tolerance from *F. arundinacea var glaucescens* into *Lolium* reported by Humphreys et al. (2005), and also from its close relative *F. arundinacea* (Humphreys and Thomas 1993; Humphreys and Pasakinskiene 1996), there are several examples of the successful use of introgression-mapping for transfers of both simple and complex traits in the *Lolium-Festuca* genome complex (Moore et al. 2005; Armstead et al. 2006; Kosmala et al. 2006; Gronnerod et al. 2004). Therefore, there is some cause for optimism that the introgression-breeding approach may be suitable for gene transfers for improved protein protection for ryegrass. The very high frequency of gene exchange at meiosis in *Lolium-Festuca* grass hybrids provides access throughout the *Festuca* genome to genes for all traits of potential value to *Lolium* (Humphreys et al. 2003).

Alm et al. (2011), conducted a QTL study in *Festuca pratensis* to locate genes associated with resistance to cold and drought stress and found a major QTL for resistance to severe drought stress on chromosome 3 that spanned the entire linkage group. Another study conducted by Turner et al. (2008) located QTL for drought resistance in *Lolium perenne* and found no association with chromosome 3. The outcome would suggest that *Festuca* chromosome 3 would be a good source of genetic variants for improved drought resistance in *Lolium*. This has proved to be the case with Humphreys and Pasakinskiene (1996) and Humphreys et al. (2005) identifying *Festuca* translocations derived from drought-tolerant fescue species onto *Lolium* chromosome 3 that led to significant improvements in resistance to severe drought stress. In field experiments in France, breeders' lines derived from these introgression lines proved as effective under drought stress as certain interspecific hybrids that had the entire *Festuca* genome present (Humphreys et al. 2011).

The alternative to introgression as a route at combining in a single plant genome the positive attributes of *Lolium* and *Festuca* species is the use of amphiploidy where entire *Lolium* and *Festuca* genomes are combined. The majority of *Festulolium* cultivars marketed currently have been produced by this method (Ghesquière et al. 2010). The stability of such hybrids would be enhanced given a functional chromosome pairing regulator that can provide for strict homologous chromosome pairing and disomic inheritance. Such a system is present in polyploid fescue species such as *Festuca arundinacea var glaucescens* (Jauhar 1993). New procedures in development at IBERS provide opportunities to exploit a functional fescue chromosome pairing regulator and thereby provide opportunity for the effective use of amphiploidy as a means to harness the protein protection mechanism present in this fescue species. Alternative breeding approaches underway at IBERS that employ either introgression or amphiploidy should answer which strategy is the more appropriate and in future

provide options to the livestock industry of use of grass cultivars that will improve ruminant nutrition through mitigating plant-mediated proteolysis in the rumen.

The assessment of the alternative breeding approaches is underway as part of a new PhD programme funded by EBLEX and HCC. The breeding approach must both optimize the expression of the *Festuca arundinacea var glaucescens* trait and maintain the positive attributes of *Lolium*. The programme will test the suggestion that protein stability in the rumen would be increased by increasing the *Festuca:Lolium* genome dosage in *Festulolium* hybrids. Hybrid combinations having different genome dosage will be compared and the variation between and within genome combinations for expression of the protein protection mechanism determined. The findings will be compared with the alternative introgression breeding approach where *Festuca*-derived genes (or regulatory regions) responsible for protein protection will be sought that may have previously been lost inadvertently by Shaw (2006) during her introgression-mapping backcross breeding programme. The current studies should provide an answer to whether introgression-mapping is appropriate for transfer of the traits that confer reduced plant-mediated proteolysis. The PhD project will also attempt to identify the mechanism responsible for protein protection in *F.arundinacea var. glaucescens*. Taken together, the Mediterranean origins of the fescue species where it is exposed regularly to heat stress and the observation by Shaw (2006) that the expression of the thermo-tolerance conferring heat shock protein (HSPs) (Vierling 1991); Howarth and Ougham (1993), HSP70 was immediate on exposure to rumen conditions in *F. arundinacea var. glaucescens* but delayed by up to five hours in *L. multiflorum*), it is plausible to suggest that the fescue HSP provides at least one potential mechanism to explain the greater resistance of the *Festuca* species compared with *Lolium*. If the hypothesis is proven correct, then gene introgression would seem a feasible route to achieve expression of the fescue trait following transfer to ryegrass. A successful outcome will provide a co-adaptive trait that will serve to improve field survival of *Lolium* during hot summers, and will also enhance rumen-use-efficiency to aid food security and to combat climate change.

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Chapter 12
Chromosomal Rearrangements in BC_1 Progeny Obtained from Crosses of Tetraploid *F. pratensis* × *L. perenne* Hybrids with Tetraploid *L. perenne*

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Abstract Physical mapping of genes responsible for quality traits requires well established cytogenetic maps and chromosome identification. Genomic *in situ* hybridization (GISH) allows to discriminate parental genomes and to track recombination between them. Fluorescence *in situ* hybridization (FISH) with 5S and 18S–5.8S–25S (35S) rDNA probes provides chromosomal landmarks, and allows to detect chromosome re-arrangements; a characteristic rDNA position provides useful markers for chromosome identification. The aim of this study was to assess the genomic constitution and chromosome arrangements in BC_1 progeny obtained from crosses of tetraploid ($2n=4x=28$) *F. pratensis* × *L. perenne* hybrids with tetraploid ($2n=4x=28$) *L. perenne*. GISH examination in BC_1 progeny showed variability in respect to somatic *Lolium* and *Festuca* chromosome number, as well as *Lolium* and *Festuca* recombinant chromosomes and homoeologous recombination breakpoints. FISH experiments showed various numbers of both rDNA loci (3–5 sites for 5S rDNA and 10–13 sites for 35S rDNA). *Lolium* chromosome 3 and *Festuca* chromosomes 2 and 3 were also involved in recombination showing rearrangements.

Keywords Chromosomal rearrangements · *Festuca pratensis* · FISH · GISH · Introgression · *Lolium perenne*

12.1 Introduction

In introgression breeding programs, intergeneric recombination can be used to transfer abiotic and biotic stress resistance traits from *Festuca* species into *Lolium* species. Meadow fescue (*F. pratensis* Huds.; Fp) and perennial ryegrass (*L. perenne* L.; Lp) can be hybridized at various ploidy levels, producing diploid, triploid and tetraploid

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intergeneric hybrids, in which homoeologous chromosomes pair and recombine freely (King et al. 1998; Zwierzykowski et al. 1998, 2006; Kopecký et al. 2009). GISH is the method of choice in the *Lolium-Festuca* complex for analysis of the parental genomes and detection of the exchanges between constituent genomes (King et al. 1998; Zwierzykowski et al. 1998, 1999, 2006; Kopecký et al. 2006). More precise analysis of a genome constitution and identification of chromosomes in various amphiploid and introgression forms of *Festulolium* can also be obtained through the use of combined FISH/GISH (Kosmala et al. 2006; Książczyk et al. 2010; Harper et al. 2011). This method utilizes *in situ* hybridization with total genomic DNA as a probe to distinguish genomes, and with chromosome specific DNA probes (e.g. rDNA probes) to identify pairs of mitotic chromosomes or to visualize pairing of homoeologues at meiosis, as well as to identify segments carrying desirable genes of one species when introgressed into another (Kosmala et al. 2006; Kopecký et al. 2008; Książczyk et al. 2010). The application of GISH/FISH techniques in F_1 hybrids of Fp×Lp allowed the identification of Lp chromosome 3 (acc. to Thomas 1981), Fp chromosomes 2 and 3 (acc. to Thomas 1981), and this approach revealed variation in their numbers which can be easily tracked in *Festulolium* hybrids (Książczyk et al. 2010). The present study aimed at characterizing the genome constitution of tetraploid introgression forms, derived from crosses of tetraploid F_1 hybrids of *F. pratensis*×*L. perenne* into tetraploid *L. perenne*, and to describe (i) somatic Lp and Fp chromosome number, (ii) number of complete and recombinant Lp and Fp chromosomes, (iii) number of homoeologous recombination breakpoints in both species, and also (iv) to identify the number and chromosomal sites of rDNA sequences.

12.2 Materials and Methods

Tetraploid ($2n=4x=28$) F_1 hybrids of *F. pratensis* ($4x$)×*L. perenne* ($4x$), generated by intercrossing autotetraploid forms of both species (Zwierzykowski et al. 2006), were backcrossed with *L. perenne* 'Solen' ($4x$) to produce tetraploid BC_1 progeny. Forty randomly chosen BC_1 plants were used for cytogenetic analyses. The cultivars of *L. perenne* ($4x$) and *F. pratensis* ($4x$) and intergeneric tetraploid F_1 hybrids of *F. pratensis* ($4x$)×*L. perenne* ($4x$) were previously studied by GISH/FISH (Książczyk et al. 2010), and were selected as control plants in our analyses.

Chromosome preparations were made from a single root per plant of the BC_1 hybrids, according to Książczyk et al. (2010). GISH was performed according to Zwierzykowski et al. (2006), using the total genomic DNA of Lp 'Solen' and Fp 'Westa' as a probe and block, respectively. FISH was performed as described by Książczyk et al. (2010), using the ribosomal DNA probes 5S rDNA and 25S rDNA (the latter being used for detection of plant 35S rDNA loci). The Lp and Fp chromosomes identified by rDNA-FISH were numbered according to Thomas (1981).

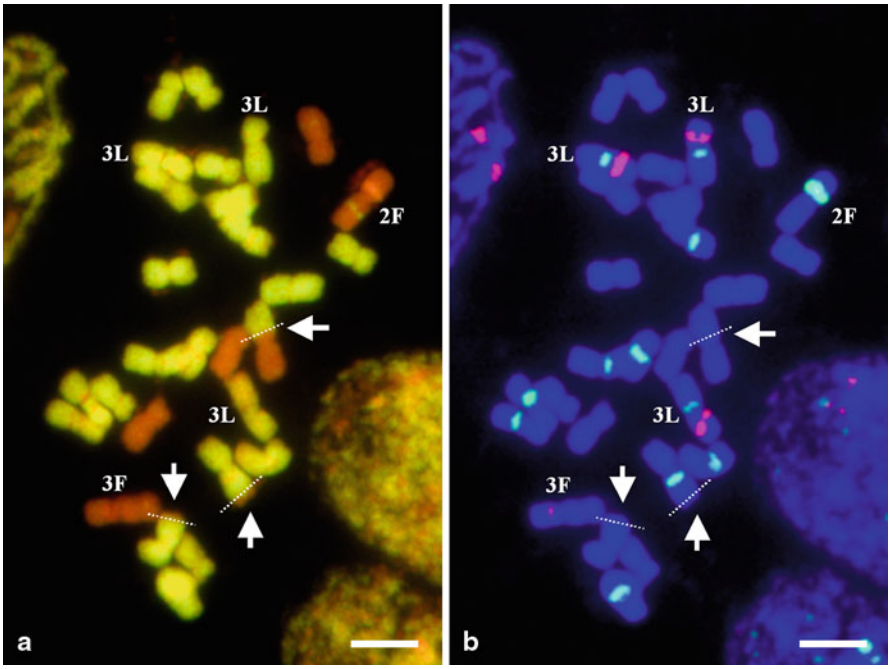

Fig. 12.1 GISH **a** (22Lp (2R) + 6 Fp (1R)) and FISH **b** analyses of the same somatic metaphase chromosome spread of a BC_1 plant. GISH image **a** was created after FISH using total genomic DNA from *L. perenne* as a probe labelled with digoxigenin and detected by anti-digoxigenin conjugated with FITC (*green*/*yellow*), with blocking genomic DNA of *F. pratensis* (*orange*/*red*); chromosomes were counterstained with propidium iodide. FISH image **b** was created using as a probe (i) 5S rDNA labelled with rhodamine (*red*) and (ii) 25S rDNA labelled with digoxigenin and detected by anti-digoxigenin conjugated with FITC (*green*); chromosomes were counterstained with DAPI (*blue*). GISH and FISH images are supplemented by the *white arrows* indicating *Lolium*/*Festuca* recombinant chromosomes (R) and by the *white lines* with intervals indicating recombination breakpoints. The nomenclature of rDNA-bearing chromosomes (Arabic numerals) follows Thomas (1981). *Uppercase letters* denote the genomic origin of tagged chromosomes. *Scale bars* represent 5 μm

12.3 Results and Discussion

The chromosome number of a total of 40 BC_1 plants ranged from 25 to 29. Among all BC_1 plants, aneuploid cells were found to be in majority (60 %) in relation to expected tetraploid cells (40 %). The number of complete Lp chromosomes ranged from 18 to 22 (mean 20.7), while the number of complete Fp chromosomes ranged from 5 to 8 (mean 6.67). The number of Lp recombined chromosomes ranged from 0 to 2 (mean 0.7), while the number of Fp recombinant ones ranged from 0 to 3 (mean 0.75). 22.5 % of BC_1 plants had no Lp and Fp recombined chromosomes, and the number of non-recombinant chromosomes of Lp ranged from 19 to 21 (mean 20.4), while the number of non-recombinant ones of Fp ranged from 6 to 7 (mean 6.77). Among all karyotyped BC_1 progeny, there were 58 recombined Lp/Fp chromosomes, of which 55 had one arm recombined (Fig. 12.1) and 3 had both arms recombined (2 Lp chromosomes and

1 Fp chromosome). Of a total of 65 recombination breakpoints observed, 58 chromosomes had single recombination breakpoint (Fig. 12.1a), 2 had double recombination breakpoints, and 1 had triple recombination breakpoints. The mean number of recombination breakpoints was similar for both Lp (0.8) and Fp (0.82) chromosomes.

FISH with both rDNA probes (Fig. 12.1b) showed inter-individual variation in number and chromosomal location of 5S and 35S ribosomal DNA sites in the BC_1 plants. The number of 5S rDNA sites ranged from 3 to 5, while the number of 35S rDNA sites ranged from 10 to 13. The number of Lp homologues of chromosome 3 also varied and ranged from 2 to 4. Variation in chromosome patterns of rDNA loci is common, and has been previously observed in diploid and tetraploid Lp and Fp cultivars and triploid and tetraploid F_1 *Festuca* × *Lolium* hybrids (Książczyk et al. 2010). As was expected in the BC_1, three Lp and one Fp large 5S rDNA sites were interstitially located, while the additional small 5S rDNA locus was always found in a distal region of one unknown Fp chromosome. The 35S rDNA sites were always located at the secondary constrictions on the Lp chromosomes (1, 2 and 3), as well as proximally located close to the centromere (7), while in the Fp chromosome (2) they were located at the secondary constriction. The rDNA-FISH revealed that Lp chromosome 3 and Fp chromosomes 2 and 3 were also involved in recombination as well as showing rearrangements. Among 58 recombined Lp/Fp chromosomes, 15 Lp and 8 Fp were rDNA-bearing, of which 5 Lp had both 5S and 35S rDNA loci (chromosome 3), 10 had Lp 35S rDNA locus (chromosomes 1, 2 or 7) (Fig. 12.1b), 5 had Fp 35S rDNA locus (chromosome 2), 2 had Fp large 5S rDNA locus (chromosome 3), and 1 had Fp small 5S rDNA locus (unknown chromosome).

12.4 Conclusions

BC_1 progeny studied by GISH showed variability in respect of Lp and Fp somatic chromosome number, recombined chromosomes and homoeologous recombination breakpoints. Variation in the number of 5S and 35S rDNA sites in BC_1 plants indicated structural karyotype heterozygosity, due to intraspecific rDNA loci variation as was observed in the parents previously studied. The combined use of GISH and FISH, together with two rDNA probes, enabled the identification of Lp and Fp recombinant rDNA-bearing chromosomes, and this technique is therefore suitable for rapid identification of the modified Lp chromosome (3) and Fp chromosomes (2 and 3). The FISH/GISH technique can now be used for monitoring Lp and Fp chromosome changes, although a larger number of chromosome-specific FISH probes are needed to identify all Lp and Fp chromosomes.

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Part III
Novel Emerging Tools

Chapter 13
Establishing Chromosome Genomics in Forage and Turf Grasses

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Abstract Analyses of large genomes are hampered by high proportions of repetitive DNA, that make the assembly of short sequence reads difficult. This is also the case for meadow fescue (*Festuca pratensis* Huds.), one of predominant grass species in temperate and Northern regions with the genome size estimated at 1C = 3,175 Mbp. This species is known for its ability to survive under freezing conditions and it has been used widely in intergeneric hybridization with various ryegrass species to produce superior Festulolium cultivars. Here we describe attempts to dissect the meadow fescue's genome into smaller fractions—individual chromosomes and groups of chromosomes. Following the methods of flow cytogenetics developed for legumes and cereals, we have developed a chromosome sorting protocol for grasses and currently we are able to sort *F. pratensis* chromosome 4 (the largest in the genome) and two groups of three chromosomes each: 2, 3, 7 and 1, 5, 6. As the first step we sequenced chromosome 4 by Illumina with 50x coverage and assembled low copy and genic regions. This facilitated detailed comparative analysis with sequenced genomes of rice, *Brachypodium* and sorghum and provided the first insight into the genome composition of this species. The possibility to purify chromosome 4 opens the way for a more efficient analysis of genetic loci on this chromosome that control important agronomic traits, such as freezing tolerance. Moreover, purified chromosomes are excellent templates for PCR screening as well as cytogenetic and physical mapping.

13.1 Introduction

Ryegrasses and fescues are among the predominant forage and turf species in temperate climates. Ryegrass species are known for their high yield, palatability, digestibility, rapid establishment, favorable nutrient characteristics, dark green color and uniformity of turf. However, they are susceptible to stress conditions such as

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winter freezing, and heat and drought during summer. Fescues have complementary characteristics to those of ryegrasses. Therefore, they are widely combined in mixtures. They have also become subjects of intergeneric hybridization. Tens of x Festulolium cultivars with various genomic constitutions have been released and are widely used in forage and turf seed industry in Europe (Kopecký et al. 2006).

Despite their agronomic importance, the progress in genetics, genomics and bioinformatics of grasses is far behind that of cereals. Among others, such studies are complicated by the outcrossing nature of these species, population-based breeding and frequent aneuploidy (Kopecky et al. 2005). Moreover, genomes of species within *Festuca-Lolium* are large and complex. The genome of meadow fescue was estimated at 1C = 3175 Mbp based on flow-cytometry, similar to that of human (Doležel et al. 2003; Kopecký et al. 2010). Ryegrass genome is just a little smaller (1C = 2623 Mbp of *L. perenne* and 1C = 2567 Mbp of *L. multiflorum*). Because of the complexity and size of genomes (full of repeats), any procedural or technical simplification would be highly welcome in genomic studies.

13.2 Chromosome Genomics

Analysis of a complex genome where a majority of DNA is present as repeats can be simplified by several approaches. Several methods were used to avoid sequencing of the repetitive parts of genomes. Sequencing of cDNAs to generate ESTs (Expressed Sequence Tags) is among the most successful. However, ESTs fail to sample rare or conditional transcripts (Martienssen et al. 2004) and other methods were proposed to target the gene space in large and complex genomes (Cot fractionation and methyl filtration) (Peterson et al. 2002; Rabinowicz et al. 1999). Unfortunately, these two methods did not provide the expected improvements.

Another alternative to overcome the complexity of large genomes is to dissect them to smaller parts and sequencing these parts individually. Working with naturally uniform and independent units—chromosomes—is perhaps the most powerful approach. In hexaploid wheat, individual chromosomes represent only 1–3 % of the entire genome (Doležel et al. 2009) and even diploid species' chromosomes (as meadow fescue) dissect the 3.2 Gbp genome into 373–543 Mbp units, each representing 11.7–17.1 % of the whole (Kopecký et al. 2010).

There are two ways to isolate individual chromosomes: microdissection and flow-sorting. Microdissection enables the isolation of any chromosome or a chromosome segment. However, the total yield is limited to only a few copies of a particular chromosome (Zhou and Hu 2007). Flow cytometric sorting relies on differences in chromosome size. It can generate samples of unlimited copy numbers of specific chromosomes with purity usually exceeding 90 %. Moreover, DNA of sorted chromosomes is suitable for further molecular analysis (Doležel et al. 2004). The output of flow cytometry analysis is a histogram of relative chromosome fluorescence intensity (reflecting chromosome size), which is called a flow karyotype (Fig. 13.1). Ideally, each chromosome is represented by a single peak on a flow karyotype. However,

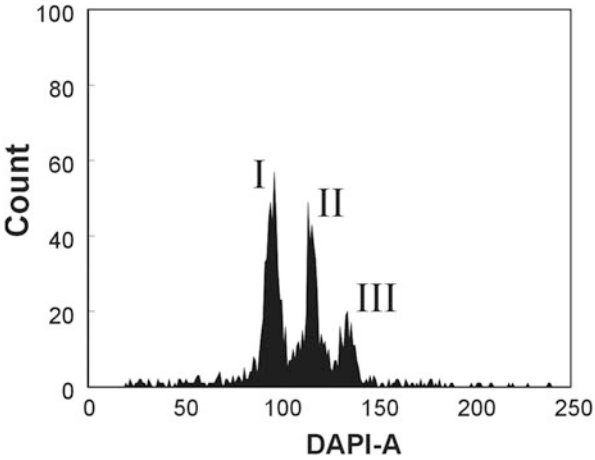

Fig. 13.1 Flow karyotyping in *F. pratensis* ($2n = 2x = 14$). The flow karyotype consists of two peaks representing groups of chromosomes and one peak representing chromosome 4F. Peak 1 represents small chromosomes 1F, 5F and 6F and Peak 2 consists of chromosomes 2F, 3F and 7F. *X axis* relative DAPI fluorescence intensity, *Y axis* number of events

chromosome size similarities frequently result in the formation of composite peaks formed by mixtures of two or more chromosomes. Doležel et al. (2009) proposed that there has to be at least 10 % difference in chromosome size to generate a separate peak on a flow karyotype. Unfortunately, in most plant species chromosomes are not that different. For example, flow cytometry analysis of hexaploid wheat (21 chromosome pairs) produced only four peaks, and only one of those contained a single chromosome, 3B (Vrána et al. 2000). All other chromosomes were present in composite peaks and could not be sorted into uniform fractions. Similarly, in barley only chromosome 1H, and in rye only chromosome 1R can be sorted into pure fractions (Suchánková et al. 2006; Kubaláková et al. 2003). However, the plasticity of plant genomes (especially in polyploids) makes it possible to develop special cytogenetic stocks with reconstructed karyotypes. Telosomic lines made it possible to sort individual chromosome arms in wheat; in instances where a telocentric overlaps in size with smaller complete chromosomes, isochromosomes provide a solution. Similarly, wheat-rye and wheat-barley addition lines enabled sorting of individual chromosomes or chromosome arms of barley and rye (Doležel et al. 2009). Nowadays, all wheat, barley and rye chromosomes/chromosome arms can be isolated in pure fractions and are being used for physical mapping, sequencing and map-based cloning.

13.3 Karyotypes of Grasses (Fescues and Ryegrasses)

Meadow fescue (*Festuca pratensis* Huds.) is a diploid species with seven chromosome pairs. The largest is metacentric chromosome 4, followed by another metacentric chromosome 3 (the one with a prominent secondary constriction), submetacentric chromosome 2 and metacentric chromosome 7. The smallest are submetacentric chromosomes 5, 6 and 1. Individual lengths (as determined at C-metaphase) vary

from 4.67 μm to 6.79 μm (Kopecký et al. 2010). Italian ryegrass (*Lolium multiflorum* Lam.) has a similar karyotype, with chromosome length ranging from 3.37 μm (chromosome 1) to 5.33 μm of chromosome 4 (Kopecký et al. 2010). The largest is the metacentric chromosome 4. Similar in length are submetacentric chromosome 2 and metacentric chromosomes 3 and 7. These three chromosomes carry secondary constrictions. The smallest are submetacentric chromosomes 5, 6 and 1. Perennial ryegrass (*Lolium perenne* L.) has a very similar karyotype. On the other hand, the karyotype of hexaploid tall fescue (*F. arundinacea* Schreb.) consists of 21 chromosome pairs of similar size.

The variation in length of diploid grass species' chromosomes was promising for separation (and sorting) of individual chromosomes using flow cytometry. The above mentioned over 10 % difference in chromosome size allowed us to predict that chromosome 4 would form separate peak on the flow karyotype and generate a pure sample, while the remaining six chromosomes would form two composite peaks.

13.4 Flow Cytometry and Chromosome Genomics in Grasses

After overcoming problems with the synchronization of cell cycle in such puny little root tips of germinating seed of meadow fescue, we were able to isolate and stain mitotic chromosomes for a flow cytometry analysis. As predicted, the flow karyotype of meadow fescue consists of three peaks (Fig. 13.1). The first (composite) peak represents three smallest chromosomes—1F, 5F and 6F. Another three chromosomes (2F, 3F and 7F) form the composite second peak. The third peak represents chromosome 4F. Thus, this chromosome is the only one which can be directly sorted from the standard karyotype. Nowadays, we routinely sort chromosome 4F with purity exceeding 92 %.

The two composite peaks of the meadow fescue flow karyotype may be partitioned into sections, as done in wheat, theoretically enabling sorting of particular chromosomes with a reasonable purity. Moreover, special cytogenetic stocks could be used to sort specific chromosome constructs, such as those used in cereals. The difference in chromosome size between *L. multiflorum* and *F. pratensis* warrants a prediction that chromosomes 2F, 3F and 7F can be sorted from disomic substitution lines, where two homologous chromosomes of tetraploid *L. multiflorum* are substituted by their homoeologues from *F. pratensis* (Kopecký et al. 2008a). We do have such lines available for every chromosome of *F. pratensis*.

13.5 Applications for Sorted Grass Chromosomes

Sorted chromosomes can be used for a wide range of applications in genomics, genetics and cytogenetics.

13.5.1 Bacterial Artificial Chromosome (BAC) Libraries

The development of a chromosome specific BAC library is one of the most attractive uses of flow-sorted chromosomes. The strategy of fingerprinting a well defined part of the genome (particularly single chromosomes/chromosome arms) and sequencing clone by clone is certainly more precise and efficient than the whole genome sequencing, especially in all cases where high proportions of repeats make contig assembly a challenging proposition. Chromosome specific BAC libraries are attractive resources for physical mapping by ordering BAC clones according to their fingerprint patterns. Such libraries have been developed for most of wheat chromosomes/chromosome arms and the short arm of rye chromosome 1 (1RS) (Šimková et al. 2008; Šafář et al. 2010). The utility of chromosome specific BAC libraries was demonstrated by the construction of a physical map of chromosome 3B of wheat (Paux et al. 2008).

Until now, only the whole genome BAC libraries have been available for species of the *Festuca—Lolium* complex. Donnison et al. (2005) and Farrar et al. (2007) developed BAC libraries for *F. pratensis* and *L. perenne*, respectively. A partial BAC library for *F. pratensis* was developed and used for cytogenetic mapping by Kopecký et al. (2008b, 2010).

Successful sorting of chromosome 4 of *Festuca* (and presumably of *Lolium*) opens the way for the development of a BAC library specific for this chromosome, BAC libraries for chromosomes 2F, 3F and 7F could be potentially created using single chromosome substitution lines. Given the above statement on partitioning of composite peaks, we can assume that enriched BAC libraries for other chromosomes (with purity of ~65 %) could be developed as well.

13.5.2 Development of Molecular Markers

Chromosome specific BAC libraries are a valuable source of molecular markers. By BAC End Sequencing (BES), Paux et al. (2008) generated over 700 ISBP (Insertion Site-Based Polymorphisms) markers for chromosome 3B, which are already used in breeding programs. Similarly, using BES, Bartoš et al. (2008) and Kofler et al. (2008) developed ISBP and SSR markers, respectively, specific for the short arm of chromosome 1R of rye. The advantage of chromosome-based approaches for marker development is that the chromosomal specificity of markers can be verified by PCR on flow-sorted chromosomes serving as templates.

Genetic maps are available for both ryegrass species (*L. multiflorum* and *L. perenne*), as well as for tall and meadow fescues. However, the genetic map of meadow fescue comprises only 466 markers (Alm et al. 2003). The development of the DArTFest array (Kopecký et al. 2009) enriched the genetic map by additional almost 150 DArT markers (Bartoš et al. 2011), however, the number of markers is still limiting for fine genetic mapping and positional cloning of agronomically important genes. Thus, a low number of molecular markers for meadow fescue calls for the

use of chromosome-based approach. Besides the development of markers by BAC-end sequencing, the example of wheat chromosome 3B shows that a combination of flow sorting and the DArT technology is fully capable of yielding high numbers of chromosome specific DArT markers (Wenzl et al. 2010).

13.5.3 Cytogenetic Mapping Using Sorted Chromosomes

Sorted chromosomes can be used as an alternative template for cytogenetic mapping, instead of traditional cytological preps squashed or dropped on a microscopic slide. The advantage of this approach is the purity of a chromosome fraction on a slide. Moreover, dropped individual chromosomes solve the interference problem brought about by the presence of cytoplasm and cell wall residues always present on squashed preparations. Spatial resolution and sensitivity of the technique can also be significantly improved by using super stretched sorted chromosomes (Valárik et al. 2004).

We used sorted chromosomes as a template for cytogenetic mapping of various DNA sequences in *F. pratensis*. Localization of several clones from a partial BAC library identified all seven chromosomes of *F. pratensis*. The detailed cytogenetic mapping of various sequences (BAC clones and repeats) on chromosome 4 showed the effectiveness of this method. In total, we localized five BAC clones and 13 repeats (centromeric and telomeric repeats and 11 microsatellites). Surprisingly, the telomeric repeat originating from wheat produced signals not only in the telomeric regions of both chromosome arms, but also in a proximal region of the long arm (4FL). Similarly, additional signals were also detected in intercalary regions of chromosome arms 2FL, 5FL and 6FS (where S and L denote short and long arms, respectively). These signals might represent positions of ancient structural rearrangements. Signals in the centromeric/pericentromeric region was also detected by FISH with BAC clones 1F21, 1G18 (additional signals on both arms), 2B14 (additional signal on the short arm), 2D4 (additional signal on the long arm) and 2N9 (additional signals on both arms). Trials with 11 microsatellites (motifs CA, GC, TA, CAG, CAT, CGG, GAA, GAC, GAG, TAA and TAC) on chromosome 4F did not provide satisfactory results. Only the microsatellite with the CAG motif provided a signal in the distal part of the short arm of 4F. No other microsatellites were detected on this chromosome.

13.5.4 Sequencing of Individual Chromosomes

Sequencing of BAC ends from a chromosome specific BAC library provides an insight into the molecular organization of individual chromosomes. By sequencing ends of almost 11,000 BAC clones of wheat chromosome 3B-specific library, Paux et al. (2008) has shown that 86 % of sequences were repeats and only 1.2 % came from coding regions. The balance were sequences of unknown role or function. Similar

results were obtained by sequencing ends of rye 1RS specific BAC clones (Bartoš et al. 2008).

Nowadays, the dramatic reduction in cost and increase in effectiveness of the Next Generation Sequencing enables sequencing of entire chromosomes (and even genomes) in a reasonable time and for a reasonable cost (Doležel et al. 2009). Mayer et al. (2011) sequenced individual chromosomes of barley using the Roche 454 technology with 1.28–2.76x coverage. This enables the prediction of total number of barley genes (~32,000) and provided a tool for fine comparative studies with model species: rice, sorghum and Brachypodium. Moreover, a virtual gene order has been designed for all seven barley chromosomes.

We sequenced chromosome 4F by Illumina HiSeq2000 with the coverage of over 50x. Using the same approach of comparative analysis as Mayer et al. (2011) and Wicker et al. (2011), we were able to identify collinear regions of *F. pratensis* chromosome 4 in genomes of barley and three model species: rice, sorghum and Brachypodium.

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Chapter 14
DArTFest DNA Array—Applications and Perspectives for Grass Genetics, Genomics and Breeding

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Abstract DArTFest is a DNA microarray developed for the *Festuca-Lolium* complex, consisting of 7,680 probes. As it offers high-throughput and low-cost screening of thousands of genomic loci, it has been successfully used in a number of projects. These include the analysis of interspecific, intraspecific and intravarietal genetic variation, genetic mapping, assigning markers to chromosomal segments, comparative analysis of the *Festuca* and *Lolium* genomes against model plant species rice and *Brachypodium*, an analysis of genomic constitution of Festulolium cultivars and

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QTL mapping of agronomically important traits such as resistance against crown rust and frost tolerance. In order to facilitate the use of DArT markers linked to traits of interests, a large number of them has been already sequenced and conversion to PCR-based markers is in progress.

14.1 The DArTFest Array

The need for a high-throughput genotyping system has led to the development of various DNA arrays and chips that can be used to screen thousands or hundreds of thousands genomic loci in a single pass. However, most of them require the availability of extensive sequence information in the target organism. In contrast, Diversity Arrays Technology (DArT) is a microarray hybridization based technique that permits simultaneous screening thousands of polymorphic loci without any prior sequence information. DArT is high-throughput, low-cost, quick and reproducible. The DArTFest is the first DArT (Diversity Array Technology) array developed for forage and turf grasses (Kopecký et al. 2009).

For the development of the DArTFest array, 40 accessions each of *L. perenne* L., *L. multiflorum* Lam., *F. pratensis* Huds. and *F. arundinacea* Schreb, plus all seven available accessions of *F. glaucescens* Boiss. were chosen to discover the maximum genetic variability within the *Lolium-Festuca* complex and included ecotypes, cultivars and parents of mapping populations. For each of the five species tested, we developed a library of DArT clones using the *PstI/TaqI* method of complexity reduction. The DArTFest array consists of 7,680 probes derived from these methyl-filtered genomic representations (Kopecký et al. 2009). This contribution reviews the uses of the DArTFest array and highlights the contribution of this platform to grass genetics, genomics and breeding.

14.2 Genetic Diversity

The DArT technology was originally developed for fast and low-cost screening of genetic variability. In line with this, the DArTFest array was initially used for the evaluation of genetic variability in five species of *Festuca-Lolium* complex. As expected, the accessions (ecotypes, cultivars and pre-breeding genotypes) belonging to individual species always clustered together (Kopecký et al. 2009). Similarly, Pratley et al. (in preparation) evaluated genetic variability among and within three ryegrass species (*L. perenne*, *L. multiflorum* and *L. rigidum*) and tall fescue. The observed intraspecific and interspecific variability was high enough to prevent identification of individual cultivars, but it was possible to separate different species. On the other hand, individual plants of five different Festulolium cultivars always clustered based on their identity (Kopecký et al. 2011). Baird et al. (2011) used DArTFest array for evaluation of genetic variability in commercial turf-type tall fescues. The analysis revealed extremely low genetic variability among cultivars being released and

propagated for the US seed market. This may be caused by either a severe genetic bottleneck during conversion to turf germplasm and/or extensive sharing of breeding material.

14.3 Genetic and Physical Mapping

DArT markers were used to enrich the existing genetic maps of three grass species. Out of 2,761 probes, which scored positively in *L. multiflorum*, 529 DArT markers were placed on its genetic map (Bartoš et al. 2011). This significantly enriched original genetic map generated with a total 352 other markers (Studer et al. 2006). In *L. perenne*, 297 DArT markers were genetically mapped with a total map length of 966 cM (Tomaszewski et al. 2011). The average spacing between markers was reduced from 7.5 cM in the original map (Anhalt et al. 2008) to 1.54 cM. The genetic map of *F. pratensis* was enriched by 149 DArT markers to a total 736 cM (Bartoš et al. 2011). In hexaploid tall fescue (*F. arundinacea var. genuina* Schreb.), a limited number of polymorphic DArT markers resulted 115 and 88 mapped DArT markers on male (1221.9 cM) and female SSR integrated genetic map (1194.3 cM), respectively, in a pseudo F_1 test cross progenies of B400 × W279 (Azhaguvel et al. unpublished). This is in agreement with findings of much lower genetic variability in fescues as compared to ryegrass species (Kopecký et al. 2009). DArT markers are also being used for development of physical map of *L. perenne* (Ian Armstead, personal comm.).

14.4 Markers for Introgression Mapping

Using seven substitution lines of tetraploid *L. multiflorum*, each carrying one chromosome of *F. pratensis* (Kopecký et al. 2010), we were able to anchor 160 DArT markers to individual *F. pratensis* chromosomes. The anchoring could potentially be made at sub-chromosomal level using recombinant lines, where markers would be anchored to small chromosome segments—bins. Chromosome substitution lines could also be developed for *Lolium* chromosomes in *Festuca* genome and anchor DArT markers to *Lolium* chromosome bins.

14.5 Identification of Hybrids and Their Genomic Constitution

DArTFest array was used for discrimination of parental species in interspecific hybrids (Kopecký et al. 2011). Hybrid origin was proved in all plants of five *Festuca* × *Lolium* cultivars. Each cultivar was represented by 20 plants. The analysis of genomic constitution revealed differences among cultivars from intermediate types to introgression forms. Interestingly, plants belonging to individual cultivars always

formed tight clusters. Such sensitivity and resolution, which was never achieved before, calls for broader use of DArTFest array, which was confirmed to be suitable for low-cost and rapid characterization and protection of existing and newly released Festulolium cultivars.

14.6 Association Mapping and Marker Assisted Selection

Marker Assisted Selection (MAS) is said to make the breeding process more efficient and faster. However, its efficient application requires markers linked to traits of interest. Bartoš et al. (2011) were able to identify 96 DArT markers with significantly different distribution between high freezing tolerant and low freezing tolerant plants, but only five of these markers could be mapped on the *F. pratensis* genetic map. Colinearity with sequenced *Brachypodium* genome indicated the localization of a freezing tolerance QTL in the central part of chromosome 4 and a distal part of the short arm of chromosome 2 in *Lolium* and *Festuca*. Similarly, Tomaszewski et al. (2011) were able to identify QTLs for crown rust resistance in linkage groups 2, 3, 4 and 7. The same approach is in progress for identification of QTLs for snow mold and drought tolerance.

14.7 Sequence Analysis of DArT Markers

Sequencing 620 genetically mapped DArT markers provided first insights into the origin and the nature of markers on DArTFest array (Bartoš et al. 2011). Among them, 398 (64.2 %) were singletons. The remaining 222 markers were redundant and assigned to 90 marker bins, which consisted of two to six markers. Blast search against an in-house built composite plant repeat database indicated that only 44 (7.1 %) of DArT markers contained repetitive elements. These observations support the notion that DArT markers represent low-copy genomic regions (Wenzl et al. 2006). Moreover, blast searches against non-redundant protein sequences and expressed sequences revealed that a majority of DArT markers were potential gene-derived sequences (Bartoš et al. 2011). The availability of sequences from DArT markers provides an opportunity to convert them to PCR-based markers for future application in breeding and gene discovery.

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Chapter 15
Using DArT Markers in *Festuca* × *Lolium* Breeding

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Abstract DArT technology was applied to a tetraploid ($2n = 4x = 28$) *L. multiflorum* × *F. glaucescens* hybrid lineage over ten generations. By selecting 151 *Lolium*- and 210 *Festuca*-specific DArT markers among the 3,884 ones developed by Kopecký et al. (Development and mapping of DArT markers within the *Festuca–Lolium* complex, BMC Genomics 10:473, 2009), it is shown that DArT polymorphism is well consistent with the history of the plant material and the events in relation with interspecific hybridisation: amphiploidisation, introgression into 4*x*- and 2*x*-*L. multiflorum*, reduction of effective size, chromosome mapping as well as parent-specific response to summer water deficit. In this respect, frequency of *Festuca* markers within a 4x-BC1 population was found to have increased on average by 2.4 % among plants having survived in sward after summer while frequency decreased by 10.2 % after three generations of seed multiplication from the initial polycross of BC1 parents.

Keywords Molecular markers · Interspecific hybridisation · Marker-assisted introgression · Drought tolerance

15.1 Introduction

Since 2004, *Festulolium* definition covers any hybrid variety between *Festuca* sp. and *Lolium* sp. in agreement with EU directive (2004/55/EC). If both *L. multiflorum* and *L. perenne* have been involved at about the same extent in *Festuca* × *Lolium* hybridisation, only *F. pratensis* has been widely used as *Festuca* parent so far. In

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particular, *F. glaucescens* ($2n = 4x = 28$) is not so well documented although, as *F. pratensis*, it is a progenitor of the modern hexaploid tall fescue, *F. arundinacea* ($2n = 6x = 42$). In amphiploid *Festulolium* hybrids, *F. glaucescens* enables more homologous chromosome paring and more stability over generation comparatively to amphiploids derived from *F. pratensis*. Better persistency provided by *F. glaucescens* relatively to *F. pratensis* was also established from recent registration of *L. multiflorum* × *F. glaucescens* amphiploid cultivars on the French National list (Ghesquière et al. 2010). Deep water extraction in summer from long-living rooting system was demonstrated to be a key-function in *Festuca* for improving summer drought tolerance in *Lolium* sp. (Durand et al. 2007).

From an evolutionary point of view, all *Festulolium* varieties including amphiploid forms from *F. pratensis* have a marked shift towards *Lolium* genome, which is even more enhanced following backcross into *Lolium* sp., further breeding and/or seed multiplication (Canter et al. 1999; Kopecký et al. 2006; Zwierzykowski et al. 2006). Although genome *in-situ* hybridisation (GISH) method is effective to describe evolution at this scale, a more high-throughput and accurate technology is lacking for a quantitative survey of genetic changes over *Festulolium* breeding. DArT technology based on DNA restriction and hybridisation on a micro-array revealed to fulfil these requirements of robust system for broad genome coverage (Jaccoud et al. 2001). It was first applied within the *Festuca*/*Lolium* complex of species by Kopecký et al. (2009) with successful application to genotyping of *Festulolium* hybrids (Kopecký et al. 2011) and to mapping in *L. multiflorum* and *F. pratensis* (Bartoš et al. 2011).

This paper reports quantitative genome changes in a *Festulolium* hybrid plant material derived from *F. glaucescens*. This was achieved by sampling plant individuals over a ten generations *L. multiflorum* × *F. glaucescens* hybrid lineage ranging from amphiploid forms up to 4x- and 2x-introgression forms into *L. multiflorum*, and by using 2149 DArT markers. The assay also included the evaluation of DArT marker response to drought by sampling, after summer, surviving plants within a dense sward of an introgression *Festulolium* population.

15.2 Materials and Methods

A 94-DNA sample microplate was elaborated from one individual of *F. arundinacea* ($2n = 6x = 42$) as control, 3 F1-hybrids between *L. multiflorum* and *F. glaucescens* ($2n = 4x = 28$) and the ten parents of the amphiploid *Festulolium* cultivar *Lueur* of F4 generation. Furthermore, 73 individuals deriving from one generation of backcrossing (BC1) amphiploid hybrids into 4x-*L. multiflorum* were added. They included nine out the ten parents of the cultivar *F99.4* and 64 individuals of the commercial seed generation, i.e. following three generations of seed multiplication the initial polycross. Thirty-three individuals among the 64 ones were randomly sampled within a population whose plants were continuously maintained in pots after germination and hence, never knew sward conditions as well as limiting resources. By contrast, 31

individuals came from a plot broadcast sown in spring 2005 which went through a severe summer water-deficit before sampling in September 2006. The last 7 individuals were diploid ($2n = 2x = 14$) after two generations of seed multiplication of a BC4 introgression mapping population of *F. glaucescens* chromosome 5 (SAGES 2004). Thus, the whole lineage encompassed ten generations, all deriving from the same original *F. glaucescens* source, either of 4x- or 2x-ploidy level and of amphiploid or introgression nature.

Ten µl of purified DNA at a concentration of 100 ng/µl each sample was provided at Diversity Array Technology Pty Ltd (DArT P/L, Yarralumla, Australia). All individuals were genotype-profiled by using the same procedure described by Kopecký et al. (2009) and a set of 2,149 DNA probes, of which 1,667 belonged to the initial DNA library of 3,884 polymorphic clones. This library was developed from 39 individuals of *Festuca arundinacea*, 40 and 7 individuals of *F. pratensis* and *F. glaucescens* (respectively) and 40 individuals of each of the two diploid *Lolium* species, *L. perenne* and *L. multiflorum*. Although unbalanced in terms of number of individuals, all species were expected to have equally contributed to the final DArT array of 3,884 polymorphic clones by the initial production of 1,536 restricted DNA-clones each species.

1,667 markers among the 2,149 assayed had individual score published (http://www.biomedcentral.com/content/supplementary/1471-2164-10-473S1.xls) which enabled to compute marker frequency within each species. A marker was hold as specific of any species when its frequency was less than 0.01 in the 4 other species. *Fa-Fg* markers, *i.e. Festuca*-specific markers but not of *F. pratensis* origin, were defined by merging scores from *F. arundinacea* and *F. glaucescens* due to unbalanced plant sampling. Ambiguous DArT scores were considered as missing data and were not further taken into account in computing. Five hundred and eleven markers among 1,667 ones were thus found to be species-specific: 205 *Fa-Fg* markers, 151 *Lm*-markers, 126 *Fp*-markers and 29 *Lp*-markers. Note that species-specific markers so defined may have mean frequency not necessarily closed to 1 within the species they are expected to be specific; mean frequency of *Fa-Fg* markers and *Lm*-markers was 0.910 and 0.740 (respectively) in the initial sampling of the DArTFest array. The rate of *Festuca* marker introgression over the *Lm* × *Fg* lineage was computed as: *Fa-Fg* score/205/(*Fa-Fg* score/205 + *Lm* score/151). A restricted set of 139 *Fa-Fg* markers and 122 *Lm*-markers was further defined on the basis to be present in the 9 parents who were available in the initial polycross of the *Festulolium* cv*F99.4*. Changes from polycross to commercial seed generation were especially surveyed at those markers. In respect with mapping, Bartoš et al. (2011) mapped 530 DArT markers in *L. multiflorum*. The present assay of 2,149 markers included 262 of those mapped markers, of which 29 belonged to the 151 *Lm*-specific markers previously identified.

Multiple correspondence analysis (MCA) was performed by using the *Corresp* procedure of SAS/STAT software (release 8.1 for 320 SunOS; SAS Institute Inc., Cary, NC, USA) to point out marker associations between individuals at any generation or plant sampling. Markers of extremely high or low frequency were used only as supplementary variables in MCA. The response of plant sampling in sward vs

Table 15.1 Number of polymorphic DArT markers and *Festuca* rate of introgression over *L. multiflorum* × *F. glaucescens* lineage. Selection of 205 *Fa-Fg* and 151 *Lm*-specific markers was performed out 1667 DArT markers; mean and standard deviation of frequency over markers refer to the original sampling of *F. arundinacea* + *F. glaucescens* and *L. multiflorum* used to produce the original DNA clone library (Kopecký et al. 2009). Number of lost DArT markers from the 122 *Fa-Fg* and 139 *Lm* markers initially present in the parent polycross of the cv *F99.4* in brackets

	Fa-Fg markers	*Lm* markers	Rate of *Fa-Fg* introgression
Total number	205	151	–
Mean frequency	0.910	0.740	–
Standard deviation	0.190	0.226	–
F. arundinacea	173	30	0.809
F1 hybrids	180	115	0.536
Cv *Lueur* parent polycross	197	129	0.529
Cv *F99.4* parent polycross	122	139	0.393
Cv *F99.4* seed			
Control	90 (32)	136 (3)	0.345
After summer	104 (18)	137 (2)	0.403
Introgression mapping population	10 (112)	118 (21)	0.086

control was tested through X^2 statistics at 1 df over all markers and for each marker individually; Fisher's exact test was performed when X^2 requirement was not met, e.g. class size higher than 5.

15.3 Results

15.3.1 Rate of Festuca-Marker Introgression Over Generation

One-hundred and seventy-three *Fa-Fg* markers gave a positive score (84.4 %) onto the *F. arundinacea* control, that is closely to the mean frequency (0.910) of those markers within the initial sampling of *F. arundinacea-F. glaucescens* (Table 15.1). On the other hand, 30 *Lm*-markers among 151 (19.9 %) gave unexpected positive response. Among F1 hybrids and parent polycross of the *Festulolium* cv *Lueur*, the *Festuca* rate of introgression was estimated to be 0.536 and 0.529 (respectively), i.e. not far from the 1:1 *Festuca:Lolium* genome balance expected from strict amphiploid hybrids. By contrast, the rate of *Fa-Fg* markers strongly decreased to 0.393 in the cv *F99.4* parent polycross, that is after four polycrossing generations following the initial BC1. Since DArT markers are of dominant inheritance, only one generation of back-crossing amphiploid hybrids into *L. multiflorum* would have not made the rate of *Festuca* introgression to depart from the 0.5 rate expected under strict disomic inheritance. In the commercial seed generation of the cv *F99.4*, the rate of introgression also contrasted according to the origin of the markers and sampling within population. Only 3 and 2 *Lm*-specific makers were lost in the seed control and in the sample of plants collected after summer (respectively) while the corresponding loss of *Fa-Fg*

Table 15.2 Change of mean frequency at the 122 *Fa-Fg* and 139 *Lm* DArT markers initially present in the parent polycross of the introgression cv *F99.4*. Allele (+) mean frequency estimated from polymorphic markers within population only, assuming gene equilibrium and dominant inheritance of the markers. Standard deviation of mean frequency in brackets

	Fa-Fg markers		*Lm*-markers	
	Mean marker frequency	Allele (+) mean frequency	Mean marker frequency	Allele (+) mean frequency
Cv *Lueur* parent polycross	0.674 (0.007)	–	0.449 (0.009)	–
Cv *F99.4* parent polycross	0.267 (0.006)	–	0.575 (0.010)	–
Cv *F99.4* seed				
Control	0.153 (0.003)	0.0647 (0.0007)	0.567 (0.004)	0.2546 (0.0015)
After summer	0.177 (0.003)	0.0647 (0.0007)	0.570 (0.004)	0.2514 (0.0015)
Introgression mapping population	0.018 (0.002)	0.1154 (0.0050)	0.494 (0.011)	0.4150 (0.0068)

markers reached 32 and 18 only (respectively). The decrease was even more enhanced as well as unbalanced in the BC4 introgression mapping population by the loss of 21 *Lm*-markers against 112 *Fa-Fg* markers. All indicates that intense selection against *Festuca* genome occurs following primary introgression in *L. multiflorum* but that sampling in the droughted sward enabled to partly reverse the rate of introgression towards its value in the initial polycross.

15.3.2 Response to Summer Drought

Mean frequency score of the 139 *Lm*-markers slightly increased from 0.449 in the cv *Lueur* parent polycross to about 0.57 in the cv *F99.4* whatever generation. (Table 15.2). On the contrary, mean frequency of *Fa-Fg* markers decreased from 0.674 to 0.267 following back-crossing. However, *Fa-Fg* marker mean frequency was found significantly higher among the plants sampled after summer (0.177 vs 0.153; $P < 0.0037$) while that of *Lm*-markers remained almost perfectly stable on average (0.570 vs 0.567; $P < 0.7972$).

Eight contrasts of frequency between sample in sward and seed control were significant ($P < 0.05$, one-tailed X^2 or Fisher's exact test) out 132 *Lm*-markers against 9 significant contrasts out 110 *Fa-Fg* markers. Global error I of significant individual tests was estimated to be <0.067 for *Fa-Fg* markers and <0.267 for *Lm*-markers. Furthermore, the 8 significant *Lm*-marker contrasts divided into 4 negative against 4 positive ones while all the 9 *Fa-Fg* marker contrasts but one were positive, suggesting that *Festuca* markers tended overall to greater positive unidirectional response than *Lolium* markers. Unfortunately, no *Lm*-marker of significant response was found already mapped by Bartoš et al. (2011).

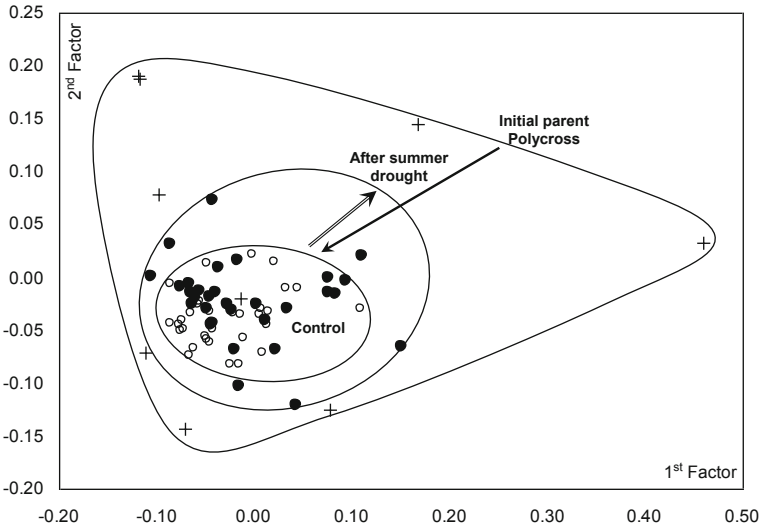

Fig. 15.1 Multiple correspondence analysis plotting on the two first factors (13.6 % and 9.4 % of total inertia, respectively) the coordinates of nine BC1 parents of the *Festulolium* cv *F99.4* (+) and 64 individuals of commercial seed generation: 33 individuals as control (○) vs 31 individuals sampled in sward after summer (●). Individual scores at 139 *Fa-Fg* and 122 *Lm* DArT markers of which, 21 and 40 markers (respectively) used as supplementary variables. *Arrows* indicate contrary variation of *Fa-Fg* marker frequency through three generations of seed multiplication vs response to summer drought within commercial seed generation

Comparison of mean frequency with rate of introgression indicates that apparent increase of *Fa-Fg* markers by sampling within the cv *F99.4* resulted from retaining numerous polymorphic *Fa-Fg* markers rather than increasing marker frequency. Multiple correspondence analysis illustrates how sampling plants after summer has made to reverse the steady loss of *Festuca* markers over three generations of seed multiplication (Fig. 15.1).

Estimate of allele frequency at polymorphic *Fa-Fg* markers, assuming gene equilibrium within population, was close to 0.065 in the cv *F99.4* against 0.115 in the diploid introgression mapping population, i.e. far, in both cases, from the 0.25 frequency expected from BC1 introgression and no selection occurring (Table 15.2). Within the tetraploid cv *F99.4*, this suggests that introgressed *Fa-Fg* markers should be predominantly present as simplex genotype (*LLLF*) rather than as genotype of higher *Festuca* dosage (duplex *LLFF*).

15.4 Discussion

The DArT markers which were experimented enabled to quantify genome changes across a diversified *L. multiflorum* × *F. glaucescens* lineage. It agreed well with the global scheme of *Festulolium* variety evolution (Ghesquière et al. 2010) based on

GISH assessment (Kopecký et al. 2006; Zwierzykowski et al. 2006). In this model, only *L. multiflorum* × *F. glaucescens* amphiploids had evolution rate considerably slow down in comparison with amphiploids from *F. pratensis*. Higher preferential homologous chromosome pairing and hence, disomic inheritance in *L. multiflorum* × *F. glaucescens* amphiploids effectively prevent that *Festuca* genome is little by little eliminated over generations. However, rapid loss of *Festuca* chromosomes occurs following backcrossing into *Lolium* sp. Using isozyme loci and 4x-BC2 introgression populations (Ghesquière et al. 2000), the rate of *Festuca* introgression was found not to decrease so strongly as from DArT makers. Possibly, stronger interspecific linkage disequilibrium in BC1 than in BC2 population enhanced higher selection rate against *Festuca* loci. Within 2x recombinant populations, similar frequency and rate of transmission over generations was found among homeoalleles at *Pgi-2* locus, whatever they come from *F. pratensis* or *F. glaucescens* (Humphreys and Ghesquière 1994). Frequency of DArT markers after two generations of seed multiplication a *F. glaucescens* chromosome 5 introgression population indicated rather stronger selection. As isozyme loci (e.g. *Pgi-2*) were not located on chromosome 5, it is possible that transmission rate varies according to *Festuca* chromosomes. Since tetraploid *F. glaucescens* is already of amphiploid nature, transmission and recombination rate into *Lolium* sp. may also differ between homologous chromosome pairs.

Response to natural selection feeds thinking about breeding for abiotic tolerance by using a *Festuca* × *Lolium* hybridization approach. Few evidences have been reported in literature. True amphiploid forms are only rarely found in nature; however, it was reported that 3x-hybrids can be continuously produced from unreduced gametes of diploid *L. perenne* and *F. pratensis* and that, depending on environmental conditions, selection makes 2x-introgression forms to develop either of predominant *Lolium* or *Festuca* genome (e.g. Humphreys and Harper 2008; Casler et al. 2002, 2009). Under artificial conditions, survival in rain-out shelters was enhanced within BC2 populations from *F. arundinacea* × *L. multiflorum* hybrids (Humphreys 1989; Humphreys and Thomas 1993). Extending this introgression scheme to independent plant material, it was found that *F. pratensis* chromosome 3 and *F. glaucescens* chromosome 5 carry favourable genes of drought tolerance (SAGES 2004), which was latter confirmed following a QTL approach within *F. pratensis* (Alm et al. 2011) and, in the field, by 2x-recombinant populations close to finished variety (Humphreys et al. 2011).

We showed herein that natural selection similarly operated within a sward of a segregating 4x-BC1 population in the span of few months of strong water deficit. However, the positive response of *Festuca* markers (+2.4 %) appeared to not balance the loss at each generation of seed multiplication (−3.4 %). Consequently, breeding for *Festuca* traits within introgression populations may be not fully effective unless it is early assisted by genotyping at a whole genome scale as provided by DArTs.

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Chapter 16
Development of an SNP Identification Pipeline for Highly Heterozygous Crops

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Abstract Next Generation Sequencing technologies significantly advance the development of molecular markers for molecular breeding. Dedicated NGS data-analysis procedures must be developed for *de novo* reference assembly and SNP discovery for crop species without a reference genome sequence. In outcrossing fodder crops, the high degree of polymorphism hampers *de novo* assembly, contig clustering, read mapping, and SNP discovery. Using selected candidate genes as case studies, we illustrate the reconstruction of a reference transcript sequence from RNA-seq data from multiple genotypes, we validate *de novo* transcript assembly by Sanger sequencing, and analyse how read mapping and SNP discovery parameters determine sensitivity and specificity during SNP discovery. Thus, we propose a general strategy to construct a non-redundant reference transcriptome for crops without a sequenced genome, using predicted proteins from a closely related model species as a guidance for clustering and annotation. This reference transcriptome is required for candidate gene discovery and exome-wide identification of polymorphisms.

Keywords *Lolium perenne* · Next generation sequencing · Gene discovery · SNP identification · Candidate gene

16.1 Introduction

The genomes of outcrossing fodder crops are characterized by a high degree of heterozygosity and heterogeneity within populations and cultivars. This high level of genetic diversity can be exploited for the development of molecular markers for linkage

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map construction, association genetics or genomic selection. Next generation sequencing (NGS) technologies significantly advance the process of gene discovery and marker development (Shendure and Ji 2008; Varshney et al. 2009). Here, we propose and validate bioinformatics procedures to identify DNA-polymorphisms in candidate genes in *Lolium perenne*. First, because a fully annotated reference genome sequence is not yet available for *L. perenne*, we performed *de novo* assembly of Illumina RNA-seq data from 14 independent *L. perenne* genotypes, which can be mined for candidate genes. Second, we selected orthologs of *TB1* and four candidate genes in the MAX/RMS/DAD pathway that act in the strigolactone biosynthesis and response pathways, which control branching and plant architecture (Leyser 2009). These candidate genes were PCR-amplified from the 14 genotypes used for NGS sequencing, cloned, and Sanger sequenced to validate *de novo* transcriptome assembly. Third, these Sanger sequence data were used to analyse the genetic diversity, and to evaluate parameters for read mapping and SNP discovery. Based on these findings, we propose a general strategy for *de novo* assembly of a reference transcriptome and subsequent SNP discovery in highly polymorphic crop species.

16.2 Material and Methods

Ilumina GAII transcriptome sequencing Fourteen *L. perenne* genotypes representing different architectural types were selected from an association mapping population of 602 genotypes. Two independent samples of basal stem tissue containing axillary meristems were collected from each genotype. After RNA extraction (RNeasy including DNase treatment; Qiagen) and quality control (Agilent 2100 Bioanalyzer), 5 μg of total RNA from the replicated samples were pooled per genotype, and used for non-normalised, indexed mRNA-Seq library preparation (mRNA-Seq Sample Prep Kit; Illumina, protocol version 1004898 Rev. D, September 2009; and Multiplexing Sample Preparation Oligonucleotide Kit; Illumina). The concentrations (Qubit fluorometer; Invitrogen), the insert size (range between 100–200 bp) and purity of the libraries were determined (Agilent 2100 Bioanalyzer). Equimolar amounts of two libraries were pooled per lane of a paired-end flow cell (v4). Clustering was conducted on an Illumina Cluster Station followed by $76 + 7 + 76$ cycle sequencing on an Illumina Genome Analyzer (version IIx, SCS 2.6.26/RTA 1.6.47.1) using the Genomic DNA Sequencing Primer, the Index Seq Primer, the Multiplexing Rd2 Seq Primer, and clustering and sequencing kits (SBS v4; Illumina). About 15–30 million paired-end reads were obtained per genotype. After trimming for low quality bases (limit 0.01; minimal length 25 bp; CLCbio), *de novo* assembly was performed with the CLCbio Genomics Workbench (v4.0.3) using default parameters, obtaining so-called CLC-contigs. A second round of clustering of the CLC-contigs was performed with CAP3 (Huang and Madan 1999) with default settings using in-house developed Perl scripts, yielding so-called CAP3-contigs.

PCR amplification, cloning, and Sanger sequencing of candidate genes For each candidate gene, primers were designed with the Primer3 software in SNP-free

regions of the consensus sequence obtained after the second round of clustering with CAP3. Fragments of candidate genes were PCR-amplified from genomic DNA and cloned into the TOPO TA cloning vector (Invitrogen). For each genotype, six colony-PCR products per amplified fragment were verified by agarose gelelectrophoresis. Of these, four colony-PCR products per genotype were randomly taken, purified, and Sanger-sequenced on both strands.

Identification of polymorphisms We determined the degree of polymorphism in the candidate genes in two independent ways. First, the quality-trimmed 76 bp paired-end reads were mapped to the full-length consensus sequence for each gene to discover SNPs (referred to as read mapping SNPs or 'RM-SNPs'). NGS read mapping parameters were: 90 % length fraction, 95 % similarity, in/del cost = 3, mismatch cost = 2. SNP discovery parameters were: window length = 11, max gap or mismatch count = 7, min average qual = 10, min central qual = 15, min read depth = 15, min SNP frequency = 14 %, 10 % or 7 % (≈4, 3 or 2 out of 28 alleles). Second, SNPs were identified by comparison of all Sanger sequences (referred to as 'Sanger SNPs'). SNPs were classified based on their occurrence in all sequenced alleles (maximal 28): occurring once (unique), or occurring at least twice (Sanger 2^{+}SNPs). We then used the Sanger SNPs to confirm the RM-SNPs, and determined the number of false negative and false positive RM-SNPs (Table 16.2). For *LpMAX2*, we could only PCR-amplify fragments from 4 genotypes representing one single allele, suggesting some hidden SNPs in the primer binding region (data not shown). So, we aligned and compared all LpMAX2 CLC-contigs instead, to confirm RM-SNPs. Likewise, the LpMAX4 CLC-contigs and RM-SNPs suggested the presence of a divergent allele in genotypes Lp16 and Lp20. However, this allele could not be cloned and sequenced, probably due to hidden SNPs that hamper PCR-amplification of this allele from those genotypes.

16.3 Results and Discussion

***De novo* assembly of transcript sequences and identification of candidate genes** The RNA-seq data from 14 individual genotypes were assembled separately to avoid mixing highly polymorphic allelic sequences during *de novo* assembly. The *de novo* assembler of the CLCbio Genomics Workbench reconstructed between 49,000 and 79,849 CLC-contigs per genotype.

Brachypodium distachyon is the most closely related species to *L. perenne* with a fully annotated genome sequence (International Brachypodium Initiative 2010). Therefore, we used *B. distachyon* as an intermediate for the identification of orthologs of candidate genes (*MAX1*, *MAX2*, *MAX3*, *MAX4* from *Arabidopsis thaliana* and *TB1*, a TCP-family transcription factor from *Zea mays*), using Plaza 2.0 for phylogenetic analysis of gene families (Proost et al. 2009). We selected all CLC-contigs (representing the different alleles in 14 genotypes) with a significant similarity (tBLASTn) to the candidate *B. distachyon* proteins, and clustered them with CAP3 as a 'second' assembly step. While the CLC-contigs of individual genotypes may

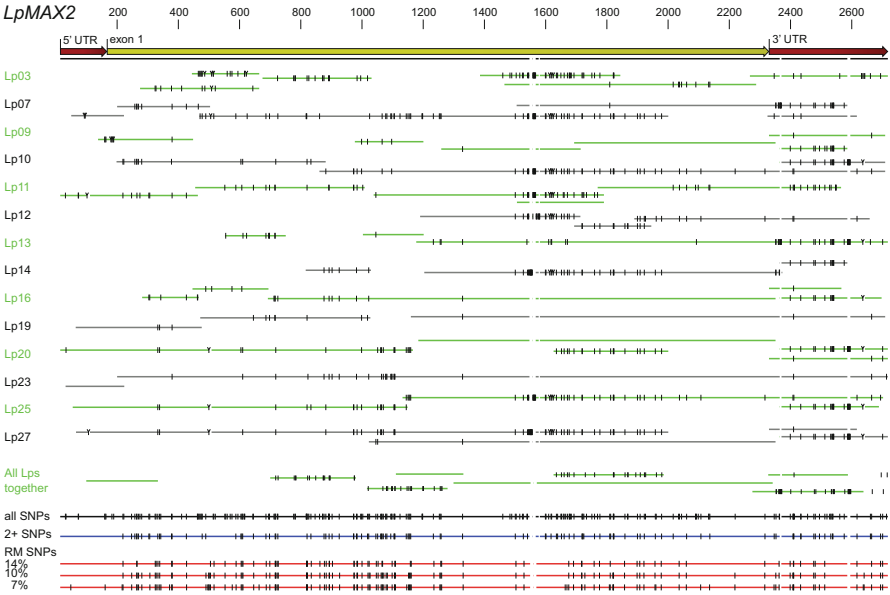

Fig. 16.1 Reconstruction of reference transcripts from RNA-seq data and identification of SNPs in *LpMAX2*. Exon (*yellow arrow*), 5′ and 3′ untranslated region (UTR; *red arrow*) and coordinates (in basepairs) are indicated on the reference sequence. CLC-contigs of different genotypes (Lp03-Lp27; coloured by alternating *green* or *black lines*) are aligned to the reference. SNP positions (*short vertical lines*) indicate different allelic variants. Contigs obtained by *de novo* assembly of all the reads from 14 genotypes together are indicated for comparison (All Lps together). SNPs identified by comparing contigs are indicated if they occur at least once (all SNPs, *black line*) or in multiple contigs (2^{+}SNPs, *blue line*). SNPs identified by mapping RNA-seq 76 bp paired-end reads to the common reference (RM-SNPs; using a SNP frequency of either 14 %, 10 % or 7 % of the reads) are indicated on red lines

contain only partial or fragmented sequence for the candidate genes, it was possible to reconstruct near full-length transcripts by aligning the available fragments from multiple genotypes (Fig. 16.1).

The CAP3 contig assembly typically yielded a few CAP3-contigs per candidate gene. Because allelic as well as paralogous CLC-contigs are selected via BLAST, CAP3-contigs derived from paralogs must be filtered out after the CAP3 clustering. To distinguish between alleles and paralogs, we compared CAP3-contigs (BLASTx) against all *B. distachyon* proteins and retained only those with a best BLAST hit with the original candidate *B. distachyon* gene. In addition, we performed phylogenetic analysis with the orthologous gene families defined in Plaza 2.0. Finally, we identified a single ortholog for *LpMAX2*, *LpMAX3*, and *LpMAX4*, two *MAX1* orthologs, named *LpMAX1-1* and *LpMAX1-2*, and a *LpTB1* transcript that is identical to *LpTB1* isolated by Brazauskas et al. (2010).

Construction of a non-redundant reference transcriptome sequence Application of RNA-seq for gene discovery, gene expression analysis, and SNP discovery

requires the construction of a non-redundant reference sequence for read mapping. This reference should contain a single representative (consensus) sequence of each locus. Otherwise, the estimated number of genes per family is wrong, and reads are divided between redundant reference contigs covering the same locus, leading to bias in expression levels and failure to detect polymorphisms.

During *de novo* assembly, regions with few polymorphisms are collapsed into a single reference sequence, representing the consensus of the alleles. In contrast, short regions of high polymorphism density tend to block contig extension, resulting in neighboring, non-overlapping contigs. In addition, allelic sequences with long stretches of high polymorphism density are considered as distinct sequences during *de novo* assembly and are consequently reconstructed as two independent contigs, despite representing the same locus. These issues hamper *de novo* assembly of highly polymorphic genome sequences (Zharkikh et al. 2008, Miller et al. 2010, Velasco et al. 2010, Donmez and Brudno 2011). Similarly, *de novo* assembly of transcript sequences in highly polymorphic species typically leads to the assembly of multiple (on average 2 to 4 in our data set) CLC-contigs per transcript in each *L. perenne* genotype, hence a highly redundant reference transcriptome. The number and position of contig breakpoints depends on the specific combination of allele-pairs in a given genotype. Therefore, allelic fragments obtained from multiple independent genotypes will overlap at least partially and can be aligned in a second step using clustering algorithms such as CAP3 (Fig. 16.1).

Paradoxically, combining RNA-seq data from multiple genotypes before the *de novo* assembly only makes the fragmentation issue worse by increasing the degree of polymorphism. For example, *de novo* assembly of reads from all genotypes together, yields eight contigs for *LpMAX2*. These are fragmented and partially redundant, and cover less than the total length of the transcript (Fig. 16.1). In contrast, combining contigs from multiple genotypes after the *de novo* assembly actually helps solve this fragmentation issue. Obtaining more sequence data from a single genotype can not solve the fragmentation problem, because it is caused by the degree of polymorphism between alleles, not by low sequence depth itself. Our results show that it is more effective to first assemble transcriptomes from multiple genotypes in parallel (k-mer based *de novo* assembly), followed by a second clustering step using a global sequence alignment algorithm (e.g. CAP3), than to increase the tolerance to polymorphisms during primary *de novo* assembly.

Validation of *de novo* transcript assembly by comparison to Sanger sequencing of candidate genes The accuracy of the transcriptome sequence and subsequent SNP identification depends on the accuracy of the *de novo* assembly. We compared CLC-contigs to individually cloned and Sanger-sequenced alleles of the respective genotypes. The number of nucleotide substitutions reflects the putative *de novo* assembly error rate. In a total aligned sequence length of 22,284 bp, 102 nucleotides (0.46 %) were different between the cloned fragments and the *de novo* assembled sequences in the respective genotypes (Table 16.1). In the case of *LpMAX4,* it is possible that failure to PCR-amplify a divergent allele from genotypes Lp16 and Lp20 precluded confirmation of CLC-contigs, leading to an inflated estimation of

Table 16.1 Validation of *de novo* transcript assembly by comparison to Sanger sequencing of candidate genes

Gene	Average read depth (number of reads/ position/genotype)	Total length of aligned sequence (bp)	Number of mismatches	Mismatches (%)
LpMAX1-1	6.1 (2.5–14.1)	8,999	37	0.41
LpMAX2	28.9 (9.4–46.6)	3,643	8	0.22
LpMAX3	4.3 (2.4–6.6)	2,623	14	0.53
LpMAX4	8.6 (3.8–12.0)	3,758	39	1.04
LpTB1	4.6 (2.6–8.8)	3,261	4	0.12
Total	*10.5*	*22,284*	*102*	*0.46*

the error rate. In general, these five candidate genes had a relatively low read depth per genotype in our NGS data (Table 16.1), and we expect that *de novo* assembly of genes with a higher read depth will be even more accurate. In any case, the high level of accuracy (>99.5 %) reflects the reliability of the *de novo* assembly, and confirms that the procedure described here can be used for gene discovery and identification of polymorphisms.

Genetic diversity in candidate genes Using the read mapping and SNP identification parameters described in the material and methods section, we detected 157 RM-SNPs that could be confirmed by Sanger sequencing, and only two false positive RM-SNPs. The SNP density in these candidate genes as estimated by Sanger sequencing of 10–14 genotypes ranges between 3–4 SNPs per 100 bp for the 2^{+}SNPs, and can reach up to more than 8 SNPs per 100 bp including unique alleles. The 18 false positive RM-SNPs of *LpMAX4* listed in Table 16.2 are probably caused by failure of PCR-amplification of the corresponding alleles from genotypes Lp16 and Lp20. So, these parameters can be used to generate a transcriptome-wide high quality RM-SNP set for the development of gene-specific molecular markers.

We also detected a high percentage of false negative RM-SNPs: up to one-third of the 2^{+}SNPs and almost all of the unique SNPs in the Sanger datasets. Two factors may cause significant underestimation of the polymorphism level. First, a substantial fraction of the diversity may reside in a large number of 'unique' alleles per gene, and these fall below the detection limit. Lowering the SNP frequency threshold (from 14 % to 10 % or 7 %) may allow detecting more SNPs, including unique alleles, as demonstrated for *LpMAX1* and *LpMAX2*, but also increases the number of false positive RM-SNPs. In the case of *LpMAX1*, disregarding low frequency SNPs implies excluding as much as five unique alleles from 14 genotypes (data not shown), and about half the number of SNPs, thus representing a substantial fraction of the genetic diversity.

Second, a high local density of SNPs (i.e. read mapping mismatches) precludes reads from divergent alleles to be correctly placed onto the reference, thus eliminating them from SNP discovery. In the case of *LpMAX2*, 36 of the 96 2^{+}SNPs were not detected in the RM-SNP set. These are mostly located in regions with high polymorphism density. In *LpMAX3*, we identified only two distinct alleles in 11 genotypes,

Table 16.2 Validation of read mapping SNPs by comparison to Sanger sequencing of candidate genes

Gene Length (bp) Read depth	SNP-type	Sanger/CLC-contig SNPs		False positive RM SNP	Total	SNP density (SNP/100 bp)	
		unique	2^+			2^+	Total
LpMAX1	Sanger	58	51		109	3.1	6.7
1637 bp	RM 14 %	0	27		27		
70.5x	RM 10 %	4	33	1	38		
	RM 7 %	8	38	7	53		
LpMAX2	CLC-contigs	81	99		180	4.4	8.6
2187 bp	RM 14 %	10	63	1	74		
354.0x	RM 10 %	14	78	4	96		
	RM 7 %	16	84	9	109		
LpMAX3	Sanger		34		34	3.8	3.8
897 bp	RM allele 1		12		12		
24.3x	RM consensus		21		21		
LpMAX4	Sanger	2	31		33	2.1	2.2
1488 bp	RM 14 %		27	2	29		
38.8x	RM 10 %		27	7	34		
	RM 7 %		27	11	38		
LpTB1	Sanger		2		2	0.2	0.2
972 bp	RM 14 %		2		2		
51.2x	RM 10 %		2		2		
	RM 7 %		2	1	3		

which differ at 34 SNPs in 897 bp (Table 16.2). Only 12 RM-SNPs are detected using allele '1' as reference for read mapping and SNP discovery, because reads derived from allele '2' are not mapped onto the reference due to high numbers of mismatches (polymorphisms). However, if the two allelic sequences are merged into a consensus sequence and then used as reference, 21 SNPs of a total of 34 Sanger 2^+SNPs are correctly detected in the RM-SNP set (Table 16.2). Clearly, this effect is independent of the read depth or the frequency of the alleles in the population, but leads to a strong local underestimation of allelic diversity in regions with the highest polymorphism density. These data illustrate that the threshold level of sequence similarity used for read mapping must be adjusted to the expected number of polymorphisms or, *vice versa*, that the reference sequence must be adjusted to optimally facilitate read mapping.

So, the selection of the reference sequence is critical. Importantly, a common reference sequence can be constructed 'on the fly' by CAP3-clustering of all alleles in the sample pool and using the resulting consensus sequence as representative allele (e.g., *LpMAX3*). This sequence may not exist as a 'true' allele in the population, but as a consensus it should have the minimal number of mismatches to all allelic variants and thus provides a more balanced approach to determine the mismatch threshold for read mapping within that specific sample pool. Using any of the existing alleles (either from within the sample pool, or from a fully sequenced reference genotype outside the current sample pool) creates more bias. All reads originating from this

allele would have a perfect match while all other reads would have increasing mismatch penalties depending on the genetic distance between alleles, hence inferring combined mismatch treshold/reference-dependent bias.

An alternative to short read mapping, is to use the CLC-contigs from *de novo* assembly for SNP identification or confirmation, as was demonstrated for *LpMAX2*. Other alternatives are to perform read mapping per genotype if sufficient read depth is available, or to perform pairwise comparisons of all genotypes.

False negative (hidden) SNPs interfere with the design and performance of SNP marker assays. For the design of primer extension assays, or probe-based genotyping assays, typically the 50 basepair sequences flanking the selected SNP should not contain neighboring SNPs. With an average density of 3–4 2^{+} SNPs per 100 bp, it may prove difficult to find such SNP-free regions. In addition, a relatively high percentage of false negative (hidden) SNPs flanking known markers can cause failure of the SNP assay. Therefore, suppressing false negative SNP identification improves the SNP assay conversion rate and SNP calling rate for genotyping. Especially in species with such high degree of polymorphism, Genotyping by Sequencing (GBS) may prove to be a valuable alternative to probe-based or primer-based SNP-genotyping assays.

16.4 Conclusion

De novo assembly algorithms were first developed for species with a relatively low degree of polymorphism, such as naturally inbreeding *Arabidopsis thaliana*, but are less suitable for highly polymorphic species. They tolerate only a low frequency of polymorphisms, as to resolve highly repetitive regions for reference genome assembly. As a consequence, *de novo* assembly introduces contig breakpoints at highly polymorphic regions in a genotype-dependent manner. We propose a two-step assembly strategy that resolves transcript fragmentation and simultaneously collapses separately assembled allelic sequences (obtained either within a genotype or across multiple genotypes) into a non-redundant consensus transcriptome required for read mapping, expression analysis and SNP discovery. We propose that a complete transcriptome can be reconstructed in *L. perenne*, by first performing *de novo* transcriptome assembly of multiple genotypes, then systematically performing BLAST searches with all proteins of a reference species, followed by CAP3 clustering of the CLC-contigs from different libraries (genotypes, treatments, and/or tissues), and filtering CAP3-contigs based on best reciprocal BLAST hits. Analysis of the *LpMAX2* and *LpMAX3* genes illustrates how the choice of the reference sequence influences the number of identified RM-SNPs, and why constructing a common reference sequence is a useful strategy to suppress false negative SNPs, while maintaining stringency for read mapping.

Based on these case studies, we optimised parameters for *de novo* assembly, contig clustering, and SNP identification. These will be integrated into a bioinformatics pipeline to reconstruct a reference *L. perenne* transcriptome, using the predicted

protein set of a closely related species, such as *B. distachyon*, as guidance for clustering and annotation. This database can then easily be searched for orthologs of candidate genes and includes allelic variants when multiple genotypes have been sequenced in parallel. Thus, read mapping onto this reference transcriptome facilitates the identification of polymorphisms in a transcriptome-wide sequence set for crop species without the need for a fully sequenced genome. This bioinformatics pipeline can be easily adapted to other highly heterozygous non-model species through the use of precomputed orthologous protein sets derived from a number of model species. In conclusion, the SNP discovery is greatly enhanced through NGS and can generate more markers at lower cost for application in molecular breeding.

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Chapter 17
First Insights into the Mitochondrial Genome of Perennial Ryegrass (*Lolium perenne*)

K. Diekmann, T. R. Hodkinson, K. H. Wolfe and S. Barth

Abstract Plant mitochondrial genomes encode the majority of genes that are involved in aerobic respiration which provides energy for the plant cell. To date only 32 plant mitochondrial genomes have been sequenced completely and only one from the Pooideae subfamily to which *Lolium* belongs. We aimed to sequence the complete mitochondrial genome of perennial ryegrass to assess its variation in comparison to other grasses and to provide insights into agronomically important traits such as cytoplasmic male sterility. We found all 33 known plant mitochondria protein-coding genes, three ribosomal RNA and 20 transfer RNA genes. However, plant mitochondrial genomes are complex in their structure and exhibit a low degree of conservation in relation to other mitochondrial genomes. Thus, a complete assembly of the perennial ryegrass mitochondrial genome was not possible due to the lack of a suitable reference genome and the high variability. However, we reduced the large number of contigs to a set of 43 that contained either protein-coding genes or consisted of more than 10 kb. Based on this data set we found evidence for intracellular gene transfer events from the chloroplast genome to the mitochondrial genome and possibly duplicated gene exons. We estimated the size of the mitochondrial genome of perennial ryegrass to be approximately 565 kb.

Keywords *Lolium perenne* · Perennial ryegrass · Mitochondrial genome

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17.1 Introduction

Mitochondria can be found in fungal, animal and plant cells. They are commonly known as the power plants of the cell because they are the location of the aerobic respiration and thus involved in major metabolic processes. Mitochondria are believed to have endosymbiotic origins. They very likely derive from the rickettsial subdivision of α-proteobacteria. The most mitochondrial-like eubacterial genome sequenced belongs to the α-proteobacterium *Rickettsia prawazekii* (Gray et al. 1999). It is believed that the ancestral mitochondrial genome either lost or transferred the majority of its genes to the nucleus. Nowadays only 50–60 genes remain within plant mitochondrial genomes. From a plant breeding perspective mitochondrial genomes are nevertheless interesting because mitochondria are, in general maternally inherited and mutations within the plant mitochondrial genome can confer cytoplasmic male sterility (CMS) which is a valuable tool in hybrid plant breeding. CMS has also great potential in *Lolium perenne* breeding. Hence gaining information about the *L. perenne* mitochondrial genome was the main objective of the present study.

To date, more than 2000 animal mitochondrial genomes have been sequenced and made publicly available. In contrast only 32 plant mitochondrial genomes are publicly available (Fig. 17.1). The first plant mitochondrial genome sequence was published in 1993 (*Marchantia polymorpha*). In 1997, the mitochondrial genome sequence of the model plant *Arabidopsis thaliana* became available. The first grass mitochondrial genome sequence, *Triticum aestivum*, was published in 2005. Differences between the number of publicly available animal and plant mitochondrial genome sequences are very likely due to the complex nature of plant mitochondrial genomes. While animal mitochondrial genomes are rather conserved in their genome size (varying from 10−50kb, ~16kb in average), plant mitochondrial genomes show a huge variation. Although not sequenced yet, the *Brassica hirta* mitochondrial genome is expected to be the smallest of the higher land plants with approximately 208 kb. *Cucumis melo*, the musk melon, on the other hand is estimated to contain the biggest mitochondrial genome. Its size is currently estimated to exceed 2,400 kb. This huge variation in genome size can not only be detected between unrelated plant species, but also within plant families. In the Cucurbitaceae family, for example, the genome size varies enourmously (~380 kb in water melon, over ~1000 kb in courgette, and more than 2,400 kb in musk melon. High variation in the genome size can also be detected within the grass family. Currently ten grass mitochondrial genome sequences are published (Fig. 17.2). So far *Triticum aestivum* contains the smallest mitochondrial genome (~452 kb) and *Tripsacum dactyloides* the biggest (~704 kb).

Plant mitochondrial genome size can be affected by several factors. One factor is the occurrence of repetitive regions that vary in size and number from one plant species to another. Horizontal and intracellular gene transfer processes also contribute to the genome size variation. In general, intracellular gene transfer events involve transfer from the chloroplast genome to the mitochondrial genome. However, while transferred ribosomal genes often remain functional in the mitochondria, protein-coding genes often become dysfunctional due to shifts in the reading frame. The

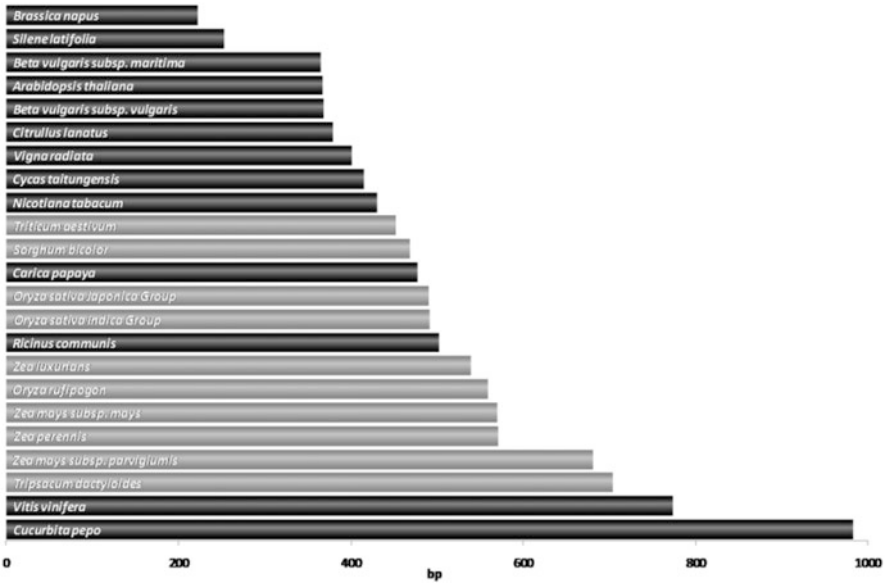

Fig. 17.1 Mitochondrial genome size of higher land plants. Highlighted are all sequenced members of the Poaceae family

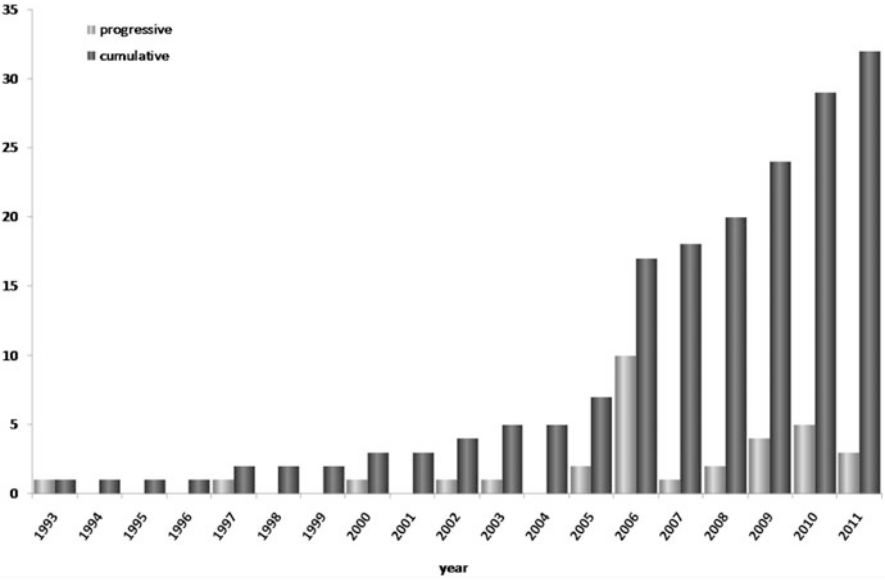

Fig. 17.2 Number of publicly available mitochondrial genome sequences from moss and higher plant species in October 2011. (www.ncbi.nlm.nih.gov)

biggest variation in genome size is very likely caused by the huge variation in the intergenic spacer regions. In all plant mitochondrial genomes the number of genes is similar. But the proportion of coding region relative to the total mitochondrial genome size varies from 3 % in the biggest so far sequenced plant mitochondrial genome (courgette) to 22 % in the smallest (rapeseed). Hence, the bigger a plant mitochondrial genome is the larger are the intergenic spacer regions. In addition, these intergenic spacer regions demonstrate a huge sequence variation. The complex nature of plant mitochondrial genomes needed to be considered when sequencing the mitochondrial genome of *L. perenne*.

17.2 Material and Methods

For sequencing the *L. perenne* mitochondrial genome, leaves of 14 days old etiolated seedlings from cultivar Shandon were harvested and the DNA isolated following a protocol of Kiang et al. (1993). Unfortunately only small amounts of DNA were obtained by this method and hence a whole genome amplification step had to be incorporated to obtain enough DNA for sequencing. To account for the repetitive and large non-conserved regions of the plant mitochondrial genome, a hybrid sequencing approach was carried out consisting of conventional Sanger sequencing with an insert size of 2.5 kb and GS FLX (454) sequencing with a read file length of around 500 bp. Based on already available grass mitochondrial sequencing information, the genome size of *L. perenne* was expected to be approximately 500 kb. Assuming no contamination of the isolated DNA, read files obtained by Sanger sequencing covered the genome around five times and 454 sequencing around 200 times. Sequencing was outsourced to the sequencing company GATC.

17.3 Results

Sanger files were firstly assembled using the program Phrap (http://www.phrap.org/), and a hybrid assembly approach using the Newbler software (454 Life Sciences, Branford, Connecticut, USA) was used to assemble read files derived from both sequencing approaches. However, both assemblies resulted in large numbers of contigs (Phrap: 227, Newbler: > 4000). Another assembly carried out in Lasergene (DNAstar, Inc., Madison, Wisconsin) resulted in even more contigs (> 8000). At this stage a complete assembly of the *L. perenne* mitochondrial genome did not seem possible. However, in order to reduce the amount of contigs and end up with a draft assembly it was hypothesized that contigs based on fewer than 100 reads are very likely based on low quality read files or possible sample contamination, while contigs based on more than 100 reads most likely contain the valuable *L. perenne* mitochondrial sequence information. Thus, a final draft assembly based on 43 contigs consisting of a total of 564,927 bp with 33 protein coding genes, three ribosomal RNA genes and 20 transfer RNA genes (14 single copy) was obtained.

Comparative analyses of the draft assembled *L. perenne* mitochondrial genome to published grass mitochondrial genomes confirmed earlier observations that mitochondrial coding regions are highly conserved, however gene order (Ogihara et al. 2005) and intergenic spacer regions (Sugiyama et al. 2005) were highly variable. A rearrangement study revealed only few regions with conserved gene order across all grass mitochondrial genomes currently available. A comparison of the *L. perenne* chloroplast genome to the draft assembly of the *L. perenne* mitochondrial genome revealed at least three chloroplast regions that were copied to the mitochondrial genome. Transferred tRNA genes are expected to be functional. However, protein-coding genes are generally dysfunctional due to frameshifts. Some evidence for potential horizontal gene transfer events were also detected. The abuscular mycorrhiza *Glomus intraradices* lives in close symbiosis to many land plants. It makes soil derived nutrients, especially phosphorus, available to plants and benefits from the photosynthetically fixed carbon. This symbiosis is expected to be as old as the earliest land plants (Karandashov and Bucher 2005). The mitochondrial genome of *G. intraradices* is publicly available and was compared to the mitochondrial sequences of *L. perenne*. Thus a 305 bp fragment was detected that showed a 76 % similarity to the *L. perenne* mitochondrial genome. This fragment can also be detected in other land plant species and could be derived from a horizontal gene transfer event.

17.4 Conclusion

The mitochondrial genome of *Lolium perenne* was sequenced and a draft assembly established. First analyses provided insights into the organization of the *L. perenne* mitochondrial genome. Evidence for intracellular and horizontal gene transfer events was also detected. The availability of *L. perenne* mitochondrial genome sequences enables the investigation of CMS in *L. perenne* and closely related grass species in the future and provides a new resource for the development of molecular markers.

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Chapter 18
Quantifying Early Vigour and Ground Cover using Digital Image Analysis

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Abstract Early vigour, or the fast leaf area development by an establishing crop is important in many breeding programs and is currently evaluated by subjective rating. The objective of this study was to test whether analysis of digital images can quantify ground cover in a fast and repeatable way in two trials where ground cover evaluations were important. Both in a greenhouse trial comparing the early vigour of different varieties of tall fescue (*Festuca arundinacea* Schreb.) as in a field trial comparing different varieties of rye (*Secale cereale* L.), Italian ryegrass (*Lolium multiflorum* L.) and lopsided oat (*Avena strigosa* Schreb.) for use as cover crops, we took pictures on regular intervals. In both trials, parameters that allowed accurate discrimination between pixels that represented bare soil and pixels that represented soil covered with plants were easily found. The Hue dimension of the Hue Saturation Brightness colour space was the parameter with the largest discriminating power between ground and plant covered pixels. Both in the field as in the greenhouse, there were significant differences in ground cover between the varieties. We found a good regression between ground cover and biomass production in the early growth stage of the cover crops in the field trial, until the vegetation reached a soil cover of ca. 50 % and a corresponding biomass of ca. 500 kg dry matter/ha ($R^2 = 88$ %, p value = <0.001). In later stages, correlation between ground cover and biomass was weak due to the presence of erect growing genotypes, with few tillers and a lower ground cover but with a good aboveground biomass production. We conclude that image analysis has a good potential to quantify early ground cover and early aboveground biomass production as it can work both fast and accurate in the field.

18.1 Introduction

Plant breeders routinely use ground cover as a parameter to measure early vigour or density of a grass sward. Trained plant breeders use discontinuous scores to assess it qualitatively. The use of digital image analysis allows quantification of the covered ground area in a repeatable way.

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The method to quantify ground cover is basically simple. A software programme counts the number of green pixels in a digital image of the crop and calculates the proportion of these green pixels in the whole image. The operator has to set limits to define which pixels are green, and thus covered by plant material, and which are not.

Richardson et al. (2001) used digital image analysis and a commercial software package to quantify ground cover in a sward of turfgrass. Image analysis produced a much lower variance than the subjective scores resulting in more significant differences. A macro was developed (Karcher and Richardson 2005) to perform ground cover analysis on batches of pictures, minimizing the time needed to analyse a high number of similar pictures.

Also Lock et al. (2004) tested the accuracy of digital image processing for quantification of ground cover in turf grass. They improved robustness by using geometric classifiers in addition to the hue, saturation and lightness (HSL) colour space. Image analysis for ground cover quantification was successfully used in a non-grass crops e.g. by Purcell (2000) and by Behrens and Diepenbrock (2006).

The first objective of this study was to test whether analysis of digital images can quantify ground cover in a repeatable way in practical trials where ground cover evaluations are important. Secondly, correlation between ground cover and biomass production was investigated.

18.2 Material and Methods

Two trials in which ground cover and early vigour were important, were selected for testing digital image analysis in practise.

The first trial compared the early vigour of eleven different varieties of tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) in flats measuring ca. 0.2 m^2 in the greenhouse (De Dauw 2009). The grass was planted in the flats and not sown in order to have an equal number of uniformly spaced plants in each flat. On three occasions in the year 2009 (early summer, late summer, autumn) we planted three flats per variety and took pictures of the flats on regular time intervals. For picture taking each flat was placed under a camera (CANON eos 50 D with lens EF-S 17–85 mm) which was mounted on a stand with a horizontal arm 75 cm above the flats in a room with artificial light. No flash was used to take the pictures, shutter speed, aperture and white balance were equal for all pictures taken in the three experiments. When the canopy of the flats was completely closed the experiments were stopped and the aboveground dry matter (DM) yield of each flat was determined.

The second trial was a field trial comparing six varieties of rye (*Secale cereale* L.) and one variety of both Italian ryegrass (*Lolium multiflorum* L.) and lopsided oat (*Avena strigosa* Schreb.) for use as cover crops with two different sowing dates (22/09/2010 and 14/10/2010) in the autumn of 2010 (Verhelst 2011). The trial was sown at a density of 300 viable seeds m^{-2} for rye and oat, and 2000 viable seeds m^{-2}

for ryegrass. The trial was organized as a split plot design with three replicates. The main factor was sowing date and the subfactor was variety/species. Individual plot size was 30 m^2. Between 24/10/2010 and 7/2/2011 each plot was photographed with an interval of ca. 20 days, using a portable stand with a horizontal arm on which a camera (FUJIFILM FinePix F470) was mounted, 1.5 m above the ground. As we worked with natural light in the field trial, camera setting were different on each occasion we took pictures. We used the setting that the camera selected automatically for the given circumstances. Flash was never used. A picture covered a ground surface of approx. 2.5 m^2. On two occasions (20/1/2011 and 21/2/2011) both ground cover and aboveground biomass of the plots were determined. Biomass production was determined, by cutting all plantlets just above the ground, on a surface of 1 m^2 per plot.

Image analysis was done with Image J, a public domain, Java-based image processing program (http://rsbweb.nih.gov/ij/). Using the colour thresholder plugin (http://www.dentistry.bham.ac.uk/landinig/software/software.html) the original colour images were transformed into binary images where all pixels covered by plant material in the original image (green) had value 1 and all other pixels (ground, litter) had value 0. Pictures were processed in the Hue (H), Saturation (S), Brightness (B) colour space. In this colour space, each pixel in a picture has a value for each of the three dimensions ranging from 0–255. Pixels with different colours can be divided in groups by putting limits on the values of H, S and B. These limits are commonly called thresholds. Threshold values for H, S and B allowing to discriminate between green pixels and other pixels, were searched by trial and error on a random subset of all pictures taken on the same occasion (called "batch" hereafter). Once the right threshold values were found for a particular batch, it was analysed in an automated way using a macro. Inputs for this macro were a batch of pictures and threshold values; output was the ground cover for each picture in the batch.

Weeds were present in all plots of the field trial. Distinction between weeds and the crop of interest was made, based on the different size and shape (circularity) of small weeds in the pictures. Mostly, weeds were smaller than the sown rye/grass plants. In addition, the rye and grass plantlets occurred as a continuous line in the picture as they were sown in rows, whereas the weeds were mostly growing between the rows. Dicot weeds had a rather round shape (or higher circularity) compared to the sown grass and rye. Values for size and shape that distinguished crop and weeds in the images were also determined by trial and error.

18.3 Results and Discussion

In both trials, discrimination between ground and plant material was better in the Hue, Saturation and Brightness (HSB) colour space than in the Red, Green and Blue (RGB) colour space. The Hue dimension of the HSV colour space was the parameter with the largest discriminating power.

As the pictures were taken under controlled, artificial light in the greenhouse trial, the same threshold values worked for most the batches taken in the different seasons. When the canopy of the flats was nearly closed (ground cover > 80 %), the threshold values needed to be altered slightly in order to ensure a good discrimination between leaves and ground.

In the field trial, light was an uncontrollable factor. Therefore new threshold values had to be searched for each batch of pictures taken. Cloudy days with no direct sunlight were preferred for picture taking in the field, as shadow caused problems in the image analysis. The presence of weeds was another factor of difference between the greenhouse trial and the field trial. The most abundant species were *Stellaria media* L. and *Poa annua* L. In the early growth stages, the distinction between weeds and crops based on size and circularity worked well (Fig. 18.1), and most of the weeds could be removed. In later stages we were no longer possible to distinguish between weeds and crops. This was not considered as problematic, as the rye and grass were far more competitive than the weeds. Only for oat, the least vigorous species, weeds were clearly present and thus contributing to the ground cover.

In both trials, significant differences in ground cover between the varieties/species were found. In the field trial, significant differences in ground cover were found until the best varieties reached a soil cover of around 80 %. This stage occurred around 80 days after sowing for the fastest growing varieties sown on the early sowing date (21/09/2010). Soil cover by oat decreased after day 100 due to frost damage (Fig. 18.2a). For the later sowing date (14/10/2010) significant differences in soil cover between varieties persisted until the end of the trial (Fig. 18.2b), and the soil cover at the end of the trial was below 50 %.

Similar results were obtained in the greenhouse trial (Fig. 18.3). Both in the trials sown in early summer and late summer, differences between the varieties were largest on the moment that the fastest growing varieties reached 80 % ground cover. In the very early stages when ground cover is very low and in the later stages of growth, when the canopy is nearly closed, differences in ground cover between the varieties were small and insignificant. Once a ground cover of 80 % was reached, the increase in ground cover decreased. Newly formed leaves overlap with the older leaves, and the plants start to grow higher with the result that the difference between the fast developing varieties and the slower developing varieties gradually disappeared.

In the field trial, a good relationship was found between biomass production and ground cover for the plots that were sown on 14/10/2010, but not for the plots sown on 21/9/2010 (Fig. 18.4a). A linear model was fitted to the data of the plots sown on 14/10/2010. The relationship found ($y = -16.1 + 1.32x, \mathrm{R}^2 = 0.88, p = 0.0000$) indicates that for a ground cover up to 50 %, an increase of 1 % in ground cover, corresponds with a biomass increase of 13 kg DM.ha^{-1} (Fig. 18.4b). In later growth stages, as it was the case for the early sown cover crops, the relationship between ground cover and aboveground biomass production is lost due to differences in growth habit between the different species/varieties. Some (tetraploid) rye varieties combined both an erect growing with few tillers resulting in a rather low ground cover and a very good biomass production. Italian ryegrass on the other hand, combined a good ground cover with a low biomass production. Once a biomass of 150 g.m^{-2}

Fig. 18.1 *Above*: picture taken on 24/10/2010 of a rye plot sown on 22/09/2010; Middle: the same picture as above after thresholding, threshold values: Hue: 36–115, Saturation: 0–255, Brightness: 109–255. Soil cover = 20.3 %; *Under*: same picture as in the middle, after removing particles with a size < 250 pixels and a circularity > 0.2. Soil cover = 18.6 %

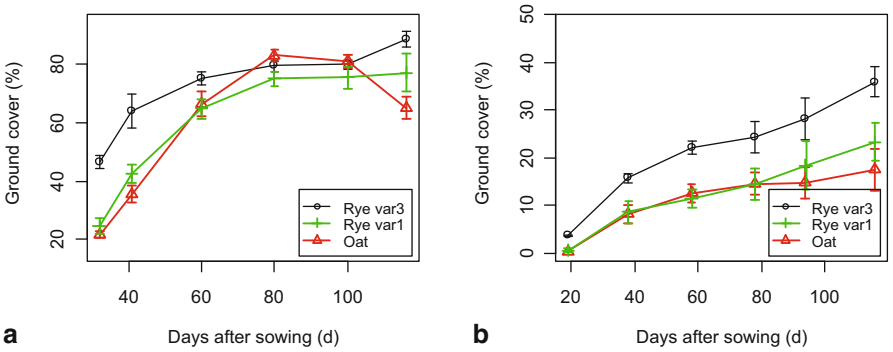

Fig. 18.2 Mean ground cover for a vigorous (var3) and a slow (var1) growing rye variety and lopsided oat sown on 22/9/2010 (**a**) and 14/10/2010 (**b**) error bars indicate standard deviation (sd)

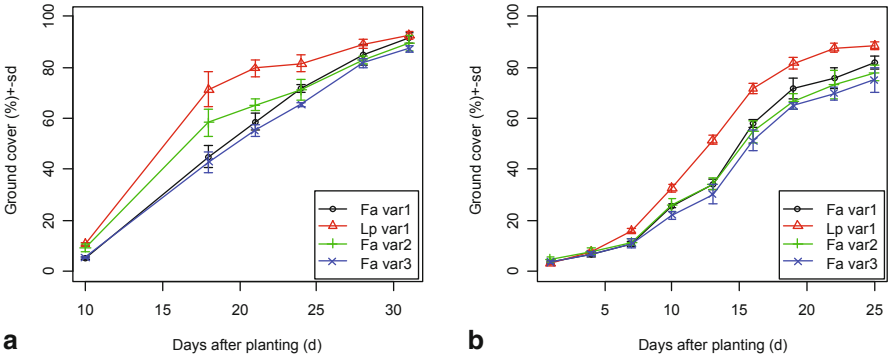

Fig. 18.3 Mean ground cover for three varieties of *Festuca arundinacea* (Fa) and a variety of *Lolium perenne* (Lp) planted in early summer (**a**) and in late summer (**b**) in a greenhouse trial. Error bars indicate standard deviation (sd)

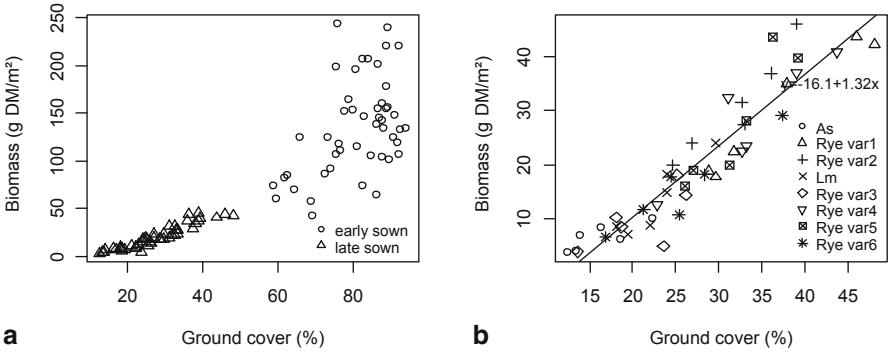

Fig. 18.4 Relationship of biomass production and ground cover for cover crops in a field trial. **a** Contrast between the early sown (21/9/2010) and late sown (14/10/2010) cover crops. **b** Regression for the late sown cover crops

was reached, the ground cover of all varieties/species, productive or not, was situated between 75 and 90 %.

In the greenhouse trial, biomass production was determined at the end of the experiments. No relationship was found between ground cover and biomass production in none of the three experiments. At the end of the experiments, ground cover was nearly complete for all grass varieties resulting in a low variation for ground cover and a much higher variation in biomass production. Moreover and similar to the field trial, genotypes with contrasting growth habits (erect, few tiller vs. prostrate, much tillers) confounded the results at the end of the experiment.

18.4 Conclusion

As in both trials, ANOVA resulted in significant differences between the varieties/species, the method can be considered as repeatable. Correlation between ground cover and above ground biomass production was good until a certain level of ground cover was reached. In the field experiment we studied, this level was at 50 % ground cover. Beyond that level, correlation was lost due to the differences in growth habit of different genotypes.

In the trails we analysed, image analysis proved suitable for quantification of ground cover by crops. Ground cover gave indirectly information about biomass production, but this relationship was restricted only to the early growth stages.

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Chapter 19
Expression of the *Lolium perenne Terminal Flower 1* Gene in Alfalfa and Tobacco

N. Ferradini, A. Nicolia, F. Veronesi and D. Rosellini

Abstract The *Terminal Flower 1* gene of *Lolium perenne* (*LpTFL1*) was overexpressed in *Festuca rubra* and *Arabidopsis thaliana*, and a delay or even the complete suppression of flowering was obtained. We have evaluated the *LpTFL1* GENE as a possible candidate to delay or prevent the flower transition process in alfalfa. This may be useful in forage crops to lengthen the vegetative phase, and in transgenic crops to control gene flow. Alfalfa was transformed via *Agrobacterium tumefaciens* using the binary vector pCAMBIA3300-*LpTFL1* (kindly provided by C. S. Jensen), in which the *LpTFL1* gene was under the control of the *Zea mais Ubiquitin* promoter. To ensure a high level of expression of the gene, in a second construct, the CaMV *35S* dual-enhancer promoter was used. RT PCR analysis confirmed the expression of *LpTFL1* in several transgenic alfalfa plants. These were phenotypically normal throughout the growth cycle, flowering was unaffected, and the plants set seed normally; the same was true for tobacco, that was transformed with the same constructs. Our results indicate that *LpTFL1* cannot be used for flowering repression in alfalfa.

19.1 Introduction

In forage crops, like alfalfa, the delay or the suppression of flowering may have a positive effects on forage quality. In addition, the manipulation of flowering transition could allow to prevent gene flow and dispersal of transgenes from genetically engineered crops. Genetic and molecular analyses have shown that several genes are involved in the control of the switch from vegetative to reproductive growth.

The *Arabidopsis Terminal Flower 1* (*TFL1*) gene is responsible for the maintenance of inflorescence meristem identity. It encodes a putative flowering signal molecule, and its mutation results in the precocious conversion of the inflorescence meristem into a flower meristem, that forms a terminal flower (Bradley et al. 1997; Ohshima et al. 1997). Constitutive expression in *Arabidopsis* produces plants with large rosettes, long, branched stem, delayed or, in extreme cases, lack of flower

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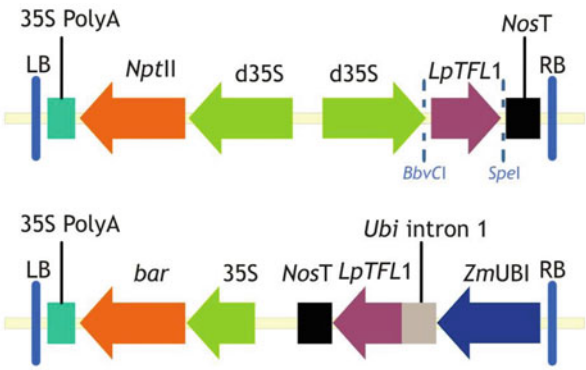

Fig. 19.1 pCAMBIA-*LpTFL*1 and pCAMBIA-2300-*LpTFL*1 binary vectors

differentiation (Ratcliffe et al. 1998). The orthologous genes were investigated to various extent in several species (Danilevskaya et al. 2010 and references therein).

The *Terminal Flower 1* gene of *Lolium perenne* (*LpTFL1*) plays a primary role in controlling the process of meristem differentiation and the transition from the vegetative to reproductive phase. It was overexpressed in *A. thaliana* and *Festuca rubra* (Jensen et al. 2001), obtaining a delay or even the complete suppression of flowering. We have evaluated the *LpTFL1* GENE as a possible candidate to delay or prevent the flower transition process in alfalfa. We also tested the effect of *LpTFL1* expression in tobacco.

19.2 Materials and Methods

The *Medicago sativa* genotype RSY1, from the RegenS-Y germplasm and the *Nicotiana tabacum* variety Petit Havanna were used in this work. The two binary vectors used for alfalfa and tobacco transformation are shown in Fig. 19.1.

The pCAMBIA 3300-*LpTFL1* vector (kindly provided by C.S. Jensen, DLF Trifolium, DK) contains the 528 bp *L. perenne Terminal Flower 1* (*LpTFL1*) gene under the control of the maize *Ubiquitin* promoter (*Zm*UBI) and its first intron, and of the *Nos* terminator. The selectable marker gene *bar*, that confers resistance to phosphinothricin, is under the control of the CaMV 35S promoter and terminator. The coding sequence of *LpTFL1* gene was amplified by PCR from pCAMBIA3300-*LpTFL1* with the primers 5′TGCTTT**CCTCAGC**GGATCCATGTCTAGGTCTGT and 5′GGTTAT**ACTAGT**TCAGCGCCTCCTGGCAGCAGT, which contain the *BbvC*I and *Spe*I restriction sites, respectively (bold). The 541 bp amplicon was digested with the same enzymes and ligated into the corresponding sites of the pCAMBIA2300-d35S-NosT vector between the dual CaMV35S promoter and the *Nos* terminator, thus obtaining the pCAMBIA2300-*LpTFL1* transformation vector (Fig. 19.1). It contains the *Npt*II selectable marker gene.

The two vectors were separately introduced into *A. tumefaciens*, strain AGL1, by electroporation and used in the transformation experiments. Alfalfa transformation and regeneration was carried out as described by Ferradini et al. (2011), whereas tobacco transformation and regeneration were performed by the standard leaf discs method. In all tissue culture phases, except rooting, the transgenic plants were selected on 7.5 mg l^{-1} (alfalfa) or 10 mg l^{-1} (tobacco) phosphinothricin (pCAMBIA3300-*LpTFL1* transformation) or 25 mg l^{-1} kanamycin (pCAMBIA2300-*LpTFL1* transformation). Cefotaxime (400 mg l^{-1}) was added in all media to prevent *Agrobacterium* growth.

Alfalfa green somatic embryos and tobacco green shoots obtained in selective conditions were cultured into fully grown plants.

The genomic DNA was extracted from young, fully expanded leaves of the putative transgenic plants (GeneElute Plant Genomic DNA Miniprep Kit, Sigma). PCR analysis was performed to detect the presence of *LpTFL1* (alfalfa primers: NosT-*LpTFL1*-For: GCGGGACTCTAATCATAAAAACC and *LpTFL1*-Rev: ACTGTATCTGTGCCTTCCTTCAG, expected amplicon 299 bp; tobacco primers: *LpTFL1*-For: CAATGACACGACCAACAATAAGAG and ZmUBI-PROM-Rev: TAATAAATAGACACCCCCTCCACA, expected amplicon 1184 bp). All PCR amplifications were performed using: 1x Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM primers, 1U Taq (Sigma).

Total RNA was extracted (*Spectrum^{TM} Plant Total RNA kit*-Sigma) from 100 mg leaf tessues in pre-blooming phase of the PCR positive plants and purified from residual genomic DNA using the On-column DNASE I Digest Set (Sigma). The complete DNA degradation was evaluated by PCR using the specific primer pair Actin-For 400 (CCACCACTCAATACGATGTTTCCA) and Actin-Rev 400 (CTAACGCACACCTTCCCGTAT). cDNA was synthesized using M-MuLV Reverse Transcription Kit (Diatheva) and used as template for amplification with the primers UBI int-For (TGGCAGCAGTCTCTCTCTGAC) and *LpTFL1*-Rev (see above). The actin cDNA was amplified as a control with the primers indicated above.

19.3 Results

In the alfalfa transformation experiment with the construct pCAMBIA3300-*LpTFL1*, 29 putative transgenic somatic embryos were regenerated with phosphinothricin selection, and 22 were cultured into plants (Fig. 19.2; Table 19.1). Seventeen plants were PCR-positive (not shown).

RT-PCR confirmed the presence of the transcript in 8 of the 17 putative *LpTFL1* plants; the level of expression showed some variation (Fig. 19.2). All *LpTFL1*-expressing alfalfa plants were phenotypically indistinguishable from non-transgenic controls. The pattern and timing of flowering was normal and fertile flowers were produced (not shown). Seed set in transgenic plants ranged between 0.17 and 3.76 seeds per floret, whereas controls produced 3.06 and 3.77 seeds per floret. The variation in seed set was within a normal range, and was not related to the expression of *LpTFL1*.

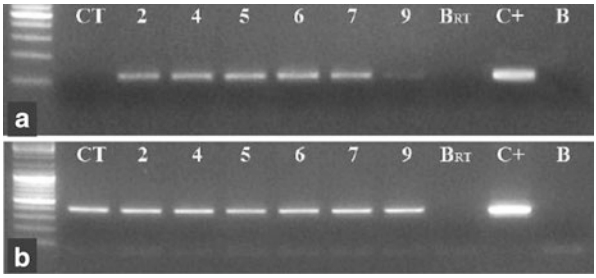

Fig. 19.2 Example of RT-PCR results in putative transgenic alfalfa plants: **a** amplification of the *LpTFL1* cDNA. **b** amplification of the actin cDNA (control). First lanes on the *left* (**a** and **b**): GeneRuler 100 bp Plus DNA Ladder (Fermentas) *CT*: cDNA of a non-transgenic plant; *2*, *4*, *5*, *6*, *7*, *9*: cDNA of transgenic plants; B_{RT}: no reverse transcriptase; *C+* : pCAMBIA 3300-*LpTFL1* (**a**) or alfalfa genomic DNA (**b**); *B*: water

Table 19.1 Results of alfalfa transformation

Vector	Leaf explants	Somatic embryos	Converted embryos	RT-PCR-positive plants
pCAMBIA3300-*LpTFL1*	184	169	22	8
pCAMBIA2300-*LpTFL1*	100	67	17	9

Table 19.2 Results of tobacco transformation

Vector	Leaf explants	Shoots	Converted shoots	PCR-positive plants
pCAMBIA3300-*LpTFL1*	140	53	23	12
pCAMBIA2300-*LpTFL1*	40	18	12	7

In the transformation experiment with pCAMBIA2300-*LpTFL1*, 67 kanamycin resistant embryos were regenerated, 17 were converted to plants, and 9 were PCR ad RT-PCR positive (Table 19.1). They were also phenotypically normal throughout development and flowering was not different from non transgenic control plants.

In tobacco, the transformation experiments produced 12 PCR-positive plants with the pCAMBIA3300-*LpTFL1* construct and 7 PCR-positive plants with the pCAMBIA2300-*LpTFL1* construct (Table 19.2).

RT-PCR analysis of the transgenic plants confirmed the presence of the transcript in 7 of the 12 pCAMBIA3300-*LpTFL1* plants (Fig. 19.3). The pCAMBIA2300-*LpTFL1* plants have not yet been tested for transcript presence. None of the plants showed phenotypic differences from the control plants. All the *LpTFL1* tobacco plants produced flowers and capsules with viable seeds.

19.4 Discussion

We have evaluated the *LpTFL1* gene of perennial ryegrass as a possible candidate in controlling the flowering process in alfalfa by overexpressing it with two constitutive promoters, ZmUBI and CaMV35S. RT-PCR confirmed the expression of *LpTFL1*

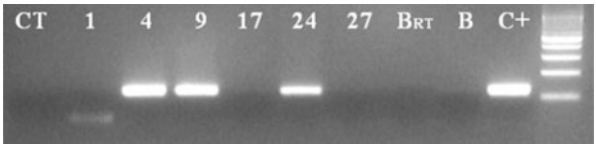

Fig. 19.3 Example of RT-PCR results in tobacco putative transgenic plants. *CT*: cDNA of a non-transgenic plant; *1*, *4*, *9*, *17*, *24*, *27*: cDNA of putative transgenic plants; B_{RT}: no RT enzyme; *C+*: pCAMBIA 3300-*LpTFL1*; *B*: water. Last lane: GeneRuler 100 bp Plus DNA Ladder (Fermentas)

gene in 17 plants. Our results show that the overexpression of the *LpTFL1* gene in alfalfa did not alter the architecture of the plants, nor it affected flowering and seed set in any visible way. The same was true for tobacco, a phylogenetically distant species.

On the contrary, *LpTFL1* overexpression in species as diverse as *A. thaliana*, *L. perenne* and *F. rubra*, resulted in a flowering delay or even suppression (Jensen et al. 2001, 2004). In *Arabidopsis*, for example, the overexpression of *LpTFL1* produced dramatic alterations in both the vegetative and reproductive phases: a significant increase in the number of nodes before and after the floral transition phase and a delay or absence of flowering (Jensen et al. 2001).

In our case, the absence of a phenotype both when the gene was driven by the ZmUBI and by the CaMV 35S promoter, suggests that the lack of impact on phenotype is not due to a low level of expression; in fact, the Dual 35S promoter is known to confer high constitutive expression in alfalfa (Samac et al. 2004). This will be ascertained by further molecular analyses.

Our negative result may be explained by the taxonomic distance between perennial ryegrass and the two recipient species, *M. sativa* and *N. tabacum*, even though *LpTFL1* did affect the phenotype in *A. thaliana*, also a taxonomically distant species. Similar to our result, overexpression of *Arabidopsis TFL1* had no effect on tobacco flower development, whereas overexpression of CEN, the *Antirrhinum TFL1* putative ortholog, caused delayed and indeterminate flowering: this indicates that sequence divergence in the *TFL* proteins among species underlies functional diversification.

Comparison between *LpTFL1*, the tobacco homologous proteins CET2 and CET4, and *M. truncatula TFL1*-a revealed 71 and 72 % similarity, respectively. However, it has been observed that overall sequence similarity does not always imply functional similarity in this gene family (Yoo et al. 2010), while a single amino acid change in the *TFL* gene can turn the encoded protein from a flowering repressor to a flowering activator (Hanzawa et al. 2005).

Genes controlling flowering in legumes have been investigated (Hecht et al. 2005, 2011), but not yet in alfalfa. Even though the flowering pathways may differ between annual and perennial *Medicago* species, the genome of *M. truncatula* is a valuable resource for isolating alfalfa flowering genes.

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Chapter 20
Morphological and Molecular Characterization of Branching in Red Clover (*Trifolium pratense*)

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Abstract Clover is an essential element of sustainable grasslands. Clover reduces the need for nitrogen fertilizer and results in improved nutritional value of grasslands. Plant architecture, which is under genetic and environmental control, may have a strong influence on traits such as forage yield, re-growth capacity, seed yield and persistence in red clover. The genetic aspect of branching has been widely studied in model plants but has received little attention in red clover. Our present aim is to translate the knowledge regarding genes involved in bud outgrowth from model plants to red clover. Branching was studied in two environments during one growing season in clonal replicates of two genotypes with contrasting architecture, a highly branched and prostrate genotype (Crossway_2), and a poorly branched and erect genotype (Diplomat_8). The number of nodes and the quantity as well as the position of bud outgrowth into branches differed greatly between genotypes and were similar across both environments. The influence of auxin and strigolactone on bud outgrowth was investigated by applying these hormones to isolated single node segments. Furthermore, genes from the strigolactone pathway were isolated from red clover and their expression was studied in various tissues of the two genotypes.

20.1 Introduction

Red clover can be used in monoculture or in mixed grasslands, and in conventional as well as organic farming. In mixtures, the addition of red clover reduces the need for nitrogen fertilization and positively affects milk production due to its high palatability and associated high intake rate. (Taylor and Quesenberry 1996; Bertilsson and Murphy 2003). However, red clover has a low persistence and disappears from grasslands after two to three seasons. Plant architecture is investigated for its contribution to persistence in this species, as it affects yield, tolerance to defoliation and re-growth capacity.

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Plant architecture is determined by the degree of branching, internode elongation, the determinacy of the shoot organ size and growth habit. Buds are formed in the axils of the leaves and may either develop into branches or stay dormant. The processes that control axillary bud formation and outgrowth are under genetic and environmental control (e.g., photoperiod, plant density, defoliation), and influence architectural differences between individual plants. The genetic aspect of plant architecture has been demonstrated in model species to be regulated by genes involved in hormone biosynthesis, signaling or response (McSteen and Leyser 2005; Ongaro and Leyser 2008, Ferguson and Beveridge 2009). Little is known, however, on the role of these different aspects in red clover.

Due to its obligate outcrossing, red clover populations are genetically very diverse and cultivars also remain heterogeneous (Dias et al. 2008). In a previous study (Cnops et al. 2010), we described the diversity of architectural types in 15 cultivars and six landraces. From that collection of plants, two genotypes (Crossway_2 and Diplomat_8) with extreme branching phenotypes were chosen for detailed morphological, physiological and molecular analyses. Crossway_2 is a highly branched genotype derived from a prostrate cultivar with improved persistence under grazing from New Zealand (Rumball et al. 2003). Diplomat_8 is a poorly branched, erect genotype selected within a French cultivar mainly used for mowing purposes. Here, we present a detailed description of the architecture of these contrasting genotypes, their response to hormones determining branching, and the expression of genes involved in the strigolactone biosynthesis, signaling and response pathways. Together, the data elucidate some of the genetic differences underlying architecture in red clover.

20.2 Material and Methods

In a first step, the two studied genotypes were clonally propagated. Cuttings containing one node, preferentially with an outgrowing bud, were rooted in soil under moist conditions. After rooting, the cuttings were transplanted to 10 L pots. For experiments carried out in the growth chamber (20 °C; 16 h light), six clones of each genotype were investigated. For experiments carried out in open air, 12 clones of each genotype were used. The plants were regularly watered and no fertilization was applied.

Analysis of Branching Patterns The number of nodes and branches and the flowering time were recorded in the growth chamber and in open air. Plants in the growth chamber were analyzed weekly during a period of three months; plants grown in open air were analyzed once every two weeks (March till July 2010).

Response to Hormone Treatment Per treatment 25–30 stem fragments, containing arrested buds derived from the nodes closest to the crown (node1 or node2), were isolated from elongated branches of non-flowering plants of both genotypes grown

under controlled conditions. The stem fragments were placed on water solidified with 0.7 % plant tissue culture agar MC29, supplemented with 20 µM GR24 or 1 µM NAA. After three days the culture medium was refreshed. The stems were photographed immediately after isolation and three, five and seven days later using a stereomicroscope. Lengths were measured using the software programme ImageJ (http://rsbweb.nih.gov/ij). Data were analyzed using ANOVA with post-hoc tests Tukey HSD or Games-Howell ($P < 0.05$) (SPSS).

Patterns of Gene Expression Tissue from first and second internode and node1 (starting from the crown) was isolated from plants grown in controlled conditions. Total RNA was extracted using the Qiagen RNeasy Mini extraction kit. A DNAse treatment was done using the DNA-free kit of Ambion, and was followed by a LiCl precipitation. cDNAs were prepared using the Superscript III First-Strand Synthesis Supermix kit of Invitrogen according to the manufacturer's instructions. Q-PCR was performed with SYBR Green I Master mix and was run on a LightCycler 480 Real-time PCR system. Per genotype and per tissue five biological repeats were analyzed. The expression of the genes *TpMAX2*, *TpMAX4* and *TpBRC1*, genes involved in the strigolactone (SL) pathway (*MAX4* = SL biosynthesis gene; *MAX2* = SL signal transduction gene and *BRC1* = SL response gene) were determined. The values were normalized using *TpACTIN*. One of the internode1 tissues was used as a reference for the calculation of relative expression values as calculated by the lightcycler480 software (1.5.0SP4).

20.3 Results and Discussion

Analysis of Branching Pattern Red clover forms a crown at soil level that consists of a main axis (MA) and lateral branches. When the plants are not mown, the MA in most genotypes remains vegetative during the first growing season. First-order branches are formed from the axillary meristems (buds) in the leaf axils of the MA. In leaf axils of first-order branches, axillary buds are formed that can develop into second-order branches. Second-order branches can produce higher order branches (Taylor and Quesenberry 1996; Cnops et al. 2010).

The number and position of nodes and branches were recorded at regular time intervals in clonal replicates of Crossway_2 and Diplomat_8 grown in the growth chamber and in open air. In Fig. 20.1, the temporal dynamics of the number of nodes and branches in each genotype is shown for growth room conditions. Crossway_2 plants had from the start of the experiment more nodes and branches. The differences between the two genotypes were evident already during the vegetative phase and increased during further development. At 198 °C.days (mid vegetative phase), Crossway_2 had produced three times more nodes and 5.5 times more branches than Diplomat_8. At flowering time (751 °C.days), 4.5 times more nodes and 6.5 times more branches had been produced in Crossway_2. Four weeks later, Crossway_2 still

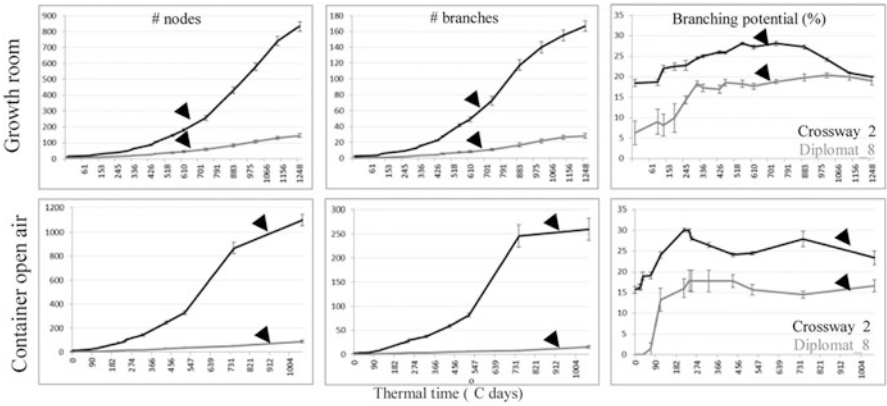

Fig. 20.1 Differences in branching complexity between Crossway_2 and Diplomat_8. The total number of nodes and branches is shown for plants grown in two different environments: growth room, (*top*) open air *bottom*. The average value obtained for six clones (*top*) or 12 clones (*bottom*) is represented; error bars are standard errors. Flowering time is indicated with an *arrowhead*

had 5.7 times more nodes and 5.9 times more branches than Diplomat_8 (Fig. 20.1). The differences found were due to the production of a much higher number of nodes and branches at all hierarchical orders, but were especially pronounced from the second order onwards in Crossway_2 compared to Diplomat_8. These differences were not due to a shift in development since both genotypes had similar flowering times under growth room conditions.

We determined the bud outgrowing potential (BOP) (calculated as number of branches/number of nodes) in both genotypes to see if the higher branching capacity in Crossway_2 was due to an higher node initiation rate or to a higher rate of bud outgrowth. The BOP was higher in Crossway_2 than in Diplomat_8 for all time points except for the last two, for which a BOP of 20 % was estimated for both genotypes (Fig. 20.1). This suggests that the differences in branching between these two genotypes are not only due to a higher node initiation rate in Crossway_2 but also due to a higher bud outgrowth capacity.

In open air, both genotypes flowered later (747 °C.days) than in the growth chamber (656 °C.days), but similar results were obtained for plants grown in both environments. The number of nodes, branches and the BOP was in Crossway_2 at all time points higher than in Diplomat_8 (Fig. 20.1). The number of nodes and branches in Crossway_2 plants grown in open air was higher compared to plants kept in the growth chamber. However, the BOP at flowering time was in Crossway_2 comparable for both environments. Diplomat_8 produced at flowering a similar number of nodes and branches in both environments, but the BOP was slightly lower in the growth chamber than in open air.

Response to Hormone Treatment To investigate if the genotypes differed in their response to strigolactones (SL), stem fragments with single nodes were isolated

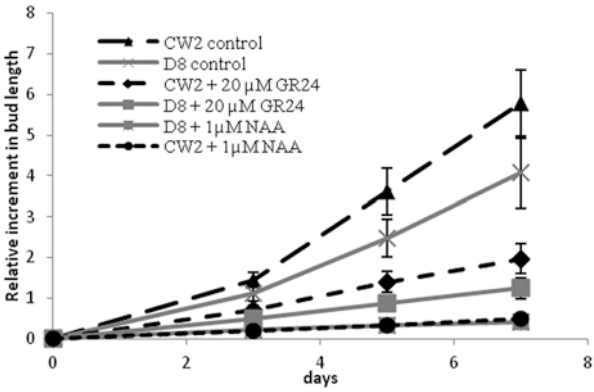

Fig. 20.2 Influence of hormone treatment on bud outgrowth of nodes on isolated stem fragments. Relative length increment [(length day 7–length day 0)/length day 0] at each time point was determined as the mean of 25 nodes. Bars are standard errors. CW2 = Crossway_2; D8 = Diplomat_8

according to Chatfield et al. (2000) and grown in petri dishes containing medium with or without the synthetic SL GR24 or NAA (Fig. 20.2). The addition of 1 µM NAA reduced bud growth almost completely. Relative bud growth was significantly reduced in both genotypes seven days after the addition of GR24. These data suggest that both genotypes react similarly to external application of auxin and SL and that they probably do not differ in their signalization. Differences in endogenous hormone concentrations will be measured in future experiments.

Patterns of Gene Expression Red clover sequences of *MAX4* (SL biosynthesis), *MAX2* (SL signaling) and *BRANCHED1* (SL response) were PCR-amplified using degenerated primers and cloned. The expression of these three genes was analyzed in the two genotypes in the first and second internode and the first node of outgrowing branches (Fig. 20.3). Strikingly, the expression of, *TpMAX2* and *TpBRC1* is higher in the tested internode and node tissues of Crossway_2 than in those of Diplomat_8. A similar expression pattern was observed for *TpMAX4* in internode1. Based on the phenotypes of Arabidopsis mutants, high *MAX2, MAX4* and *BRANCHED1* are expected to be associated with less branching phenotypes, which is not the case in the studied (dormant) tissues of red clover. These results need to be challenged in further experiments addressing more tissues and additional genes; for example the single node system is an ideal system to follow gene expression during bud outgrowth under well-defined conditions.

20.4 Conclusion

The two genotypes described in this paper are a good model to study architectural differences in red clover. We established an assay for bud growth in isolated stem fragments, which gave us a powerful tool to study the influence of hormones on bud outgrowth under controlled conditions and to look for individual differences in

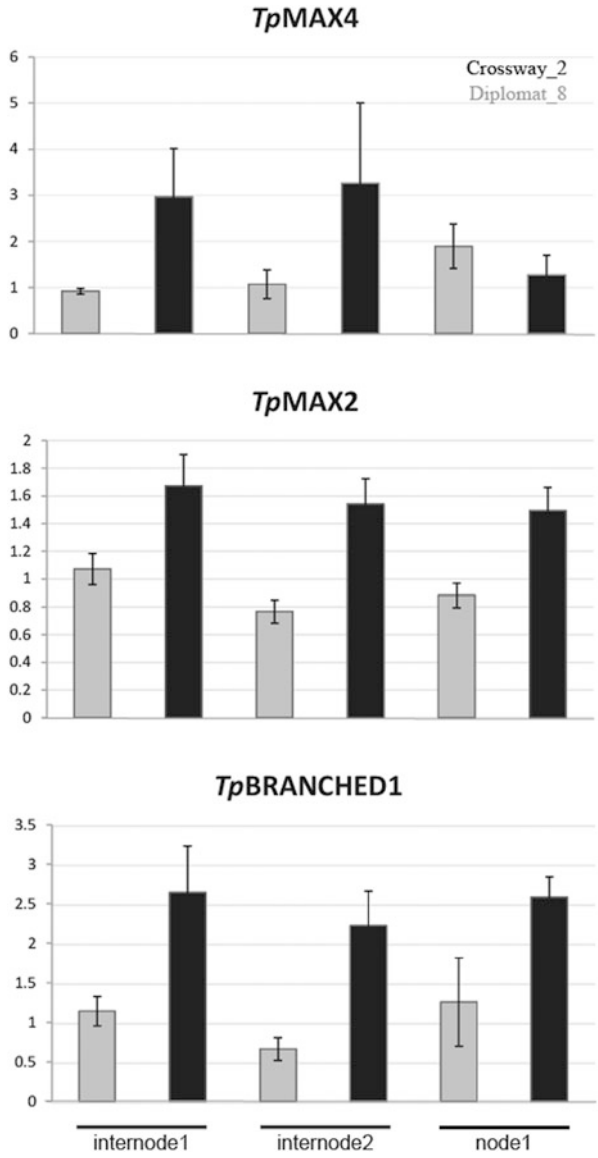

Fig. 20.3 Expression analysis of strigolacton related genes in *red* clover. Relative expression pattern of genes in the first and second internode and node1 red clover tissues. Data are the average of 5 biological repeats with standard errors. The values were normalized according to TpACTIN

red clover gene expression during this process. Further research with the expression pattern of a broader collection of genes and re-growth under mowing conditions is required to fully understand the architectural differences observed in red clover genotypes. The knowledge obtained from the molecular, physiological and molecular study will be applied to a broader collection of genotypes representing a wide variety

of architectural characteristics. Using allele mining, we hope to identify valuable genotypes for introduction into breeding applications.

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Part IV
Breeding Towards Breeding Objectives

Chapter 21
Designing Grass Cultivars for Droughts and Floods

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Abstract Temperate productive grasslands are often located in areas of high rainfall prone to flooding, but even here moderate summer droughts occur with regularity causing significant yield reductions. Grasslands capable of resisting both water excess and deficit are required. Alternative breeding technologies are employed to combine as *Festulolium* cultivars the desirable traits of *Lolium* and *Festuca* species, and also through their enhanced root systems, improve soil structures and hydrology. An amphiploid *L. perenne* × *F. pratensis* cultivar can significantly reduce rainfall runoff compared to either its parental species. Evidence suggests this was due to an initial intensive root growth followed by extensive root senescence. This appears to alter soil structure and increase soil porosity and moisture retention providing an ecosystem service by both combating run-off subsequent to heavy rainfall and increasing soil water supply during dry periods.

In a second programme aimed at improving drought resistance in *Lolium*, genes for drought resistance were transferred from *Festuca arundinacea var glaucescens*. These significantly increased water-use-efficiency and forage yield of *Lolium* under soil water deficit conditions with no compromise to forage quality.

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21.1 Introduction

The *Lolium-Festuca* complex comprises diverse, heterogeneous, and primarily outbreeding species that hybridise naturally having genomes that are functionally interchangeable following chromosome exchange and their intrinsic propensity for extensive interspecific chromosome pairing and recombination (Humphreys et al. 2003). As a consequence, genome combinations may be constructed capable of providing a virtually unlimited supply of genetic variation and a large range of contrasting grass phenotypes relevant to traits such as ontogeny, persistency, stress tolerance, water and nutrient uptake and use, and agronomic value available to plant breeders ready for evaluation for their suitability for incorporation in crop improvement programmes (Ghesquière et al. 2010).

It is accepted widely that the diploid ryegrasses *Lolium perenne* and *L. multiflorum* in terms of their rapid and prolonged seasonal growth that provides large foliar yields and forage with high nutritive value and palatability are the most suitable grass species for sustainable livestock agriculture. However, they lack generally the stress resistance and water- and nutrient-use-efficiency common to many related fescue (*Festuca*) species and ecotypes (Ghesquière et al. 2010). Their evolution, speciation and subsequent widespread dispersal provides the opportunity for identifying genotypes that confer the specific adaptations necessary for growth in the diverse edaphic and climatic conditions that span globally most if not all temperate grasslands (Humphreys et al. 2011). From early in forage grass cultivar development, the potential and desirability of combining the complementary characters found in *Lolium* and *Festuca* species within stable hybrid forms has been recognised and over the years a range of intergeneric cultivars has been developed (Humphreys et al. 2006; Ghesquière et al. 2010). However, only recently following a change to EU legislation has it been possible to develop commercially as cultivars under their own unique cultivar category; *Festulolium*, any *Lolium* × *Festuca* species hybrid combination with the provision this was obtained by conventional plant breeding technologies.

Two breeding approaches are employed, amphiploidy where entire *Lolium* and *Festuca* genomes are combined or introgression-breeding approaches where selected traits are transferred from a donor species, usually *Festuca* with little disruption to the host genome (Thomas et al. 2003). The former amphiploid approach has been used more widely and various cultivars have been marketed, particularly in Central Europe. The development of genome sequencing and marker-assisted breeding technologies makes introgression and precision-led gene transfer another option, as evidence from many sources would indicate that alien *Festuca* genes when transferred onto *Lolium* chromosomes function effectively and are transmitted normally in accordance with Mendelian expectations and in many cases without incurring linkage drag (Humphreys et al. 2005).

The current work describes the use of both breeding approaches to develop *Festulolium* cultivars capable of providing both sustainable crop production and an environmental service during a time of climate change.

21.2 Materials and Methods

21.2.1 Root Ontogeny and its Impact on Soil Structure and Hydrology

Details of this project, SuperGraSS funded by a BBSRC are described elsewhere (Macleod et al. 2007; Gregory et al. 2010; Humphreys et al. 2010).

The species and species hybrids used comprise six cultivars They were: *Lolium perenne*, (Lp) cv AberStar, *Lolium multiflorum*, (Lm) cv AberEpic, *Festuca pratensis*, (Fp) cv Bf993 (all $2n = 2x = 14$), *Festuca arundinacea* (Fa) cv Dovey ($2n = 6x = 42$), and *Festulolium* cultivars Lp $\times$ Fp cv Prior, and Lm $\times$ Fa cv 99-1 (both $2n = 4x = 28$). Five individual tillers of equal size of each species were established in 1 m deep pipes. A total of eight identical replicates were established for each plant genotype. The pipes were lined with 153 mm wide 250 gauge lay-flat polythene tubing (LBS Horticulture, Colne, Lancs. UK) heat-sealed at the bottom and punched with four drainage holes. A 3 cm layer of horticultural grit was added to the base to maintain good drainage and the polythene tubing was filled with Humax John Innes No3 potting compost with wetting agent. Five tillers were planted in each pipe in September 2006. The pipes were held in frames in a block design and maintained in a heated (minimum temperature 8 °C), unlit glass house throughout the year. Water was applied evenly and this was confirmed with rain gauges placed within the blocks.

Below ground growth was investigated in mid-April and again in mid-September in 2007 and 2008. The polythene sleeve was slid out of the pipe to enable non-destructive observations of roots around the sides of the soil column. The maximum depth at which roots were visible was recorded. The column of potting compost was marked into 10 cm sections and root density was scored for each section. Root system size was the sum of scores for all the sections, and root profile was the regression coefficient for a line fitted through the root scores from the lowest section with roots to the top section. These were calculated for the total and new root system. Root persistency between spring and autumn in years 1 and 2 was also recorded.

The same six grass cultivars were established and maintained in the field as in the root-pipe experiment over an identical time-frame and with the same cutting regime and fertiliser applications. The cultivars were grown as four replicated 10 $\times$ 3 m hydrologically-isolated plots, with rainfall and run-off measured over the course of 31 rainfall events that subsequent to an initial six months establishment phase, spanned all changes in grass growth over the two year field study (as in Gregory et al. 2010).

21.2.2 Development of Lolium Cultivars with Enhanced Water-use-efficiency and Drought Resistance

Drought resistant *L. multiflorum* lines were developed by transfer of genes for drought resistance from a Mediterranean fescue, *F. arundinacea var glaucescens* onto a ter-

minal location on chromosome three of ryegrass without further disruption to the *Lolium* genome (Humphreys et al. 2005). Subsequently in a DefraLINK programme LK0688 using a sequence-tagged-site (STS) marker described by Humphreys et al. (2005), the fescue genes were transferred into a breeders' line producing population Bb2540. In a field trial with three replications, performance over two consecutive severe summer droughts (2009–2010) and consistent high temperatures (circa 38 °C day/25 °C night) was assessed at INRA, Lusignan, France, and also under artificial drought conditions in a glasshouse at IBERS over one summer over a three consecutive-month period with no irrigation. Recovery following irrigation was evaluated at both locations. For the artificial drought assessment at IBERS, 3 × 1 m^2 plots, were sown of Bb2540 at 3.3 g/m^2 and compared with a *L. multiflorum* control cv Atalja sown, established, and maintained as with Bb2540. All glasshouse plots were maintained at field capacity until start of the drought (15-06-10) with soil water content monitored twice weekly in two locations in each drought bin at four 10 cm soil depths (0–40 cms). The methodologies used in the drought experiments were standard IBERS protocols (e.g. Alm et al. 2011) with the incorporation of the soil water measures. These were carried out using a Delta-T PR2 soil moisture sensor, data captured by a Delta-T HH2 Moisture Meter (Delta-T Devices, Ltd., Cambridge, UK). Forage yield was measured over four cuts prior to the start of drought, two cuts during drought, followed by a final cut on 08-10-10 4 weeks into the recovery phase subsequent to completion of the drought treatment when soil water content was returned to field capacity. Water-use-efficiency (WUE) was calculated as g DM/unit H_2O consumed.

Forage yield was measured in 3 × 10 × 3 m plots at INRA Lusignan over two cuts in 2009 and three cuts in 2010 and compared between various *Festulolium* introgression populations and amphiploid cultivars, the control cultivar *L. multiflorum cv* Abys, hybrid ryegrass cultivars, Delicial and Lifema, and a *Festulolium* amphiploid cultivar Leuer that contained entire genomes of *F. arundinacea var glaucescens*. Regrowth and persistency was scored (0–5 scale) on 01-09-10 after two consecutive dry summers.

Statistical analyses were performed on all according to standard procedures within GenStat® Version 11.1 (Payne et al. 2008).

21.3 Results

21.3.1 Root Ontogeny and its Impact on Soil Structure and Hydrology

21.3.1.1 Year 1

Species' cultivars and hybrids differed significantly ($P < 0.001$) for annual forage production, and spring and autumn root depth, root size, new root production and

root profile. Root longevity between spring and autumn also differed significantly ($P = 0.009$). For forage production, Lm cv AberEpic was most productive followed by Lp × Fp cv Prior, Fa cv Dovey and Lp cv AberStar. The most substantial root system was developed by Lp × Fp cv Prior followed by Fa cv Dovey, Lm cv AberEpic and Lp cv AberStar.

21.3.1.2 Year 2

Significant differences ($P < 0.001$) in forage production between cultivars persisted during Year 2 with Lp cv AberStar most productive largely due to its high tiller number. Fa cv Dovey was also high yielding but this was due to leaf size rather than tiller number. The two *Festulolium* cultivars Lp × Fp cv Prior and Lm × Fg cv 99-1 were ranked intermediate. Root depth in spring and autumn differed significantly between genotypes ($P < 0.001$), but in Year 2, well developed root systems and growth were particularly evident in Lp cv AberStar. However, the extensive root system present in the *Lolium* cultivars, which was superior to the *Festulolium* cultivars in the spring of Year 2, was due primarily to development of new roots within 20 cms of the soil surface. Root persistency between spring and autumn, as Year 1, differed amongst genotypes ($P < 0.05$), having increased in Fp cv Bf993 by 44 %, but having decreased in *Lolium* spp. especially Lm cv AberEpic. Reduced root persistency was particularly evident in Lp × Fp cv Prior (circa 50 %) and this was due mainly to extensive senescence of its well developed root system, particularly at depth during the summer of Year 2. In spring Year 2, the root profile of the two *Festulolium* cultivars Prior and 99-1 was superior to the other plant species. This changed during the summer months of Year 2 with significant reductions in root growth and increased root turn-over especially at depth in the soil column, reflected by both root profile and deepest root measurements. By autumn Year 2, most new root growth in the *Festulolium* cultivars was confined to the upper soil regions. At this time, overall root growth was similar within cultivars of the same species, but differences in root distribution were evident.

21.3.2 Development of Lolium Cultivars with Enhanced Water-use-efficiency and Drought Resistance

The two year INRA field trial was an effective measure of the drought resistance of population Bb2540 having two consecutive prolonged summer droughts (no rains throughout August) with accompanying high temperatures (circa 37 °C). With a total yield over three cuts in 2010 (Year 2 of the field, is considered a better indicator than Year 1 of crop performance under drought stress), diploid *L. multiflorum* Bb2540 yielded 9.23 TDM/ha which was not significantly different ($P < 0.05$) from that generated by amphiploid hybrid ryegrass cultivars, Delicial nor Lifema. Interestingly, there was also no significant difference ($P < 0.05$) in total yield of Bb2540 compared

with the amphiploid *Festulolium* cultivar Leuer (8.63 TDM/ha) which was selected in France for its persistency under drought. The cultivar comprised two entire genomes of *F. glaucescens var arundinacea* compared to the single translocated *Festuca* chromosome 3 segment from the same species present in Bb2540. There was indeed no difference in total yield between the three amphiploid cultivars and Bb2540 over the entire two Year field trial. The subsequent persistency scores (0–5 scale) taken on 1st September 2010 indicated a greater persistency for the *Festulolium* cv Leuer ($P < 0.05$) over Bb2540 and the amphiploid hybrid ryegrasses which all shared a common persistency (score = 3). These were all significantly more persistent ($P < 0.05$) than the control *Lolium* variety Lm cv Abys (2x) (score = 1).

The forage quality of Bb2540 (with two other IBERS *Festulolium* introgression lines included in the INRA field trial) measured as %water-soluble-carbohydrate (WSC) over all three cuts in 2010 was significantly greater ($P < 0.05$) than all other cultivars used in the field trial (including Lm cv Abys) at all three forage cuts. The same outcome was obtained in field trials (2009 and 2010) at IBERS where Bb2540 was the highest ranked cultivar for %WSC (over three cuts/yr) being significantly higher ($P < 0.05$) than UK Nationally Listed cultivar Lm cv Ligrande. %WSC of Bb2540 was not significantly different to that of other Nationally Listed *Lolium* cultivars; Lm cultivars Alamo (in 2010), and DaVinci, and Belluna (in both 2009 and 2010).

At IBERS, in a simulated drought experiment in a purpose-built glasshouse, water-use-efficiency was calculated during a three month period with no irrigation and also during a subsequent one month recovery phase when water was returned sufficient to restore soil water to field capacity. $3 \times 1\ m^2$ replicated swards of Bb2540 (and two alternative introgression populations) and also as control, Lm cv Atalja were sown, established and compared for their forage yields taken over four cuts between March and June 2010 prior to the start of the drought treatment. Over these cuts and given an optimal water supply (field-capacity) Bb2540 out-yielded the control Lm cultivar Atalja ($P < 0.001$), particularly in those cuts made early in March and June. During the drought treatment, cuts were made in July 4 weeks (moderate drought) and in September 12 weeks (severe drought) and water-use-efficiency (WUE) calculated as Total Dry Matter (TDM)/unit H_2O consumed. The drought treatment had a highly significant impact on forage yield in all grass populations tested. However, total yield from Bb2540 over the two cuts during the drought treatment was twice that of Atalja with an 88 % improvement in WUE ($P < 0.001$). Water uptake by Bb2540 overall was 1 % higher than by Atalja, but this was not statistically significant suggesting that it was more the water utilisation capabilities of Bb2540 rather than more efficient water-uptake that gave Bb2540 an adaptive advantage. Over all glasshouse plots, soil water content declined rapidly from an initial field capacity of 38 % to a 10 % level that was maintained consistently during the latter half of the drought treatment (circa 6 weeks) when foliar growth had declined significantly ($P < 0.001$). On re-watering during the one month recovery period, field capacity at 38 % and optimal water supply was once again restored. The yield of Bb2540 after 1 month recovery was > 100 % greater that of the control, Lm cv Atalja.

21.4 Discussion

Given a changing climate with grasslands being subjected at increasing frequencies to episodes of either extreme rainfall or persistent drought, there is a pressing requirement for grass breeders and geneticists to respond to ensure a maintained food security by increasing the resilience of currently productive cultivars, especially *Lolium perenne* and *L. multiflorum*. Hybridisation with *Festuca* species to produce *Festulolium* cultivars provides us that opportunity and as shown here also provides us with additional important examples of an ecosystem service. Two alternative approaches to *Festulolium* breeding are described, the use of amphiploidy that generated *Festulolium loliaceum* cv Prior, where entire genomes of *Lolium* and *Festuca* were combined, and the alternative more targeted gene introgression approach that generated diploid *L. multiflorum* population Bb2540 that contained a single translocated *Festuca*-derived genome sequence in an otherwise intact *Lolium* genome (Humphreys et al. 2005).

Gregory et al. (2010) and Humphreys et al. (2010) describe a part of the multidiscipline BBSRC-funded programme SuperGraSS. Here it is demonstrated how the initial extensive root growth and the subsequent senescence of *Festulolium loliaceum* cv Prior, particularly at depth was likely to have had a major impact on soil structure and hydrology and led to the reduced rainfall run-off reported in the SuperGraSS programme. In brief, it was shown in the BBSRC-funded project that Prior had a significant impact on plot scale rainfall runoff compared to either its parental species. Over a two-year period, Prior (4x) reduced runoff by 51 % compared to the UK National Listed *L. perenne* cv AberStar, and by 43 % compared to *Festuca pratensis* cv. Bf993, a progenitor of cultivar S215 the fescue parent of Prior. A number of previous laboratory studies have described how roots change soil hydraulic properties (e.g., Whalley et al. 2005). These reports have demonstrated how changes to the water release characteristics of soils tend to be associated with either an increased number of larger pores in the rhizosphere or an increase in water repellence. Root activity tends to increase the number of large pores in the soil, and as it is known that certain fescue species such as *Festuca arundinacea var glaucescens* produce larger deeper rooting systems than ryegrass (Durand et al. 2007), this it is thought will contribute to their proven overall greater drought resistance both by their capability to access water deep in the soil and by their ability to restructure soil to aid in its water retention.

Humphreys et al. (2005) described the introgression breeding programme that led to the transfer of genes for drought resistance from *F. arundinacea var glaucescens* into diploid *L. multiflorum*. The *Festuca* genes were transferred onto a distal location on the satellite region of *Lolium* chromosome 3. A major QTL for resistance to severe drought stress has been identified on linkage group 3 in *F. pratensis* that would include the *F. arundinacea var glaucescens* translocation (Alm et al. 2011) The *Festuca* translocation had been transferred into a breeders' line (Bb2540) using a sequence-tagged-site (STS) marker described by Humphreys et al. (2005) and the heritability and continued function of the *Festuca*-derived drought resistance genes confirmed in

a previous Defra-funded programme (Humphreys and Humphreys 2007). To the authors knowledge the report here is the first ever detailed study of the potential impact on forage yield and WUE to be achieved by any forage grass by the incorporation of a single alien chromosome segment from a wild-related species. A highly significant increase ($P < 0.001$) in WUE of 88 % in Bb2540 compared with the control *Lolium* cultivar Atalja demonstrates very well the possible impact and advantage such a cultivar might have in drought-prone grasslands. In its natural habitat, *F. arundinacea var glaucescens* undergoes a phase of summer quiescence (Humphreys et al. 1997) which would be a highly unsuitable agronomic trait for livestock agriculture in European grasslands, where continued grassland growth and production in summer is considered an essential requirement. The high yields of Bb2540 both in irrigated and in droughted conditions are proof of its excellent agronomic performance. In field trials at IBERS and at INRA, Lusignan, where summer droughts are a major constraint to grassland persistency and productivity, Bb2540 performed consistently well, outperforming most other cultivars tested. Over 2 years, the cultivar had productivity consistent with the amphiploid cultivar Leuer which utilized entire genomes of *F. glaucescens* to confer its drought resistance traits. No convincing evidence was found that the drought resistance achieved in Bb2540 was due to the presence of a superior root system and water uptake to that found in the *Lolium* control which was evidence that it resulted from a more effective retention and use of the water it obtained.

A major advantage of the introgression-breeding approach was the ability to select for improved drought resistance without compromise to forage quality. WSC measures in Bb2540 both at IBERS and INRA were consistently high and equivalent to, or better than, current UK Nationally Listed *L. multiflorum* cultivars.

The *Festuca* genes from chromosome three are currently being transferred by marker-assisted breeding into *L. perenne* where their impact on WUE will be assessed. For future grassland agriculture in the UK, it will be the impact that *Festuca* genes for drought resistance can bring into *L. perenne*, the agricultural grass species of choice, that which will determine their ultimate value and use in sustaining future grassland production in a time of climate change.

Although not apparently relevant to Bb2540, the generally superior root systems of *Festuca* species compared with those of *Lolium* offer a range of potential uses in regard to environmental safeguards. We cite here using as example *Festulolium loliaceum* cv Prior, the potential use of *Festulolium* hybrids to modify soil structures to encourage improved rainfall retention and reduce flooding and run-off of soil nutrients. The substantial root growth and turn-over exhibited in the cultivar Prior will also likely support effective C-sequestration properties. Large *Festuca* roots will also stabilize soils, and safeguard against soil erosion. They will be more effective than those of *Lolium* for penetration of hard compacted soils. There is also accumulating evidence to indicate roots of *Festuca* species are more efficient in nutrient and water capture.

To conclude, given positive support from all the relevant stakeholders, commercial use of *Festulolium* cultivars will provide new opportunities for European grasslands

where they should demonstrate a truly multifunctional value sustaining both productive and persistent grassland agriculture at a time of climate change whilst also providing additional environmental safeguards.

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Chapter 22
Variation and Heritability of α-Linolenic Acid Content and Rumen Escape Protein Fraction in Fodder Grass and Clover

J. Baert, M. Vandewalle, J. De Riek, J. De Boever, V. Fievez and C. Van Waes

Abstract α-Linolenic acid (C18:3) in forages enhances ω-3 fatty acid and conjugated linoleic acid content in milk and meat of ruminants with beneficial effects on human health. An increase of the fraction of rumen escape protein (REP) in grass and clover may reduce nitrogen losses by cattle. To determine the variation of C18:3 and REP, field plot trials under conservation management were set up. The trials included cultivars of perennial ryegrass, Italian ryegrass, meadow fescue, tall fescue, timothy, cocksfoot and red and white clover. For the grasses we found the highest C18:3 content in timothy and the lowest in Italian ryegrass. Tetraploid ryegrass varieties had on average a higher C18:3 content than diploid varieties. The linolenic acid content in white clover was higher than in red clover and the content in both clovers was higher than in the grasses. For all species the C18:3 content was highly positively correlated with the protein content. Among the grasses the REP was highest in cocksfoot and tall fescue and lowest in perennial ryegrass. Diploid ryegrass cultivars had a higher REP fraction than tetraploid cultivars. The REP in red clover was higher than in white clover and the REP in both clovers was lower than in the grasses. REP was highly negatively correlated with dry matter digestibility and the content of digestible protein. For the estimation of the heritability we determined the C18:3 content and REP of 300 single plants of each of the species perennial ryegrass, tall fescue and red and white clover, grown in pots. We carried out positive and negative selections for both parameters in the four species. The results of their offspring suggested a moderate to good heritability of both parameters and opportunities for breeding. Improved grass/clover mixtures may be an important source of C18:3 in ruminant feeding. However because of the negative correlation between REP and

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the digestible protein content, breeding for improved nitrogen efficiency in cattle by increasing the fraction of rumen escape protein in the forage is not obvious.

Keywords Grass · Clover · Linolenic acid · Rumen escape protein · Variation · Heritability

22.1 Introduction

The nutrition of cattle may affect the quality of the meat and milk as well as their environmental impact. In order to breed grass and clover varieties that meet current sustainable requirements we studied the variation and heritability of two quality parameters in grass and clover: the content of α-linolenic acid (C18:3) and the fraction of rumen escape protein (REP).

α-Linolenic acid is the major fatty acid in grass and clover. This poly-unsaturated ω-3 fatty acid and the derived conjugated linoleic acid (CLA) in ruminant milk and meat may be beneficial to human health (prevention of cardiovascular diseases and cancer).

Protein of grass and clover is extensively degraded in the rumen. The degraded protein is partly transformed by the rumen bacteria into useful microbial protein and partly lost in the urine to the environment causing ammonia volatilization and nitrate leaching. The fraction of the protein that escapes rumen degradation may be digested in the intestine. The levels of excreted nitrogen may be reduced by increasing the availability of energy for rumen fermentation (e.g. water soluble carbohydrate content, Humphreys et al. 2010) or by enhancing the REP fraction of the forage (Tamminga et al. 1994).

Using fast and reliable screening procedures we determined the variation of the C18:3 content and REP of grass and clover species and varieties. Within a few species we carried out a divergent selection for both parameters and estimated their heritability.

22.2 Material and Methods

To study the variation of C18:3 and REP of the grasses a strip plot experiment in three replicates with two levels of nitrogen application rate (350 and 230 kg N/ha/year) and 24 varieties belonging to six species was sown in September 2006 in Merelbeke (Belgium). The variation of the clover was tested in a random block design with four varieties of red and three varieties of white clover. In 2007 and 2008 the grass and clover plots (size: 8.1 m^2) were cut five times a year. The species and varieties in the trials are presented in Table 22.1.

The heritability of C18:3 and REP was estimated for the species: perennial ryegrass (diploid), tall fescue, red clover (diploid) and white clover. In 2008, 300 single plants (12 cultivars × 25 plants per cultivar) from each of the four species were

Table 22.1 Species and varieties in variation trials

Species	Variety	Ploidy	Type
Lolium perenne	Rebecca	2x	Early heading
	Indiana	2x	Early heading
	Aberdart	2x	Intermediate heading
	Premium	2x	Intermediate heading
	Barnhem	2x	Late heading
	Merks	2x	Late heading
	Merlinda	4x	Early heading
	Lacerta	4x	Early heading
	Aberglyn	4x	Intermediate heading
	Graciosa	4x	Intermediate heading
	Abercraigs	4x	Late heading
	Floris	4x	Late heading
Phleum pratense	Comer	6x	Hay type
	Motim	6x	Pasture type
Festuca pratensis	Merifest	2x	
	Pradel	2x	
Festuca arundinacea	Barolex	6x	
	Barelite	6x	
	Bariane	6x	
Dactylis glomerata	Barmoral	4x	
Lolium multiflorum	Bellem	2x	
	Davinci	2x	
	Gemini	4x	
	Barmega	4x	
Trifolium pratense	Merula	2x	
	Lemmon	2x	
	Milvus	2x	
	Larus	4x	
Trifolium repens	Barblanca	4x	Large leaves
	Riesling	4x	Intermediate leaves
	Barbian	4x	Small leaves

grown in 12 L pots. Two cuts of the sowing year were analyzed for the determination of the C18:3 content and the REP. For each species the 30 plants with the highest and lowest C18:3 content or REP were selected. In total 16 isolated polycrosses with these plants were installed. In 2009 we harvested the seeds of each of the divergent selection groups. These progenies (300 plants per selection group) were again sown in 12 L pots in 2010 together with seeds of three of the original cultivars as reference. Two cuts were harvested in 2010 and analyzed for C18:3 and REP. The heritability of both parameters for each species was calculated as: (the difference between the divergent progeny groups)/(difference between the divergent parental groups).

In both the variation and heritability study we determined at each cut the fresh yield and the content of dry matter (DM), crude protein (CP), water soluble carbohydrates (WSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), C18:3 and the in vitro dry matter digestibility with cellulase (DMD). Except for yield all these parameters were estimated by near infrared reflectance spectroscopy (NIRS) on dried samples of the grass and clover. NIRS calibrations of C18:3 were developed

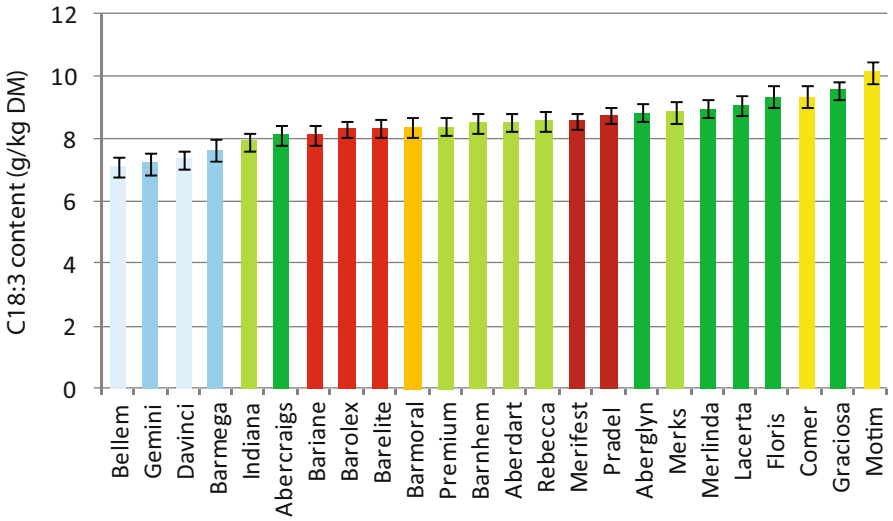

Fig. 22.1 C18:3 content (average ± s.e. of 2 years, 5 cuts a year, 2 N rates) of 24 varieties belonging to the grass species perennial ryegrass (*green*), Italian ryegrass (*blue*), tall ferscue (*red*), meadow fescue (*brown*), timothy (*yellow*) and cocksfoot (*orange*). (*dark*: tetraploid, *light*: diploid)

for grass and clover separately. The REP was estimated by means of a multiple regression equation based on DMD, DM and ADF with a determination coefficient of 84 % and a residual error of 3.1 %-units (Vandewalle et al. 2010). To derive this prediction equation REP was determined on a limited sample collection of 56 fresh grass and clover samples through *nylon bag* incubations in the rumen of fistulated cows according to the CVB protocol (CVB 2003), whereas corresponding dried and ground samples were chemically analyzed. The content of protein digestible in the intestine (PDI) was estimated by an equation including DMD, CP, DM and NDF also derived from the limited sample set.

22.3 Results

22.3.1 Variation of C18:3

For the grasses there were significant effects of the harvest year, the cuts within each year, the N fertilization level, the species and the varieties on the C18:3 content. At the high N application rate the C18:3 content was 0.4 g/kg DM higher than at the low rate. The mean C18:3 content of the varieties ranged from 7.1–10.1 g/kg DM (Fig. 22.1) with an average of 8.5 g/kg DM. The C18:3 content of timothy was significantly higher and the C18:3 content of Italian ryegrass significantly lower than of the other species. For both ryegrass species tetraploid varieties had on average a higher C18:3 content than the diploid varieties.

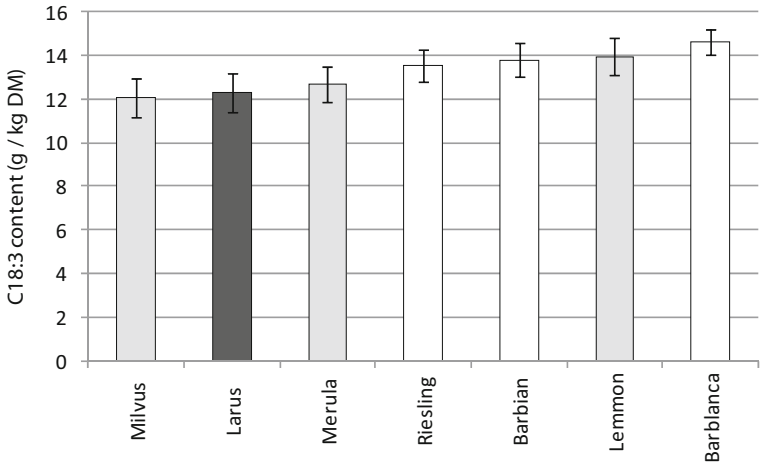

Fig. 22.2 C18:3 content (average ± s.e. of two years, five cuts a year) of seven clover varieties belonging to *white* clover (*white*) and *red* clover (*black*: tetraploid, *grey*: diploid)

The C18:3 content of the clover (mean: 13.3 g/kg DM) was much higher than that of the grasses. White clover had a significantly higher C18:3 content (14.0 g/kg DM) than red clover (12.8 g/kg DM) (Fig. 22.2). There were no significant differences among varieties within the species. For both the grasses and the clovers the C18:3 content was highly positively correlated with the protein content.

22.3.2 Variation of REP

For the grasses, REP was significantly affected by harvest year, cuts within each year, species and variety. The N application rate had no significant effect. The mean REP of the varieties ranged from 38.0–44.6 % (Fig. 22.3) with an average of 41.2 %. The REP of cocksfoot and tall fescue was significantly higher and that of perennial ryegrass significantly lower than the other species. For both ryegrass species diploid varieties had a higher REP than the tetraploid varieties.

The REP of the clover (mean: 34.2 %) was much lower than the REP of the grasses. Red clover had a significantly higher REP (36.2 %) than white clover (31.7 %) (Fig. 22.4). There were almost no differences between varieties within the species.

22.3.3 Heritability of C18:3 and REP

Table 22.2 shows the C18:3 content and REP of the positive and negative selections that were made for both parameters of each species and of their offspring. Based on these divergent selection the heritability was calculated (Table 22.3).

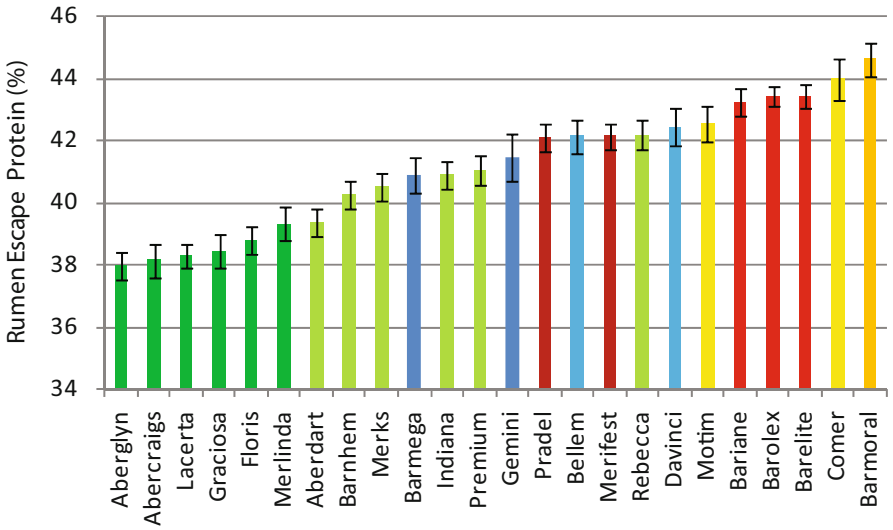

Fig. 22.3 REP (average ± s.e. of two years, five cuts a year, 2 N rates) of 24 varieties belonging to the grass species perennial ryegrass (*green*), Italian ryegrass (*blue*), tall ferscue (*red*), meadow fescue (*brown*), timothy (*yellow*) and cocksfoot (*orange*). (*dark*: tetraploid, *light*: diploid)

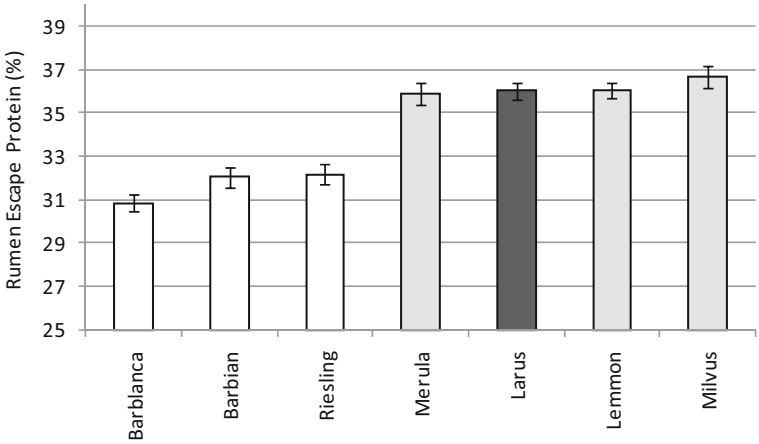

Fig. 22.4 REP (average ± s.e. of 2 years, 5 cuts a year) of 7 clover varieties belonging to *white* clover (*white*) and *red* clover (*black*: tetraploid, *grey*: diploid)

By selecting the 10 % best plants the progress of the positive selection group of the parents was higher for C18:3 than for REP because of the higher variation in C18:3. Selection for higher REP could not be done without decreasing protein digestible in the intestine.

Differences between the positive and negative groups were still observed in the offspring but to a lower extent than in the parents. The heritability of C18:3 and REP

Table 22.2 C18:3 content, REP and PDI (in % of the mean of 3 reference varieties) of the positive (+) and negative (−) selections of the parents and offspring of 2 grass and 2 clover species

Generation (year)	Species	C18:3		REP (PDI)	
		−	+	−	+
Parents (2008)	Tall fescue	77	125	91 (104)	110 (96)
	Perennial ryegrass	76	127	92 (103)	109 (96)
	White clover	84	117	93 (107)	107 (99)
	Red clover	81	121	91 (100)	110 (99)
Offspring (2010)	Tall fescue	96	115	95 (104)	113 (91)
	Perennial ryegrass	72	112	96 (105)	107 (99)
	White clover	94	104	97 (105)	104 (100)
	Red clover	86	102	100 (96)	104 (93)

Table 22.3 Heritability of C18:3 content and REP for 2 grass and 2 clover species

Species	C18:3	REP
Tall fescue	0.39	0.96
Perennial ryegrass	0.79	0.64
White clover	0.28	0.45
Red clover	0.41	0.25

for clover was on average lower than for the grasses. Perennial ryegrass showed a good heritability for C18:3 content and tall fescue for REP.

22.4 Discussion

Although the α-linolenic acid content in dried grass and clover is low, there is a significant variation between and within species with a good heritability. Elgersma et al. (2003) also found consistent differences in the C18:3 content among cultivars of perennial ryegrass throughout the season. Fresh perennial ryegrass may contain about 15 g C18:3/kg DM. Thanks to the variation and heritability of C18:3, a variety with a 10 % higher C18:3 content may be bred. This variety may produce 15 kg C18:3 more per ha (let dry matter yield be 10 t/ha/year). The intake of 10 kg dry matter of this variety offers the cow 165 g C18:3 which is similar to 1.4 kg of an enriched concentrate (12 % C18:3) based on linseed. This is about the quantity of concentrate that is supplemented in winter to cows for the production of high ω-3 milk. Because of the higher C8:3 content in white clover, mixtures of improved grass and clover varieties may replace even higher quantities of the linseed concentrates.

Due to the strong negative correlation between REP and digestibility, breeding for higher REP doesn't hold many prospects. Lower digestibility may reduce microbial protein production. Among the grasses tall fescue has a high REP with a high heritability. Some small progress in REP should be possible without loss of PDI. Clovers have a high CP content with a much lower REP than grasses. In this respect tall fescue may very well complement red clover as a high yielding mixture for the production of home grown protein roughage under conservation management.

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Chapter 23
Similarities and Differences in Leaf Proteome Response to Cold Acclimation Between *Festuca pratensis* and *Lolium perenne*

A. Kosmala, A. Bocian, M. Rapacz, B. Jurczyk, Ł. Marczak and Z. Zwierzykowski

Abstract *Lolium perenne* is used extensively as a forage and turf grass due to its high nutritive values, rapid establishment and persistence. *Festuca pratensis*, a species closely related to *L. perenne*, has lower nutritive values but is one of the most winter-hardy species within the *Lolium-Festuca* complex. Frost tolerance is the main component of winter-hardiness and the plant species growing in temperate regions can acquire it through exposure to low, non-lethal temperatures, a phenomenon known as cold acclimation. Herein, we review our recent results of two proteomic projects, focused on *F. pratensis* and *L. perenne*. Each project involved the comparison of leaf protein accumulation profiles during cold acclimation between plants with different levels of frost tolerance by the use of two-dimensional electrophoresis and identification of differentially accumulated proteins by mass spectrometry. In the present paper similarities and differences in leaf proteome response to cold acclimation between *F. pratensis* and *L. perenne* are summarized.

Keywords Cold acclimation · *Festuca pratensis* · Frost tolerance · *Lolium perenne* · Proteome

Abbreviations

CA	Cold acclimation
HFT	High frost tolerant
LFT	Low frost tolerant

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MS Mass spectrometry
PPFD Photosynthetic photon flux density
2-DE Two-dimensional electrophoresis
T_{EL50} The temperature at which 50 % of total electrolytes were released from leaf tissues
%Vol Relative volumes of spots

23.1 Introduction

Perennial ryegrass (*Lolium perenne* L.) is a high quality forage and turf grass species. However, its ability to perform in harsh winter climates is limited. Meadow fescue (*Festuca pratensis* Huds.), a species closely related to *L. perenne*, has lower nutritive values but is one of the most frost tolerant species within the *Lolium-Festuca* complex. Frost tolerance is the main component of winter-hardiness and one of the best indicators of plant ability to survive harsh winter conditions. The plant species growing in temperate regions can acquire frost tolerance through exposure to low, non-lethal temperatures, a phenomenon known as cold acclimation (CA). This process is associated with altered expression patterns of a specific set of genes (Thomashow 1999; Sandve et al. 2011).

The analysis of the cellular proteome complement during CA could help to understand the mechanisms involved in cell responses to low temperature stresses. The tools most frequently used for visualizing protein components of protein samples and their identifications are two-dimensional electrophoresis (2-DE) and mass spectrometry (MS), respectively.

Herein, we review the results derived from two proteomic projects, one focused on the proteome of *F. pratensis* (Kosmala et al. 2009), other on the proteome of *L. perenne* (Bocian et al. 2011). In each project, the comparison of leaf protein accumulation profiles during CA between plants with different levels of frost tolerance within each species was carried out. The 2-DE technique was adopted, followed by the identification of proteins which were accumulated differentially between the selected plants by MS.

Here, we summarize and emphasize the similarities and differences in leaf proteome response to CA between *F. pratensis* and *L. perenne*.

23.2 Dynamics of Frost Tolerance Level During Cold Acclimation of *F. pratensis* and *L. perenne*

High frost tolerant (HFT) and low frost tolerant (LFT) genotypes within each species were selected from populations of *F. pratensis* cv. Skra and *L. perenne* cv. Arka. The way of selection was similar for both species and involved a two-step procedure. Pre-hardening was performed during seven days at 12 °C, 8/16 h photoperiod, and

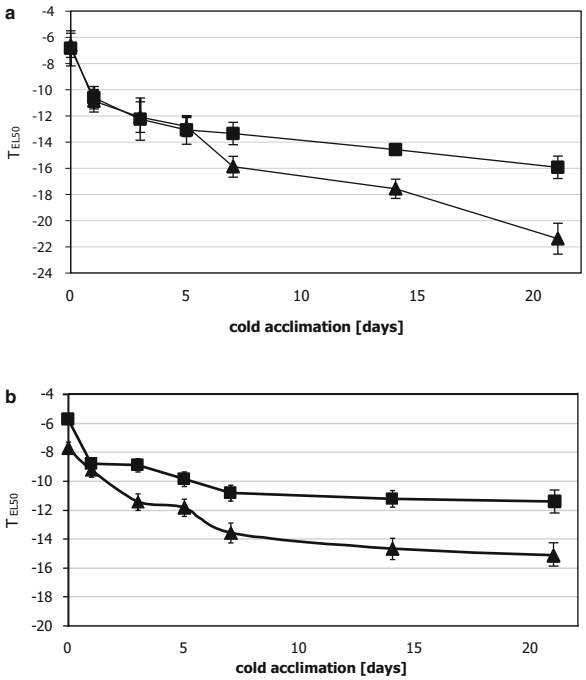

Fig. 23.1 Changes in the level of frost tolerance of high frost tolerant (▲) and low frost tolerant (■) genotypes during cold acclimation of *Festuca pratensis* **(a)**, *Lolium perenne* **(b)**. T_{EL50} means the temperature at which 50 % of total electrolytes were released from leaf tissues. Values of T_{EL50} and least significant differences for $P = 0.05$ were calculated on the basis of freezing tests made in two replicates at five freezing temperatures: −3, −6, −9, −12 and −15 °C

200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and CA during 21 days at 4/2 °C, 10/14 h photoperiod, and 200 μmol m^{-2} s^{-1} PPFD. The 1st step of plant selection involved the estimation of the regrowth after freezing at −8 °C, −11 °C and −14 °C; the 2nd step, involved the estimation of the T_{EL50} values (temperature causing a 50 % electrolyte leakage) one day before CA and after 2, 8, 26 h, and 3, 5, 7, 14 and 21 days of CA. Two genotypes from each species with the extreme values of T_{EL50} after 21 days of CA, a low frost tolerant (LFT) and a high frost tolerant (HFT) plant were chosen for further proteomic research. In Fig. 23.1 we present the physiological traits demonstrating the level of frost tolerance during CA for HFT and LFT genotypes of both *F. pratensis* (a) and *L. perenne* (b).

The comparison of the diagrams revealed differences in the dynamics of frost tolerance during CA of the analyzed species. Differences in the values of T_{EL50} between HFT and LFT genotypes of *F. pratensis* appeared after the 5th day, and increased continuously until the 21st day of CA. The HFT ($T_{EL50} = -21.4$ °C) and LFT ($T_{EL50} = -15.9$ °C) *F. pratensis* plants differed by as much as −5.5 °C in their frost tolerance after 21 days of CA. On the other hand, CA between the 7th and 21st day only slightly (statistically not significantly) increased frost tolerance in *L. perenne*. In fact, the differences in frost tolerance between the analyzed plants appeared on the 3rd day of CA and frost tolerance reached its final levels on the 7th day of CA. The HFT ($T_{EL50} = -15.13$ °C) and LFT ($T_{EL50} = -11.42$ °C) *L. perenne* plants differed by as much as −3.7 °C in their frost tolerance after 21 days of CA.

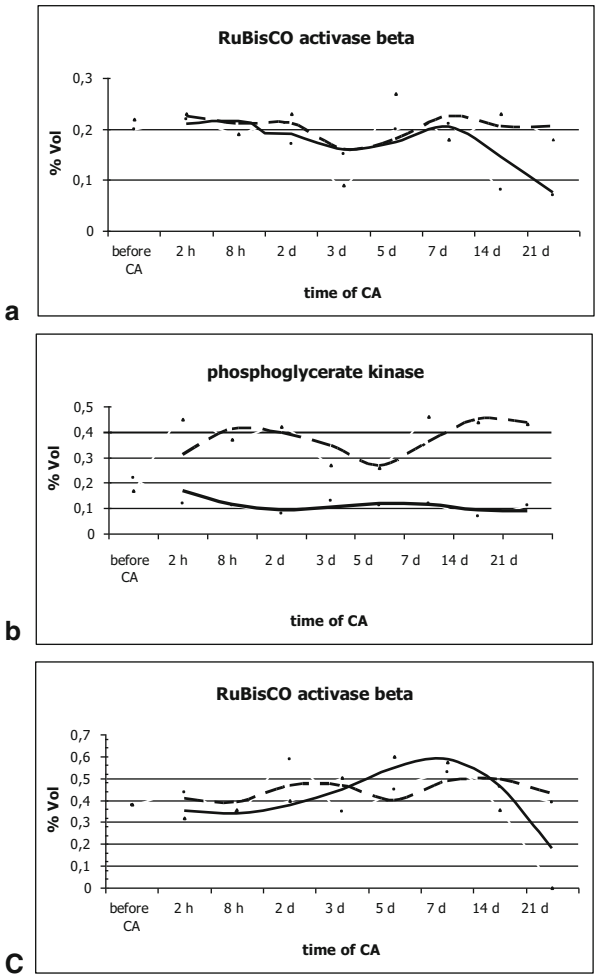

Fig. 23.2 Comparisons of the accumulation profiles of RuBisCO activase beta **a**, **c**, **d** and phosphoglycerate kinase **b**, **e** during cold acclimation (CA) of *high frost tolerant* (**- – -**) and *low frost tolerant* (—) genotypes of *Festuca pratensis* **a**, **b** and *Lolium perenne* **c**, **d**, **e**. %Vol means relative volumes of spots. Three biological replicates were used to calculate mean %Vol. The trend *curves* are shown

23.3 Leaf Proteome Response to Cold Acclimation in *F. pratensis* and *L. perenne*

2-DE was performed on the leaf samples harvested from three clones of each selected genotype (three biological replicates) one day before CA and after 2, 8, 26 h, and 3, 5, 7, 14 and 21 days of CA. The proteomic experiments were carried out in the same conditions for both species. Following electrophoresis the gels were stained

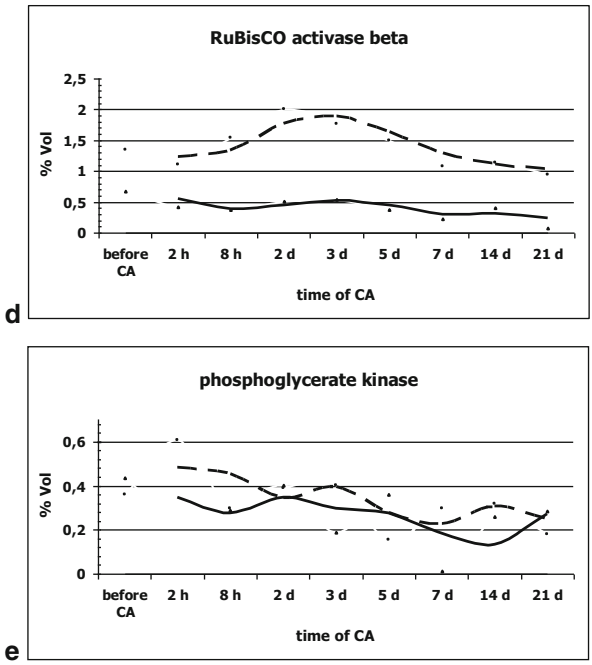

Fig. 23.2 (Continued)

with colloidal Coomassie Brilliant Blue G-250. Spot detection and image analyses (normalization, spot matching, protein accumulation analyses, statistics) were performed with Image Master 2-D *Platinum* software (GE Healthcare). Relative volumes (%Vol) of spots were used to create the detailed protein accumulation profiles for HFT and LFT genotypes of each species during CA. The protein abundance for each spot at a corresponding time point before and during CA between HFT and LFT plant was compared. The spots with at least 1.5-fold differences ($P \leq 0.05$) in protein abundance or spots present only in one genotype (HFT or LFT) and absent in the other genotype at a minimum of one time point of CA, were subjected to MS analyses and protein identification. Fragmentation spectra and/or peptide masses were measured using a Matrix Assisted Laser Desorption/Ionization Time of Flight MS or electrospray ionization-MS/MS tandem MS. The data were exported to the Mascot software for MSDB, NCBI or SwissProt database search (www.matrixscience.com).

The detail proteomic results can be found in our recent papers (Kosmala et al. 2009; Bocian et al. 2011). Herein, we indicate only the general similarities and differences in leaf proteome response to CA, which could be noticed between *F. pratensis* and *L. perenne*.

First of all, the time points of CA at which the differences in protein accumulation between HFT and LFT genotypes were most frequent, were different for *F. pratensis* and *L. perenne*, and involved the 26th hour of CA in *F. pratensis* and both the 5th and

the 7th day of CA in *L. perenne*. Interestingly, as mentioned earlier, frost tolerance reached its maximum levels in *L. perenne* genotypes exactly on the 7th day of CA (Fig. 23.1b). Similar numbers of differentially accumulated proteins were observed: 41 for *F. pratensis* and 42 for *L. perenne*. However, the proportions of those proteins to the total protein numbers detected on 2-D maps were different for the two species, 5.1 % (41/800) and 7.2 % (42/580) for *F. pratensis* and *L. perenne*, respectively. Generally, different proteins were found to be differently accumulated between LFT and HFT *L. perenne*, compared to LFT and HFT *F. pratensis* genotypes; the only exceptions were, chloroplastic phosphoglycerate kinase and RuBisCO activase beta, common for both species (Fig. 23.2a–23.2e). However, in both *F. pratensis* and *L. perenne*, chloroplast proteins, including proteins directly involved in the process of photosynthesis, were the major group selected as differentially accumulated during CA between genotypes differing in frost tolerance levels. On the other hand, chloroplast proteins are a major component of all the proteins found in leaves.

Both phosphoglycerate kinase and RuBisCO activase beta are involved in the reactions of the dark phase of photosynthesis. In the case of *F. pratensis*, a decreased accumulation of RuBisCO activase during the last two weeks of CA in the LFT genotype (Fig. 23.2a) and an increased accumulation of phosphoglycerate kinase during the whole period of CA in the HFT genotype (Fig. 23.2b) were observed. In the case of *L. perenne*, two forms of RuBisCO activase were observed, one showed higher abundance in the HFT genotype during the whole process of CA (Fig. 23.2d), and the other decreased accumulation level after 21 days of CA in the LFT genotype (Fig. 23.2c). Lower accumulation level of phosphoglycerate kinase was observed at most time points of CA also in LFT plant (Fig. 23.2e). The accumulation patterns of both enzymes observed in *F. pratensis* and *L. perenne* may suggest that HFT genotypes are more efficient in photosynthetic carbon metabolism. The increasing rate of this metabolism could be one of the mechanisms protecting plants against photoinhibition of photosynthesis. It was demonstrated that plants more tolerant to cold-induced photoinhibition are often also more frost tolerant (Rapacz et al. 2007). However, at this stage of the research this hypothesis is speculative.

23.4 Conclusions

Further research, involving other methods of differential protein quantification (instead of 2-DE), e.g. isobaric tags for relative and absolute quantification (iTRAQ), should be applied to better dissect the proteome dynamics of *F. pratensis* and *L. perenne* during cold conditions. Phosphoglycerate kinase and RuBisCO activase beta were found to be differentially accumulated during CA between HFT and LFT genotypes, both in *F. pratensis* and *L. perenne*, and this prompts further study on these proteins.

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Chapter 24
Multi-population QTL Detection for Flowering Time, Stem Elongation and Quality Traits in *Medicago truncatula*

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Abstract *Medicago truncatula*, as a model species, is useful to study the genetic control of flowering time, stem elongation and quality (protein content and digestibility) in legume crops. These traits were measured in four mapping populations originating from five parental lines. Single and multi-population quantitative trait locus (QTL) detections were carried out. A large variation was observed within populations and transgressive segregations were noted. On average, genotypes with long primary branches and a high branch elongation rate showed an early flowering date and a long main stem and had lower digestibility and protein content. Ninety QTLs for morphogenesis and 27 QTL for quality were identified and localized over all eight chromosomes. All QTLs for quality traits were located in genomics regions showing QTL for morphogenesis, suggesting common regulation and/or colocation of genes responsible for their variation. Using genomic resources publicly available, a list of candidate genes that could control variation for aerial morphogenesis and quality was established.

Keywords Morphogenesis · Heritability · Model species · Legume · Gene

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24.1 Introduction

Vegetative growth, flowering date and forage quality are major breeding traits in forage legumes. They are known to have a quantitative inheritance so the analysis of their genetic determinism is quite complex in alfalfa (*Medicago sativa*) or clovers (*Trifolium* sp.). With the assumption that genes or genomic regions responsible for trait variation in a model species will also contribute to explain genetic variation in a related crop species, we have studied genetic determinism of breeding traits in the model species *Medicago truncatula*. In this diploid and autogamous species, recombinant inbred lines (RILs) mapping populations, markers and genomic sequences are available to detect QTLs and to identify candidate genes that may explain trait variation.

Four mapping populations were studied for stem elongation, flowering date, energy value and protein content. QTL were detected in each cross and a multi-population QTL detection was carried out.

24.2 Material and Methods

Four RIL populations of *M. truncatula* involving a total of five parental lines were studied: LR4 (Julier et al. 2007), LR5 (Ameline-Torregrosa et al. 2008), LR1 (Pierre et al. 2008) and LR6 (Fig. 24.1). Each RIL population was composed of 173 to 233 lines. The populations were analysed for aerial morphogenetic traits and quality traits in experiments conducted in 2002, 2003, 2004 (for LR4), spring and autumn 2005 (for LR1 and LR5), 2007 and 2008 (for LR6). Plants of the RILs populations and the five parental lines were grown in individual pots in a greenhouse at INRA Lusignan (France). All lines of LR4 in 2002 and 2003 were repeated three times but, in order to maximise the number of RILs under study, there was no repetition for LR4 in 2004, LR1, LR5, LR6, except for 15 RILs randomly taken and five parental lines. These 20 lines were repeated three times. Each repetition was composed of one plant. The flowering time (FT) was recorded when a plant had one open flower on a primary branch, and transformed in degree-days with a temperature basis of 0 °C. The length of the first two emerging primary branches (LPB) was measured twice a week during the growth period. When all the plants of the trial had flowered, the length of the main stem (LMS) was measured. The plants were harvested, dried and grounded. Energy value, evaluated through dry matter enzymatic digestibility (DIG) and protein content (PC) were measured using NIRS equations. The curve of branch elongation as a function of sums of degree-days showed a linear phase. For each RIL, branch elongation rate (BER) was calculated as the slope of this linear phase.

Framework maps of each cross included about 60 SSR markers and covered about 600 cM. QTL mapping was performed using QTL Cartographer (Basten et al. 1994; Basten et al. 2002) with the composite interval mapping (CIM) procedure. The threshold for adding a QTL, determined at 5 % risk by a permutation test method (1000 replications), was set to 11.33 (LOD $\geq$ 2.46). The limits of the confidence

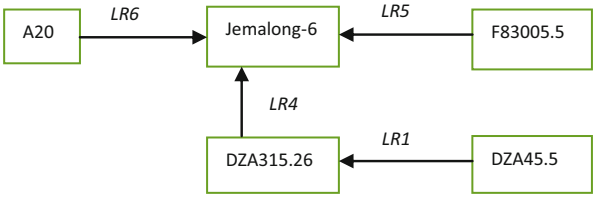

Fig. 24.1 Crossing design between five *M. truncatula* parents to obtain the four mapping populations LR1, LR4, LR5 and LR6. The *arrows* indicate the direction of the cross, from the male to the female parent

interval of QTL position were estimated at the positions where the LOD value drop-off was equal to one (Lander and Botstein 1989).

To better estimate the position of the QTLs that were common to different populations and their effects, a multi-population QTL analysis was carried out with the MCQTL software package (Jourjon et al. 2005). First a consensus genetic map was built from the genetic maps of LR1, LR4, LR5 and LR6 by using BioMercator software (Arcade et al. 2004). Each QTL position and its support interval were projected on the consensus map. In a second step, adjusted means of aerial morphogenetic and quality traits per RIL were calculated. Adjusted means (Blanc et al. 2006) and the consensus map were used to launch the multi-population QTL analysis with the "connected" option.

The version 3.5 of *M. truncatula* genome sequence (http://www.medicagohapmap.org/?genome) was used to detect the BACs that belong to the support interval of the major QTLs. Among the genes annotated on the BACs, we searched out those involved in plant growth or quality.

24.3 Results and Discussion

Significant variation among lines was observed in each population for all traits. In most cases, transgressions towards lower and higher values than those of parents were observed.

Positive and significant correlations were observed between BER and LPB in all seasons; negative and significant correlations were observed between FT and LMS and between FT and LPB except for LR5 in autumn 2005. These correlations indicate that on average, genotypes with long primary branches and a high branch elongation rate showed an early flowering date and a long main stem. Correlations between digestibility and protein content were highly significant. Digestibility and protein content were negatively correlated with stem elongation.

In the QTL detection in each population, a total of 90 QTLs for BER, LMS, FT, LPB were identified and localized over all eight chromosomes, with a greater concentration on chromosomes 1, 2, 7 and 8. The bottom of chromosome 7 carried QTLs for FT, BER, LMS, and LPB on populations LR1, LR4 and LR5 with high R^2 values. No QTL was found in this region in LR6. The strongest QTLs for FT in LR6 were located on chromosome 6 at the positions 15.4 –21.1 cM, explaining 15–19.4 % of the variation. For quality traits, 19 QTLs were detected on chromosomes 1, 4, 5,

6, 7 and 8 for digestibility and 8 QTL were detected on chromosomes 1, 2, 3, 7 and 8 for protein content. In fact, all QTL for quality traits were located in regions where QTLs for morphogenetic traits were also present. Either morphogenesis explained most of the variation for quality, or genes involved in both types of traits co-located in the genome.

The multipopulation QTL analysis produced four QTLs (herein called mcQTLs) for LPB, two for BER, six for LMS, and two for FT, DIG and PC (Table 24.1), each explaining between 3.0 and 22.9 % of the variation. For LPB and FT, the mcQTLs on chromosome 7 had higher effects than the others mcQTLs, explaining 10.2 and 22.9 % of variation, respectively. For LPB, the mcQTL on chromosome 1 explained almost 12 % of the variation. For BER, DIG and PC, the two mcQTLs had similar R^2. This part of the variation explained by the mcQTLs was often lower than that of the initial QTLs because not all populations × seasons produced these QTLs. QTLs that were specific to one single cross such as the QTL for FT on chromosome 6 in LR6 were not revealed with this multi-population analysis, as if its effect was "diluted" in the whole design. In most cases, the alleles of each parent had either positive or negative effects, explaining the transgressions in the progenies. However Jemalong6 alleles except one induced long branches, high BER, low digestibility and early FT and DZA315.16 alleles induced short branches, low BER, early FT and high PC. All the mcQTLs were found in a position where at least one QTL was found in a population. But some QTLs that seemed to co-localise in several populations (FT on chromosome 1, BER on chromosome 1, LPB on chromosomes 4 and 8) were not recovered in the multi-population analysis. The same positions for the mcQTLs were observed for LPB and LMS on chromosome 1, for LPB, LMS and BER on chromosome 2, for FT, LPB and LMS on chromosome 7, and for FT and LMS on chromosome 8, suggesting partially common genetic regulation.

The *in silico* analysis of BACs included in the confidence interval of major mcQTLs for FT on chromosome 7 and LMS on chromosome 1 revealed three and ten candidate genes related to these traits, respectively. In the confidence interval of the mcQTL for FT on chromosome 7, genes related to floral induction were identified: zinc finger protein CONSTANS-like, FLOWERING LOCUS T and PEBP genes. CONSTANS-like gene proved to contribute to variation for stem length and flowering date in alfalfa (Herrmann et al. 2010). At the bottom of chromosome 1, where a mcQTL for LMS was identified close to QTLs for LPB, eight genes were detected (COP1, CLAVATA1, SBP, ARF/SAR, Auxin-binding protein, EMBRYOGENIC FLOWER 2, Aux/IAA protein and NAM) related to shoot and branching development through hormone response and signalling. In the genomic region revealed on chromosome 6 for FT, a gene FAR1 involved in the far-red responses controlled by phytochrome A, and a member of SKP1 gene family, homologue of ASK1 in *Arabidopsis* are annotated. In the QTL regions of quality traits, genes related to cell wall polysaccharides and protein biosynthesis, lignin pathway or plant development metabolism were revealed on chromosomes 1, 3, 7 and 8. It is established that stem growth contributes to decrease both stem digestibility because of an increase in lignified tissues and plant protein content through reduction of the proportion of leaves in the biomass. The presence of genes involved in quality pathways

Table 24.1 Location of QTLs for aerial morphogenesis and quality traits, proportion of explained variation (R^2) and effects of parents in a multi-population QTL analysis

Trait	Chromosome	Position	Confidence interval	R^2	Effect of parents				
					Jemalong6	DZA315.26	DZA45.5	A20	F83005.5
Length of primary branches (LPB), (cm)	1	65.3	64–70	5.5	−1.72	−0.32	3.19	−1.67	0.51
	2	52.3	49–58	6.0	2.40	−0.61	−1.31	2.16	−2.65
	3	52.6	51–55	4.3	2.06	−1.27	−1.69	−1.36	2.27
	7	56.5	54–60	10.2	3.29	−2.60	−4.42	2.17	1.56
Branch elongation rate (BER), (10^{-3} cm.day^{-1})	2	52.3	46–59	3.0	0.8	−1.9	2.6	0.2	−5.7
	4	72.7	68–77	3.5	0.8	2.8	−1.2	−0.6	−1.8
Length of main stem* (LMS), (cm)	1	62.5	55–64	11.7	−3.18	−0.49	0.49	3.68	−0.50
	2	57.3	48–58	5.0	−0.59	−0.55	0.55	3.59	−3.00
	7	22.9	17–26	8.5	−2.55	1.17	−1.17	4.25	−1.69
	7	50	41–54	4.9	2.13	1.19	−1.19	−1.49	−0.64
	8	5	1–9	5.2	1.16	1.09	−1.09	2.89	−4.06
	8	31.5	28–37	7.8	2.37	−2.51	−1.50	−0.87	2.51
Flowering time (FT), (°C.D)	7	51.5	50–55	22.9	−76.6	57.7	149.7	−68.8	−62.0
	8	5	0–9	3.9	−31.5	20.9	33.1	−36.2	13.7
Digestibility (DIG), (%)	7	56.5	53.9–59.3	10.9	−0.77	0.35	0.88	−0.93	0.47
	8	5.0	3.2–20.2	7.6	−0.42	−0.23	0.41	−0.68	0.92
Protein content (PC), (%)	1	70.0	59.3–70.0	4.1	0.24	0.08	−0.27	−0.17	0.12
	7	57.4	54.6–63.6	4.5	−0.32	0.21	0.40	−0.33	0.04

*Analysis conducted on three populations (DZA315.26 × DZA45.5, Jemalong6 × F83005.5 and Jemalong6 × A20)

in QTL regions of quality traits is consistent with the hypothesis that an intrinsic genetic regulation of quality exists besides the physiological relationship between morphogenesis and quality. The genomic regions or the genes detected in the model species are candidates to explain phenotypic variation in cultivated legume species. They could be the targets of new studies aiming at checking their role in a specific crop and identifying the alleles conferring positive effects. These alleles could then be used in marker-assisted selection.

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Chapter 25
Role of the *RCT1* Gene in Anthracnose Resistance in Alfalfa

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Abstract Anthracnose, caused by *Colletotrichum trifolii*, is a severe disease of alfalfa (*Medicago sativa*). The *RCT1* gene, isolated from the model legume *M. truncatula*, is a candidate gene to explain genetic variation for anthracnose resistance in alfalfa. A bulk segregant analysis was carried out to test this hypothesis: from each of eight alfalfa varieties, 15 resistant (R) plants and 15 susceptible (S) plants were selected and DNA was extracted. The whole gene including the upstream and downstream regions (a total of 14 kb) was amplified by PCR for each individual and the R and S plants were pooled for each variety. Sequencing was carried out using the next generation sequencer 454 (Roche). The sequence reads, that averaged 295 bp, were assembled to produce consensus sequences that can be considered as alleles. Considering the five exons of the gene, five regions contained clear deletion/insertion polymorphism but these polymorphisms were present in both the R and S pools. Individual genotyping for these indels indicated that different alleles were present but no specific allele was associated with the phenotype. These polymorphic regions in *RCT1* seemed not to explain the variation of anthracnose resistance in alfalfa. However, the presence of one rare allele inducing a lack of function was associated with the resistance. A divergent selection for this allele would test its interest in breeding programs.

Keywords *Medicago sativa* · *Medicago truncatula* · *Colletotrichum trifolii* · Candidate gene · Bulk segregant analysis · Next-generation sequencing

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25.1 Introduction

Anthracnose, caused by *Colletotrichum trifolii*, determines severe yield losses in alfalfa (*Medicago sativa*) forage production (Barnes et al. 1969; Raynal et al. 1989). This fungus attacks stems and crowns and kills the plants, contributing to stand decline. Three races have been described (Ariss and Vandemark 2007). Alfalfa resistance to anthracnose is conferred by two independent dominant genes, *An1* and *An2* (Elgin and Ostazeski 1985). Resistance to race 1 and likely race 4 is conferred by *An1* (Mackie et al. 2003), and to race 2 by *An2*. Breeding has focused on the selection of resistant varieties, using tests in controlled conditions based on artificial inoculations (Gondran 1984; O'Neill 1991). Despite an oligogenic inheritance, quantitative methods are used to select resistant plants, specifically because the autotetraploidy of alfalfa makes genetic analyses complex. QTL studies were conducted to localise the resistance genes on the genome, both in alfalfa (Mackie et al. 2007) and in the model plant *M. truncatula* (Ameline-Torregrosa et al. 2008; Yang et al. 2007) in which susceptible and resistant plants were identified. Partially consistent results were obtained with QTLs found on chromosome 4 but also a QTL with strong effect on chromosome 8 in alfalfa.

In *M. truncatula*, diploidy and the available genomics tools give the opportunity to conduct fine mapping strategies aiming at discovering the gene responsible of a QTL. Fine mapping analysis in a large population resulted in the identification of the *RCT1* gene, a TIR-NBS-LRR resistance gene (Yang et al. 2008). The function of this gene was not validated in *M. truncatula* because of the difficulty to transform some genotypes, but it was introduced in susceptible alfalfa genotypes, and conferred resistance to anthracnose (Yang et al. 2008). Transgenic varieties are not generally accepted by the European society, and transgene escapes risks are high in alfalfa. Thus, the use of *RCT1* to breed resistant transgenic cultivars is not possible for the European market.

Given the results of Yang et al. (2008), we hypothesized that the alfalfa *RCT1* ortholog could be responsible for anthracnose resistance in this species. The objective of this study was to test the relationship of *RCT1* sequence polymorphism with alfalfa resistance to anthracnose. The polymorphisms related to resistance could be used to develop markers useful to breeders to select for plants carrying high doses of resistant alleles. The underlying hypothesis is that a gene identified to explain trait variation in the model species would explain the variation for the same trait in alfalfa (Julier and Meusnier 2010).

25.2 Material and Methods

The *RCT1* gene of *M. truncatula* spans more than 3.5 kb. Upstream and downstream regions as well as intronic sequences showed polymorphisms that could be related to resistance expression (Yang et al. 2008). As a consequence, we sequenced the whole gene, including non-coding regions. Because of the low sequence conservation in

non-coding sequences but the high sequence conservation in coding regions between alfalfa and *M. truncatula*, we designed primers in the genes flanking *RCT1* in *M. truncatula*.

The heterozygosity of alfalfa implied the need to sequence the four alleles of the gene. Direct sequencing of small gene portions is feasible (Pierre et al. 2011) but time-consuming and expensive. Next Generation Sequencing technologies offer the possibility to sequence a large set of genomic sequences at a reasonable cost, and have been adopted to get the complete sequence of *RCT1*, with its four alleles, in a set of genotypes.

Eight varieties with different resistance level were chosen and anthracnose tests were carried out on 100 seedlings of each variety with the strain C86-2 of *C. trifolii*. For each variety, 15 resistant (R) plants and 15 susceptible (S) plants were selected, and the genomic DNA was extracted. Two primer pairs were defined to amplify the whole gene and the upstream and downstream regions (a total of about 14 kb). A bulk segregant analysis (BSA) approach was used with the objective to identify gene polymorphisms associated with resistance (Michelmore et al. 1991). The 454 (Roche) sequencing technology was used. The PCR products of the 15 R and 15 S plants were pooled for each variety. DNA libraries were tagged for each variety and the libraries were then pooled. Consequently, up to 60 different alleles could be present in each pool. The full *RCT1* gene was cloned and Sanger-sequenced in one alfalfa plant to produce a reference sequence.

The raw sequences were assembled based on the reference sequence to produce consensus sequences that could be considered as alleles. As a very large sequence polymorphism was present, the rest of the analysis was restricted to the five exons of the gene. These consensus sequences were translated into proteins and R and S sequences were compared. In the regions showing deletions, primer pairs were designed to genotype the individuals of the R and S pools. The relationship between genotype and phenotype was analysed.

25.3 Results and Discussion

Resistance is dominant over susceptibility, therefore susceptible genotypes should only carry recessive alleles (r), their genotype is rrrr. By contrast, the resistant genotypes carry at least one allele for resistance (R) and may be RRRR, RRRr, RRrr or Rrrr. This notation is schematic because we do not know how many alleles of susceptibility or resistance exist. If susceptibility corresponds to inactive allele, several alleles of susceptibility may be observed due to accumulation of mutations. To summarize, the susceptible (S) pools should only contain alleles for susceptibility, except if a resistant individual has been misclassified as susceptible. The resistant (R) pools should contain both alleles for resistance and susceptibility, their proportion depending on the frequency of resistance alleles in the variety (Julier et al. 2004).

A last point could modify the analysis: the alleles of resistance or susceptibility could be different in the varieties. These varieties were bred by different breeders

Table 25.1 Evaluation of the level of resistance of the eight alfalfa cultivars to anthracnose, expressed as the percentage of resistant plants

Variety	Resistant plants (%)
Galaxie	20.0
Bar 2	27.0
Prunelle	28.6
Kali	41.8
Canelle	42.0
Symphonie	44.9
Bar 1	47.4
Marshall	83.9

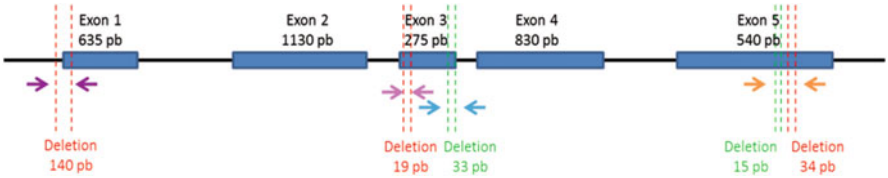

Fig. 25.1 Scheme of the *RCT1* genomic region. Deletions indicated in red induce a reading frame shift; those in *green* do not produce a reading frame shift. The *arrows* indicate the position of the primers

who use partly different genetic backgrounds. However, the low level of genetic differentiation among European varieties is favorable to the hypothesis that the genetic determinant of resistance is the same in all varieties.

Resistance Test The eight varieties were evaluated for their resistance. As expected, Marshall was the most resistant variety. Kali was chosen for its low level of resistance, but had unexpected 42 % resistant plants. The other six varieties, known as of intermediate resistance level, had among 20 and 47 % resistant plants. In all varieties, 15 resistant plants and 15 susceptible plants were chosen, except in Marshal in which only seven susceptible plants were identified (Table 25.1).

***RCT1* Sequencing** The whole gene with the upstream and downstream regions was cloned and sequenced for a single individual with Sanger method. It comprised a 14.5 kb sequence, with the same five exons structure as in *M. truncatula*. Within this individual, polymorphism was identified, with some long insertions/deletions. A reference sequence was built and exons were identified.

With the NGS method, the same genomic region was sequenced for 240 individuals, pooled as described above (potentially 960 alleles). A total of 5,70,819 sequences was obtained with a mean size of 295 bp. Raw sequences were assembled based on the reference sequence to produce consensus sequences that can be considered as alleles. As a very large sequence polymorphism was present, the rest of the analysis was restricted to the five exons of the gene. Five clear deletion/insertion polymorphisms were found: one in the ATG region of exon 1, two in exon 3 and two in exon 5 (Fig. 25.1).

Table 25.2 Number of alleles of 102, 241 and 244 bp in the polymorphic region of exon 1

Phenotype	Number of individuals	Allele 102	Allele 241	Allele 244	Missing data
R	130	28	405	61	6
S	126	13	409	54	7

In all pools, both deleted and non-deleted alleles were found, the misclassification of a single R individual in a S pool could induce this situation. However, this case should be rare because only the plants showing clear phenotypes were used to build the pools.

Polymorphism at the Individual Level For the deletions on exon 1, beginning and end of exon 3, primers were defined to amplify a portion of less than 300 bp (Fig. 25.1). As the two deletions of exon 5 were close to each other, a single primer pair was used for both. Polymorphism was evidenced for each region with 2–5 different alleles. One, and the same, allele per region was highly frequent, whatever the variety or the R or S pool. The difference in allele frequency between R and S plants was never significant for the frequent alleles. For the deletion of exon 1 that was the most promising because of ATG deletion, the deleted allele (102 bp) had a too low frequency to be associated with resistance in this population (Table 25.2) even if the χ^2 test reached $P = 0.06$. Surprisingly, the deleted allele that probably induced a lack of function was associated with the resistance, a case already described in the literature in other pathosystems. A divergent selection for the presence or absence of this allele would test if the lack of function of *RCT1* confers the anthracnose resistance in alfalfa.

25.4 Conclusion

The polymorphic regions of *RCT1* exons identified through the sequencing of the whole gene with a bulk-segregant analysis did not explain the variation of anthracnose resistance in the alfalfa varieties studied, even if a deleted rare allele was weakly associated with the resistance. Non coding sequences of this gene or even other genes may control the trait. In this specific case, the gene detected in *M. truncatula* for anthracnose resistance may not be the one responsible for the same trait in alfalfa. Indeed, a QTL study indicated that the position of a QTL for anthracnose resistance did not correspond to the position of *RCT1* (Cazaux 2008). A previous study on a CONSTANS-like gene involved in flowering date and stem elongation (Herrmann et al. 2010) showed that translational genetics between *M. truncatula* and alfalfa may be efficient. However, this study shows the power of next-generation sequencing technologies for genome analysis of a polyploid and heterozygous species.

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Chapter 26
The EUCARPIA Multi-site Rust Evaluation—Results 2010

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Abstract The EUCARPIA multi-site rust evaluation was repeated in 2010 for the fourth time. The trials were sown at 24 sites in 11 countries in Europe. The 20 Italian/Hybrid (*Lolium multiflorum* and *L. boucheanum*, respectively) and 34 perennial ryegrass (*L. perenne*) cultivars were sown in separate trials. The cultivars were grown as rows in a completely randomized block design with four replicates. Rust incidence was scored in the year of seeding by each of the participants. The method used to test

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cultivars for rust resistance was based on an estimation of the percentage of leaf area affected. Crown rust (*Puccinia coronata* f. sp. *lolii*) was again the most frequently observed rust on both ryegrass species. Stem rust (*P. graminis* f.sp. *graminicola*) was reported at only one site for Italian and at four sites for perennial ryegrass. Variation in resistance to crown rust among cultivars was significant at 17 sites for Italian and at 16 sites for perennial ryegrass. The cultivars 'Tarandus', 'Gosia', 'Caballo' and 'Domino' showed the highest level of resistance of all the Italian ryegrass cultivars tested. 'Gwendal' and 'Bocage' were the most crown rust resistant perennial ryegrass cultivars. The ranking of the mean crown rust susceptibility of the cultivars was highly correlated with the corresponding ranking of cultivars in the 2001, 2004 and 2007 trials, respectively. This was true for both perennial and Italian ryegrass. Coefficients of rank order correlations of average cultivar disease scores between the years were greater than $r_s = 0.97$ ($p < 0.05$) for Italian and $r_s = 0.86$ ($p < 0.05$) for perennial ryegrass, respectively. Therefore, there is no evidence that crown rust resistance

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of an individual cultivar was overcome by the rust pathogen over the 9 years of experimentation.

26.1 Introduction

In 2000, the EUCARPIA Fodder crops and Amenity grasses section initiated a multisite rust evaluation trial. The aim of the trial was to determine the susceptibility of different Italian (*Lolium multiflorum*), Hybrid (*L. boucheanum*) and perennial (*L. perenne*) ryegrass cultivars to the most important rust species throughout Europe. In repeating this trial every 3rd year, starting in 2001, we wanted to assess if rust resistance of individual cultivars breaks down and is overcome by the rust pathogen over the course of time. The results of the 2001, 2004 and 2007 trials were published in the proceedings of the "Eucarpia Fodder crops and Amenity grasses" meetings in Braunschweig in 2002, Perugia in 2006 and La Rochelle in 2009, respectively (Boller et al. 2003; Schubiger et al. 2007, 2010a). In addition, a summary and a comparison of the three trials were published in 2010 (Schubiger et al. 2010b). The present paper reports on the results of the experiments undertaken in 2010.

26.2 Material and Methods

The trial was sown at 24 sites in 11 European countries. At 16 of these sites the trial was carried out during each of the 4 years of evaluation (2001, 2004, 2007 and 2010). The same 33 perennial, 15 Italian and 3 Hybrid ryegrass cultivars were tested as in the previous three trials. In addition, the Italian ryegrass cultivars 'Crema' and 'Gosia' and the perennial ryegrass cultivar 'Maja' were included as they were in the trials of 2007. Nine Italian, two hybrid and 15 perennial ryegrass cultivars were tetraploid, the other cultivars were diploid.

Twelve grams of seed of each cultivar were forwarded to each participant in the form of encoded seed lots. At each site the seed was sown in spring in a completely randomized block design with four replicates. The cultivars were grown as rows (3 m long and 0.5 m apart). The perennial and Italian ryegrass cultivars were sown in separate trials. The hybrid ryegrass cultivars were included in the Italian ryegrass trial. The trials were cut and fertilized as was customary at each site.

The trials were scored for rust incidence between July and October one to three times during different growth cycles and periods of abundant rust development. Participants were asked to score the cultivars for each rust species occurring in the field, separately. A scale from 1 to 9 was used: with 1 = no rust disease, 2 = trace of rust, $3 = 5\ \%, 4 = 10\ \%, 5 = 25\ \%, 6 = 40\ \%, 7 = 60\ \%, 8 = 75\ \%$ and 9 = more than 75 % of the foliage covered with rust. The rating values represented a relative estimate of leaf area occupied by rust pustules, and not reaction type. For any particular site, scoring data with an average score of at least two were included

Table 26.1 Analysis of variance of mean rust disease scores. All F-values are significant at the $p < 0.001$ level

Ryegrass species	*Lolium multiflorum*		*Lolium perenne*	
Rust species	*P. coronata*	*P. graminis*	*P. coronata*	*P. graminis*
No. of cultivars	20	20	34	34
No. of sites	17	1	16	4
F-value for cultivars	137.8	23.7	66.6	28.5
F-value for sites	132.9	–	253.7	143.9
F-value for cv. x site interaction	4.4	–	4.7	3.1

in the analysis, provided that there were significant differences between the cultivars at the site. If there were sites with more than one valid scoring per year, means of the scorings (per row) were calculated and used in further analysis.

26.3 Results

Crown rust (*Puccinia coronata*) was the most serious and the most frequently observed rust disease of Italian/Hybrid and perennial ryegrass. A relevant incidence (average score of at least two and significant differences between cultivars) of crown rust on Italian ryegrass was observed at 17 out of 23 sites and on perennial ryegrass at 16 out of 24 sites, respectively. Stem rust (*P.graminis*) occurred on Italian ryegrass at only one site (Les Rosiers, data not shown) and on perennial ryegrass at four sites, respectively.

Over all sites, there was a highly significant difference ($p < 0.001$) in mean crown rust scores among Italian/Hybrid ryegrass cultivars (Table 26.1). The cultivars 'Tarandus', 'Gosia', 'Caballo' and 'Domino' showed the highest level of resistance of all the Italian/Hybrid ryegrass cultivars tested (Table 26.2). Despite the occurrence of significant interactions of cultivars with sites, the Spearman rank order correlation between the data of a particular site and the mean of all sites was always significant (Table 26.2).

Mean crown and stem rust susceptibility scores of perennial ryegrass cultivars at each site are presented in Tables 26.3 and 26.4. Analysis of variance revealed highly significant differences in crown and stem rust susceptibility over all sites (Table 26.1). 'Gwendal' and 'Bocage' were the most crown rust resistant cultivars. 'Gwendal' and 'Pastoral' were the most stem rust resistant cultivars. The ranking of the cultivars in respect to crown or stem rust susceptibility was very consistent. The Spearman rank order correlations between the data of each site and the mean of all sites were significant in all but four cases for crown rust (Druelle, Gumpenstein, Montours and Radzikow). The rank order correlation between the mean disease scores of the perennial ryegrass cultivars for the two rust pathogens was not significant at $p < 0.01 (r_s = 0.41)$.

Table 26.2 Crown rust (*Puccinia coronata*) disease scores of 17 Italian and 3 hybrid ryegrass cultivars at 17 sites. Data are means of one to three scorings per site. Scores are made in a scale of 1–9; 1 = no rust. Cultivars are ranked according to the mean over sites. 4x = tetraploid; 2x = diploid

Cultivar	Asendorf D	Aston UK	Bornhof D	Druelle F	Gumpenstein A	H. Zivotice CZ	Lelystad B. NL	Les Rosiers F	Lodi I	Merelbeke B	Montours F	Ottersum NL	Perugia I	Pulling D	Radzikow PL	Swifterband NL	Zurich CH	Mean of all sites
Tarandus (4 x)	1.8	1.9	1.3	4.9	1.6	2.3	1.3	2.5	3.9	1.8	1.5	1.3	2.5	1.0	2.7	2.2	1.1	2.1
Gosia[b] (4 x)	2.8	1.5	1.5	3.3	2.1	4.2	1.0	2.0	3.3	1.5	1.1	1.0	1.8	2.0	3.8	2.0	1.1	2.1
Caballo (4 x)	2.3	2.0	2.0	4.6	1.6	2.6	1.3	3.0	3.6	1.3	1.8	1.8	2.7	1.0	2.8	1.5	1.3	2.2
Domino (4 x)	1.8	1.8	1.3	4.5	1.8	2.3	1.3	2.8	4.9	1.5	1.9	1.5	2.9	1.3	3.1	1.8	1.5	2.2
Zorro (4 x)	2.3	1.6	1.3	5.1	1.6	3.3	1.3	3.0	5.8	1.8	1.8	1.5	2.5	1.0	3.3	1.7	1.5	2.4
Bolero (4 x)	1.8	1.6	1.5	5.4	1.6	2.3	1.3	2.5	5.4	3.3	1.8	1.3	2.5	1.5	3.4	2.3	1.5	2.4
Tonyl (4 x)	2.0	2.9	1.0	4.9	1.8	2.8	1.8	2.5	4.6	2.0	2.5	2.0	2.0	1.5	3.5	2.0	1.4	2.4
Barprisma (2 x)	2.5	2.1	1.3	4.6	2.3	2.8	1.5	3.3	3.3	3.3	1.8	2.5	2.9	1.3	3.8	2.3	1.3	2.5
Aberexc[b] (4 x)	3.3	2.5	1.5	3.4	2.0	3.1	2.3	3.3	4.4	3.0	1.1	1.8	2.5	2.0	3.3	2.6	2.1	2.6
Fastyl (2 x)	2.8	2.5	2.0	5.0	2.3	2.3	1.5	2.3	3.6	3.5	3.1	1.8	2.8	1.5	3.6	2.3	1.4	2.6
Ellire (4 x)	2.3	2.1	1.5	5.5	1.5	2.8	1.5	3.0	6.3	2.5	3.5	1.8	3.7	1.8	3.5	2.1	2.0	2.8
Pirol[b] (2 x)	4.5	2.3	1.8	5.1	2.5	3.0	2.0	3.0	5.7	4.3	3.6	2.8	3.4	2.5	3.7	3.4	2.5	3.3
Meryl (2 x)	3.8	2.0	3.3	5.3	2.3	3.1	2.0	4.0	4.8	5.0	3.6	3.5	3.3	2.3	4.4	3.2	2.8	3.4
Crema (2 x)	5.8	5.5	2.3	5.0	3.4	3.0	3.0	2.5	5.6	4.8	7.4	3.5	3.6	2.0	4.1	3.8	2.5	4.0
Danergo (4 x)	2.8	2.0	4.0	6.4	2.0	5.2	3.3	5.8	5.9	7.0	4.3	5.0	3.8	2.5	4.2	2.3	3.3	4.1
Lolita (4 x)	3.5	1.9	5.0	6.9	2.1	5.8	3.0	5.3	8.0	4.0	4.0	4.8	4.5	2.5	4.7	2.2	3.5	4.2
Ligrande (2 x)	5.3	2.8	3.0	6.9	2.6	5.0	6.0	4.0	6.3	7.8	4.8	5.5	4.0	4.0	4.8	3.5	4.5	4.7
Gumpen[b] (2 x)	5.3	2.6	4.3	6.6	3.3	4.8	3.5	4.5	7.3	8.3	5.6	5.8	4.8	3.5	5.3	3.4	5.3	4.9
Lema (2 x)	5.0	2.1	4.3	6.8	2.8	4.8	4.5	4.8	7.0	7.8	6.9	5.8	3.8	4.8	5.2	3.8	4.5	5.0
Gordo (2 x)	4.8	2.4	4.0	7.0	2.6	5.9	4.3	6.0	6.9	7.8	5.1	6.5	5.2	4.8	5.3	3.8	5.8	5.2
Mean	3.3	2.3	2.4	5.4	2.2	3.6	2.4	3.5	5.3	4.1	3.4	3.1	3.2	2.2	3.9	2.6	2.5	3.2
LSD ($P < 0.05$)	1.0	0.5	1.8	0.8	0.4	0.6	1.1	1.6	1.6	1.3	0.7	1.3	1.2	0.9	0.7	0.7	1.0	
Correlation[a]	0.82	0.54	0.82	0.82	0.73	0.72	0.94	0.75	0.78	0.94	0.89	0.94	0.88	0.89	0.87	0.78	0.94	

[a]Spearman rank order correlation with mean of all sites (all values are significant at $P < 0.05$)
[b]Hybrid Ryegrass

Table 26.3 Crown rust (*P. coronata*) disease scores of 34 perennial ryegrass cultivars at 16 sites. Data are means of one to three scorings per site. Scores are made in a scale of 1–9; 1 = no rust. Cultivars are ranked according to the mean over sites. 4x = tetraploid; 2x = diploid

Cultivar	Asend-orf D	Aston UK	Bornhof D	Druelle F	Gumpen-stein A	H. Zivot-ice CZ	Lelystad B. NL	Lodi I	Malc-how D	Merelb-eke B	Monto-urs F	Ottersum NL	Pulling D	Radzi-kow PL	Swifter-band NL	Zurich CH	Mean of all sites
Gwendal (4 x)	2.8	1.8	2.5	2.8	2.3	2.3	2.7	1.3	2.8	2.7	2.5	1.0	2.0	2.5	3.2	1.3	2.3
Bocage (4 x)	2.8	2.3	4.3	2.5	2.0	2.5	2.7	2.0	2.3	3.3	3.1	1.3	1.0	2.5	2.9	1.8	2.4
Carrera (2 x)	3.5	2.0	3.3	3.3	2.6	2.5	3.3	1.4	2.3	3.3	2.6	1.8	1.5	3.5	3.7	1.3	2.6
Lacerta (4 x)	2.5	3.0	5.5	3.3	2.5	2.5	2.8	3.7	1.8	2.3	4.1	1.0	1.5	2.3	1.8	1.3	2.6
Pastoral (4 x)	3.8	2.3	5.0	3.1	2.4	1.8	3.3	2.6	2.3	3.3	3.1	1.0	1.3	2.3	3.7	2.5	2.7
Orval (4 x)	3.3	2.3	4.0	3.0	2.6	2.0	5.1	4.6	3.0	3.5	3.5	1.0	1.3	3.3	1.8	1.6	2.9
Aubisque (4 x)	3.8	2.8	6.0	3.5	2.0	2.3	3.5	1.4	3.3	2.8	2.8	1.0	1.5	2.5	4.3	2.8	2.9
Vincent (2 x)	4.0	2.5	3.8	4.0	3.3	2.3	4.1	1.8	2.8	4.3	3.3	2.0	1.3	3.5	2.4	1.3	2.9
Option (2 x)	4.0	2.8	5.3	4.4	3.3	2.3	3.8	2.4	2.5	4.0	4.5	1.5	1.5	3.0	3.3	1.3	3.1
Heraut (2 x)	4.5	2.8	4.8	4.1	3.0	2.3	4.1	2.1	2.8	4.5	3.6	2.5	1.3	4.0	2.5	2.1	3.2
Elgon (4 x)	5.0	3.0	7.3	2.6	2.5	2.3	5.4	2.2	4.0	3.0	2.5	1.5	1.8	3.0	3.8	3.1	3.3
Kells (2 x)	4.5	2.8	5.8	4.4	2.9	2.8	5.0	1.6	3.3	4.8	2.4	2.5	1.8	3.5	3.6	2.6	3.4
Roy (4 x)	3.3	3.0	6.5	2.5	2.1	3.0	5.0	3.1	3.3	4.5	3.6	2.0	1.8	2.5	4.8	3.1	3.4
Guru (2 x)	3.3	3.0	2.5	4.9	3.4	3.5	4.6	3.7	2.0	4.8	3.4	4.8	1.3	4.0	2.7	3.4	3.4
Barnhem (2 x)	5.3	3.0	5.3	3.4	3.6	2.3	5.8	2.2	3.0	4.8	2.5	3.0	1.3	3.8	4.3	1.8	3.4
Aberdart (2 x)	5.3	3.3	6.5	3.8	2.6	2.0	5.6	2.9	2.0	5.0	2.0	2.3	1.8	3.3	4.4	3.0	3.5
Corbet (2 x)	4.8	2.5	5.5	4.5	3.0	2.3	4.8	1.6	3.0	5.0	4.3	2.0	2.3	4.3	3.9	2.0	3.5
Fennema (2 x)	4.0	3.3	6.5	4.6	3.1	2.8	4.5	2.6	3.3	5.0	2.5	2.8	1.5	3.8	3.4	2.6	3.5
Kentaur (4 x)	5.0	3.3	7.5	2.5	2.1	4.3	4.9	4.4	5.0	3.5	2.4	1.8	2.3	2.3	5.1	3.1	3.7
Maja (4 x)	5.3	2.3	6.0	2.9	2.3	3.5	5.5	3.6	4.3	4.0	2.9	2.8	2.3	2.8	5.7	3.5	3.7
Weigra (2 x)	4.3	3.0	6.3	4.3	3.3	3.0	4.5	4.0	3.3	4.8	5.3	2.5	2.0	3.5	3.3	2.4	3.7
Sponsor (2 x)	4.8	3.5	7.5	4.5	3.0	2.3	5.5	1.3	2.8	5.5	2.8	2.8	1.8	3.5	4.8	3.4	3.7
Litempo (4 x)	4.5	3.3	7.3	3.4	2.3	3.5	5.2	4.1	3.8	3.3	3.0	1.8	1.8	3.5	5.5	3.9	3.7
Arabella (2 x)	4.8	3.3	6.8	4.6	3.3	2.8	5.1	3.4	2.8	5.3	4.5	2.3	1.8	4.0	4.0	2.0	3.8

Table 26.3 (continued)

Cultivar	Asendorf D	Aston UK	Bornhof D	Druelle F	Gumpenstein A	H. Zivotice CZ	Lelystad B. NL	Lodi I	Malchow D	Merelbeke B	Montours F	Ottersum NL	Pulling D	Radzikow PL	Swifterband NL	Zurich CH	Mean of all sites
Gladio (2 x)	5.5	3.0	7.5	3.9	3.3	2.5	6.1	2.4	2.8	6.0	2.8	2.3	2.8	3.0	4.4	3.4	3.8
Terry (4 x)	5.5	4.0	8.3	2.9	2.4	3.0	5.8	4.9	4.3	4.0	2.9	2.0	2.8	3.0	5.0	3.1	4.0
Tivoli (4 x)	5.5	4.8	8.5	2.8	2.6	2.8	5.5	3.5	4.8	5.3	2.5	2.8	2.3	2.3	5.3	4.9	4.1
Foxtrot (2 x)	6.8	2.5	7.3	3.6	2.6	2.8	6.0	2.6	3.8	5.5	4.0	3.3	2.3	3.3	6.1	3.6	4.1
Aristo (2 x)	6.8	3.3	7.8	3.4	3.0	2.5	7.0	2.6	4.0	5.8	2.8	4.0	2.5	3.3	7.2	3.9	4.3
Sirocco (4 x)	6.0	3.5	8.0	3.4	2.5	3.8	6.2	5.8	5.3	5.0	5.3	1.8	2.5	3.3	6.1	4.0	4.5
Helmer (4 x)	7.0	4.0	8.5	3.0	2.3	3.5	6.7	6.6	4.0	4.5	3.3	3.3	2.0	3.0	6.3	4.6	4.5
Lipresso (2 x)	5.5	3.3	7.8	4.6	3.5	4.3	6.3	5.1	2.8	6.5	5.0	4.8	4.3	4.0	4.2	4.4	4.8
Condesa (4 x)	6.8	4.3	8.3	3.0	2.9	2.5	6.3	4.7	4.3	7.3	4.8	4.0	3.5	3.3	7.5	5.9	4.9
Aurora (2 x)	6.5	4.3	8.8	5.8	3.8	6.8	6.9	2.5	6.8	8.5	5.8	7.8	5.5	4.8	8.2	8.5	6.3
Mean	4.7	3.0	6.2	3.6	2.8	2.8	5.0	3.1	3.3	4.6	3.4	2.5	2.0	3.2	4.4	3.0	3.6
LSD ($p = 0.05$)	0.9	0.7	1.6	0.9	0.6	1.0	0.7	1.8	1.3	1.3	0.8	1.4	1.0	0.8	0.9	1.3	
Correlation[a]	0.88	0.79	0.88	ns	ns	0.61	0.88	0.55	0.64	0.78	ns	0.73	0.80	ns	0.80	0.85	

[a]Spearman rank order correlation with mean of all sites (all values are significant at $p < 0.05$ except ns = not significant)

Table 26.4 Stem rust (*Puccinia graminis*) disease scores of 34 perennial ryegrass cultivars at four sites. Data are means of one to two scorings per site. Scores are made in a scale of 1–9; 1 = no rust. Cultivars are ranked according to the mean over sites. 4x = tetraploid; 2x = diploid

Cultivar	Les Rosiers F	Montours F	Radzikow PL	Malchow D	Mean of all sites
Gwendal (4 x)	1.0	2.5	2.6	2.3	2.1
Pastoral (4 x)	1.0	2.0	2.9	2.8	2.2
Roy (4 x)	1.0	2.3	3.0	2.8	2.3
Tivoli (4 x)	1.3	2.0	3.0	2.8	2.3
Bocage (4 x)	1.0	2.8	2.9	2.5	2.3
Maja (4 x)	1.0	2.3	3.3	2.8	2.3
Aubisque (4 x)	1.0	2.8	2.9	2.8	2.3
Aberdart (2 x)	1.3	2.3	3.5	2.5	2.4
Carrera (2 x)	1.5	2.5	3.4	2.8	2.5
Terry (4 x)	1.3	2.8	3.4	2.8	2.5
Elgon (4 x)	1.0	3.5	3.3	2.5	2.6
Orval (2 x)	1.0	3.5	3.3	2.5	2.6
Kentaur (4 x)	1.3	2.8	3.1	3.3	2.6
Lacerta (4 x)	2.0	3.0	3.0	2.8	2.7
Condesa (4 x)	1.5	2.3	3.6	3.5	2.7
Litempo (4 x)	1.5	2.5	3.5	3.5	2.8
Aristo (2 x)	2.0	3.0	3.5	3.0	2.9
Helmer (4 x)	2.3	3.0	3.3	3.0	2.9
Sirocco (4 x)	1.3	3.8	3.6	3.5	3.0
Foxtrot (2 x)	2.0	3.3	3.6	3.5	3.1
Gladio (2 x)	2.5	3.5	3.8	3.3	3.3
Guru (2 x)	4.0	3.0	3.5	3.8	3.6
Option (2 x)	3.5	3.3	3.5	4.3	3.6
Barnhem (2 x)	2.0	4.3	3.8	4.8	3.7
Vincent (2 x)	3.0	4.0	3.8	4.8	3.9
Heraut (2 x)	2.8	4.8	3.5	4.8	3.9
Weigra (2 x)	4.0	3.3	4.0	4.5	3.9
Kells (2 x)	3.0	5.3	4.0	4.3	4.1
Fennema (2 x)	2.8	5.5	3.9	4.5	4.2
Sponsor (2 x)	3.8	4.8	3.9	4.5	4.2
Arabella (2 x)	4.0	4.5	3.9	4.8	4.3
Lipresso (2 x)	4.0	4.0	4.5	4.8	4.3
Aurora (2 x)	4.3	3.8	4.4	6.0	4.6
Corbet (2 x)	4.8	5.8	4.3	5.3	5.0
Mean	2.2	3.4	3.5	3.6	3.2
LSD ($p = 0.05$)	0.9	0.9	0.5	1.0	
Correlation[a]	0.92	0.87	0.91	0.92	

[a]Spearman rank order correlation with mean of all sites (all values are significant at $p < 0.05$)

The ranking of the Italian/Hybrid and perennial ryegrass cultivars in terms of crown and stem rust resistance was consistent over the whole experimentation period of nine years. The Spearman rank order correlations of average cultivar disease scores between the years 2001, 2004, 2007 and 2010 are presented in Table 26.5. For perennial ryegrass, the correlations involving 2007 were consistently lower than those among 2001, 2004 and 2010.

Table 26.5 Coefficients of Spearman rank order correlations among mean crown (*Puccinia coronata*) and stem rust (*P. graminis*) disease scores of Italian (*Lolium multiflorum*) and perennial ryegrass (*L. perenne*) in 4 years of evaluation

	Italian ryegrass	Perennial ryegrass	
	Crown rust	Crown rust	Stem rust
2001 *vs*. 2004	0.99*	0.97*	0.95*
2001 *vs*. 2007	0.98*	0.92*	0.94*
2001 *vs*. 2010	0.99*	0.96*	0.94*
2004 *vs*. 2007	0.97*	0.90*	0.98*
2004 *vs*. 2010	0.99*	0.95*	0.92*
2007 *vs*. 2010	0.98*	0.86*	0.94*

*significant at $p < 0.01$

26.4 Discussion

In 2010, the results of the Eucarpia multisite rust evaluation trial revealed that several Italian and perennial ryegrass cultivars are still resistant to crown and stem rust in a wide range of sites across Europe.

As a consequence, the 2010 ranking of the ryegrass cultivars in terms of crown and stem rust resistance was very consistent with the ranking of the trials in 2001, 2004 and 2007, respectively.

The trends for a breakdown of crown rust resistance of some cultivars in 2007 were not confirmed. In 2010, for example, 'Orval' ranked again in the 6th position despite its 20th position in 2007. Therefore, there is no evidence that crown rust resistance of an individual cultivar was overcome by the rust pathogen over the 9 years of experimentation.

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Chapter 27
The Main Topics of Resistance Breeding in Grasses in the Czech Republic

B. Cagaš and M. Svobodová

Abstract In the years 1995–2009 the incidence of important diseases in grasses grown for seed in the Czech Republic was investigated. The goal of the study was to find out whether there has been a significant increase in the level of resistance. Each year a total of 500 samples of 19 cultivated grass species were taken on 2,000–4,500 ha in various regions of the Czech Republic. Parasitic silver top (*Fusarium poae*) was detected in 13.8–48.1 % of the locations under observation, with a statistically insignificant downward trend of 0.19 % per year (P-value = 0.7518). Powdery mildew (*Blumeria graminis*), occurring in 9.9–40 % of the locations showed a significant downward trend of 1.2 % per year (P-value = 0.0373). Rusts (*Puccinia* spp., *Uromyces* spp.) were recorded on 4.0–33.4 % of the sites; the trend remained constant. Choke (*Epichloë typhina*) was found in 0.1–3.8 % of the locations, the incidence of choke showed a significantly upward trend of 0.19 % per year (P-value 0.0030). Leaf spots (predominantly caused by *Cladosporium* spp., *Drechslera* spp., *Mastigosporium* spp. etc.) occurred in 7.5–35.4 % of the locations (an decreasing incidence by 2 % per year, P-value = 0.0001).

Keywords Silver top · Leaf diseases · Rusts · Powdery mildew · Choke

27.1 Introduction

The land area of grasses grown for seed in the Czech Republic was subjected to strong fluctuations in the last years, like in other European countries, and at present it is estimated to be 13,000 ha. Grass seed production is still a very interesting branch of plant production and grass seeds are mostly exported. However, their amounts and quality are often significantly affected, especially in some years, by the action of specific pests and diseases (Cagaš 2010). The spectrum of cultivated grass species and varieties is unprecedentedly wide and has considerably increased since the year

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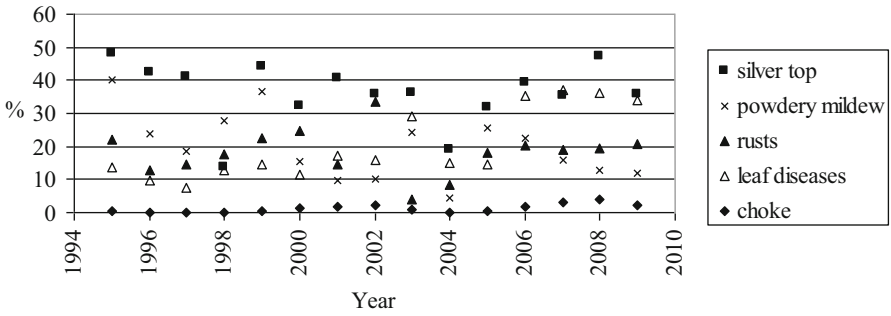

Fig. 27.1 Incidence of important diseases in grasses grown for seed in the years 1995–2009 in the Czech Republic (%)

1995. In a number of species the spread of some diseases can be controlled by traditional methods, in others by growing varieties with a higher resistance. The goal of this study was to assess on the basis of long-term monitoring the prospects of occurrence of some causal agents of diseases, to find out whether there has been some progress in developing resistance to some diseases, to show the success rate of resistance breeding in varieties grown for seed production in the Czech Republic and to outline potential future trends of breeding for resistance.

27.2 Materials and Methods

Data on the incidence of the most serious diseases of grass seed stands (Fig. 27.1) were obtained from the written reports of inspectors of Central Institute for Supervising and Testing in Agriculture (ÚKZÚZ) whose task was to assess the occurrence of pests and diseases during the growing season. In the period of study (1995–2009) each year a total of 500 samples were collected to determine the incidence of a particular disease. This study was carried out on 2,000–4,500 ha in 19 cultivated grass species in different regions of the Czech Republic. Data on the number of varieties of both forms of Italian ryegrass and meadow fescue grown in the Czech Republic in the years 1995–2009 were obtained from the List of varieties of those particular years. Data on the incidence of the most important diseases were processed by regression analysis in the program Statgraphics version XV.

27.3 Results and Discussion

The incidence of important pests and diseases occurring on grasses grown for seed in the Czech Republic in the years 1995–2009 is shown in Fig. 27.1. It reveals that parasitic silver top caused by the fungus *Fusarium poae* and transmitted predominantly by the meadow plant bug (*Leptopterna dolobrata*) was detected in the observation period

in 13.8–48.1 % of the locations under study, powdery mildew (*Blumeria graminis*) occurred in 4.2–40 % of the locations, grass rusts (*Puccinia* spp., *Uromyces* spp.) in 4.0–33.4 % of the sites, leaf diseases (predominantly *Cladosporium* spp., *Drechslera* spp., *Mastigosporium* spp., *Pyrenophora* spp.) in 7.5–35.4 % of the locations and choke (*Epichloë typhina*) was detected in 0.1–3.8 % of the locations.

Parasitic silver top can be controlled by a combination of non-chemical and insecticide treatments. Choke still remains a hard-to-solve problem; however, with respect to the narrow range of the host species it is considered a minor problem (Cagaš 2009). Out of the three remaining grass mycoses the most serious problem with respect to high economic harmfulness is graminicolous rusts, mainly crown rust (*Puccinia coronata* Corda var. *coronata*) and stem rust (*Puccinia graminis* subsp. *graminicola* Urb.). Regression analysis of the incidence of the most serious diseases, where y = % of localities with disease incidence, x = year 1–15, shows interesting facts. The incidence of silver top (*Fusarium poae*) showed a slightly downward trend of 0.19 % per year ($y = -0.19x + 37.693$), but under the same conditions in the future a marked decline cannot be expected probably due to non-consistent chemical control of grass seed stands. Moreover, the dependence is not significant (*P*-value = 0.7518). A significant downward trend in incidence (*P*-value = 0.0373) was recorded in powdery mildew (*Blumeria graminis*)—1.2 % per year ($y = -1.2186x + 29.735$). In contrast, a significantly upward trend of 0.19 % per year was seen in choke (*Epichloë typhina*) ($y = 0.1914x - 0.2314$, *P-value* 0.0030) and especially in leaf diseases—almost 2 % per year ($y = 1.9936x + 4.3181$, *P-value* = 0.0001). The incidence curve of graminicolous rusts suggests a steady trend ($y = 0.0311x + 17.845$) and it is expected that the incidence of rusts will be at the same level as it is now (Fig. 27.1). The dependence, however, is not significant (*P*-value = 0.9436).

The findings suggest the necessity to aim breeding at increased resistance to stem rust and crown rust in their major host species—perennial ryegrass, Italian ryegrass and meadow fescue. To other important reasons belong the incidence of favorable conditions supporting the overwintering of the spores and early rust infection. Breeding for resistance to rust diseases has been a priority of research teams for a long time and according to Heijden and Roulund (2010) good progress has been made in this area and the resistance of present-day perennial ryegrass varieties to *Puccinia coronata* has markedly increased. Has this trend become evident also in the collection of varieties of the genus Lolium grown for seed in the last 15 years in the Czech Republic? The number of varieties of both forms of Italian ryegrass, perennial ryegrass and meadow fescue listed in the State Variety Book increased from 22 varieties (in the year 1995) to 114 (in the year 2011). The non-registered varieties which are grown for seed also had a similar increase in tendency. The incidence of rusts as given in Fig. 27.1 was studied in the years 1995–2009 in a large population of varieties of several species. The study did not make any distinction between *P. coronata* and *P. graminis* and included also other grass rusts. In this complex the increase in resistance in ryegrasses and fescue was not very dramatic, as confirmed by the regression line (Fig. 27.1). The multi-site rust evaluation trials organized on different European localities can help to detect not only the widespread of both rusts

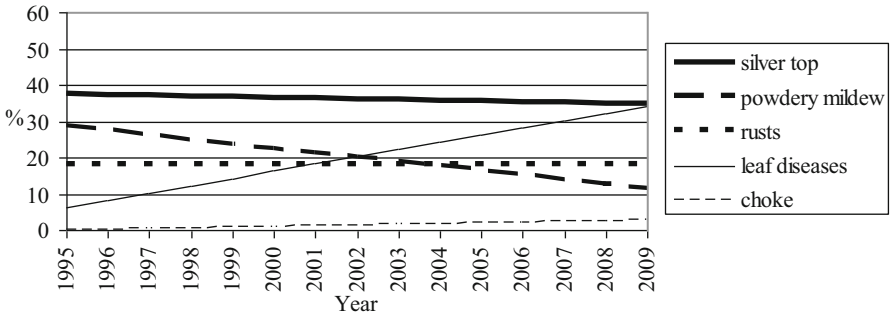

Fig. 27.2 Regression lines of selected diseases incidence (%) (x = 1–15)

but possible sources of resistance (Schubiger et al. 2010). Breeding for resistance to grass rusts, especially stem rust, which has been spreading very quickly in Central Europe after the year 1999, is a vital task for Czech grass breeders (Fig. 27.2).

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Part V
Genetic Variation and Adaptation

Chapter 28
Origins of Diploid Dactylis from the Canary Islands as Determined by DNA Sequencing

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Abstract Two diploid forms of Dactylis are known to occur on the Canary Islands, *Dactylis smithii* at low altitude and the newly discovered *Dactyls metlesicsii* at high altitude. Nuclear ITS sequences of these two diploid forms were compared with a wider set of diploids from Spain, Portugal and North Africa. The results suggest that Dactylis has a dual origin in these islands. The newly discovered high altitude form *metlesicsii* was found to have originated from the Iberian Peninsula unlike the low altitude form *smithii* which originated from nearby North Africa. It is also likely that hybrid forms occur where their ranges overlap.

28.1 Introduction

Dactylis is a genus represented by diploids, tetraploids and a hexaploid. It is distributed from China in the east to the Canary and Cape Verde Islands in the west, over a wide range of climatic conditions, including temperate, sub-tropical, tropical, Mediterranean and sub-alpine. Tetraploids cover almost the full range of the genus while diploids are often little more than relic populations with very restricted ranges. A hexaploid population occurs in Libya and Egypt. Many authors consider the genus to be a single large and very diverse species, *Dactylis glomerata* L. (Stewart and Ellison 2011), while other authors divide the genus into many species and/or sub-species, often in an inconsistent manner. Many authors divide the tetraploid forms into *glomerata* and *hispanica* types; the larger green productive *glomerata* types from high rainfall regions and smaller bluish *hispanica* types from dry Mediterranean climates. Unfortunately there is no consistent taxonomic treatment of the genus as a whole.

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In 1988, 15 diploid forms were reviewed by Lumaret (1988) but today 18 geographic diploids are known (Stewart and Ellison 2011). Although some forms may not warrant their current taxonomic status the names still serve to provide a valuable terminology for such geographically distinct and often isolated populations.

- *altaica* from the Alatau Mountains of Kazakhstan;
- *aschersoniana* in northern Europe and the Caucasus;
- *castellata* in Algeria;
- *himalayensis* in the Himalayan mountains;
- *hyrcana* on the Talish plain of North West Iran;
- *ibizensis* on the Balearic Islands near the East Coast of Spain;
- *izcoi* in North west Spain, formerly known as "Galician";
- *judaica* in the mountains of Israel and Lebanon;
- *juncinella* at altitude in the Sierra Nevada mountains of Spain;
- *lusitanica* on the Sintra near Lisbon, Portugal;
- *mairei* in Algeria;
- *metlesicsii* at high altitude in the Canary Islands;
- *parthiana* at high altitude in the Elburz mountains of Iran;
- *reichenbachii* on the dolomite soils of the European alps and French Massif Central;
- *santai* in Morocco;
- *sinensis* in the mountains of Xingjian Province of China;
- *smithii* at sub-tropical mid-altitudes of the Canary and Cape Verde Islands;
- *woronowii* on the steppes of Iran;

While the diploid forms cross very readily, the resulting hybrids often have a reduced fertility with nuclear and cytoplasmic differences (Parker and Borrill 1968) but few major structural chromosome differences (Wetschnig 1991). This partial sterility is indicative of partial speciation, consistent with the large number of sub-species known.

There have been a number of attempts to understand the historical relationships among the diploid subspecies using morphological differences (Stebbins and Zohary 1959), isozymes (Lumaret 1988), phenolic compounds (Fiasson et al. 1987) and chromosome karyotypes (Wetschnig 1991) and it is only recently that molecular techniques have been employed.

Stewart and Ellison (2011) used nuclear ITS and chloroplast trnL intron sequences to clarify the molecular phylogeny relationships. This showed that the genus is relatively recent with the first diploids originating in central Asia over 150,000 year ago, and during the subsequent interglacial period migrating west to Portugal and south into Israel. As the northern climate cooled in the last glaciation, this continuous distribution became disjunct, leaving many small remnant populations in milder southern glacial refugia. At the same time the central Asian forms were able to migrate from Israel across North Africa through the expanding grasslands as far as Morocco, and on to the Canary and Cape Verde Islands. As the climate warmed again in the post glacial period, the North African grassland forms became restricted to small disjunct remnants, while the forests of Northern Europe were re-colonised by *aschersoniana*

Table 28.1 ITS sequence differences between Dactylis forms

		Accessions	55	92	425	571	601
izcoi	Spain	5	C	G	G	A	A
lusitanica	Portugal	6	C	G	G	A	A
metlesicsii	Canary Islands	1	C	G	A/G	A/G	A/G
smithii	Canary Islands	6	T	C	A	G	A/G
castellata	Morocco	2	T	C	A	G	G
mairei	Algeria	2	T	C	A	G	G
santai	Algeria	6	T	C	A	G	G

from the Caucasus glacial refuge. Surprisingly many phylogenetic lineages consist of both productive temperate forms as well as xeromorphic dryland forms indicating Dactylis is quite plastic in its historical response to climate.

This paper presents molecular data on the recently reported *Dactylis metlesicii* Schönfelder & Ludwig (Schönfelder and Ludwig 1996), a diploid form from high altitudes in the Canary Islands, and compares it to the lower altitude *Dactylis smithii* Link as well as to the phylogeny of the other diploids.

28.2 Materials and Methods

A single accession of *Dactylis metlesicsii* was collected on the Ruta de las Siete Cañadas, Tenerife Island, (Herbario de la Universidad de la Laguna, TFC 44983). DNA was extracted from approx. 10 mg of dried leaf material using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The ITS region was amplified using the primers EC-1 and EC-2 (Williams et al. 2001), and the trnL (UAA) intron was amplified using the primers "c" and "d" (Taberlet et al. 1991). PCR products were purified and sequenced directly as described in Ellison et al. (2006). DNA sequences were aligned, with manual adjustment to optimise alignments where necessary, using MegAlign (DNASTAR). The tree was rooted using the closely related species *Lamarckia aurea* (Catalan et al. 2004). Ploidy levels were confirmed using flow cytometry of leaf tissue in comparison to a known diploid standard. For each accession of each subspecies 3 or more genotypes were sampled.

28.3 Results

The results of the ITS sequencing for a range of forms, as presented in Table 28.1, show that the clear differentiation between Iberian forms and North African forms of Dactylis as reported by Stewart and Ellison (2011). The *smithii* forms share similar sequences with the North African forms except at position 601 where three out of four accessions expressed a dual sequence, indicative of introgression from the Iberian

form. In contrast the single accession of *metlesicsii* expressed a greater similarity with Iberian forms with three dual sequences (425, 571 and 601) indicative of some introgression from the North African forms *castellata* and *mairei*. All forms in this regions including *metlesicsii* share the same chloroplast trnL sequence (Stewart and Ellison 2011).

28.4 Discussion

These results show that *metlesicsii* shares a greater molecular affinity with Spanish forms of Dactylis in contrast to the lower altitude *smithii* which shares a greater molecular affinity with adjacent North African forms of Dactylis.

This molecular result is consistent with the marked differentiation of phenolic compounds found in high and low altitude tetraploid forms on Gran Canary Island (Jay and Lumaret 1995). It is most probable that these tetraploids are based upon their sympatric diploids as geneflow is usually from diploid to tetraploid in Dactylis (Borrill and Lindner 1971; Lumaret and Barrientos 1990).

It is probable that the Canary Islands have been colonised by Dactylis forms from both North Africa and the Iberian Peninsula, with the *smithii* African derivatives predominating at low altitude and the *metlesicsii* Iberian derivatives dominating at high altitudes. There are also indications that hybridisation has occurred between these two forms, as could be expected, where they come into contact.

These results add the *metlesicsii* form to the overall phylogeny of diploid Dactylis developed by Stewart and Ellison (2011) and provide a clear indication of historical adaptive radiation within diploid Dactylis, from which the more common tetraploids have developed. This knowledge will allow breeders to resynthesise many new and unusual tetraploid forms not available in nature.

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Chapter 29
Introduction and Adaptation of *Cynodon* L. C. Rich Species in Australia

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Abstract *Cynodon* L. C. Rich comprises warm season grass species that are valuable as turf and forage in warm climates across the globe. Traditionally, *Cynodon* breeding programs have aimed to produce varieties with improved turf and forage grass quality characteristics. More recent goals, such as improved abiotic stress tolerance, aim to address challenges associated with climate change. Germplasm collections representing a fraction of the global distribution of *Cynodon* comprise the plant resource for these breeding programs. We compared a core collection consisting of 116 Australian genotypes with an international core collection of 59 genotypes representing *Cynodon's* global distribution. Our aims were to determine whether unique genetic diversity exists amongst Australian *Cynodon* that may enhance international breeding resources. This research will facilitate the incorporation of Australian *Cynodon* genetic resources, which have expanded and naturalized under harsh Australian climates, into existing germplasm collections.

29.1 Introduction

Cynodon spp. comprise perennial, widely adapted, warm-season grasses that serve multiple purposes in all continents of the world and are reported to have excellent heat and drought tolerance (Taliaferro 2003; Zhou et al. 2009). Enormous morphological variability exists within the genus (Harlan and de Wet 1969; Harlan et al. 1970). *Cynodon* breeding programs aim to improve quality characteristics, such as yield, vigour, and uniformity, as well as abiotic stress tolerance, which requires sampling and characterization of germplasm collected from diverse environments around the world.

There a currently nine recognised *Cynodon* species (Harlan et al. 1970). Cosmopolitan *C. dactylon* var. *dactylon* (2n = 4x = 36; common bermudagrass) is the

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most widespread variety. Other cultivars include triploid hybrids produced by crosses between *C. dactylon* var. *dactylon* and diploid species, such as *C. transvaalensis* Burtt-Davy (Taliaferro 2003). Most *Cynodon* species apparently originated from eastern and tropical areas across the southern half of Africa and are currently accepted as being introduced in the USA (Merril 1940), Canada (Macoun 1902), New Zealand (Cheeseman 1925; Langdon 1954), and Australia. However, the question of whether *Cynodon* spp. were present in Australia prior to European settlement has not been resolved (Brown 1814 #56; Langdon 1954 #72).

Cynodon germplasm collection began in the early 20th Century (Taliaferro 2003) and a number of collections have since been developed (Anderson and Wu 2007; Wu 2011). Despite the interest in retention of *Cynodon* resources for breeding, the extent of genetic diversity within these collections represents only a fraction of the diversity that is available in the wild (Wu 2011). Further collection of the genus across its current range is likely to sample genotypes that have evolved under stresses associated with diverse environments.

The broad aim of this study was to assess whether *Cynodon* genetic diversity in Australia will expand international breeding resources. Specific goals were to determine whether unique genetic diversity exists in Australia and to increase knowledge of origins and introductions of Australian *Cynodon*.

29.1.1 Materials and Methods

29.1.1.1 Plant Materials, EST-SSR Genotyping and Core Collection Development

The plant materials used in this study comprised a core collection of 116 genotypes representing 1070 *Cynodon* genotypes from Australia (Australian core collection; AC). Core collection selection was based on EST-SSR marker diversity, climate, ploidy, morphology, environment type (managed or wild), and performance under a range of abiotic stresses (Jewell et al. 2012). Compared to the source germplasm (the germplasm present in the entire source population), the AC contains increased gene diversity ($H = 0.18$ versus $H = 0.14$), >95% of the total number of alleles, and similar proportions of accessions representing specific climates, ploidy levels, and environment type. DNA extraction procedures and EST-SSR analyses have been reported previously (Jewell et al. 2010; Kearns et al. 2009; Zhou et al. 2009). DNA from 59 international accessions representing global distribution was provided by the USDA-ARS in Tifton, GA (international collection; IC; Anderson et al. 2009). These accessions were a subset of a core collection of 600 accessions maintained at the Crop Genetic and Breeding Research Unit (USDA-ARS Coastal Plain Experiment Station, Tifton, GA, USA). This core collection was selected to maximize the phenotypic and geographic diversity among these 600 accessions (Anderson 2005). The 59 accessions utilised in this analysis encompassed the geographic and putative species diversity present in the international core collection.

Table 29.1 Sample size (*n*), Nei's heterozygosity (*H*), number of alleles (*A*), number of genotypes (*G*) and proportion of polymorphic bands (*PPB*) for the Australian core collection and the international collection

Parameter	Australian	International
n	116	59
H	0.17 ± 0.03	0.18 ± 0.03
A	114	112
G	116	57
PPB	0.84	0.74

29.1.1.2 Genetic Variation and Population Structure

EST-SSR bands were scored as either present or absent and treated as dominant markers. Total number of alleles, rare alleles (alleles present in $< 5\%$ of individuals), and private alleles (exclusive to either the AC or the IC) were calculated in Excel (2003). Nei's heterozygosity (*H*), number of genotypes (*G*) and proportion of polymorphic bands (*PPB*) were calculated in R software version 2.12.1 (The R Development Core Team 2005) using the aflpdat package (Ehrich 2006). Tess software, which defines sub-populations (clusters; K) according to the likely number of ancestral genotypes represented in the population, was used to infer population structure (Francois and Durand 2010). Q values give proportional memberships of individuals to each cluster. Ten runs of K = 2–20 were performed using the BYM admixture model with a neighbour-joining tree as the starting configuration. Default values were used for all parameters. The most likely K (K_{max}) was the K corresponding to the lowest mean deviance information criterion (DIC). Clumpp (Jakobsson and Rosenberg 2009) was used to permute Q matrices from ten runs of Kmax and bar plots were constructed from permuted Q matrices using Distruct (Rosenberg 2004). Diva-gis software (Hijmans et al. 2005) was used to generate maps showing the global distribution of the accessions used in this study.

29.1.2 Results

29.1.2.1 Genetic and Allelic Variation

Genetic divergence between the IC and the AC was negligible ($F_{ST} = 0.039, \text{sd} = 0.009$). Heterozygosity (*H*) and number of genotypes (*G*) were similar between the AC and the IC (accounting for sample size differences; Table 29.1). However, *PPB* was slightly higher in the AC than in the IC. The number of alleles detected was similar between the AC (114) and the IC (112). Almost half the total number of alleles across both collections were considered rare. Of these, eight were found to be common in the AC but rare in the IC, while only five were found to be rare in the AC but common in the IC. There were 16 alleles that were only present among Australian genotypes and one of these was also common in Australia (Table 29.2).

Table 29.2 Total number of alleles, rare alleles, alleles that were rare in both the Australian and international collections, alleles that were common in Australia but rare in the international collection, alleles that were rare in Australia but common in the international collection, alleles that were private in Australia and alleles that were private in the international collection

Marker	Alleles	Rare Alleles	Australian Rare/International Rare	Australian Common/International Rare	Australian Rare/International Common	International Private	Australian Private
Tri-63	19	12	3	0	2	5	2
Tri-69	6	3	0	1	1	1	0
Tri-72	7	2	0	0	1	2	1
Tri-58	6	2	1	0	0	1	0
Tri-76	7	4	0	0	0	1	3
Tri-10	8	4	3	0	0	0	1
Tri-87	4	2	1	0	0	0	1
Di-35B	2	1	1	0	0	0	1
Tri-79	12	7	6	1	0	1	0
Tetra-8	8	2	1	1	0	1	0
Tetra-3	7	2	1	0	0	1	0
Tri-91	12	4	1	3	1	0	2
Tri-17	8	6	4	1	0	0	1
Tri-56	12	6	4	0	0	1	1
Tri-74	2	0	0	0	0	0	0
Tri-88	8	4	1	1	0	0	3
Total	*128*	*61*	*27*	*8*	*5*	*14*	*16*

29.1.2.2 Population Structure

Five sub-populations were detected using Tess (Fig. 29.1a). The ratio of IC to AC individuals was highest in clusters 1 and 2 and lowest in clusters 3 and 5 (Fig. 29.2). Cluster 3 contained the highest proportion of admixture and was represented entirely by IC individuals. However, one of these was an Australian accession of the IC. Cluster 5 consisted almost exclusively of non-admixed IC individuals. The only exception was one AC individual which shared $<50\%$ ($Q = 0.43$) homology with the cluster 5 ancestral genotype. All other AC individuals were found in clusters 1, 2 and 4. Sub-populations did not separate species into discrete clusters. The global distribution of clusters is shown in Fig. 29.1b.

29.1.3 Discussion

This study showed that allelic diversity was similar in the Australian and international collections. This does not indicate that a population bottleneck associated with introduction and colonisation processes contributed to the current distribution of *Cynodon* in Australia. Introduced populations arising from bottleneck events are usually characterised by lower levels of genetic diversity compared with source populations. This

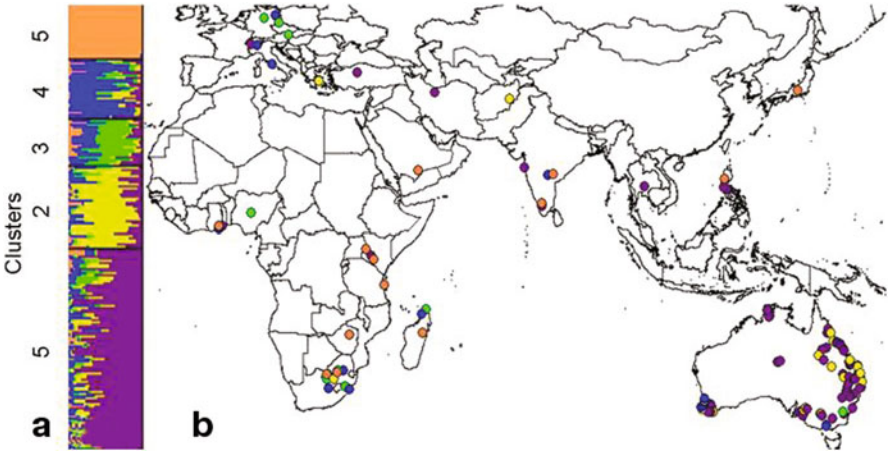

Fig. 29.1 Distribution of *Cynodon* in terms of (**a**) clusters identified using the spatially explicit Bayesian population structure program TESS and (**b**) geographic proximity identified using DIVA-GIS software with individuals colored according to TESS–inferred clusters

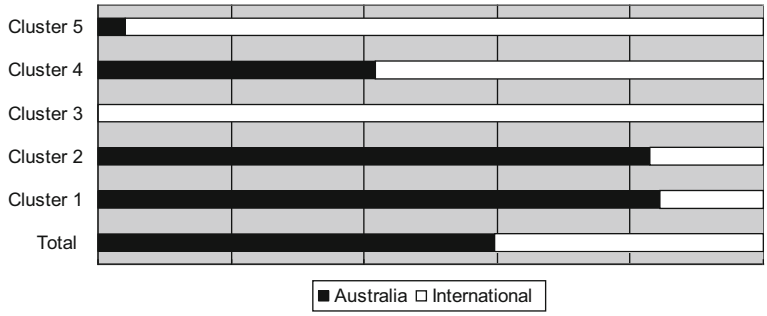

Fig. 29.2 Ratio of Australian and international accessions in each of Five clusters

was the reported cause of reduced levels of genetic diversity in introduced populations of the invasive weed *Rubus alceifolius* Poir. (Rosaceae) compared with that of populations representing the native range of this species (Amsellem et al. 2000). Similar observations have reportedly been associated with genetic bottlenecks during domestication of many crop species including sunflower, (Mandel et al. 2011), maize (Vigouroux et al. 2005), soybean (Kuroda et al. 2010), and barley (Matus and Hayes 2002). It is possible that multiple introductions have led to the distribution of *Cynodon* in Australia and hybridisations between individuals arising from these introductions have led to maintenance of genetic diversity within the genus.

The collection clustered into five sub-populations. However, these did not correspond to distinct clusters of species. This may be explained by the extensive multiplicity of *Cynodon* species and sub-species nomenclature, which has led to enormous taxonomic confusion within the genus (The Plant List 2010). Previous

population structure analyses of the international collection have also found that species did not form discrete clusters. This was reportedly due to mis-naming of accessions prior to Harlan et al.'s (1970) revision of the genus, contamination among nursery plots since establishment of the collection in the 1940's, and taxonomic confusion during species classification (Anderson et al. 2009).

Despite these confusions, the population structure analysis, which shows that Australian germplasm is largely restricted to only 3 of the 5 Tess clusters, suggests that fewer ancestral populations contributed to the current distribution of Australian than global germplasm. This implies that the origins of Australian *Cynodon* may involve colonisation by species endemic to other countries. These findings will need to be further investigated through analyses investigating the phylogenetic species diversity of *Cynodon* in Australia.

The identification of rare and private alleles within Australian *Cynodon* shows that there may be genetic diversity present in Australia that may not be represented in current international breeding resources. Furthermore, the presence of alleles that are common in Australia but rare in the international collection suggests that *Cynodon* in Australia may be undergoing range expansion and adaptation (Barret and Schluter 2008). The presence of private, but not rare, alleles in Australia may also represent adaptive responses involving mutations occurring since *Cynodon's* introduction to Australia. However, the more parsimonious explanation for these observations is associated with the insufficient sample size of the international germplasm. Therefore, these speculations will need to be confirmed through further analyses, such as adaptation and association mapping studies.

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Chapter 30
Variation in Traits Associated with Carbon Sequestration for a Range of Common Amenity Grass Species

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Abstract Carbon sequestration in the soil profile depends on a number of soil, plant, climatic and management factors, and their interactions. Plants affect the quantity, quality and placement of carbon in the soil profile. Ecological studies have demonstrated considerable variation in soil organic matter (SOM) under different types of grassland but the potential for exploiting genetic variation amongst grasses for carbon deposition in the soil has not yet been explored. Neither has there been any attempt to select and breed amenity or forage grass genotypes exhibiting enhanced carbon sequestration.

30.1 Introduction

Carbon sequestration is the relatively long term storage of carbon in the stabilised soil organic matter (SOM) fraction and depends on a number of soil, plant, climate and management factors, and their interactions (Bardgett 2011). Besides constituting one of the measures identified under article 3.4 of the Kyoto Protocol as necessary for mitigation of global climate change, efforts to enhance carbon sequestration are justified by the decline in SOM reported, irrespective of soil type and land-use, across England and Wales (Bellamy et al. 2005).

Long term equilibrium levels of SOM are substantially higher in grassland soils, especially permanent pasture, compared with arable cropped soils. Soussana et al. (2004) calculated a figure of 25 t carbon ha^{-1} for the average difference in soil organic carbon stock (0–30 cm) between temperate lowland cropland and pasture. Consequently, options for enhancing carbon storage in grassland systems have so far focused on conversion from temporary to permanent grassland and alterations in N input (Soussana et al. 2004). In general, the kinetics of carbon accumulation following a change in vegetation or management are non-linear: rapid change occurs

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Table 30.1 List of varieties established, grouped by species

Lolium perenne	*Festuca rubra ssp. littoralis*	*Festuca rubra ssp. commutata*	*Festuca rubra ssp. rubra*	*Agrostis capillaris*	*Deschampsia caespitosa Koeleria cristata*
AberImp	AberFlight	Bl 3051	Adinda	AberRoyal	Abercampsia
Ace	AberCharm	Bargreen	Boreal	BarKing	Barcampsia
Ba13516	AberGem	Baroxi	Cindy	AberRegal	
Bargold	Barcrown	Center	Greenvie	Heriot	BarKoel
Bizet	Barpearl	Raisa	Hollywood	Lance	–
Brightstar	Cezanne	–	–	Avalon[c]	–
Cadix	Count	Pintor[a]	Aberfleece[b]	Vespa[c]	–
Greenfair	Mocassin	–	Quatro[b]	Highland[d]	–

[a] *F. longifolia*
[b] *F. ovina*
[c] *A. canina*
[d] *A. castellana*

during the early years followed by an asymptotic approach to a new equilibrium value over many decades (Post and Kwon 2000).

A number of candidate traits associated with carbon sequestration can be identified from experimental and modelling studies (Jones and Donnelly 2004). These can be classified according to whether they affect:

a. Quantity, achieved through the balance between photosynthesis and respiration, the net primary production.
b. Quality, through tissue composition, for example lignin content affecting the rate of decay.
c. Placement of carbon in the soil profile, including leaf litter production, root depth, turnover and decomposition.

The objective of this preliminary study is to assess variation in selected traits likely to affect net carbon sequestration in amenity grasses, grown under field conditions and receiving contrasting levels of fertiliser application.

30.2 Materials and Methods

The study utilised existing field-plots, established 18 months previously. In total 40 cultivars were grown representing five species groups, see Table 30.1. The trial consisted of 1 m^2 plots randomised in two split blocks replicated twice. From establishment onwards two blocks received zero fertiliser and two blocks 360 kg N/ha/year, plus maintenance phosphate and potash in six applications. Prior to this trial beginning, all plots were cut once a week throughout the growing season to a height of 30 mm, with the clippings removed.

Measurements were made over a ten week period. All plots were cut immediately prior to the start and again after four and ten weeks, for dry matter yields. Following the final cut, detailed measurements were confined to perennial ryegrass and strong

Table 30.2 Herbage dry weights for five species at final harvest. Means of cultivars

Species	Cultivar Number	Herbage Dry Weight (g/m^2)	
		No Nitrogen	Nitrogen
Agrostis ssp	8	17.5	230.8
Festuca rubra ssp rubra	5	16.9	121.7
F. rubra ssp littoralis	8	17.3	175.2
F. rubra ssp commutata	5	14.0	112.9
Lolium perenne	8	3.0	23.0

creeping red fescue plots. This involved taking duplicate soil cores (0–30 cm depth) from each plot, for separation into live shoot and root biomass. Leaf litter was also collected from quadrats within the plots over the ten week period. Fractions were oven dried and weighed.

30.3 Results

It is clear from this study that there are differences in above ground biomass between species especially at the high nitrogen application level (Table 30.2). Detailed measurements of leaf litter and root biomass were confined to the *Lolium* and *Festuca*

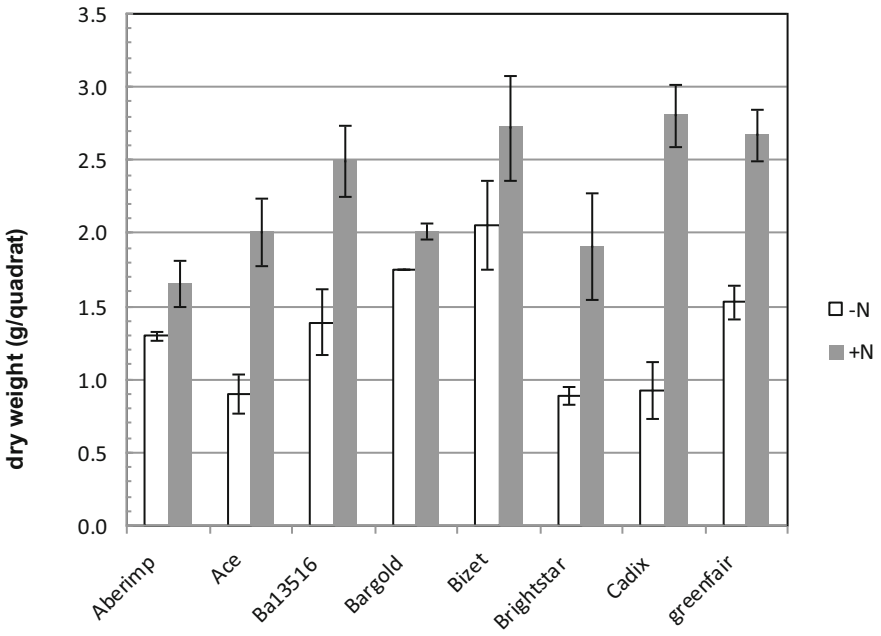

Fig. 30.1 Leaf litter production by cultivars of *Lolium perenne* over 10 week period

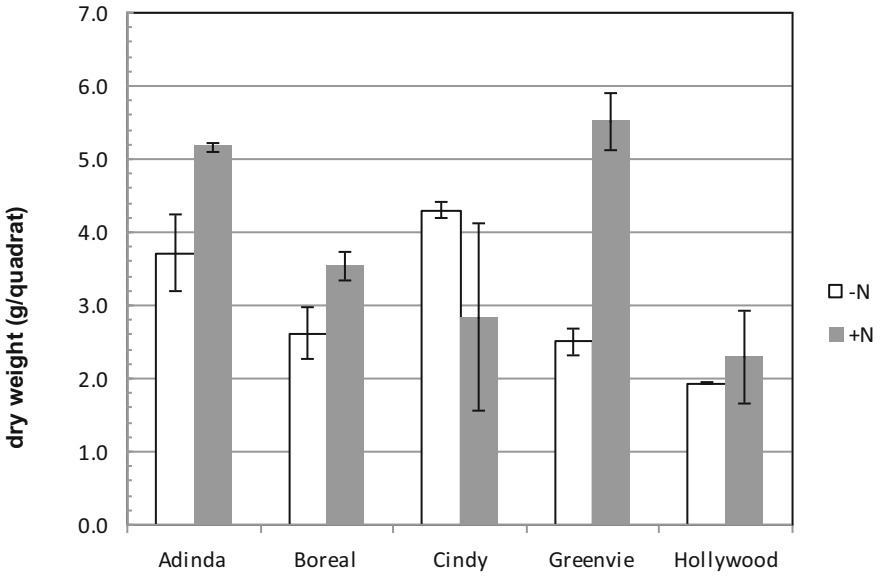

Fig. 30.2 Leaf litter production by cultivars of *Festuca rubra* ssp *rubra* over 10 week period

rubra plots, and provide evidence of variation between cultivars (populations) of the same species (Figs. 30.1–30.4). In particular the *Lolium* cultivars show variation with and without nitrogen for leaf litter production, (Fig. 30.1). For the *Festuca rubra* cultivars Adinda and Greenvie produced more leaf litter in the high nitrogen treatment, but the cultivar 'Cindy' appeared to produce more leaf litter at low levels (Fig. 30.2). The data for root mass was less conclusive and only Bizet and the stay green variety, Ba 13516 stand out as having a greater root mass at higher nitrogen levels. There were no significant differences between the varieties in the zero fertiliser treatment for root mass for either species (Figs. 30.3 and 30.4).

30.4 Conclusion

The scope of this trial was to provide an initial comparison of potential carbon sequestering traits across different species and genotypes. This variation has the potential to be exploited to enhance the effectiveness of future grass varieties in the storage of carbon in the soil.

The dynamics of carbon sequestration suggest that both the enhancement of short-term carbon accumulation (0–5 years) and long-term equilibrium level of soil organic carbon (>50 years) are targets that warrant consideration with respect to genetic improvement of amenity grasses. Enhancing the short-term rate of carbon accumulation would benefit arable to grassland conversion schemes and the restoration of brown-field sites.

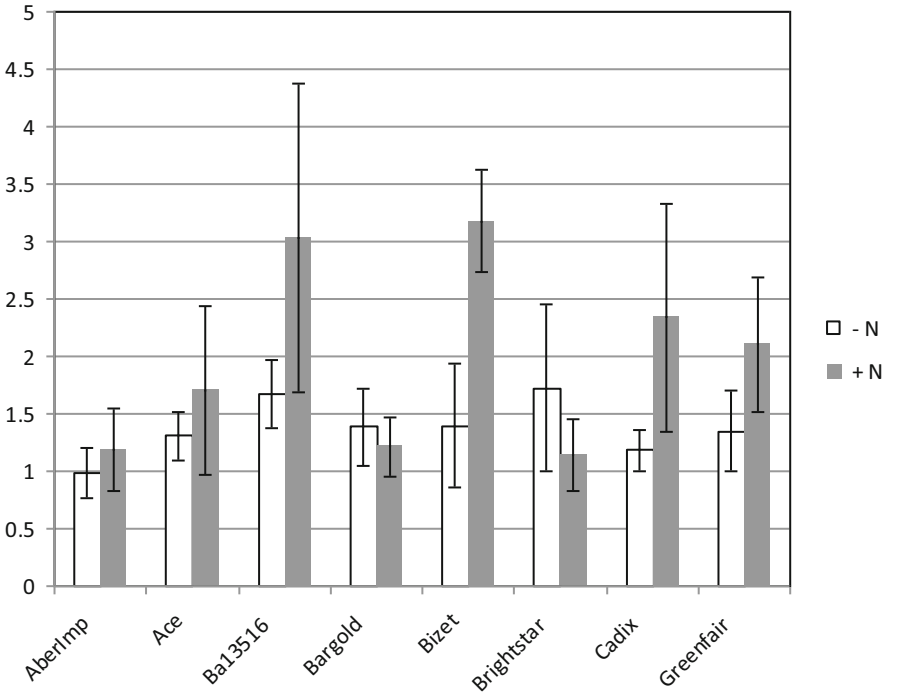

Fig. 30.3 Average *Lolium perenne* root material in soil cores

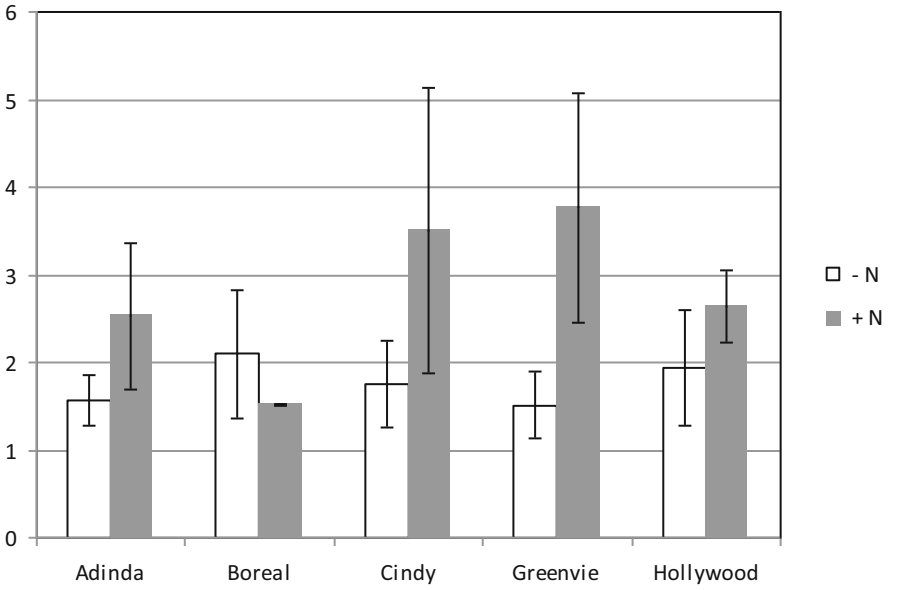

Fig. 30.4 Average *Festuca rubra* ssp *rubra* root material in soil cores

Candidate traits associated with carbon sequestration can be classified according their effect and further detailed work could be targeted at

- Quantity, biomass produced above and below ground, including leaf/root turnover
- Quality, resistance to decomposition and chemical form, including C:N ratio
- Placement in the soil profile and the risk of loss through decomposition and release of carbon

Future work will require measurement of SOM levels under field conditions over many years to determine more thoroughly the variation within grass species and the subsequent development of enhanced carbon sequestering varieties.

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Chapter 31
Suitability of Grass Species for Phytoremediation of Soils Polluted with Heavy-metals

G. Żurek, M. Pogrzeba, K. Rybka and K. Prokopiuk

Abstract Highly heavy metal (HM) polluted soil from non-ferrous mine and smelter in Poland (further named as "Waryński") were used in pot experiment for six grass varieties. Growing media with three different amounts of "Waryński' soil (0, 33 and 100 %) mixed with unpolluted field soil were used. The grass varieties showed significant differences in ability to accumulate HM from soil. Performed measurements proved proportional reduction of plant growth with increasing amount of polluted soil in the growing medium: 100 % Waryński soil inhibited plant growth so strongly that it was not useful for further analysis. Depending on the variety, it has been estimated that using different grass varieties, it could be possible to extract: 2.6–10.2 g of lead, 10.2–34.2 g cadmium and 250–2,562 g zinc, as calculated for total grass yield from 1 ha.

Keywords Bioaccumulation · Lead · Cadmium · Zinc

31.1 Introduction

The term 'phytoremediation' refers to the environmental cleanup techniques, where plants stabilize, extract or degrade water, air or soil pollutants. It offers a coast-effective and environment-friendly alternative or complementary technology for conventional remediation such as soil incineration or excavation and pump-and-treat system (Pilon-Smith 2005).

Pollutants could be of organic (i.e. fuels, solvents, explosives, pesticides, herbicides, chemicals etc.) or inorganic (salts of heavy metal ions) nature. Presence of high levels of pollutants as heavy metals in soil is stress factor for plants, and may produce deleterious effects on many physiological processes (Seregin and Ivanov 2001). Finally, plants reduce growth, yield and generative reproduction.

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Soils contaminated by inorganic pollutants are serious problem, and it is estimated that ca. 1.5 mln places in Europe need to be remediated, from which 300,000 pose a potential threat to surroundings (Kucharski et al. 2005). After sufficient plant growth and metal accumulation, plants are harvested and removed, resulting removal of metals from polluted site. Despite HM uptake, plants cover the soil and erosion and pollutants leaching will thus be reduced (Chhotu and Fulekar 2009). Biomass removed from polluted areas should be used only for non-food purposes, i.e. conversion to energy. In some cases metals can be extracted from the HM-rich ash and serve as a source of revenue, thereby offsetting the expense of remediation (Ghosh and Singh 2005). Variety of techniques and number of plants species need to be evaluated for cleanup of polluted areas. From many species examined for phytoremediation of HM, trees and grasses seems most interesting. Trees, such as *Salix* are high biomass producers, and also have effective nutrient uptake, high evapotranspiration rate and a pronounced clone specific capacity for HM uptake (Pulford and Watson 2003). But, due to its high water consumption and specific harvest equipment, its practical use is limited to areas, located in the vicinity of water on large and flat fields. But this is not always in the case of polluted areas. Therefore, we tested grass species with relatively high biomass yields and low site and harvest requirements. The aim of our studies was to evaluate the suitability of grass species for phytoremediation of HM polluted soils by evaluation of the effect of HM ions contamination on plant growth and determination of Pb, Cd and Zn ions concentration in biomass.

31.2 Materials and Methods

Six grass varieties were selected for pot studies: tall fescue (*Festuca arundinacea* Schreb.) 'Terros' and 'Rahela', reed canary grass (*Phalaris arundinacea* L.) 'Keszthelyi', tall oat grass (*Arrhenatherum elatius* J. et C. Presl.) 'Wiwena', tall wheatgrass (*Elytrigia elongata* (Host) Nevski)'Bamar' and switchgrass (*Panicum virgatum* L.) 'Shelter'. Metal pots (18.8 dm^3) were filled with soil mixture composed of typical field soil and highly HM-polluted soil from non-ferrous mine and smelter in Poland (further named as "Waryński" soil). Different amounts (0, 33 and 100 %) of "Waryński" soil were used. Mentioned soil was previously used in other experiments, as source of man-made, HM contamination (Kucharski et al. 2005; Japenga et al. 2007). Soil mixtures were further analyzed for pH, soil organic matter (SOM) contents and concentration of lead, cadmium and zinc (Table 31.1).

Grass seed was sown in April of 2010 and aboveground plant biomass was harvested at the end of the season. Considering low quantity of biomass harvested from 100 % "Waryński" soil, biomass for analysis of concentrations of Pb, Cd and Zn was harvested from pots with 0 and 33 % of "Waryński" soil. Bioaccumulation factor (BAF) was expressed as ratio of concentration of HM in plant biomass to the concentration of mentioned chemicals in soil.

Table 31.1 Selected chemical parameters of soil mixtures used in experiment

Amount of "Waryński" soil in mixture (%)	Soil mixtures characteristics				
	pH_{KCl}	HM concentration (ppm)			SOM (%)
		Pb	Cd	Zn	
0	6.1	7.7	0.2	17.7	1.00
33	6.3	659.0	36.5	1966.0	1.80
100	6.4	1412.0	90.2	4685.0	3.63

Table 31.2 Dry matter yields, concentration of HM and BAF of tested grass varieties

Species and variety name	"Waryński" soil in mixture (%)	DM yield (g/plant)	HM in biomass (ppm)			BAF		
			Pb	Cd	Zn	Pb	Cd	Zn
Festuca	0	17	0.73	0.19	19.5	0.095	1.188	1.102
arundinacea	33	7.2	0.52	4.96	95.75	0.00078	0.136	0.049
RAHELA	100	2	–	–	–	–	–	–
Festuca	0	9.1	1.65	0.31	30.8	0.216	1.906	1.74
arundinacea	33	7.9	0.99	2.64	85.85	0.00149	0.072	0.044
TERROS	100	1.5	–	–	–	–	–	–
Elytrigia	0	10.5	0.25	0.14	18.45	0.033	0.875	1.042
elongata	33	7.8	1.2	1.77	42.6	0.00182	0.048	0.022
BAMAR	100	1.5	–	–	–	–	–	–
Phalaris	0	6.1	0.38	0.1	34.5	0.049	0.625	1.949
arundinacea	33	7.7	0.59	1.49	259.0	0.0009	0.041	0.132
KESZTHELYI	100	2.4	–	–	–	–	–	–
Arrhenatherum	0	5.4	0.31	0.1	20.45	0.041	0.594	1.155
elatius	33	7.4	0.68	2.47	71.25	0.00102	0.068	0.036
WIWENA	100	2.5	–	–	–	–	–	–
Panicum	0	13.7	0.22	0.05	13.9	0.028	0.281	0.785
virgatum	33	12.7	0.54	0.75	44.45	0.00082	0.020	0.023
SHELTER	100	2.4	–	–	–	–	–	–
Mean	0	10.3	0.59	0.15	22.93	0.077	0.911	1.296
	33	8.45	0.75	2.34	99.82	0.00114	0.064	0.051
	100	2.0	–	–	–	–	–	–
$LSD_{\alpha = 0.05}$	0	3.42	0.649	0.081	6.233	0.085	0.507	0.352
	33	3.61	0.423	0.414	17.45	0.0006	0.0113	0.0089
	100	>0.001	–	–	–	–	–	–

31.3 Results and Discussion

Reduction of plant growth, finally expressed in decreasing values of dry matter of plants was proportional to the increase of HM concentration in soil mixture (Table 31.2).

Despite of high concentration of Pb in polluted soil mixture, its concentration in plant biomass was low. It was only 28 % more than in plants grown in unpolluted soil. It has been mentioned by Kucharski et al. (2007) that Pb bioavailability in

"Waryński" soil was relatively low as compared to Cd or Zn. It has been shown in our results that Cd and Zn concentration in plant biomass grown on polluted soil was 1,507 and 335 %, respectively, higher than concentration in plant biomass from unpolluted soil. Bioaccumulation factors (BAF) for plants grown on polluted soil were lower than grown on unpolluted soil. Such relation for Zn and Cd has also been reported by Baran and Jasiewicz (2009). Values of BAF for zinc from polluted soil was close related to BAF for unpolluted soil ($r = 0.80, \alpha < 0.001$). It is therefore possible that tested varieties exposed some genetic properties for accumulation of zinc, which were not so clear in case of cadmium ($r = 0.53, \alpha < 0.05$) and lead ($r = 0.24$, ns). HM may also interact—for example, Zn^{2+} ions may counteract Cd^{2+} absorption (Seregin and Ivanov 2001).

The highest concentrations of HM as well as BAF-s for Pb, Cd and Zn were noted for *E.e.* 'Bamar', *F.a.* 'Rahela' and *P.a.* 'Keszthelyi', respectively. Considering reduction of biomass yields on HM polluted soils, it is possible to extract from 2.6 g (*F.a.* 'Rahela') to 10 g (*F.a.* 'Terros') of lead, from 10 g (*P.v.* 'Shelter') to 34 g (*A.e.* 'Wiwena') of cadmium and from 320 g (*E.e.* 'Bamar') to 2,562 g (*P.a.* 'Keszthelyi') of zinc in biomass harvested from 1 ha.

Grass species used in this study are not the highest effective HM accumulators. For example in case of willow, cadmium accumulation more than 200 g per hectare per year was reported (Porębska and Ostrowska 1999). But in case of phytoremediation, research should focus not only on the absolute amount of HM yearly extracted but also on diversity of species used for mentioned process, due to relatively long time scale and numerous, currently unpredictable factors involved in future success.

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Chapter 32
Targeting Lucerne Cultivars to Saline-soil Environments

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Abstract Lucerne (*Medicago sativa* L.) is rated as moderately susceptible to soil salinity, a major stress whose incidence is increasing in drought-prone regions because of irrigation with saline water. Three-year dry-matter yield (DMY) of 14 populations (cultivars or landraces) of different origin was assessed across ten agricultural environments of Algeria, Italy, Morocco and Tunisia whose electrical conductivity (EC_e) in the 0–30 cm soil layer ranged between 0.2 and 6.0 dS m^{-1}. The adaptive responses of the populations were modeled by factorial regression as a function of EC_e and one or two additional significant covariates. Ameristand 801S (a benchmark variety for salt tolerance) and the Moroccan landrace Erfoud 1 displayed positive genotype × environment (GE) interaction in environments with high soil salinity. Nine populations and one additional Algerian landrace (Tamantit) underwent a seed germination test under saline conditions, to verify the ability of this test to predict the field-based adaptive responses to soil salinity of the populations. The salt concentration required to inhibit germination of 50 % of viable seeds (IC(50)) ranged from 1.13 % NaCl of Prosementi to 2.61 % NaCl of Ameristand 801S. IC(50) values of the populations displayed moderately high correlation ($r = 0.70$; $P < 0.03$) with the genotype slope as a function of EC_e in the factorial regression ($\beta\, EC_e$). Salt-tolerant genetic resources could be found in north African landraces which evolved in less favourable oasis environments, such as Tamantit and Erfoud 1.

Keywords Factorial regression analysis · Genotype × environment interaction · Germination test · *Medicago sativa* · Salt tolerance

32.1 Introduction

Lucerne (*Medicago sativa* L.) is the main perennial forage legume grown in the Mediterranean basin and other semi-arid areas. Soil salinity is a major stress affecting crop production in drought-prone regions whose incidence is increasing because of irrigation with saline water. Lucerne is rated as moderately susceptible to soil

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salinity, with 30 % yield reduction occurring under salinity that corresponds to a soil electrical conductivity (EC_e) of 6.1 dS m^{-1} (Maas 1986). An increase of salt tolerance through selection is feasible, as genetic variation exists for this trait among populations (Smethurst et al. 2008). Narrow-sense heritability estimates for this trait were reportedly between 0.23 and 0.61 depending on the germplasm (Al-Khatib et al. 1994). Salt tolerance is a complex trait involving the integrated contribution of several physiological and biochemical mechanisms at various levels of the plant structure. Smethurst et al. (2008) identified two major mechanisms of tolerance in lucerne, one based on sodium exclusion from leaves and the other implying effective sodium compartmentation in cell vacuoles. Early screening for salt tolerance has been proposed by means of seed germination testing under saline condition, but the consistency of its results with those for the adult plant is still controversial (Johnson et al. 1992; Smethurst et al. 2008). The North American Alfalfa Improvement Conference (NAAIC) has defined a standard test for assessing cultivar salt tolerance from seed germination under saline conditions (Rumbaugh 1986).

The main objective of this study was to verify whether field-based adaptive responses to soil salinity of lucerne populations could partly be predicted just by screening for seed germination under saline conditions. As a first step of this investigation, three-year dry-matter yield (DMY) of 14 lucerne populations was assessed across ten agricultural environments which varied widely for soil salinity as expressed by soil EC_e values. Factorial regression modeling of the population responses, whose results are reported in detail elsewhere (Annicchiarico et al. 2011), indicated soil EC_e as one of the main environmental variables affecting the site-specific adaptation of the populations as expressed by their genotype × environment (GE) interaction for DMY. Field-based salt tolerance of the populations was estimated by their regression parameter for response to soil EC_e.

32.2 Materials and Methods

Fourteen populations, including six farm landraces from north Africa and Italy and eight commercial varieties from Italy, France, Australia and the USA (Table 32.1), were evaluated for three-years in ten agricultural environments encompassing six locations of Algeria, Italy, Morocco and Tunisia. Crop management in the ten environments implied rainfed conditions, irrigation with 9-week summer suspension, or continuous irrigation (Annicchiarico et al. 2011). Soil electrical conductivity in the 0–30 cm soil layer ranged between 0.2 and 6.0 dS m^{-1} across environments. Two locations (Alger in Algeria, and Médenine in Tunisia) approached the threshold of 2 dS m^{-1} for salinity damage, while the Algerian location of Hmadna featured fairly high soil salinity (5.2 and 6.0 dS m^{-1} in its rainfed and irrigated environments, respectively). Each experiment was designed as a randomized complete block design (RCBD) with four replications. DMY was recorded over three-years on a 2.4 m^2 harvest area of the 5 m^2 large plots. The total number of harvests varied depending on the growth potential of the environment, ranging between nine (Mateur rainfed in

Table 32.1 Name, code, germplasm type, origin, code, slope of genotype × environment interaction for dry-matter yield as a function of soil electrical conductivity (EC_e), and NaCl concentration required to inhibit germination of 50 % of viable seeds (IC(50)), for 14 lucerne cultivars evaluated in 10 Mediterranean environments and 10 populations tested for seed germination under saline conditions

Population	Code	Type	Origin	$\beta\, EC_e$ (dS m^{-1})	IC(50) (% NaCl)[a]
ABT 805	Ab	Com	SE USA	−0.005	–
Ameristand 801S	Am	Com	SW USA	0.713*	2.306 a
Coussouls	Co	Com	S France	0.172	–
Demnat 203	De	Lan	Morocco	0.114	–
Erfoud 1	Er	Lan	Morocco	0.575†	1.588 bc
Gabès 2355	Ga	Lan	Tunisia	0.329	1.367 d
Magali	Mg	Com	W France	−0.369	1.200 ef
Mamuntanas	Ma	Lan	Sardinia	−0.082	1.202 e
Melissa	Me	Com	S France	0.110	–
Prosementi	Pr	Com	N Italy	−0.696*	1.130 f
Rich 2	Ri	Lan	Morocco	−0.156	1.433 cd
SARDI 10	Sa	Com	S Australia	−0.608*	1.303 de
Sicilian ecotype	Sc	Lan	Sicily	0.257	1.355 d
Siriver	Si	Com	S Australia	−0.353	–
Tamantit	Ta	Lan	Algeria	–	1.785 b

Com commercial variety, *Lan* farm landrace

†, *: regression slope different from zero at $P < 0.06$ and $P < 0.05$, respectively; data from Annicchiarico et al. (2011)

[a]Values followed by same letter do not differ at $P < 0.05$ according to Probit analysis

Tunisia) and 23 (Oued Tessaout under continuous irrigation in Morocco). GE interaction effects for DMY were modeled by factorial regression (Denis 1988). The slope of GE interaction for DMY as a function of EC_e ($\beta\, EC_e$) in the factorial regression model estimated the adaptation to saline-soil conditions of each population. Positive $\beta\, EC_e$ values indicated positive GE interaction for DMY in saline-soils environments and, hence, salt tolerance of the population.

A subset of nine populations, including four with contrasting $\beta\, EC_e$ value, and one additional Algerian landrace (Tamantit), were chosen for the following assessment of salt tolerance based on seed germination under saline conditions (Table 32.1). We adopted the standard NAAIC test (Rumbaugh 1986), which contemplates seed germination in each of eight concentrations of NaCl: 0.00, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 % (wt/wt) in deionised water. The eight concentrations corresponded to 0.0, 85.6, 128.3, 171.1, 213.9, 256.7, 299.4 and 342 mM NaCl solutions, or to 0.0, 8.3, 11.6, 15.5, 17.2, 23.1, 26.7 and 30.0 dS m^{-1} electrical conductivity. Differently from the protocol by Rumbaugh (1986), 30 scarified seeds per Petri plate were germinated instead of 25, and three replications in a RCBD were used instead of two. The plates were placed in the dark in a germination cabinet maintained at 25 °C, counting germinated seeds after seven days. Germination data, corrected for possible hard seeds, were analysed by Probit analysis. Population mean values and the respective 95 % confidence intervals were estimated for the NaCl concentration (%)

required to inhibit germination of 50 % of viable seeds (IC(50)). The correlation between βEC_e and IC(50) values of the populations was assessed for data of the nine populations in common between field evaluation and germination test.

32.3 Results and Discussion

Population and environment main effects and GE interaction for total DMY across the ten agricultural environments were significant ($P < 0.01$). The highest mean yield (48.12 t ha^{-1}) was recorded in Oued Tessaout under continuous irrigation, and the lowest (12.16 t ha^{-1}) in Hmadna rainfed, where the crop was exposed to severe drought and salinity stress. Environment mean yield was closely correlated with annual and spring-summer water availability, and tended to positive association with milder winter temperatures, lower soil salinity and higher number of harvests (Annicchiarico et al. 2011).

The best factorial regression model included number of harvests, soil electrical conductivity and spring-summer (April-September) water available for the crop as significant ($P < 0.05$) environmental covariates. It explained 53 % of GE interaction variation for DMY. A simpler model including only spring-summer water available and soil electrical conductivity accounted for 42 % of the GE interaction sum of squares (Annicchiarico et al. 2011).

The two factorial regression models provided similar estimates of population salt tolerance as expressed by their βEC_e parameter. The βEC_e values for the three-covariate model which are reported in Table 32.1 were used for correlation with data of seed germination under saline water. Ameristand 801S was the population featuring the highest salt tolerance on the basis of its high positive βEC_e value (implying large positive GE interaction in environments with high soil salinity). Indeed, this cultivar has been considered as a benchmark variety for salt tolerance in lucerne (Smethurst et al. 2008). Also the Moroccan landrace Erfoud 1 showed positive βEC_e value ($P < 0.06$), unlike the remaining cultivars and particularly Prosementi and SARDI 10, which were highly susceptible on the basis of their negative βEC_e value (Table 32.1).

The two factorial regression models allowed to scale up to other environments the indications on site-specific, top-yielding populations. In particular, they highlighted the outstanding adaptation of Ameristand 801S to saline-soil environments, as well as the specific adaptation of Erfoud 1 to saline-soil environments without severe drought stress (Annicchiarico et al. 2011).

Four populations with contrasting βEC_e value, namely, Ameristand 801S, Erfoud 1, Prosementi and SARDI 10, and five populations with intermediate field response to saline-soil environments, underwent the seed germination test under saline conditions along with the Algerian landrace Tamantit. The IC(50) values of the populations ranged from 1.13 % of Prosementi to 2.61 % of Ameristand 801S (Table 32.1). The latter cultivar confirmed also here its outstanding salt tolerance, which was obtained through several cycles of phenotypic selection for this trait and is reportedly related

to a sodium exclusion mechanism (Smethurst et al. 2008). According to indications by Rumbaugh (1986), cultivars with IC(50) greater than about 1.6 % NaCl should be considered as salt tolerant, and those with IC(50) lower than about 1.45 % NaCl as salt susceptible. In the current investigation, Ameristand 801S and the Algerian landrace Tamantit exceeded the threshold value for stress tolerance, and Erfoud 1 was just near it. All other populations had IC(50) values indicating susceptibility, with the Moroccan landrace Rich 2 being close to the threshold value of 1.45 % (Table 32.1).

The correlation between the factorial regression coefficient βEC_e and IC(50) of the nine common populations was moderately high, namely, $r = 0.70$ ($P < 0.03$), supporting the adoption of the germination test as a rapid means to roughly predict the adaptation to saline-soil conditions of lucerne populations. In contrast, Johnson et al. (1992) found that selection based on seed germination in saline water was not correlated with forage yield in saline soils of adult plants. However, as also inferred from Smethurst et al. (2008), the concentration of the 80 mM NaCl solution applied for the screenings by Johnson et al. (1992) was likely insufficient for an efficient discrimination between tolerant and susceptible germplasm.

The adaptation to salt stress of Erfoud 1 and Tamantit may be related to the specific conditions of the oasis environments in which these landraces evolved. Our results suggest that useful lucerne germplasm for salt-tolerance breeding might still be substantially untapped. Novel sources of tolerance could provide a valuable genetic base for the improvement of the crop, or could be used in target crosses between germplasms characterized by different tolerance mechanisms, once their tolerance mechanisms were revealed.

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Chapter 33
Comparison of Seed Mixtures for Technical Revegetation at High Altitude

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Abstract With the aims of improving the success of sowings, enhancing the biodiversity, and reducing the impact of interventions (both in terms of energy input and genetic pollution), the use of seed mixtures including 'site-specific' species is increasingly recommended for revegetation interventions at high altitude. This germplasm is ecologically adapted to the prevailing pedoclimatic conditions and native to the target region. In this three-year study performed in Alpe Palù (Chiesa in Valmalenco, Sondrio, Italy) at 2,020 m a.s.l. elevation, ground cover and botanical composition of native seed mixtures were compared versus those of commercially available mixtures. Both a simpler and a more complex version (with greater number of species) were evaluated for both native and commercial mixtures. The commercial mixtures colonised faster the bare soil in the year following the sowing, but in the subsequent years the native mixtures reached over 80 % of ground cover being no longer different from the former ones. The complexity of mixture had only slight effects on the soil cover. A relative prevalence of grasses was evident in the commercial mixtures, and of non-legume dicots in the native ones. The better botanical balance featured by the native mixtures is likely to result in a more-lasting stability of the covers and an increase of biodiversity in the long term.

Keywords Alps · Ecological restoration · 'Site-specific' species

33.1 Introduction

Mountain areas altered by infrastructures for winter tourism need revegetation to restore the landscape appearance as well as preventing environmental damages due to water run-off and soil erosion (Florineth 1992; Krautzer et al. 2001; Peratoner 2003).

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Table 33.1 Composition and species complexity of the five seed mixtures sown at high altitude

Seed mixture	Grasses	Legumes	Other dicots
Local, simple (A)			
Composition (%)	76	18	6
No. of species	4	3	2
Local, complex (B)			
Composition (%)	76	18	6
No. of species	8	3	2
Commercial, simple (C)			
Composition (%)	80	20	0
No. of species	4	3	0
Commercial, complex (D)			
Composition (%)	88	11	1
No. of species	9	5	1
Commercial, site-specific (E)			
Composition (%)	82.3	16.5	1.2
No. of species	7	5	2

Species and varieties selected for lowland turf or forage purposes are often used for sowing at high altitude because of their large availability of seed at reasonable price. However, this germplasm may prove unsuitable to the peculiar environmental conditions, while requiring costly maintenance (as fertilisation and mowing) and affecting the local biodiversity (Peratoner 2003). To improve the success of sowings, enhancing the biodiversity, and reducing the impact of interventions, the use of seed mixtures including 'site-specific' species is increasingly recommended (Krautzer et al. 2004). This germplasm is native to the same environmental context and, therefore, adapted to the prevailing pedoclimatic conditions and compatible with the existing ecosystems.

In the current study, seed mixtures obtained from local populations of site-specific species were evaluated on a high-altitude ski slope of the Italian Alps, and compared with commercial mixtures including either site-specific or non-site-specific germplasm. Aim of the work was to assess the behaviour of the mixtures in terms of ground cover and contribution to the site biodiversity.

33.2 Materials and Methods

The experiment was established in Alpe Palù, Chiesa in Valmalenco (Rhaetian Alps, Italy) at 2,020 m a.s.l. elevation. Seed of site-specific species was obtained from native populations collected in three valleys of the same mountain district, viz. Valchiavenna, Valmalenco and Upper Valtellina, and two mixtures of different species complexity were assembled with this germplasm (coded as A and B in Table 33.1). The local seed mixtures were evaluated together with three commercial mixtures. Two of them (coded as C and D) were also characterised by different complexity and mostly included non-site-specific species and varieties. The third one was a mixture

Table 33.2 Mixture (over 3 test years) and year mean values of ground cover and botanical family composition of cover

	Ground cover (%)	Family contribution to cover (%)		
		Grasses	Legumes	Other dicots
Seed mixture				
Local, simple (A)	75.3	34.0	38.6	16.7
Local, complex (B)	72.6	31.2	37.0	20.0
Commercial, simple (C)	84.1	47.3	48.5	0.3
Commercial, complex (D)	84.6	39.8	43.5	10.8
Commercial, site-specific (E)	83.4	47.6	37.8	6.4
Year				
2007	75.6 b	35.7 c	32.7 b	7.1 b
2008	75.9 b	44.7 a	44.1 a	11.1 a
2009	88.5 a	39.5 b	46.3 a	14.2 a
ANOVA probability				
Mixture (M)	<0.05	<0.05	ns	<0.001
Year (Y)	<0.05	<0.01	<0.001	<0.01
Block (B)	ns	<0.05	ns	ns
M × Y	<0.05	<0.05	<0.01	<0.001
M × B	ns	ns	<0.01	<0.05

Means followed by different letters differ at $P < 0.05$ according to Duncan's test
ns not significant ($P > 0.05$)

complex mostly including site-specific germplasm (code E). The seed rate adopted for mixtures C and D (30 g m^{-2}) was within the common range of 20–50 g m^{-2} for revegetation of ski slopes (Peratoner 2003). The site-specific seed mixtures were sown at lower rate (15 g m^{-2}) as suggested by previous experiences with this kind of germplasm (Krautzer 2005). Hand sowing took place on November 15, 2006 and was facilitated by applying 6 g m^{-2} of a commercial polyvinyl acetate water suspension as bonding material to the seed. Plots were 2 × 2 m and arranged in a randomised complete block design with four replications. Data were recorded in August of each of the three years following that of sowing, visually estimating the percentage of ground cover per plot and the contribution to cover of botanical families as grasses, legumes and other dicots. An analysis of variance (ANOVA) was carried out according to a three-way mixed factorial design, with block and year as random factors and mixture as fixed factor. The latter factor was tested roughly on a combination of variances, as suggested by Cochran (1951). Single year data were submitted to two-way ANOVA. Means were compared by Duncan's multiple range test at $P \leq 0.05$.

33.3 Results and Discussion

A ground cover close to 90 % was reached across all mixtures by the third year of evaluation (Table 33.2). The local site-specific seed mixtures had lower ground cover percentage over the three test years compared with the commercial mixtures

(Table 33.2). A significant mixture × year interaction for this character was observed, though (Table 33.2). The commercial mixtures colonised faster the bare soil, with a covering percentage in the first year exceeding 75 versus about 65 % of the native mixtures. In the subsequent years, however, the commercial mixtures substantially maintained the initial cover, while the native ones steadily spread, reaching 85 % of soil cover by the third year, being no longer different from the former ones (Fig. 33.1a). Although sown at a halved seed rate compared with the other commercial mixtures, the mixture E based on site-specific species showed similar ground cover, confirming the usefulness of this kind of germplasm for sowings at high altitude (Krautzer 2005). The local mixtures, also sown at 15 g m^{-2} rate, had a slow start, which could be partly explained by their lower seed quality compared with that of commercial material. Peratoner (2003) found that lower competitive ability of site-specific species played an important role in the establishment phase, while specific ecological features became relevant later on. In the current study, after just three years from the sowing the local mixtures filled their gap resulting in no difference between site-specific-species mixtures sown at lower seed rate and mixtures sown at higher seed rate.

The mixtures also differed over the years in the contribution of grasses and dicots other than legumes to the cover vegetation (Table 33.2). A variable percentage between about 4 and 11 % of ground (depending on the mixture) was covered by non-angiosperm vegetation such as mosses (mostly) and lichens. The three commercial mixtures had higher percentage of grasses than the two local mixtures, whereas the latter had, in turn, greater proportion of other dicot species (Table 33.2). Because of its richness in grasses, the site-specific mixture E resembled more the other commercial mixtures than the two local ones. These latter mixtures featured better botanical balance, which might result in a more-lasting stability of the covers and an increase of biodiversity in the long term, as observed by Peratoner (2003).

Despite significant mixture × year interactions (Table 33.2) for the botanical family contributions, some trends were consistent across years, with mixtures A and B being always different from mixtures C and E for the relative contribution of grasses and of other dicot species to the ground cover (Fig. 33.1b, c). The behaviour of the complex commercial mixture D was peculiar in this respect, showing a drop of cover provided by grass species in the third year of growth, that was compensated by an increase of other dicots (Fig. 33.1b, c). Peratoner (2003) reported a similar trend in the German Alps in plots sown with commercial seed mixtures, where lowland forage species decreased suddenly by the third year.

The different botanical composition of mixtures C and D at the third year was the only case of inconsistent behaviour between mixtures of same germplasm kind and different species complexity. Apart from this, indeed, the complexity of seed mixtures had only slight effects on their covering ability or botanical composition. However, a proper assessment of possible effects of the mixture complexity could be better made over a longer period of time than the current one.

In conclusion, the mixtures including native materials appeared, despite the lower seeding rate and the possibly lower seed quality, to be suitable for a profitable use

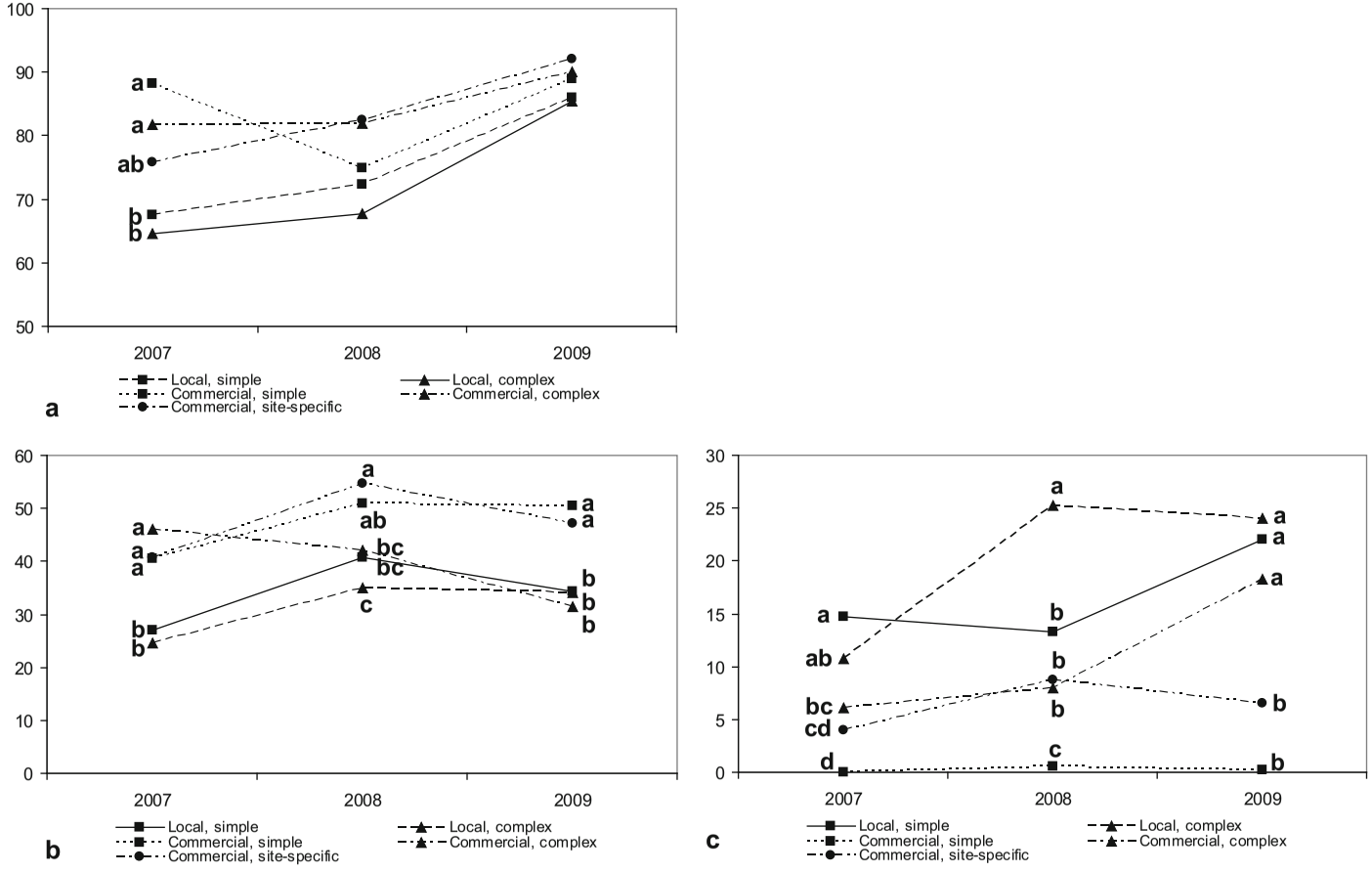

Fig. 33.1 Ground cover percentage (**a**), and contribution percentage of grasses (**b**) and other dicot species (**c**) to the cover of five seed mixtures of different nature and complexity, in 3 years following that of sowing at 2,020 m a.s.l. In each graph and for each year, values with different letters differ at $P < 0.05$ according to Duncan's test. No lettering in a year meant no significant variation ($P > 0.05$) among mixtures according to F test in the two-way analysis of variance

in the revegetation at high altitude, especially for areas of high natural value, where the absence of genetic pollution is a preliminary requirement to any intervention.

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Chapter 34
Genetic Diversity for Cell Wall Digestibility in a Diverse *Lolium perenne* Collection

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Abstract The digestibility of the cell wall has a significant impact on the fodder quality in *Lolium.* This is especially the case at reproductive maturity, when the lignification of the cell wall and the proportion of stems increase. The relevance of this factor becomes clear if we take into consideration that a 1 % increase of the digestibility of the fodder improves the daily intake per cow by 0.2 kg and results in an increase of the daily milk production of 0.4 kg per cow (Gilliland, Quality Counts on Northern Irish Recommended List, 2007). Detailed information on the variability available for cell wall digestibility in individual ryegrass genotypes and how it differs for different organs is currently not available. This information could, however, be exploited to breed ryegrasses with a higher feeding value. In a preliminary study we identified significant differences in the cell wall digestibility of the leaf fraction of ten *Lolium perenne* genotypes. The glucose release varied between 2.58 and 3.89 g glucose/20 g dry biomass. Significant differences for glucose release were identified between leaf and stem samples. To get a better estimation of the variability available for cell wall digestibility in *L. perenne*, a subset of 30 genotypes selected within a broader collection of 300 genotypes from current varieties were analyzed in detail. Preliminary results indicated that improving total digestibility might be achieved through enhanced cell wall digestibility in stems. However more genotypes should be analysed before firm conclusions can be drawn.

34.1 Introduction

Total digestibility is a major selection criterion in *Lolium* breeding. The relevance of this factor becomes clear if we take into consideration that a 1 % increase of the digestibility of the fodder improves the daily intake per cow by 0.2 kg and results in

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an increase of the daily milk production of 0.4 kg per cow (Gilliland 2007). Recently, major advances have been achieved in increasing water soluble sugar content (WSC) as a means to enhance total digestibility (Humphreys et al. 2010). Another approach to improve total digestibility is increasing the cell wall digestibility. The cell wall is a major component of the feedstock and its digestibility has a significant impact on the fodder quality in swards, especially at reproductive maturity, when the lignification of the cell wall increases (Groot et al. 2003). The contribution of cell wall digestibility to total digestibility has been studied in several forage crops as maize, orchardgrass, bromegrass and canarygrass (Barriere et al. 2009; Casler et al. 2008). In *Lolium,* little is known on cell wall digestibility, especially at the individual genotype and organ level. This knowledge is however essential to facilitate breeding for this trait.

Therefore, the objectives of this study were to estimate the variability in cell wall digestibility present in a limited set of genotypes and to estimate differences in cell wall digestibility in relation to plant organ. For this we used a small-scale saccharification assay requiring little amounts of plant material. In a second step we analyzed the correlation between cell wall digestibility and quality parameters in a larger collection of 30 genotypes varying in WSC and total digestibility, using NIRS (Near InfraRed Spectroscopy). The relationship between heading date and cell wall digestibility was also estimated in these 30 genotypes.

34.2 Material and Methods

Plant Material Ten diploid perennial ryegrass genotypes (Set 1) were used for a preliminary small-scale experiment. Four of these genotypes (10370-22, 10370-45, Ba12938-40 and Ba12990-6) were derived from three wild accessions of the ECPGR core collection of *Lolium* (10370, Ba12938 and Ba12990). Four genotypes were selected among advanced ILVO breeding material (1554-1, 5297, 5311 and KC98R-138-1) and two genotypes (ABE and OPT) are the parents of an ILVO mapping population (Studer et al. 2010). These two parental genotypes, ABE and OPT, were selected from the varieties Aberdart and Option, respectively.

Thirty genotypes (Set 2) were selected out of a collection of 300 individual genotypes from 12 varieties (25 plants/variety), displaying a wide range in WSC and total digestibility (Table 34.1). Selection of Set 2 was based on the quality parameters measured on two consecutive cuts in spring 2008 on non vernalised plants without clonal replicates of the genotypes. Set 2 was used to estimate correlations between NDFD (NDF digestibility) and total digestibility, ADL (lignin fraction) and heading date.

Growth Conditions Plants of Sets 1 and 2 were cloned in three replicates in autumn 2009 and grown in the glasshouse during winter 2009–2010 in 12 L containers that were transferred to open air by the beginning of March 2010.

Biomass Harvested The plants were harvested on April 26th and June 9th 2010. The June 2010 cut was used for quality analysis. Stem and leaf material was separated manually.

Table 34.1 WSC (percentage of dry matter), and total digestibility (percentage of dry matter) of Set 2. Values are averages of two cuts (spring 2008) of non-vernalized clones without clonal replications of genotypes

Genotype nr.	Variety	WSC_{2008}	Tot. $Dig._{2008}$
301	Merks	36.5	82.8
302	Merks	20.5	70.7
303	Merks	27.9	79.3
304	Melways	23.3	77.0
305	Melways	32.1	79.8
306	Barnhem	32.8	80.1
307	Barnhem	35.4	82.0
308	Carillon	36.6	82.0
309	Carillon	28.8	78.7
310	Asturion	30.8	78.1
311	Asturion	27.8	77.9
312	Asturion	21.7	72.0
313	Tomaso	29.4	74.8
314	Tomaso	26.7	71.0
315	Tomaso	27.0	77.0
316	Meloni	33.0	80.9
317	Meloni	34.8	81.1
318	Barata	31.1	79.3
319	Barata	16.8	67.6
320	Barata	19.0	68.3
321	Gandalf	25.0	73.1
322	Gandalf	25.8	73.9
323	Sibasa	25.0	76.2
324	Sibasa	29.8	76.2
325	Sibasa	21.8	73.1
326	Orantas	20.5	70.1
327	Orantas	23.7	75.2
328	Aberzest	25.7	74.2
329	Aberzest	39.6	83.6
330	Aberzest	40.8	83.1
Mean		28.3	76.6
Min.		16.8	67.6
Max.		40.8	83.6

Saccharification Assay Cell wall digestibility of Set 1 was determined using a small scale saccharification assay derived from Gomez et al. (2008). 100 mg of dried plant materials were ground in a Retch Tissuelyser (Qiagen) for 6 minutes at 30 Hz. Twenty mg per genotype were taken in four replicates and suspended in 1 ml 70 % ethanol. Samples were incubated in a thermoshaker at 700 rpm for 4 hours at 50 °C. After a short spin, the supernatant was discarded and the washing step was repeated 3 more times. Finally, samples were suspended in acetone after which the supernatant was removed. The samples were dried overnight and subsequently the weight of dry biomass remaining (cell wall) was recorded. Enzymatic hydrolysis was carried out using the enzyme mix Accellerase™ 1,000 (Genencor) in 0.1 M NaOAc buffer pH 4.8. The samples were incubated at 50 °C and 700 rpm during 72 hours. Glucose release was measured at specific time intervals (0 and 72 hours) using the GOD POD assay (Bergmeyer 1974).

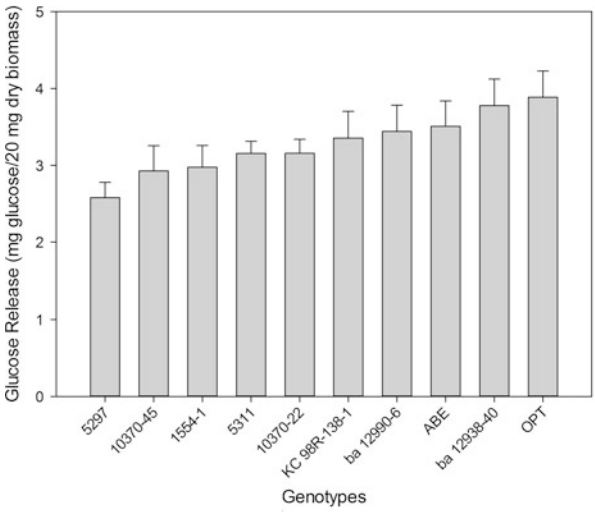

Fig. 34.1 Glucose release (mg glucose/20 mg dry material) from leaf material harvested in June 2010 of ten genotypes using a small-scale saccharification assay

Determination of Quality Parameters For Set 2 NDF, ADF, ADL, NDFD and total digestibility were determined using NIRS calibration lines available at ILVO (Plant Sciences Unit—Crop Husbandry). Reference methods for NDF, ADF, ADL and NDFD were carried out according to the methods described by Goering and Van Soest (1970), and Van Soest et al. (1991). Total digestibility was analysed by the cellulase method of De Boever et al. (1988).

Statistical Analysis Phenotypic data were subjected to analysis of variance to identify significant differences among genotypes. Pearson correlation coefficients were calculated to determine the relationships between the different parameters. The software *STATISTICA v9* (*Statsoft*, USA) was used for these calculations.

34.3 Results and Discussion

In a preliminary experiment, we used a small scale saccharification assay to determine cell wall digestibility on small amounts of tissue. Significant differences in cell wall digestibility were identified among the ten genotypes of Set 1. Glucose release of the leaf material (June 2010 cut) varied between 2.58 and 3.89 g glucose/20 g dry biomass (Fig. 34.1).

The June 2010 cut of genotype ABE contained stem material and therefore, for this genotype, the harvested biomass was divided in three fractions: leaf, sheath and stem. The cell wall digestibility of these three organs was determined separately. Glucose release was significantly different for the three fractions (3.51, 2.95 and 2.57 g glucose/20 g dry biomass for leaf, sheath and stem respectively). This small scale test proved to be reproducible (results not shown) and able to identify significant differences among the ten genotypes of Set 1 and between different organs in one

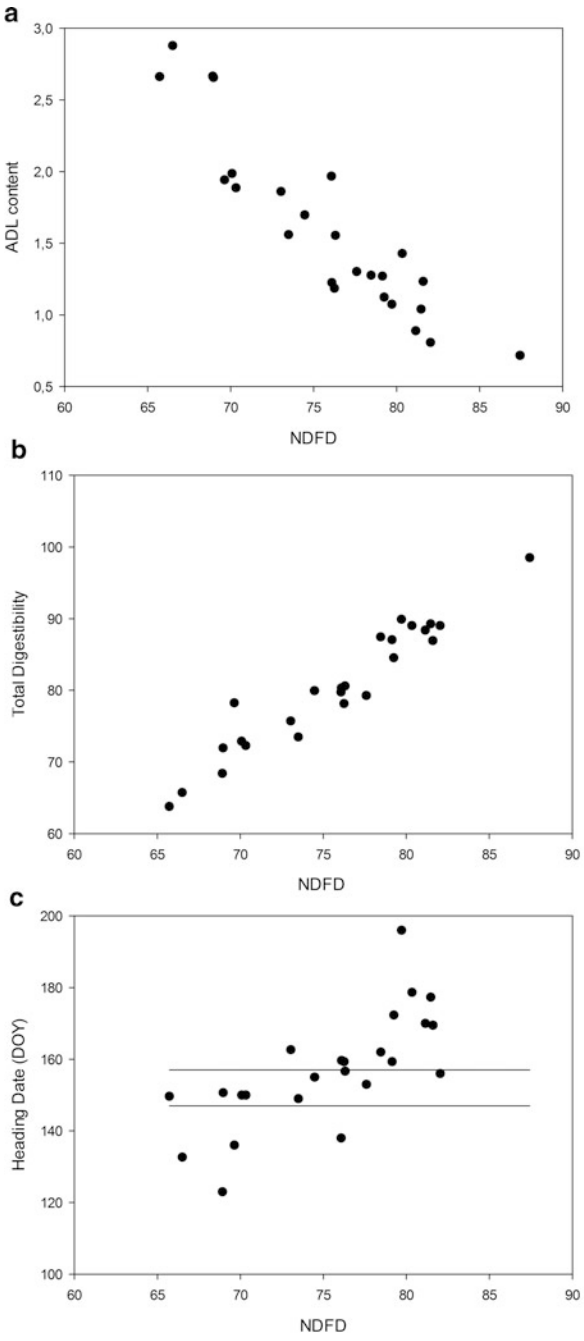

Fig. 34.2 Correlation between NDFD values (percentage of total NDF) of stem material of the June 2010 cut of 30 genotypes. **a** ADL content (percentage of absolute dry weight); **b** total digestibility (percentage of absolute dry weight); **c** heading date (DOY: day of the year recorded in 2011). *Dotted horizontal lines* represent the division into early, intermediate and late heading genotypes

genotype (ABE). This method allows thus to study spatio-temporal differences in cell wall digestibility within one plant and among genotypes.

Given the identified (and expected) lower cell wall digestibility in stem material in the ABE genotype, 30 genotypes (Set 2) covering a broad a range of total digestibility and WSC values was studied more closely in order to identify the range of cell wall digestibility in the stem fraction. Our aim was to identify prototypes with extreme values for quality parameters as WSC, total digestibility and NDFD. The cut made in June 2010 on Set 2 was separated in leaf and stem material. NIRS was used to determine NDFD (NDF digestibility), total digestibility, NDF (cell wall fraction), ADF and ADL (lignin fraction) of stem material. NDFD values varied between 66 and 87 % and were significantly correlated (Fig. 34.2) with other quality parameters such as lignin content ($r = 0.905$) and total digestibility ($r = 0.927$). A lower, but significant correlation was identified also between NDFD and heading date ($r = 0.721$). Despite the correlation found between NDFD and heading date, variation useful for breeding purposes could be observed within each maturity class. For example, the NDFD of intermediate heading genotypes (between DOY [Day of the Year] 147 and 157) varied between 65.7 and 82.0 %, indicating available genetic variation for NDFD within this maturity class.

34.4 Conclusion

The small-scale saccharification assay used here was able to identify significant differences in cell wall digestibility among genotypes and among different plant parts. This will enable the study of the spatio-temporal evolution of cell wall digestibility in single genotypes, and to compare genotypes.

Significant variation for NDFD was observed in stem material of a June cut in a set of 30 genotypes. A high correlation was identified between NDFD and heading date, indicating that caution is necessary when comparing results for cuts of plants of different maturity classes. On the other side, our data suggest that sufficient variability for NDFD might be available within maturity classes, and that selection for higher NDFD is possible. In addition, Set 2 has been harvested in 2011, taking into account maturity (results not shown). If differences among genotypes are confirmed in 2011, we will be able to conclude that genetic variation for NDFD exists in *Lolium* that can be explored for breeding purposes.

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Chapter 35
Variability Among Accessions of Forage Vetch for Basic Agronomic and Morphological Traits under Agro-Ecological Conditions of Serbia

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Abstract The vetches are important annual forage crops in Serbia. Forage and seed production, as well as morphological traits of 33 accessions of common vetch (*V. sativa*) and eight accessions of hairy vetch (*V. villosa*), were estimated in the agro-ecological conditions of Serbia. The accessions were obtained from South Australia where they had been bred or cultivated in the past 20 years. Field nurseries were established at the Institute of forage crops in Krusevac in the autumn of 2008 and 2009, with 40 spaced plants per accession. In the spring of 2009 and 2010, morphological traits, and forage and seed yield components were measured. The results for both years for all traits were analyzed by ANOVA. A cluster analysis was performed on the basis of all investigated traits, using the complete linkage method with Euclidean distances. The results indicated a high level of variability of the accessions and a high potential for genetic improvement of all investigated traits.

Keywords Variability · Agronomic traits · Morphology · Vetch

35.1 Introduction

Among annual legumes, peas and vetches are a very significant source of quality forage in Serbia, ranking directly after soybean. They are grown on about 35,000 hectares (SYB 2009). Their importance is based on a high adaptability to different growing conditions, the possibility of sowing in different periods of the year, and the production of relatively stable yields. Vetch forage is especially important for small and medium farms, where grown in mixtures with cereals, it is used to produce hay, haylage or silage (Djordjević and Dinić 2003). Breeding of forage vetches in Serbia began in the seventies and has resulted in a significant number of cultivars of winter and spring forms of common vetch (*Vicia sativa* L.) and winter hairy vetch (*Vicia vilosa* Roth.). The main aims in vetch breeding have been to increase forage yield, improve biomass quality and increase tolerance to different

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biotic and abiotic stresses. In breeding programs, local wild populations and domestic landraces were most used, while the usual methods include mass selection, and pedigree and bulk methods from hybrid populations (Mihailović et al. 2010). The aim of this paper is to examine the potential variability of the main agronomic traits of 33 accessions of common vetch and eight accessions of hairy vetch of different origins, but which were grown and had been involved in breeding programs in South Australia for the last 20 years.

35.2 Materials and Methods

The study included a total of 41 vetch accessions. 33 were common vetch (1–33) and eight were hairy vetch (34–41) obtained from SARDI (South Australian Research and Development Institute), Adelaide. Sowing was carried out in the autumn of 2008 and 2009 in space plants nurseries with plant to plant distance 60 × 60 cm and 30 plants per accession. In order to assess winter survival, the number of surviving plants (SP) was determined in the following spring. In the phase of full flowering in the next year, the following measurements on individual plants were made: plant height (PH), green matter yield per plant (GMY), dry matter yield per plant (DMY) and number of lateral branches (NLB). Seed yield components, number of pods per plant (NPP), number of grains per pod (NGP) and number of grains per plant (NGPL) were determined in ten randomly chosen individual plants. For each accession, the weight of 1,000 seeds (1000SW) was determined. Average results for both years for all traits were analyzed by ANOVA. Cluster analysis was performed on the basis of all investigated traits, using the complete linkage method with Euclidean distances (Statistica 5.0, Stat Soft Inc.).

35.3 Results and Discussion

The results presented in Table 35.1 indicate a very high variability of the accessions for all traits, as indicated by the high variation coefficients. As pointed out by Matić et al. (2010), the existence of accessions with desirable traits offers the possibility of crossing and recombination to create new cultivars. Percentage of wintered plants was largely dependent on the accession and ranged from 35 % (34) to 96 % (16), suggesting that a large number of accessions has sufficient resistance to low temperatures and winter conditions. The average height of plants of all accessions was 56.61 cm, which is significantly less compared to the results for the cultivars grown in dense swards under agro-ecological conditions of Serbia (Erić et al. 2007). The average 2-year results for GMY and DMY (67.25 and 20.45 g/plant) indicate high variability and high potential of the accessions for this trait. Most common vetch accessions showed better results than hairy vetch, and accessions no. 2, 3, 6, 12 and 14 had extremely high forage yields which were significantly higher compared

Table 35.1 Average 2-year values (2009–2010) of studied accessions

Accession No.	SP (%)	PH (cm)	GMY (g)	DMY (g)	NLB	NPP	NGP	NGPL	1000 SW (g)
Common vetch									
1	75	64.10	110.06	34.87	10.25	9.25	4.50	41.63	75.00
2	81	85.56	218.13	65.40	11.90	49.00	6.87	336.63	83.90
3	81	76.80	193.16	62.43	8.00	27.67	6.22	172.11	79.90
4	78	75.20	131.66	36.33	9.00	34.30	6.87	235.64	69.30
5	77	78.53	186.8	56.43	8.08	37.08	5.45	202.10	70.70
6	97	76.86	187.56	57.23	10.22	35.67	7.22	257.54	82.30
7	75	64.43	80.93	20.16	9.86	32.00	5.24	167.70	79.60
8	83	61.46	104.73	24.06	10.69	55.54	6.77	376.01	80.10
9	86	55.13	80.40	25.20	11.92	39.58	5.50	217.70	77.20
10	92	47.56	77.60	19.53	8.00	26.39	5.62	148.31	92.00
11	71	45.80	46.96	11.86	8.50	18.00	5.78	104.04	59.90
12	88	64.30	124.73	35.36	11.71	25.57	6.19	158.28	69.90
13	74	63.66	78.06	15.70	9.76	25.71	6.90	177.40	77.00
14	91	82.30	124.23	35.50	8.23	12.62	4.50	56.80	90.90
15	92	65.36	111.50	29.00	7.20	18.40	4.53	83.35	79.60
16	96	52.60	53.20	13.46	6.86	13.00	5.10	66.30	81.10
17	82	53.83	74.83	20.90	8.67	19.78	6.89	136.28	85.70
18	87	55.80	77.80	18.80	9.80	10.60	5.00	53.00	72.50
19	72	53.46	46.86	12.66	6.17	9.50	4.33	41.14	75.50
20	81	50.80	48.66	15.76	9.83	19.67	6.28	123.53	52.40
21	75	41.63	39.96	13.13	7.00	14.43	5.86	84.56	79.90
22	77	42.50	22.6	8.60	6.57	7.43	4.57	34.00	74.20
23	51	46.00	21.93	7.713	7.40	9.60	4.13	39.65	75.70
24	76	45.20	22.26	9.13	8.00	10.80	4.93	53.24	77.10
25	85	52.23	46.33	13.36	8.88	14.25	5.33	75.95	84.00
26	57	56.76	46.50	18.26	8.71	16.57	4.26	70.59	80.10
27	63	50.70	43.73	17.73	7.71	13.57	5.48	74.36	73.60
28	51	44.03	19.86	9.43	9.75	12.00	4.88	58.56	69.90
29	48	50.63	30.63	15.10	7.50	10.00	4.17	41.70	56.90
30	63	53.90	30.13	11.63	8.60	17.80	5.40	96.12	43.50
31	80	54.86	42.33	15.30	12.18	23.55	5.50	129.53	68.00
32	72	48.50	46.20	18.20	7.30	16.80	5.92	99.46	88.00
33	66	51.10	70.20	23.86	7.50	16.83	4.78	80.45	76.50
Hairy vetch									
34	45	43.80	18.36	6.433	11.13	132.13	3.31	437.45	44.80
35	71	70.06	36.70	13.46	14.44	68.11	3.18	216.59	36.60
36	41	42.23	8.20	3.166	12.29	7901	349	161.00	42.35
37	80	50.66	6.90	3.533	1122	8118	351	285.60	41.12
38	90	61.93	28.73	10.06	9.14	90.71	3.05	276.67	44.50
39	47	78.43	44.96	16.46	15.67	65.33	2.59	169.20	40.60
40	63	57.80	15.84	5.70	12	58.67	2.85	167.21	42.90
41	46	61.23	24.36	8.16	10.27	58.00	2.61	151.38	35.40
X		56.61	67.25	20.45	9.23	31.81	4.89	141.86	
CV[a]		21.36	80.28	76.71	24.77	86.49	30.67	69.09	
$LSD_{0.05}$		2.37	3.84	2.02	1.65	1.96	1.59	23.72	
$LSD_{0.01}$		3.13	5.09	2.67	2.18	2.59	2.12	31.39	

SP surviving plants, *PH* plant height, *GMY* green matter yield per plant, *DMY* dry matteryield per plant, *NLB* number of lateral branches, *NPP* number of pods per plant, *NGP* number of grains per pod, *NGPL* number of grains per plant, *SW* weight of 1,000 seedsa

[a]CV refers to variation among plants (genotypes) within accessions

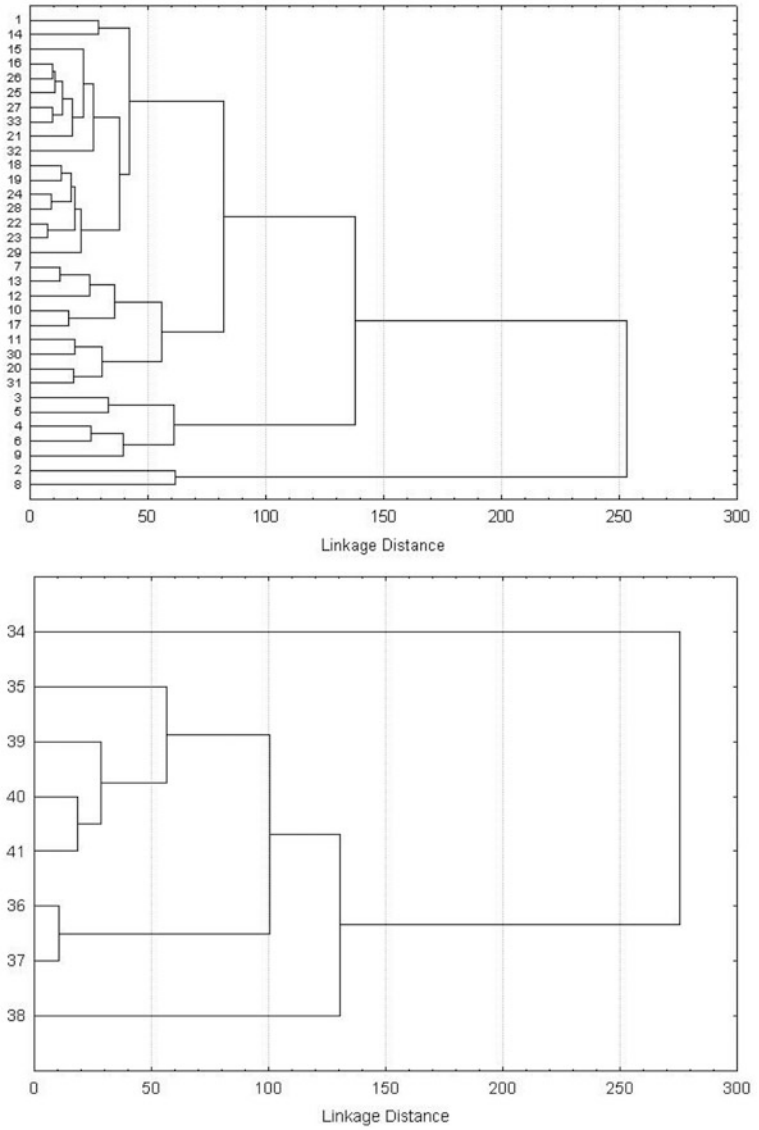

Fig. 35.1 Cluster diagrams for common vetch (*above*) and hairy vetch (*below*) accessions based on the studied traits

to the results for spring vetch by Mihailović et al. (2004). In contrast, hairy vetch accessions showed higher values for parameters of seed yield per plant. Although all accessions of hairy vetch had fewer grains per pod (NGP), due to the large number of pods per plant (NPP) they produced larger number of seeds per plant (NGP). Also, large differences among the accessions for 1,000 seed weight were determined, and

all common vetch accessions had significantly larger seeds than the hairy vetch. The cluster diagram based on the studied traits showed four groups of populations of common vetch. Within breeding germplasm accessions of vetch, populations two and eight were phenotypically the most distant from the rest of the collection and will be considered during further studies. In the dendrogram for hairy vetch, population 34 (SA Haymaker) was clearly distinct from other populations which showed a series of linkage distance (Fig. 35.1).

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Chapter 36
Genetic Variation of Root Characteristics and Deep Root Production in Perennial Ryegrass Cultivars Contrasting in Field Persistency

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Abstract The identification and breeding of genotypes with improved drought tolerance play an important role in developing grasses with better performance and persistence. Plants should maintain some root contact with ground water to maintain cell turgor and to survive during drought periods. Plants with longer roots can better exploit the available, deeper, soil water. Breeding idea is to increase soil water uptake and drought tolerance of perennial ryegrass by improving root architecture with preserved above ground biomass yield. The objectives of this study were to determine variability of root dept and distribution, shoot dry matter yield (DMY) and root/shoot ratio of three *Lolium perenne* cultivars contrasting in filed persistency. The trial was conducted in 0.9 m sand plastic tubes with 30 plants per cultivar in three replications. Plants were irrigated daily with complete nutrient solution and trimmed once after 80 days of growth. The roots were washed out three months after planting, cut in 10 cm increments, dried and weighed. Data were analyzed by standard ANOVA. There were significant differences between cultivars and those with better persistency showed higher proportions of deep roots and 8 % heavier roots in total. Similar root/shoot ratios for all cultivars have showed that plants which have invested more dry matter into root have not automatically had less shoot yield. Genotypes with deeper root systems can be used for drought resistance improvement in *Lolium perenne*.

Keywords Drought resistance · Perennial ryegrass · Root depth · Variability

36.1 Introduction

In recent years long periods of drought have been experienced regularly during summer mounts in Serbia. Therefore soil water deficit tolerance has become an extremely important breeding criterion, especially in all rain fed forage plants such as perennial grasses. Improvement of this complex trait of perennial forage grasses can be achieved in many ways, since plants have many strategies to cope with drought.

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They can "escape" the dry season (with storage organs, rhizomes, dormant buds), adjust growth and development patterns (reduce leaf area, regulate stomatal control, enhance water use efficiency and extract more water) and successfully recover after drought (minimal growth during drought then regrow rapidly when stress had been removed with maintained tiller and plant density) (Ludlow 1980). Very few of these options have been explored within perennial forage grasses. Most attempts to develop more drought-tolerant plants have concentrated on determination of survivors after drought or evaluating genotypes in dry conditions, both in the field, which is time consuming and expensive process. Thought this procedure is initially an efficient process, the later steps in drought breeding should involve methods to accelerate further selection cycles and make them more efficient.

Perennial ryegrass, which is one of the most important forage grasses with many superior characteristics in fodder production, suffers from drought susceptibility which makes it less suitable as a component of Serbian grasslands. However, there are a lot of genotypes found across the Serbian landscape which show better performance in production (Sokolovic et al. 2003, 2007), and which have been incorporated in local cultivars with better persistency (Sokolovic et al. 2010).

Resistance to drought in Serbia in forage perennial grasses has generally been evaluated through observation of prolonged field persistency, determined via the number of plants surviving after several warm and dry summers. This mechanism and strategy is of most benefit to select plants which survive the normal dry periods with high regrowth rates when it rains. Grass plants need to keep some root contact with ground water to survive because they need water constantly to maintain cell turgor. Therefore, our goal was to increase the efficient use of available water through improved root architecture and soil water uptake by looking for perennial ryegrass plants with root systems which can better exploit the available, deeper, soil water.

Drought tolerance is closely related to the distribution and penetration of root systems in the deeper portions of the soil for a number of grass species (Carrow 1996; Huang et al. 1997). Due to relatively high narrow sense heritability for deep root growth (Lehman and Engelke 1991) it is likely that selection for deeper root portions is possible. One important factor what should be taken into account in grass breeding is the high positive correlation between root and shoot characteristics, especially dry matter yield, in a number of species (Palazzo and Brar 1997). Nevertheless, it is possible to obtain, after two cycles of selection, gains both in root production (367 %) and in shoot weight (79 %) in forage type perennial ryegrass (Bonos et al. 2004). It is possible that incorporation of genetic markers associated with rooting characteristics would accelerate such a selection process (Guthridge et al. 2001).

An argument against selecting for a massive root system is that it might consume carbon at the expense of harvestable herbage, either for root growth or maintenance. However, Parsons (1988) has shown that only a small proportion (10–15 %) of assimilate is diverted to roots. Furthermore, during drought there is no shortage of carbohydrate reserves, which can account for up to 30 % dry matter in *Lolium perenne* (Thomas 1991) and 50 % in *Dactylis glomerata* (Volaire 1995). It is known that there is useful variability for root mass and distribution, or root:shoot ratios in perennial ryegrass (Crush et al. 2005, 2007) and other grasses (Bullitta 1996), and

that any genotype which invests more dry matter into root is more drought resistant. Carrow (1996) found a negative correlation between roots in the top 10 cm of soil and drought resistance. Roots at depths of up to 30 cm may mainly play a role in absorption of nutrients, which are concentrated in the topsoil. Deep roots tend to be more important for absorbing water and those plants growth may be constrained by nutrient deficiency, but at least the shoot tissues will receive enough water to keep them alive. Even a few deep roots may be all that is necessary for survival.

Even though results are not totally consistent across all experiments about this topic, the central premise of our breeding scheme for perennial ryegrass in Serbia is to explore root characteristics of persistent cultivars and consequently increase soil water uptake and drought tolerance by improving root architecture, while maintaining above ground biomass yield. The objectives of this study were to determine variability of root dept, root distribution and root/shoot ratio within and between three cultivars of *Lolium perenne* found to contrast in field persistency and performance in previous research (Sokolovic et al. 2010). Given the Serbian climate, the final long-term goal is to select plants that can survive the normal dry periods characteristic of the Serbian climate, and which exhibit with high regrowth rates subsequently when it rains.

36.2 Material and Methods

36.2.1 Plant Material

Three diploid perennial ryegrass cultivars contrasting in field persistency were objects of this research. Serbian cultivar K11 and East European cultivar Mara showed in previous experiments good field persistency and performance, while Cashel showed higher drought susceptibility and plant density reduction (Sokolovic et al. 2010). Seed was sown in containers in fertile potting mix Klasmann TS1® in glasshouse in March 2010. Seedling were transferred in pots after 4 weeks when plants were of an adequate size and grown till late summer 2010. Individual plants of all cultivars were clonally divided on 15th of August into a minimum of four small parts—ramets, each with three tillers. Three ramets were leaf and root trimmed to 2 cm and transferred to the plastic root-screening tubes. Remaining parts of plants were kept in a stock nursery.

36.2.2 Plastic Root Tube Experimental System

The method of root length and deep density evaluation was developed by Bonos et al. (2004) and Crush et al. (2005). The trial was conducted in 0.9 m long polyvinyl chloride (PVC) tubes, 75 mm in diameter (2 mm wall thickness) under greenhouse conditions with 30 randomly chosen plants per cultivar in three cloned replications. All tubes were cut in half lengthwise and rejoined with adhesive tape. Tubes were

inserted in three tables with wire net, each holding 90 tubes. Tubes were put on 10 mm polythene foam on the floor, at an approximate angle of 25° from vertical, filled 5 cm from bottom with gravel for drainage and with washed mortar sand progressively to the top using a spray of water for uniform packing. Mortar sand particles were from 0.5–2 mm in size. Three tiller ramets were planted in the center of tubes and supplied with drip nozzles for irrigation. Sand in every tube was covered with plastic foil to decrease evaporation. Each tube received 100 ml day^{-1} of low strength complete nutrient solution (Blamey et al. 1991, modified for Serbian conditions) in two separate doses (morning and evening) through a pipe system. The shoots were trimmed once after 80 days of growth, just before root analyses, dried and weighed. The roots were extracted after three months of growth by laying the tubes down on plastic 5 × 5 mm mesh and splitting them in two halves. Sand-root columns were rolled out, washed under a water shower and cut into pieces 10 cm in length. Plants with 1 cm of roots were weighed and planted in stock. Root parts were paper dried and weighted, then dried in fan oven at 65 °C over night and weighed.

36.2.3 Statistical Analyses

Measurements and calculations covered shoot dry matter (DM) weight, root DM weight in 10 cm increments and root/ shoot ratio. The data were analyzed by standard ANOVA. Root/shoot ratio has been calculated by ratio of total root DM weight and shoot DM weight. Deep root/shoot ratio has been defined as the weight (g) of roots that are deeper than 30 cm g^{-1} shoots.

36.3 Results and Discussion

There were significant differences in root extension and growth rates between cultivars and those with better persistency showed higher deep root portions (Fig. 36.1).

Evaluated within cultivar variability was very high for all investigated root traits and the cv for root DMY ranged between 22 % in cv. Mara and 28 % in cv. Cashel. There were no statistically significant differences between cultivars for root DM and root/shoot ratio. Significant differences between K11 and Cashel, were scored for proportion of root DM in top10 cm, deep root/shoot ratio and deep roots DM, while significant difference for shoot DM weight between Mara and Cashel was obtained. Statistically high significant differences among K11 and other two cultivars were detected for proportion of plants with roots below 80 cm.

Within cultivars with improved persistency more plants had root portions below 80 cm than Cashel, especially in cv. K11 (40 %; Table 36.1). This indicates that those genotypes should maintain root contact with deep soil water longer during drought period and those few deep roots may play important role in drought survival. Also they

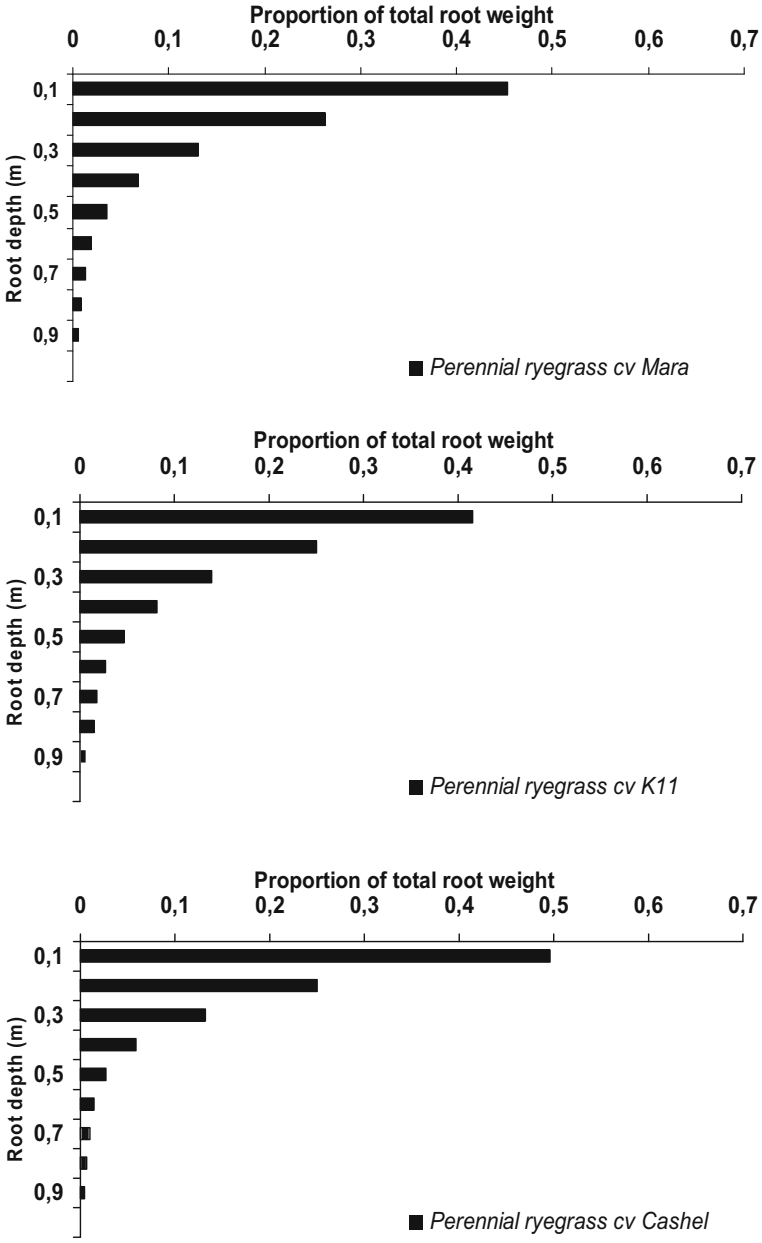

Fig. 36.1 The proportion of total root DM obtained from 10 cm increments from 0.9 m deep plastic sand tubes

Table 36.1 Average shoots and roots weights, proportions and ratios of perennial ryegrass cultivars

Traits cultivars	Shoot DM weight (g)	Root DM weight (g)	Root/ shoot ratio	Proportion of root DM top 10 cm	Deep root/ shoot ratio	Deep roots DM (mg) (below 60 cm)	Plants with root below 80 cm (%)
Mara	0.54	0.64	1.21	45.4	0.18	16.22	43.33
K11	0.51	0.65	1.23	41.6	0.23	22.06	76.67
Cashel	0.48	0.6	1.27	49.6	0.14	9.44	36.67
$LSD_{0.05}$	0.06	0.07	0.11	7.06	0.06	9.6	19.1
$LSD_{0.01}$	0.11	0.11	0.18	11.7	0.1	15.9	31.8

showed 8 % heavier roots in total, but also higher shoot DM yield in similar proportion (both differences are not statistically significant). Consequently, root/shoot ratio was similar for all cultivars, showing that plants which invest more dry matter into root do not have automatically decreased above ground DM yield.

Proportions of roots up to 10 cm deep showed that almost 50 % of roots in cultivar Cashel laid in the shallow soil, which may be important in better nutrients consumption, but it is negatively correlated with drought resistance (Carrow 1996).

More important is that cultivars Mara and especially K11 had about 100 % increased deep roots DM (Table 36.1) which resulted in higher deep root/shoot ratio. These differences obviously resulted in better field persistency, drought tolerance and subsequently in higher DMY of cultivars K11 and Mara. Those two cultivars also had high percentage of plants with root reaching below 80 cm, respectively. All such plants are fitted in selection model and can be used for *Lolium perenne* drought resistance breeding in Serbia.

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Chapter 37
The Study of Similarities Among *Medicago sativa* L. Accessions

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Abstract *Medicago sativa* is grown on 65,000 ha of agricultural land in the Czech Republic. To produce high quality forage with high protein content *Medicago sativa* is a crucial fodder species and thus takes priority by Czech breeders. Descriptions of forage species of the family *Fabaceae* have been carried out in the Research Institute for Fodder Crops, Ltd. under the project "National Programme on Conservation and Utilization of Plant Genetic Resources and Agro-biodiversity". 32 accessions (varieties) of Medicago sativa from four states (Czech Republic, France, Yemen, Hungary), were evaluated as spaced plants. 30 plants of each variety were planted on the field in the year 2009. In the year 2010 51 morphological, yield and qualitative characters were evaluated on ten plants of each origin. The average values of the observations per character and accession created the input matrix for evaluation by the method of cluster analysis. Similarities among accessions were determined on the basis of these results and correspond more or less to the origin of accessions and the geographical distance.

37.1 Introduction

Nowadays alfalfa covers 65,000 ha of agricultural land in the Czech Republic (Czech Statistical Office 2011). Despite the present trend towards a reduction of the alfalfa growing areas, alfalfa along with corn belongs to the high-yielding crops in the Czech Republic. Its advantages are predominantly relatively stable and high yield, drought resistance and persistence. From the nutritional point of view alfalfa is appreciated for its high digestible protein yield per ha and its low cost per kg produced digestible (Hrabě et al. 2004). In alfalfa breeding programs the knowledge of similarities between cultivars is very valuable. Genetic similarities of alfalfa were studied, for example by Wang et al. (2011), Julier (2010), Tucak (2008) and morphological similarities were examined by Benabderrahim et al. (2009) and others.

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37.2 Materials and Methods

In the year 2010 a population of 32 alfalfa varieties from four countries was assessed. The population included 14 varieties from the Czech Republic, five varieties from France, five varieties from Yemen and eight varieties from Hungary. The evaluations were made both in individual plantings and in the stand. In individual plantings 10 plants of each accession were evaluated. Stands were evaluated in the parcels of 10 m^2, established by the method of randomised blocks. The varieties from Yemen were evaluated only in individual plantings because they are winterkilled in these climatic conditions. The evaluation was based on 51 characters (Table 37.1). Weighed, calculated and measured traits were converted using a classifier into numbers on a nine point scale and the other traits were assessed directly using a classifier (Vacek et al. 1985). Cluster analysis was performed in the software Statistica for Windows (Statsoft, Inc. 2003) for all the accessions together. The complete linkage method was used for clustering and Euclidean distance as the measure of distance.

37.3 Results and Discussion

As you can see in Fig. 37.1 the greatest similarity was found between the varieties Reimance and Saatry from Yemen, followed by two pairs of Czech varieties. These were the pairs of Holyna and Denisa; and Litava and Niva. In contrast, significantly different from the entire population of varieties was the Czech variety Palava. In fact, five clusters were formed. The first of them contained only the varieties from Yemen. Another cluster was composed of two Czech, two Hungarian and two French varieties. The varieties of this cluster excelled in leaf area, number of pods per fruit and dry weight of bunch. The largest cluster included ten Czech varieties and the French variety Julia. In another cluster there were Hungarian varieties and the Czech variety Jitka and the French variety Harpe. The varieties in this cluster exceeded the other clusters in terms of terminal leaflet length, number of pods per 100 flowers and also height and width of pod spiral. The last cluster was composed of the Hungarian variety Alexandra, the French variety Exquise and the Czech variety Palava. Table 37.2 shows that the lowest variation in three traits was achieved by the Czech varieties Holyna (green biomass weight of bunch, dry weight of bunch and number of pods per fruit) and Morava (number of internodes, length of central internodes and seed weight per plant). The French variety Timbale and the Hungarian varieties KM-Maraton, Alexandra and KM-Gyongy showed the lowest variation in two traits. On the other hand, the greatest non-uniformity was found in the variety Wasany from Yemen (leaf area size, stem thickness, number of internodes and number of lateral branches), the Czech variety Denisa (length of central internodes, inflorescence length and seed weight per plant) and the Czech variety Jarka (terminal leaflet length, stem length and pod spiral width).

Table 37.1 Weighed, calculated, measured and scored characters

Morphological, biological and economic characters	Weighed, calculated and measured characters	Scored characters
Shape of leaf rosette in autumn		X
Density of leaf rosette		X
Number of stems in a bunch	X	
Stem length	X	
Stem thickness	X	
Shape of stem in cross section		X
Stem hollowness		X
Stem colouration		X
Number of internodes on stem	X	
Length of the middle internode	X	
Number of lateral branches on stem	X	
Shape of the terminal leaflet		X
Margin of the terminal leaflet		X
Terminal leaflet tip		X
Terminal leaflet length	X	
Terminal leaflet width	X	
Terminal leaflet area	X	
Leaf colour		X
Leaf pubescence		X
Leaf area	X	
Occurrence of compound leaves	X	
Inflorescence shape		X
Inflorescence length	X	
Number of inflorescences per stem	X	
Number of florets per inflorescence	X	
Inflorescence colour		X
Number of pods on the stem	X	
Number of pods per inflorescence	X	
Number of pods per 100 flowers	X	
Pod shape		X
Pod colour		X
Height of pod spiral	X	
Width of pod spiral	X	
Number of seeds per pod	X	
Seed shape		X
Seed colour		X
1,000-seed weight	X	
Resistance to winterkill		X
Stand height at the start of blooming	X	
Stand height in full bloom	X	
Stand height 20 days after the first cut	X	
Stand re-growth	X	
Number of cut per year	X	
Total green biomass weight	X	
Green matter yield per plant	X	
Total hay yield of stand	X	
A ratio of hay yield at the first cut to total hay yield per year	X	
Seed yield per stand	X	
Seed weight per plant	X	
Content of nitrous substances	X	
Fibre content	X	

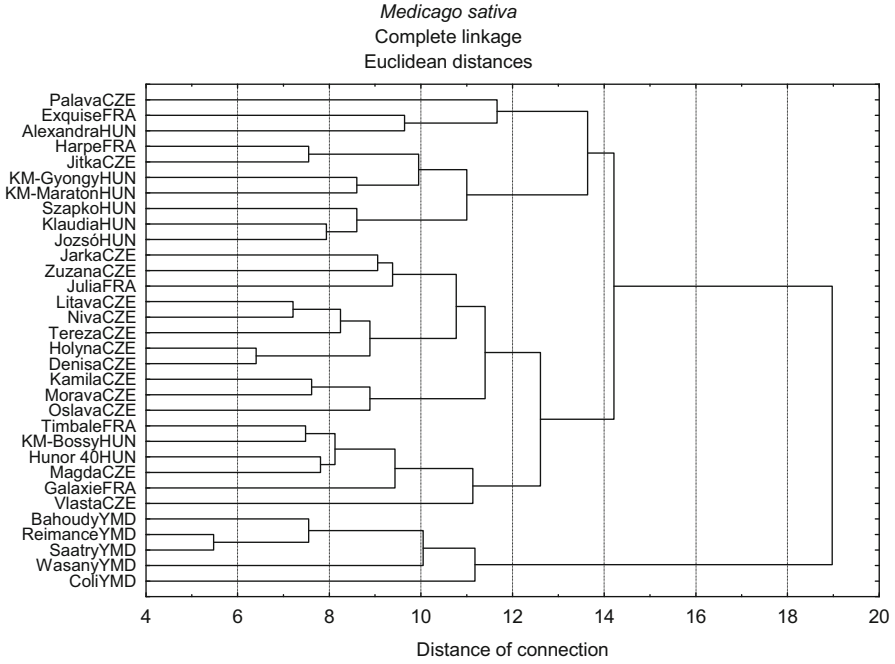

Fig. 37.1 Dendrogram of accessions of *Medicago sativa* L.

Table 37.2 Survey of maximum and minimum values of coefficients of variation as level of uniformity in measured, weighed and calculated traits

Characters	Variety	V_x (%) min	Variety	V_x (%) max
Terminal leaflet length (mm)	KM-Maraton	5.28	Jarka	19.98
Terminal leaflet width (mm)	KM-Gyongy	8.33	Tereza	27.19
Leaf area (cm^2)	Alexandra	13.40	Wasany	35.64
Terminal leaflet area (cm^2)	Alexandra	12.57	Jozsó	31.98
Stem length (cm)	Zuzana	6.09	Jarka	31.61
Stem thickness (mm)	Timbale	7.38	Wasany	34.48
Number of internodes on stem	Morava	9.13	Wasany	30.02
Length of the middle internode (cm)	Morava	12.30	Denisa	37.00
Number of lateral branches on stem	Jozsó	11.28	Wasany	43.70
Green matter yield per plant (kg)	Holyna	17.07	Saatry	72.8
Number of stems in a bunch	KM-Maraton	19.73	Bahoudy	75.96
Dry matter yield per plant (kg)	Holyna	16.54	Saatry	84.35
Inflorescence length (cm)	Timbale	13.41	Denisa	50.03
Number of inflorescence on stem	Kamila	20.24	Vlasta	74.36
Number of florets per inflorescence	Galaxie	20.60	Reimance	53.91
Number of pods on the stem	Litava	24.78	Palava	101.91
Number of pods per inflorescence	Holyna	22.05	Vlasta	59.83
Number of pods per 100 flowers	Jitka	6.69	Palava	22.21
Height of pod spiral (mm)	KM-Gyongy	11.13	Morava	39.95
Width of pod spiral (mm)	Szapko	70.94	Jarka	20.62
Number of seeds per pod	Denisa	21.88	KM-Bossy	57.68
Seed weight per plant (g)	Morava	38.73	Denisa	118.43

37.4 Conclusions

On the basis of morphological and yield characteristics the studied population may be split into clusters, which correspond more or less with the countries of origin. These results can be further used in crop breeding programmes.

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Chapter 38
Genetic Structure and Agronomic Value of Italian Lucerne Landraces: A Synopsis

P. Annicchiarico

Abstract Because of their long history of cultivation and good characterization of collecting sites, lucerne landraces from northern Italy are particularly useful for studies of genetic structure, evolutionary adaptation to specific growing conditions and comparison with modern germplasm, which can drive the efficient management and exploitation of landrace germplasm. This work aims to summarize the information from such studies. Each study included ten to 13 farm landraces belonging to the seven former commercial ecotypes of northern Italy, and four to seven well-adapted varieties. Each landrace was represented by over 250 single plants grown in dense stand under field conditions or in four artificial environments created by the combination of almost nil or high summer drought with sandy-loam or silty-clay soil. On average, landrace germplasm was at least as performing as variety material for forage yield and persistence over two or three years. Landraces exhibited specific adaptation towards drought-stress levels similar to those of their environment of origin. No mean difference between landrace and variety germplasm emerged for forage quality (as estimated by the leaf:stem ratio) or seed yield. The estimated genetic variance within population was always much larger than that among populations. These results support the exploitation of landrace germplasm through breeding procedures able to exploit its remarkable within-population diversity and its adaptation to specific environments.

Keywords Genetic resources · Genetic variation · Genotype × environment interaction · Forage quality · Forage yield · *Medicago sativa* · Plant adaptation · Seed yield

38.1 Introduction

Lucerne (*Medicago sativa* L. subsp. *sativa*, 2n = 4x = 32) was introduced to Italy from the Middle East around the second century BC and then, after its extensive cultivation during the Imperial Age and its disappearance during the Barbarian invasions, was introduced again from Spain in the sixteenth century (Prosperi et al. 1995).

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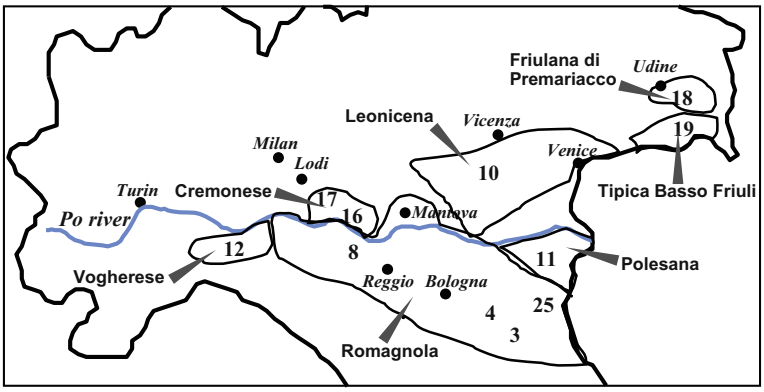

Fig. 38.1 Site of origin of eleven farm landraces and production area of seven commercial ecotypes of lucerne

Lucerne landraces were extensively grown in Italy until recently. Landrace seed, grouped into 14 commercial ecotypes depending on its area of production, accounted for about 62 % of the Italian seed market in 1997 (Annicchiarico et al. 2007). The ban to commercialization of landrace seed from 2003, justified by the lack of effective control on its origin, was accompanied by the collection of landraces with a long track of multiplication in the same farm. The Italian history of landrace cultivation contrasts with that of most European countries, whose earlier ban to marketing of landrace seed (e.g. from 1970 in France) has led to much greater loss of indigenous genetic resources (Julier 1996).

Because of their long history of cultivation and the large availability of accessions with well-known environment of origin, lucerne landraces from northern Italy are particularly useful for studies of genetic structure, evolutionary adaptation to specific growing conditions, and comparison with modern germplasm. The objective of this work was to summarize the available information provided by such studies, whose results can drive the efficient management and exploitation of landrace genetic resources.

38.2 Materials and Methods

Each relevant study included at least one landrace for each of the seven former commercial ecotypes of northern Italy, along with various well-adapted varieties. The landraces were collected from farms that had long been registered as seed donors for the relevant ecotype and had been multiplying their own seed for at least 20 years. The collecting site of eleven landraces and the production area of the seven commercial ecotypes are reported in Fig. 38.1. Dense planting was adopted in all studies, given the only moderate reliability of spaced planting for assessing production traits (Annicchiarico 2006a).

One study reported in Annicchiarico (2006b) included 4480 unreplicated genotypes that derived from seed of eleven landraces and seven varieties, as well as 128 clones of a single control genotype used to estimate the environmental variance. Each landrace was represented by 256 or 512 plants. Seedling and clonal material was subdivided into 128 different units (grids), each including one clone and 35 plants with a fixed proportion for each population. The 128 units were arranged in four parallel columns of 32 adjacent units, readjusting the data for the effects of column of units, row of units and column of plants as described in Annicchiarico (2004). The adopted plant density was 100 plants/m^2. This study allowed comparing landrace and variety germplasm for mean value and for among-population and within-population genetic variance relative to dry matter (DM) yield and final survival over 2 years and other agronomic traits. A second study based on a subset of this material, i.e. 10 landraces and 6 varieties, investigated the genetic variation for forage quality as estimated by the leaf:stem ratio (Annicchiarico 2007a).

Another study including 13 landraces and 4 varieties compared landrace and variety germplasm for three-year DM yield, survival and adaptation pattern across 4 large artificial environments ($24.0 \times 1.6 \times 0.8$ m deep) created by the factorial combination of almost nil or high summer drought with sandy-loam or silty-clay soil. These environments were able to reproduce the adaptive responses of lucerne cultivars across agricultural environments of northern Italy (Annicchiarico and Piano 2005; Annicchiarico 2007b). Each environment included four replications, and each plot comprised 70 plants grown at the density of 178 plants/m^2. The genetic variation for seed yield of this material was investigated in one environment similar to those frequently used for seed production (Annicchiarico et al. 2007).

The genetic structure for adaptation pattern of landrace germplasm was estimated here from DM yield data reported in Annicchiarico (2007c). These data relate to 18 parent genotypes, 6 for each of 3 landraces with contrasting adaptation pattern, evaluated on the basis of their half-sib progenies grown in the 4 artificial environments with contrasting soil type and drought stress level. Among-population and within-population components of variance relative to main effects and genotype × environment (GE) interaction of the 18 parents were estimated by a REML procedure, considering the GE interaction variation for yield response as an indicator of the variation for adaptation pattern.

38.3 Results and Discussion

On average, landrace germplasm was at least as performing as variety germplasm for forage yield and final persistence over 2 or 3 years (Table 38.1). The assessment of forage yield components in one experiment indicated the trend of landraces towards less stems per plant and shorter stems which was compensated, however, by greater plant survival (Table 38.1). The study in artificial environments revealed large variation in adaptation pattern among landrace populations which was mainly due to cultivar × drought stress interaction (Annicchiarico and Piano 2005). Better yield

Table 38.1 Mean value, and ratio of within-population (s^2_W) to among-population (s^2_A) genetic variance, for dry matter (DM) yield and other traits of landrace (LAN) and variety (VAR) germplasm groups

Trait	No. of entries		Mean value[a]		s^2_W/s^2_A ratio		
	LAN	VAR	LAN	VAR	LAN[b]	VAR[b]	Reference[c]
DM yield (2 years; g/plant)	11	7	9.51	8.75**	25.6**	11.1**	*A*
Main stem length (cm)	11	7	57.5	58.8*	18.8**	15.1**	*A*
No. stems/plant	11	7	6.01	6.46**	10.3**	6.6*	*A*
Plant survival (2 years; %)	11	7	79.8	64.2**	–	–	*A*
No. florets/inflorescence	11	7	17.2	17.1 ns	70.5**	4.5*	*A*
Leaf-to-stem ratio	10	6	1.04	1.00 ns	48.0**	7.3**	*B*
DM yield (3 years; t/ha)	13	4	36.5	36.9 ns	–	–	*C*
Plant survival (3 years; %)	13	4	40.1	41.8 ns	–	–	*D*
Seed yield (t/ha)	13	4	1.11	1.06 ns	–	–	*E*
Adaptation pattern[d]	3	–	–	–	21.8**	–	*F*

[a]Mean comparison: *ns* not significant $*p<0.05$; $**p<0.01$
[b]Within-group comparison of s^2_W vs. s^2_A $*p<0.05$; $**p<0.01$
[c]*A* Annicchiarico (2006b), *B* Annicchiarico (2007a), *C* Annicchiarico and Piano (2005), *D* Annicchiarico (2007b), *E* Annicchiarico et al. (2007), *F* estimated from data in Annicchiarico (2007c)
[d]As genotype × environment interaction for DM yield in 3 harvests spanning over 2 years

response under stress conditions was closely related to the level of summer drought at collecting sites (Annicchiarico and Piano 2005). Greater plant survival tended to parallel the landrace adaptation to specific environments (Annicchiarico 2007b), but was also related to more frequent mowing at collecting sites (Annicchiarico 2006b).

Landrace and variety germplasm performed comparably also for forage quality (as expressed by the leaf:stem ratio), seed yield, and one seed yield component, i.e. the number of florets per inflorescence (Table 38.1). Landrace seed yield was positively associated with frequent mowing and earlier year of seed production on its farm of origin (Annicchiarico et al. 2007).

Within-population variation was distinctly larger than among-population variation in both landrace and variety germplasm but its relative extent was larger in landraces (Table 38.1), as expected from their wider genetic base. Despite the large variation among landraces for adaptation pattern, within-population variation was much larger also for this trait on the grounds of half-sib responses of the 18 genotypes sorted out from 3 landraces with contrasting adaptive response (Table 38.1).

The good agronomic value of Italian landrace germplasm in specific agricultural environments has been confirmed by studies including also landraces and varieties from central Italy (Russi and Falcinelli 1997; Annicchiarico et al. 2012), or landraces from Sardinia and Sicily and varieties from Europe, Australia and USA tested in north-African and European locations (Annicchiarico et al. 2011). On the whole, these findings suggest that the widespread historical adoption of landraces by Italian farmers was also due to their good forage yield and persistence besides the lower cost of their seed. The difficulty of modern varieties to distinctly outperform landraces

and old cultivars emerged not only in Italy but also elsewhere, for example in USA, where lucerne breeding programmes improved mainly the tolerance to diseases while failing to achieve substantial progress for intrinsic forage yielding ability or forage quality (Lamb et al. 2006). Various factors may account for the low rate of yield progress in lucerne relative to other crops, including its out-crossing mating system, autotetraploidy, perennial growth cycle, and high rate of non-additive genetic variance arising from gene interaction (Bingham et al. 1994; Brummer 1999).

Another factor hindering lucerne breeding progress is the high GE interaction, which is widespread also in varieties and whose magnitude has been revealed also by the site-specific nature of quantitative trait loci for forage yield (Robins et al. 2007). The adaptation of Italian landraces and varieties to moisture-favourable or drought-prone environments was related to different and partly incompatible adaptive traits, complicating the selection for wide adaptation (Annicchiarico 2007b).

On the whole, the information summarized here supports the exploitation of landrace germplasm through breeding procedures able to exploit its remarkable within-population diversity and its adaptation to specific environments, especially moisture-favourable or drought-prone ones.

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Chapter 39
The Use of Genebank Accessions in the Breeding Programme of *Lolium perenne*

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Abstract Using genetic resources could add some valuable genes to the genepool used in the breeding programme. In 2005 we carried out paircrosses between material from the breeding pool and 30 accessions from the ECP/GR *Lolium* core collection with the aim of enhancing the heterosis and introducing valuable genes. The accessions were selected based on geographical spreading of the collection sites, on the genetic diversity analysed by AFLP markers and on the data generated on the *Lolium* core collection experiment. The F1 seeds from the paircrosses were multiplied to synthetic progeny families in 2007. From 23 multiplications we obtained enough seeds to sow a yield trial in May 2008. The trial was cut five times in 2009 and 2010. The dry matter yield was measured and the rust resistance and sod density were evaluated. In 2009 samples were taken at all cuts to determine the contents of water soluble carbohydrates, digestible organic matter and crude protein. None of the progenies performed better than the standard varieties (Aberavon, Barata, Cancan and Premium). Only one of the progenies had a total dry matter yield that was similar to the total dry matter yield of the standard varieties. The progenies of the crossings between the genebank accessions and the breeding material showed no heterosis effect on dry matter yield. Within the five best performing progenies we will carry out further selections.

39.1 Introduction

There are thousands of accessions of forage crops stored in genebanks all over the world. In most cases the description is limited to collection data and no evaluation data are available. Due to the huge amount of accessions and the limited information of these genetic resources choosing the appropriate accessions for breeding purposes is difficult. Nevertheless, using genetic resources could add some valuable genes to the genepool used in the breeding programme. In this study we crossed accessions from the ECP/GR *Lolium* core collection with perennial ryegrass material from our breeding programme. The progenies were tested in a yield trial.

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39.2 Material and Methods

Under the aegis of the Forages Network of the ECP/GR (European Cooperative Programme for Crop Genetic Resources Networks), a core collection of 162 populations of perennial ryegrass has been formed and evaluated at 19 sites, including at ILVO (Sackville Hamilton et al. 1998). None of the plants of this evaluation trial were used in crossings in the breeding programme at ILVO. In 2005 we decided to carry out paircrosses between material from the breeding pool and 30 accessions from the core collection with the aim of enhancing the heterosis and introducing valuable genes. The accessions were selected based on geographical spreading of the collection sites, on the genetic diversity analysed by AFLP markers (Calsyn et al. 2005; Ghesquiere et al. 2003) and on the data generated during the *Lolium* core collection experiment (Sackville Hamilton et al. 1998; Edmondson 2005 and Humphreys, personal communication). Since there was no seed available from the Polish accessions, we had to exclude these from the experiment. All the early and intermediate heading accessions were crossed with plants of the intermediate heading ILVO variety Plenty, the late accessions were crossed with late material from our breeding programme. From each accession we performed three paircrosses in crossing cells. The seeds were harvested in the summer of 2005. Crosses from 5 accessions were discarded because of a too large difference in heading date between the two plants in the crossing cell, a too high disease susceptibility or a poor seed set. The three paircrosses of each of the 25 accessions of the *Lolium* core collection with material from our breeding pool were multiplied together. Therefore we have grown 72 F1-seedlings per cross, half of them harvested on the three plants of the genebank accession, the other half on the three plants of the breeding pool material. These 72 plants were allowed to freely interpollinate in collective isolations, yielding 3 F1 based synthetic seed, which was harvested in bulk in the summer of 2007. Seed yield, thousand seed weight and germination capacity of the seeds were measured. From 23 multiplications we obtained enough seeds to sow a yield trial. Table 39.1 shows the country of origin of the accessions used in the paircrosses, in the 3 F1 based synthetics and in the yield trial.

In May 2008 we have sown a yield trial to evaluate the performance of the 23 progenies. The trial was cut 5 times in 2009 and 2010. The dry matter yield (DMY) was measured at all cuts and the rust resistance and sod density were evaluated. In 2009 we took samples at all cuts to determine the contents of water soluble carbohydrates, digestible organic matter and crude protein. These analyses were performed by Near Infrared Spectroscopy (NIRS).

39.3 Results and Discussion

The mean seed yield per plant harvested on the 3 paircrosses varied from 16 to 894 seeds (a crossing between a late French population and a late breeding population and between an intermediate French population and Plenty respectively). In the 3 F1 based synthetics there was also a large variation in the number of germinating seeds

Table 39.1 Number of accessions per country of origin used in paircrosses with material from the breeding programme, in the 3 F1 based synthetics and in the yield trial

Country of origin	Number of accessions		
	In paircrosses	In synthetics	In yield trial
Belgium (BE)	1	1	1
Bulgaria (BG)	1	1	1
Czech Republic (CZ)	1	1	1
France (FR)	5	5	5
Germany (DE)	4	4	3
Greece (GR)	1	0	0
Hungary (HU)	3	3	2
Ireland (IE)	1	0	0
Italy (IT)	1	1	1
Norway (NO)	1	1	1
Romania (RO)	3	3	3
Spain (ES)	2	2	2
Switzerland (CH)	1	0	0
The Netherlands (NL)	1	1	1
United Kingdom (GB)	4	2	2

harvested per plant, varying from 185 (Hungarian population) to 2482 (Norwegian population).

On average the total DMY was significantly lower for the progenies of the ecotypes than for the standard varieties (Aberavon, Barata, Cancan and Premium) in 2009 (9.08 and 10.29 ton/ha respectively). In 2010 the mean DMY did not differ significantly: 8.22 ton/ha for the progeny families compared to 8.48 ton/ha for the standard varieties. Figure 39.1 shows the total DMY in both testing years. The progenies of a crossing between an intermediate Spanish accession and Plenty (ES2) was yielding as much as the standard varieties in both years.

The sod density in early spring 2010 was on average similar for the varieties and progenies of the ecotypes. The Hungarian, Norwegian and Romanian progenies tended to have a better sod density after the winter. Since the sod density and the DMY are similar for the standard varieties and the progenies of the ecotypes in the second harvesting year, we can assume that most of the ecotypes have a good persistency.

On average there was no difference between the rust resistance of the progenies of the ecotypes and the standard varieties. One German, two French and the Norwegian progenies were more susceptible than the standard varieties.

The digestible organic matter (DOM) was similar for the varieties and the progenies of the accessions (80.9 and 80.3 respectively). The crude protein content (CP) tended to be higher for the progenies versus the standard varieties (13.3 and 12.5 respectively), but the water soluble carbohydrates (WSC) tended to be higher for the varieties (20.0 versus 18.5). There was a large variation in WSC ranging from 18.6 (Barata) to 21.4 (Aberavon) for the standard varieties and from 15.1 (Romanian progeny) to 20.7 (Spanish progeny) for the progenies of the ecotypes. Table 39.2 gives an overview of the results of the rust resistance and the sod density evaluation and the mean content of WSC, DOM and CP.

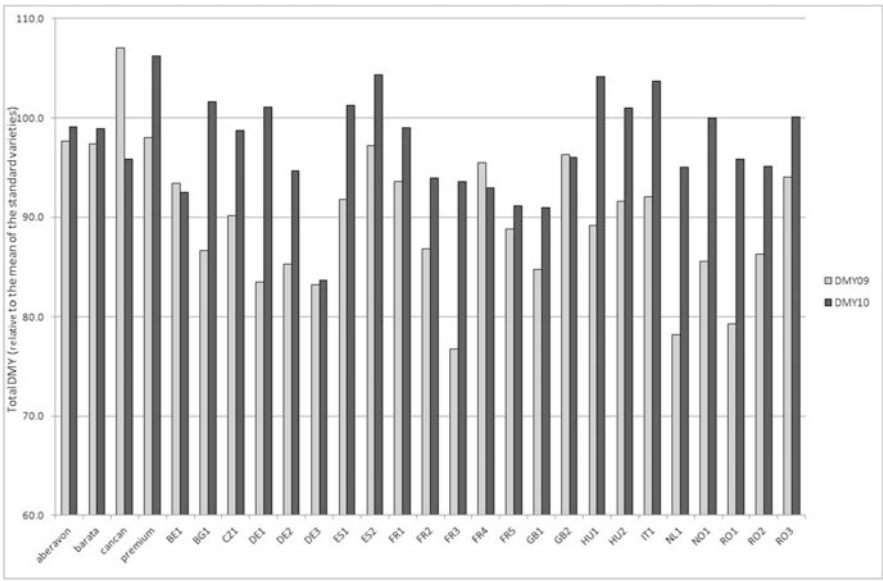

Fig. 39.1 Total dry matter yield (relative to the mean of the standard varieties Aberavon, Barata, Cancan and Premium) of the standard varieties and the progenies of the crossings between European accessions and material from our breeding programme in 2009 and 2010

Table 39.2 Results of the rust resistance and the sod density evaluation (scoring from 1 to 5; 5 = good) and the content of water soluble carbohydrates, digestible organic matter and crude protein of the standard varieties (Aberavon, Barata, Cancan and Premium) and the progenies of the crossings between European accessions and material from our breeding programme

	Standard varieties		Accessions	
	Mean	Range	Mean	Range
Rust resistance	3.3	3.0–3.7	3.0	1.0–4.3
Sod density	3.2	2.0–4.0	3.2	2.3–4.3
Digestible organic matter	80.9	80.0–82.9	80.3	78.0–82.1
Water soluble carbohydrates	20.0	18.6–21.4	18.5	15.1–20.7
Crude protein	12.5	12.0–12.9	13.3	12.5–14.5

None of the progenies performed better than the standard varieties and we will not create directly new candidate varieties. Within the five best performing progenies (CZ1, ES2, GB2, HU2, RO3) we will carry out further selections.

39.4 Conclusion

Using genetic resources in the breeding programme can add some valuable genes to the genepool. Due to the limited information of these genetic resources it is hard to choose the appropriate accessions. No heterosis effect on dry matter yield after

crossing diverse accessions with material from our breeding pool was observed. In this study the progenies of some crossings had a good persistency and will be used for further selections.

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Chapter 40
Characterization and Evaluation of Genebank Accessions as a Pre-selection Instrument for Plant Breeding Objectives and Strategies

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Abstract The federal German *ex-situ* collection of agricultural and horticultural plants at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)—the IPK Genebank—with its headquarters in Gatersleben and a satellite collection for fodder crops at Malchow/Poel, is one of the most comprehensive plant genetic resources (PGR) collections worldwide. In addition to collection, conservation, documentation and distribution activities, the characterization and evaluation comprises a major part of the work at the IPK Genebank, particularly in field trials of collected material with common geographical origin or habitat. Evaluated traits of grasses include plant development before and after winter, growth type, length and width of flag leaf and plant height or resistance to diseases. We assessed Irish *Lolium perenne* accessions for relevant breeding traits to give an example of IPK Genebank activities. On the basis of the monitored traits we are able to predict the behavior of plants against abiotic stress (e.g. drought, late frost and winter hardiness), serving as a pre-selection instrument for different plant breeding objectives and strategies.

Keywords *Lolium perenne* L. · Diseases · Abiotic stress

40.1 Introduction

The Malchow site of the IPK Genebank harbours the oilseed and fodder crop collections of the Leibniz Institute of Plant Genetics and Crop Plant Research. The Malchow collection has more than 14,000 accessions with 17 genera and 155 species of grasses, crucifers and legumes. In addition to maintenance and supply, a very important aim of the genebank is the scientific characterization and evaluation of the plant genetic resources over decades, in order to provide breeders and scientists with high-quality and well described seed/plant material. The *L. perenne* collection contains some of the best characterized and evaluated accessions of the IPK Genebank.

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In the last two decades, an initiative between the IPK Genebank and German grass breeders was started to cooperate in collecting trips and plant material multiplication, as well as in characterization and evaluation trials. As result of this initiative, the IPK Genebank obtained new seed samples (ecotypes) that increased the range of diversity within the genebank collection. The grass breeders, on the other hand, benefitted from this cooperation by receiving new potential (well-adapted) starting material (Boller et al. 2010).

40.2 Material and Methods

153 Irish *L. perenne* accessions from two different collection trips by Dr. V. Conolly/Teagasc (1981/1982) and by Dr. U. Feuerstein/Euro Grass Breeding and E. Willner/IPK Genebank in 2002 were evaluated and characterized in the Malchow Satellite Collection of the IPK. The material was split into three parts: 98 accessions from the 1981/1982 trip, 55 genebank accessions from the 2002 collection trip and 8 cultivars as standards (Aberelf, Barcley, Cancan, Fennema, Gladio, Juwel, Respect and Sambin). The plants were cultivated in the field in 2008–2010. For each genotype, 10 plants were grown and repeated four times in randomised blocks.

Quality traits were evaluated each year, including disease susceptibility, growth before and after winter, spring development, heading and flowering date, homogeneity and green matter yield. For evaluating the collected material the most important forage breeding aspects like rust susceptibility, green matter development, before winter and spring development were screened using a 1–9 scale (1 = minimum, 9 = maximum).

40.3 Results and Discussion

153 Irish *L. perenne* accessions were categorized into five maturity groups (MG) according to the Federal Plant Variety Office in Germany (BSA). The five categorized maturity groups, depending on heading date, were MG 1 (n = 84) for very early, MG 2 (n = 31) for very early to early, MG 3 (n = 9) for early, MG 4 (n = 27) for early to middle, MG 5 (n = 2) for middle to late. Due to the large number of accession numbers in the maturity groups only the most important (according to minimum, maximum) and interesting accessions of MG 1, 2 and 4 were selected for the results presented here. Because of the lower number of accessions, the maturity group 5 was not compared with MG 1–4.

For the comparisons between the individual MGs, the results were shown using box plots.

For rust susceptibility (Fig. 40.1a) the only difference was detected between MG 1 and 4 showing that early to middle maturity types are less susceptible to rust. There were no differences detectable between the very early and MG 2 and 3. Genotypes in maturity group 3 and 4 revealed a low susceptibility to rust. For green matter

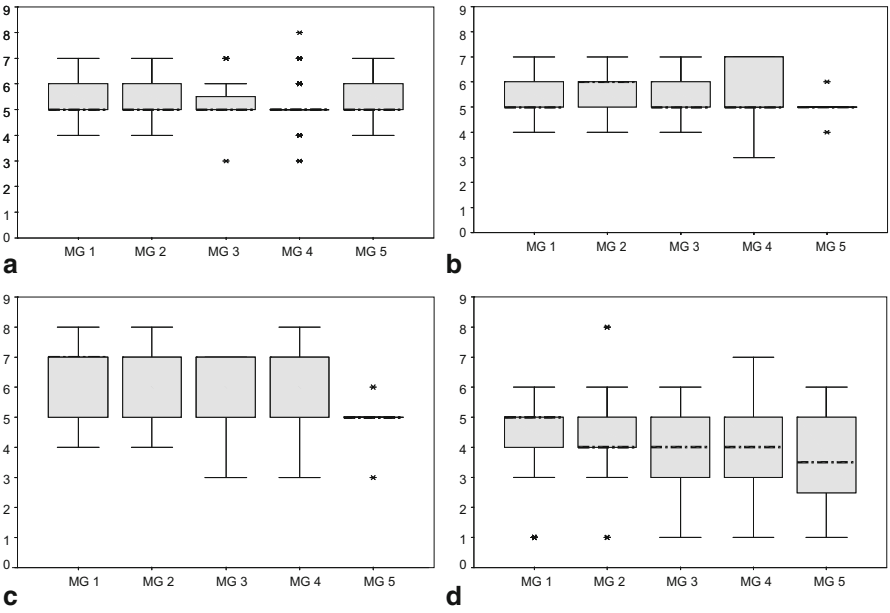

Fig. 40.1 *Box* plots presenting the variability of evaluated traits between the five maturity groups. **a** Rust susceptibility, 2009, **b** *Green* matter estimation in 2009, **c** Plant development before winter, 2009, **d** Plant development in spring, 2010

(Fig. 40.1b), no differences between the groups (MG 1–3) were observed. However, accessions of group 4 displayed a higher variation compared to the other groups. For the trait plant development before winter, there was considerable variability between groups 1–4, with evaluation scores from low (3) to high (8) plant growth (Fig. 40.1c). For the plant development in spring, a huge amount of variability was found (Fig. 40.1d) in the second winter season with a long cold period in 2010. Very early to early maturity groups (MG1, 2 and 4) were particularly variable and differed in their evaluation values from 1 to 8.

Thus, the analysis of rust susceptibility, green matter development, growth before winter and in spring within the MGs showed a huge variability according to the evaluated traits and in comparison to the standards.

Within the first maturity group (Fig. 40.2a) some accessions (1, 4 and 5) indicated better performance with respect to all of the analysed traits, while accessions with a high susceptibility to rust showed reduced biomass production and a delayed or minimum spring development (No. 26 and 28) in comparison to the standards Fennema, Juwel and Sambin. In the second maturity group (Fig. 40.2b), compared to Respect as a standard variety, several entries of Irish material showed better results for some traits. For example, accession number 5 for plant development after winter and in spring. In the third maturity group (Fig. 40.2c) only a small amount of variation was observed between the accessions. The rust susceptibility ranked between 4 and 6. The fourth maturity group (Fig. 40.2d) contained some better performing accessions

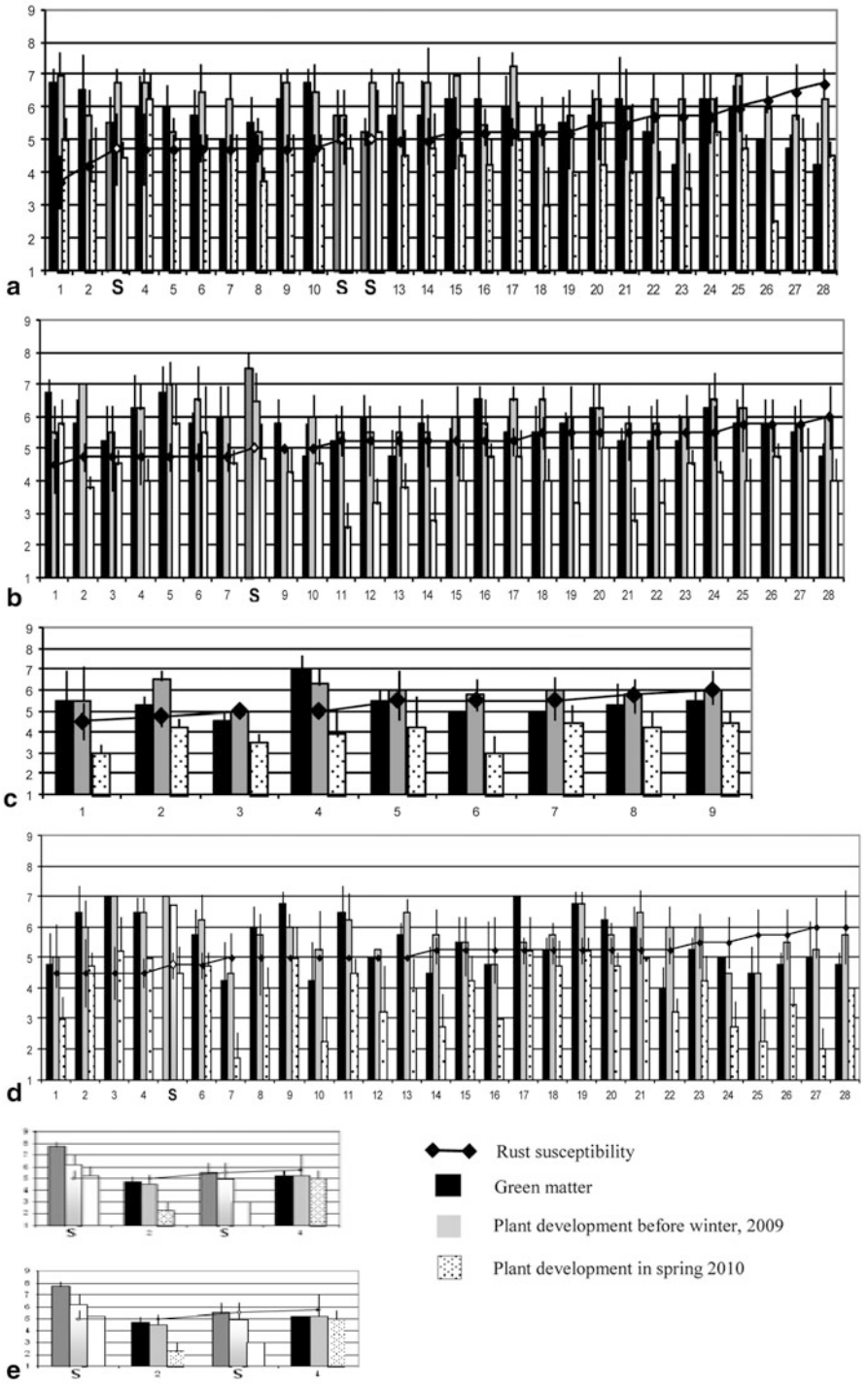

Fig. 40.2 Selected Irish *L. perenne* accessions of the five maturity groups compared to cultivars as standards, which are marked with S compared to rust susceptibility, green matter development, growth before winter and in spring for maturity group 1 (**a**), 2 (**b**), 3 (**c**), 4 (**d**), 5 (**e**)

compared to the standard (Gladio). Accession numbers 2–4 produced high biomass, had a good plant development before winter and spring and a moderate rust susceptibility (between 4 and 5). In the fifth maturity group (Fig. 40.2e) only two accessions were observed. In comparison to the standards (Barcley and Cancan) there were no differences in this group.

The results demonstrate that some of the collected material had superior performance in forage breeding traits compared to the standard varieties. The Irish ecotypes, collected in meadows and pastures, are presumably well-adapted to the Irish climate. However, for German cultivation conditions, harder and longer winter periods have to be considered as factores influencing plant development and crop growth (Figs. 40.1a, 40.1c). Regarding plant development before winter and in spring, the results varied enormously and it can be expected that this material will provide some useful accessions suitable for the German climate. In contrast, rust susceptibility between the maturity groups hardly varied.

Similar results were observed with Romanian collected plant material in the four regions of Crisana, Transylvania, Carpathians and Subcarpathians. Willner et al. (2010) described 455 Romanian ecotypes for rust susceptibility and only detected slight differences between and within the four regions. However, in comparison to standard varieties and traits, collected plant material had partly better economical qualities and climate adapted traits than standard varieties.

40.4 Conclusions

Promising candidates for breeding objectives could be identified with a wide range of variability between accessions that were partly better than standard varieties, especially in the very early to middle maturity group. High variability within maturity groups concerning green matter, rust susceptibility, and growth before winter and in spring was observed. An extensive variability for breeding and research purposes exists within and between maturity groups. Based on these results, plants with desired performance against abiotic stress (e.g. winter hardiness) can be pre-selected by IPK.

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Chapter 41
Exploitation of 'Site-Specific' Alpine Grass Germplasm for Revegetation at High Altitude

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Abstract The use of 'site-specific' germplasm, that is, species adapted to the prevailing pedo-climatic conditions and native to the same geographic context, is increasingly recommended for revegetation interventions at high altitude. Germplasm of two Alpine grass species, viz *Phleum rhaeticum* and *Poa alpina*, collected at 12 and 15 sites, respectively, across three valleys of the Rhaetian Alps, Italy, was evaluated for a set of morpho-physiological characters in a mountain (1,300 m a. s. l.) and a lowland location (81 m a. s. l.), with the main goal of identifying superior populations for further activities of selection and the secondary aim of assessing the effect exerted by an environment markedly different from those of origin on growth and seed production of site-specific species. One valley featured a slight trend of better seed yield and more suitable plant morphology. However, individual collection sites were by far the most important source of variation in both species. The collection of natural populations across a given mountain district seems an appropriate strategy to gather useful genetic variation in site-specific species. The environment of evaluation interacted with the germplasm, affecting the morphology, seed yield, disease susceptibility and survival of site-specific germplasm. Under the Italian conditions, growth and seed multiplication in mountain sites is advised.

Keywords Alps · Ecological restoration · *Phleum rhaeticum* · *Poa alpina* · 'Site-specific' species

41.1 Introduction

Revegetation of mountain landscapes altered by infrastructures for winter tourism is necessary to restore their aesthetical appeal and prevent possible environmental damages due to increased water run-off and soil erosion. Suitable germplasm

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for revegetation at high altitude must be able to combine successfully the technical requirement of ground cover with good adaptation to the peculiar pedoclimatic conditions. To reduce the risk of failure of revegetation at high altitude, the use of 'site-specific' germplasm, that is, species adapted to those conditions and native to the same geographic context, is increasingly recommended (Krautzer 2005). A set of key site-specific species belonging to the Poaceae, Fabaceae and a few other families have been identified for the formation of suitable seed mixtures (Krautzer et al. 2004).

Alpine cat's tail [*Phleum rhaeticum* (Humph.) Rauschert] and Alpine bluegrass (*Poa alpina* L.) are included among site-specific species. Better knowledge is needed on the existing level of variation within species for morpho-physiological traits, including seed production, which may affect their adoption, to drive possible further activities of germplasm collection and selection. In this study, germplasm of these two species was evaluated for a set of morpho-physiological characters in a mountain and a lowland location, with the main goal of identifying superior populations and the secondary aim of assessing the effect exerted by an environment markedly different from those of origin on growth and seed production of site-specific species.

41.2 Materials and Methods

Twelve populations of Alpine cat's tail and 15 of Alpine bluegrass collected above 1,800 m a. s. l. in three valleys of the Rhaetian Alps, Lombardy, Italy (namely, Valchiavenna, Valmalenco and Upper Valtellina) were evaluated in a mountain location of the same Alp district (Bormio, 1,300 m a. s. l.) and in a lowland location (Lodi, 81 m a. s. l.). The trials were laid according to randomised complete block designs with four replications. Originally collected seeds were sown in plug trays and seedlings were subsequently field transplanted. Each single-row plot per block included eight spaced plants in Bormio (35 cm apart) and six in Lodi (50 cm apart) for the Alpine cat's tail, and ten plants in both locations (35 cm apart) for the Alpine bluegrass. Rows were in all cases 70 cm apart. On all plants of both species and in both locations heading time (as days from 1 April to the complete emergence of three inflorescences) and length of the main culm including its inflorescence were recorded in the second year of growth. The number of seeds per plant was counted at maturity in *Ph. rhaeticum* while it is still being processed in *P. alpina*. In the latter species, the seed yield potential was visually estimated on a 9-level scale from 1 (minimum) to 9 (maximum). The susceptibility to rust (mostly *Puccinia coronata* Corda, but some populations also showed susceptibility to *P. graminis* Pers.) and the spring plant diameter were further recorded in *P. alpina* in the first and second year, respectively. The disease reaction was recorded as individual plant score from 1 (no symptoms) to 9 (> 75 % of foliage covered with rust) in Lodi, and as percentage of plants per plot with score ≥ 5 (up to 25 % of affected foliage) in Bormio. An analysis of variance (ANOVA) was carried out for all traits in both species (except susceptibility to rust) including the fixed factors 'location of evaluation' and 'valley

of origin', and the random factors 'population within valley' and 'block within location'. Susceptibility to rust in *P. alpina* was analysed by ANOVA separately for the two locations, testing the variation of valleys and populations, and a Spearman rank correlation coefficient was computed between the population mean values of the two disease assessments.

41.3 Results and Discussion

As expected, the location of evaluation generally was a significant source of variation (Table 41.1). Plant development was slower in the colder location of Bormio relative to Lodi, as indicated by the delay of 33 days in mean heading time for the Alpine cat's tail, and 25 days for the Alpine bluegrass, in the former compared with the latter site. Seed yield, either actual (in *Ph. rhaeticum*) or potential (in *P. alpina*), was greater in the mountain than in the lowland location (Table 41.2). It is a common finding that natural populations grown outside their environments of origin undergo sizeable changes in morphology and physiology, including reproductive traits (Merrell 1981). Successful seed production of site-specific species in lowland sites of other European countries has been reported, though (Krautzer 2005), and the low seed production in Lodi was likely to be accounted for by the level of summer heat and drought of this location rather than by just its altitude.

Differences among valleys of origin were moderate at most (Table 41.1). The *Ph. rhaeticum* germplasm from Valmalenco was slightly later heading than those from the other valleys. A trend of Valchiavenna to produce more seeds per plant in Bormio (+ 6 % than Upper Valtellina and + 33 % than Valmalenco) did not reach the $P < 0.10$ significance level. This trend was consistent with the significantly ($P < 0.05$) greater seed yield potential also observed in *P. alpina* germplasm from Valchiavenna at Bormio (32 % greater mean score than Valmalenco and 59 % greater mean score than Upper Valtellina). Germplasm from Valchiavenna tended to larger plant diameter in both locations, with differences among valleys significant at $P < 0.05$ in Bormio and $P < 0.15$ in Lodi (data not reported). Greater differences among valleys for *P. alpina* in Bormio than in Lodi accounted for the sizeable location × valley interaction for most characters in this species (Table 41.1).

Individual populations were by far the most important source of variation in both species (Tables 41.1 and 41.2). Selective pressures in the individual sites of origin were likely to cause the diversity among populations observed for most traits, in accordance with the genecological assumption that genetic differences among natural populations are related with the environments in which they evolved (Sackville Hamilton et al. 2002). The reported apomictic reproductive system of *P. alpina* (Huff 2010) might also account for the great separation among populations. The collection of natural populations across a given mountain district seems an appropriate strategy to gather useful genetic variation for introduction and/or breeding purposes, being aware that possible drawbacks may occur when using non-local germplasm sources in revegetation interventions (e.g., misadaptation; 'genetic pollution' of resident

Table 41.1 Probability levels of the main sources of variation in the analysis of variance for a set of morphological and seed production traits in two grass species from the Rhaetian Alps, Italy, evaluated in two altitude-contrasting locations

Source of variation	Heading time	Length of main culm	No. seeds per plant	Plant diameter	Seed yield potential
Phleum rhaeticum:					
Location of evaluation (L)	<0.001	ns	<0.001	–	–
Valley of origin (V)	<0.05	ns	ns	–	–
Population within valley [P(V)]	ns	<0.001	<0.05	–	–
L × V	ns	ns	ns	–	–
L × P(V)	ns	ns	ns	–	–
Poa alpina:					
Location of evaluation (L)	<0.001	<0.001	–	<0.001	<0.10
Valley of origin (V)	ns	ns	–	<0.05	<0.05
Population within valley [P(V)]	<0.001	<0.001	–	<0.001	<0.001
L × V	<0.10	ns	–	<0.01	<0.001
L × P(V)	<0.001	<0.05	–	<0.001	<0.001

ns not significant ($P > 0.10$)

Table 41.2 Mean and minimum and maximum individual values among 12 populations of *Phleum rhaeticum* and 15 populations of *Poa alpina* evaluated in two altitude-contrasting environments. Only characters with significant ($P < 0.05$) variation among populations are reported

	Bormio (1,300 m a. s. l.)			Lodi (81 m a. s. l.)		
	Mean	Min	Max	Mean	Min	Max
Phleum rhaeticum:						
Length of main culm (cm)	41.0	26.4	47.5	38.5	30.2	51.7
No. seeds/plant	7,200	1,680	12,165	1,256	56	3,072
Poa alpina:						
Heading time (dd > Apr 1)	41.8	40.1	46.9	16.3	13.6	21.4
Length of main culm (cm)	29.2	22.6	36.8	20.8	17.4	26.7
Plant diameter (cm)	13.4	9.7	18.8	21.4	19.3	24.1
Seed yield potential (1–9)	5.1	2.8	7.7	4.5	3.0	6.2
Susceptibility to rust[a]	64.9	30.0	90.0	4.2	1.8	6.4

[a]Recorded: (i) on a scale from 1 (no symptoms) to 9 (>75 % foliage covered with rust) in Lodi; (ii) as the plot percentage of plants with score ≥ 5 (up to 25 % of affected foliage) in Bormio

germplasm). The risk of such drawbacks is limited when there is close ecological correspondence between the target area for revegetation and the sites of origin of the germplasm used for sowing (McKay et al. 2005).

The environment of evaluation largely interacted with the population responses in Alpine bluegrass, affecting the phenology, morphology and seed yield potential (Table 41.1). The population response to rust was also prone to interaction effects

with the location of evaluation, as indicated by the nil rank correlation coefficient ($r = -0.11$; $P = 0.70$) between the population values of the indexes of disease susceptibility in the two locations. Remarkable inversions of ranking were observed in some cases between locations, such as for the populations 'Campolungo', that was among the most susceptible populations in Bormio and among the least susceptible ones in Lodi, and 'Val Loga 2', that was among the most susceptible populations in Lodi and among the least susceptible ones in Bormio. The race-specificity of the resistance determinants in *P. alpina* is unknown. Studer et al. (2007) found differences in position and magnitude of QTLs among individual evaluation locations in Italian ryegrass (*Lolium multiflorum* Lam.), suggesting a differential quantitative response to local pathogen races. In addition to race specialisation, environmental effects might also influence rust resistance (Roderick et al. 2000).

Promising ecotypes were identified in both species, which will be used in the ultimate selection of adapted high-seed-yielding germplasm for sowing at high altitude. One of the aims of the funding institution is, in fact, the implementation of a seed production chain of site-specific species involving smallholders of the Alpine area. Information has been added by this study on suitable locations for the seed multiplication of site-specific species. Under the Italian conditions, growth and seed multiplication of this germplasm in mountain sites is advised.

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Part VI
Agronomy/Performance and Compositional Analysis

Chapter 42
The Impact of Perennial Ryegrass Variety Throughout the Growing Season on *in vitro* Rumen Methane Output

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Abstract The selection and feeding of perennial ryegrass varieties may affect enteric methane (CH_4) output due to changes in rumen fermentation dynamics as a result of differences in herbage chemical composition. Thus, the objective of this study was to determine the effects of perennial ryegrass variety (PRV) harvested throughout the growing season on herbage chemical composition and on *in vitro* rumen fermentation variables and CH_4 output. Seven PRV (Alto, Arrow, Bealey, Dunluce, Greengold, Malone, Tyrella), managed under a simulated grazing regime, were incubated in a batch culture for 24 h with rumen fluid and buffer. PRV had no effect ($P > 0.05$; SEM 0.41) on CH_4 output per gram of DM incubated (CH_4i; mean values for Alto, Arrow, Bealey, Dunluce, Greengold, Malone, Tyrella were 23.9, 24.0, 24.7, 25.3, 25.2, 24.2 and 24.7 (SEM 0.41) ml CH_4 g^{-1} DM incubated, respectively). Although PRV had an effect ($P < 0.001$; SEM 1.3) on total gas production per gram of DM incubated, the scale of the effect was small (range of mean values among PRV was 148–160 ml), and PRV had no effect ($P > 0.05$) on apparent DM disappearance during the *in vitro* rumen incubation. Thus, the lack of an effect of PRV on CH_4i reflected the small scale or lack of effects on herbage composition and *in vitro* rumen fermentation variables. Hence, these results provide no encouragement that choices among the PRV examined, produced within the management regimes operated, would reduce enteric methane production.

42.1 Introduction

Grassland is the dominant (ca. 0.9) crop on agricultural land in Ireland, and any enteric methane (CH_4) mitigation strategies for ruminants must be effective within the predominantly grass-based production systems used. Purcell et al. (2011a) showed

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that altering grazing management strategy by making specific changes to herbage mass and sward allowance had little effect on *in vitro* rumen CH_4 output due to limited effects on herbage chemical composition. However, Davies et al. (1991) found differences in water soluble carbohydrate (WSC) concentration and organic matter digestibility (OMD) between perennial ryegrass varieties (PRV). Changes in the composition of herbage can affect the amount of CH_4 produced in the rumen (Janssen 2010). Thus, it is feasible that including PRV of higher nutritive value within a predominantly grass-based animal production system may decrease *in vitro* rumen CH_4 output. The objective of this study was to determine the effects of PRV, using varieties of differing country of origin (New Zealand or Ireland bred), ploidy (diploid or tetraploid) and heading date (15 May–5 June), managed under a simulated grazing regime, and thus harvested at successive stages of the growing season, on herbage chemical composition, rumen fermentation variables and CH_4 output using an *in vitro* rumen gas production technique. This *in vitro* batch culture technique was used because it is relatively low cost, rapid, and permits the simultaneous assessment of a large number of treatments compared to *in vivo* methodologies.

42.2 Materials and Methods

Seven perennial ryegrass (*Lolium perenne* L.) varieties (Alto, Arrow, Bealey, Dunluce, Greengold, Malone, and Tyrella) were sown (2006) in a randomised complete block (n = 3) design with repeated sampling (n = 10) of each of the plots (n = 7) per block at Teagasc Moorepark (Fermoy, Co. Cork, Ireland). The plots (each 1.5 × 5 m) were managed under a simulated grazing regime, being harvested on 10 sequential occasions during the 2009 growing season (19 March, 8 April, 30 April, 22 May, 9 June, 1 July, 5 August, 4 September, 5 October and 10 November—defined as Cuts 1–10, respectively). The plots received a total of 375 kg N ha^{-1} (60 kg N ha^{-1} 9 weeks prior to Cut 1, and 35 kg N ha^{-1} after all cuts except Cut 10). The fertiliser applied was calcium ammonium nitrate (CAN; 275 g N kg^{-1}). Plots were harvested to a 4 cm stubble height and, after thorough mixing, were stored at −18 °C prior to herbage chemical composition analysis.

When required, herbage samples were thawed at 4 °C for 24 h and bowl-chopped. Samples were dried (40 °C for 48 h) and milled through a 1 mm sieve prior to chemical analysis. Determination of OMD was carried out using the Tilley and Terry (1963) technique, where the final residue was isolated by filtration rather than centrifugation. Both ADF (acid detergent fibre) and NDF (neutral detergent fibre) were analysed using the filter bag techniques (Ankom 2006a, b). The crude protein (CP; N × 6.25) concentration was determined using a Leco FP 528 N analyser (AOAC 1990) and the concentration of WSC was determined using the anthrone method (Thomas 1977).

For the collection of the rumen microbial inoculum, solid and liquid phase rumen fluid (RF) samples were taken 1 h prior to feeding from four rumen fistulated steers (682 kg mean steer liveweight) individually offered a restricted allowance (0.9 of *ad libitum* intake) of a 60:40 (DM basis) grass silage and concentrate diet.

For the *in vitro* rumen fermentation incubation, approximately 0.5 g of each dried, milled sample was weighed into 160 ml fermentation bottles. Buffered mineral solution (artificial saliva) was prepared according to McDougall (1948). The RF and buffered mineral solution were maintained at 39 °C and under a constant stream of carbon dioxide (CO_2) at all times. The RF was added to the buffered mineral solution at a ratio of 1:4 (RF: buffered mineral solution) after which 50 ml of this buffered RF was dispensed into each bottle. The gas headspace pressure inside each of the bottles was recorded at the end of the 24 h incubation period using a detachable pressure transducer, and the total gas produced (TGP) in each bottle was estimated using the equation:

$$TGP(mL) = \left(\frac{bottle\ headspace\ volume\ \ [mL]}{atmospheric\ pressure\ \ [hPa]}\right) \times\ bottle\ headspace\ pressure\ (hPa)$$

A 0.8 ml sample of gas was then transferred to a pre-evacuated 2 ml screw-top glass vial for determination of CH_4 concentration. A 0.8 ml sample of liquid medium was obtained from each bottle and placed in 2 ml Eppendorf tubes with 20 µl of a 9 M H_2SO_4 solution. The volatile fatty acid (VFA) and CH_4 concentrations were measured by gas chromatography, using iso-caproic acid (0.04 M) as an internal standard for the VFA as described by Ranfft (1973). After the 24 h incubation the amount of sample disappearance, expressed as *in vitro* apparent dry matter (DM) disappearance (aDMD), was estimated as the difference between the DM weight incubated and the DM weight of the filtered (by sintered Pyrex glass crucibles; porosity number 1) residue following oven drying at 98 °C for 48 h. Both TGP and aDMD for each sample were corrected for gas yield and particulate contamination by inclusion of blank fermentation bottles containing buffered RF.

Data were statistically analysed as a randomised complete block design with repeat sampling (n = 10) of each plot (n = 7) per block (n = 3) using the Proc MIXED procedure in SAS. Pre-specified hypotheses were tested using a multiple comparison procedure (Tukey).

42.3 Results and Discussion

Although some PRV by cut interactions were found for both herbage chemical composition and rumen fermentation variables in this study, the effects of PRV within individual cuts were either in agreement with or did not markedly contradict the overall main effect of PRV averaged across all cuts throughout the grazing season for any variable. Therefore, the overall effects of PRV and of Cut are discussed separately.

42.3.1 Herbage Composition

For the PRV, the NDF concentration was higher ($P < 0.05$) for the diploid varieties Alto and Arrow than for the tetraploid varieties Bealey, Dunluce, Greengold and

Malone, possibly due to diploid varieties generally having a higher cell wall to cell content ratio (Wilkins and Sabanci 1990). The WSC and ADF (mean annual value across PRV of 234 g kg^{-1} DM; pooled SEM of 2.0) concentrations were also affected by PRV ($P < 0.05$), but PRV had no effect ($P > 0.05$) on CP concentration (218 g kg^{-1} DM; SEM 3.4). Overall, the range in composition between PRV in this study was much smaller than the range among cuts (e.g. the mean NDF values across PRV and cuts ranged from 454–483 g kg^{-1} DM and 406–505 g kg^{-1} DM, respectively, with corresponding WSC ranges of 94–122 and 59–190 g kg^{-1} DM). Thus, the small scale of the effects of PRV on the concentrations of NDF, ADF and WSC were not sufficient to create an effect on OMD ($P > 0.05$; 773 g kg^{-1}; SEM 8.8). Thus, the overall effect of PRV on herbage chemical composition was relatively small. The generally high CP concentrations found in this study reflect the relatively high input of N fertiliser applied.

The NDF, ADF, CP and WSC concentrations, and OMD, were all affected by cut ($P < 0.001$). General increases in NDF and ADF concentrations were found from Cuts 1–8, followed by general decreases for Cuts 9 and 10. These findings may reflect changes in the overall ratio of leaf to stem within the sward and most likely caused the concomitant general decrease in OMD found for Cuts 1–7 and general increase for the remaining cuts, which is in accord with Dent and Aldrich (1968). The CP concentration varied considerably through the season, being highest ($P < 0.05$) for Cuts 2 and 10, and lowest ($P < 0.05$) for Cut 7. The concentration of WSC was higher ($P < 0.05$) for Cut 1 than for all other cuts, which was most likely due to the longer time interval between N fertiliser application and cutting time for Cut 1 (approximately 9 weeks) compared to all other cuts (no more than 5 weeks). The application of N fertiliser can greatly reduce the WSC concentration of grass, especially fructans, due to accelerated growth rate following application (Nowakowski 1962; Jones et al. 1965). No clear trend in WSC concentration was found throughout the remainder of the growing season.

42.3.2 In vitro Rumen Fermentation Variables

The lack of an effect of PRV on the OMD of the herbage resulted in no effect ($P > 0.05$) on aDMD (0.72 g g^{-1}; SEM 0.012) after the 24 h fermentation. Although ml of TGP per gram of DM incubated (TGPi) and the total VFA concentration (tVFA) were both affected by PRV ($P < 0.05$), the overall scale of the effect on both variables was small (ranges of mean values among PRV of 148–160 ml for TGPi and 42.5–46.7 mmol l^{-1} for tVFA). Thus, the lack of an effect on aDMD and the small scale effects on TGPi and aDMD indicate that the effect of PRV on the extent of the *in vitro* rumen fermentation was small. This outcome explains the lack of an effect on CH_4i ($P > 0.05$; Table 42.1), as the extent of *in vitro* rumen fermentation has been shown to greatly influence CH_4 output expressed relative to substrate incubated (Lovett et al. 2004; Purcell et al. 2011b). PRV had no effect ($P > 0.05$) on ml of CH_4 output per gram of DM disappeared during the *in vitro* rumen incubation (CH_4d; Table 42.1). This finding reflects the lack of an effect of PRV on the direction of the *in vitro* rumen

Table 42.1 Effects of perennial ryegrass variety (PRV) and stage of the growing season (Cut) on ml of methane output per gram of dry matter incubated (CH_4i) and disappeared (CH_4d) after 24 h of *in vitro* rumen incubation

Perennial ryegrass variety								
Cut	Alto	Arrow	Bealey	Dunluce	Greengold	Malone	Tyrella	Mean
CH_4i								
1	28.5	29.4	30.7	30.0	27.4	29.2	29.3	29.2[a]
2	23.6	26.6	27.3	27.7	29.1	26.9	27.8	27.0[ab]
3	25.8	24.2	23.4	24.0	24.9	25.9	24.5	24.7[cd]
4	24.0	22.7	20.6	23.6	24.7	24.1	22.9	23.2[cde]
5	21.7	22.0	21.6	21.5	23.0	19.2	22.5	21.6[e]
6	23.4	22.0	22.7	23.1	23.1	23.1	22.2	22.8[de]
7	23.2	22.2	23.9	25.0	23.5	24.1	23.2	23.6[cde]
8	21.6	23.3	24.4	23.2	25.4	22.9	24.0	23.5[cde]
9	23.0	24.5	26.3	28.0	24.3	23.7	24.9	25.0[bc]
10	24.3	22.8	26.1	27.0	26.3	23.1	25.5	25.0[bcd]
Mean	23.9	24.0	24.7	25.3	25.2	24.2	24.7	–
CH_4d								
1	35.9	38.1	37.0	35.5	35.0	37.7	36.8	36.6[a]
2	31.1	31.8	32.1	33.2	33.3	31.9	32.6	32.3[d]
3	33.8	31.5	35.1	31.0	31.6	33.9	32.9	32.8[bcd]
4	34.8	33.1	31.4	34.2	35.8	37.1	33.1	34.2[abcd]
5	30.9	32.7	31.4	29.5	33.6	28.4	32.1	31.2[cd]
6	36.1	36.0	33.1	36.3	34.9	35.0	33.2	34.9[abc]
7	36.8	36.4	36.2	37.6	35.2	35.9	35.8	36.3[a]
8	33.1	35.9	37.3	37.9	37.1	33.8	36.5	36.0[a]
9	33.6	38.4	33.3	37.9	32.2	30.2	36.4	34.6[ab]
10	33.4	29.8	35.6	37.4	35.8	36.2	34.4	34.6[abcd]
Mean	33.9	34.4	34.3	35.1	34.4	34.0	34.4	–

Significance

	CH_4i		CH_4d	
	SEM	Sig	SEM	Sig
PRV	0.41	NS	0.70	NS
Cut	0.43	***	0.65	***
Cut × PRV	1.15	*	1.71	***

NS not significant ($P > 0.05$)
SEM standard error of the mean
Sig significance
***$P < 0.001$; *$P < 0.05$
[a–e]Means within a column within a variable with common superscripts do not differ ($P < 0.05$). Cuts 1–10, 29 March, 8 April, 30 April, 22 May, 10 June, 1 July, 6 August, 8 September, 5 October and 10 November, respectively

fermentation after 24 h, as evidenced by the absence of an effect on the acetic acid to propionic acid (A:P) ratio (2.59; SEM 0.020) and the non-glucogenic to glucogenic VFA ratio (NGGR; (acetic acid + 2 butyric acid)/propionic acid; 3.35; SEM 0.032), which influences the amount of hydrogen and, thus, CH_4 produced per unit of feed DM digested (Janssen 2010).

Cut had an effect on TGPi, aDMD and tVFA ($P < 0.001$). The CH_4i was also affected ($P < 0.001$; Table 42.1) by cut, and there was a general decrease in CH_4i from Cut 1 to Cut 5 after which no clear trend was observed. This outcome reflects the apparent decrease in the extent of the fermentation for these cuts, as evidenced by the general decrease in TGPi and tVFA observed from Cuts 1–5 and the general decrease in aDMD between Cuts 2–4. Although cut had a significant effect ($P < 0.001$) on the A:P ratio, NGGR and CH_4d (Table 42.1), no clear trend was found during the growing season for any of these variables. Thus, it appears that the lack of a trend in CH_4d reflected the similar finding for the direction of the *in vitro* rumen fermentation. The finding that the direction of the *in vitro* rumen fermentation appeared to have a greater influence on CH_4d throughout the growing season than the extent of the fermentation, which appeared to have a greater influence on CH_4i, is in accord with other similar *in vitro* rumen studies (Purcell et al. 2011b; Navarro-Villa et al. 2011).

42.4 Conclusion

No differences in *in vitro* rumen CH_4 output were found between the PRV examined, reflecting their small scale effects on herbage composition and *in vitro* rumen fermentation variables. Hence, these results provide no encouragement that choices among the PRV examined, produced within the management regimes operated, would reduce enteric methane production. However, the technique utilised did not take account of animal by PRV interactions that may occur under farm conditions.

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Chapter 43
Origin and Yield of European Perennial Ryegrass (*Lolium perenne* L.) Varieties in Ireland

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Abstract The Department of Agriculture, Fisheries and Food have sown 63 Intermediate and Late heading perennial ryegrass varieties in combined National List/Recommended List evaluation trials at five locations in Ireland each year from 2004 to 2008. Breeder's seed of candidate and commercial varieties originating from breeding companies in eight European countries were included. Each sowing year/experiment was harvested for two successive years under a six cut 'general purpose' protocol. Annual total yields of dry matter per hectare were sorted by origin of germplasm and averaged for each country. Intermediate varieties had an average yield of 14.8 T DM/ha, 0.3–0.4 T DM/ha greater than Late varieties (14.5 T DM/ha) from all sources except Belgium. Intermediate varieties from Germany, Denmark, Ireland, Northern Ireland and the UK were 0.4 T DM/ha higher than varieties from France and the Netherlands, while in the Late category varieties from Germany, Ireland and the UK had an average yield of at least 0.5 T DM/ha greater than varieties from Denmark, France and the Netherlands.

Keywords Perennial ryegrass · Variety evaluation · Ireland

43.1 Introduction

The climatic conditions of Ireland supports a predominately grassland dependent agri-business. Grassland covers approximately 85 % of the arable area of the island and is by far the most important agricultural land use. Given the wide climatic range of growing conditions across the European market, selection criteria in grass breeding and evaluation programmes need to focus on specific ecozones if breeding advances are to be achieved and passed on to farmers. The use of dormancy zones,

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Table 43.1 Site details of NL/RL Variety Evaluation Trials for perennial ryegrass in Ireland 2004–2010

Site	Geographic Coordinates	Altitude (m)	Soil Type (loam)	Org. Matter (g/kg)	pH
Athenry	53°18′N 8°45′W	35	Peaty	79	7.1
Backweston	53°22′N 6°30′W	50	Clay	63	7.2
Fermoy	52°08′N 8°17′W	53	Med	59	5.8
Piltown	52°21′N 7°20′W	15	Clay	49	5.5
Raphoe	54°52′N 7°36′W	65	Med	62	5.6

based on Lucerne adaptation, has been found to be a useful means of classifying such regions (Long and Gilliland 2010). Ireland is within Zone 6, which is a maritime region that includes Britain and the coastal regions of north west France and Spain. There is ample evidence that ryegrass breeding programmes focused on this agri-environmental zone have achieved notable success in the past (Van Wijk and Reheul 1991; Wilkins and Humphreys 2003). Recent work has indicated that strong genotype by environment interactions are present in identifying superior genotypes under Irish conditions (Conaghan et al. 2008; Grogan and Gilliand 2010). The objective of this study is to establish the extent of variation in total annual yield in recent Irish national perennial ryegrass evaluation trials when varieties are examined by country of origin.

43.2 Materials and Methods

Each year from 2004 to 2008, thirty Intermediate (or mid-season heading), and thirty-three Late heading perennial ryegrass varieties from eight European countries were evaluated by the Department of Agriculture, Fisheries and Food in combined National List and Recommended List trials. These groups were sown separately in a randomised complete block design with four replications at five locations (Table 43.1.)

Plots of 11.4 m^2 were broadcast sown in the autumn at a seed rate of 30 kg/ha for diploids and 40 kg/ha for tetraploids. Fertiliser N was applied annually at 350 kg/ha, and P, K and S were applied as indicated by annual soil analysis to meet growth requirements. These fertilizer rates were intended to simulate intensive grassland use. Each sowing was harvested by Haldrup plot harvester (Logstor, Denmark) for two successive years under an annual six cut 'general purpose' cutting protocol. Total plot yields in kilograms were recorded and a subsample (approx 300 g) oven dried at 800 °C for 16 h to determine Dry Matter yield. Results of each cut from each location were subjected to ANOVA using Genstat and/or AgroBase. Annual average total DM yield for each variety was sorted by origin of seed as declared on application forms used for entry to NL/RL trials.

Table 43.2 Number of perennial ryegrass varieties in Irish NL/RL evaluation trials 2004–2010

	Year sown/experiment											
Origin	04 Inter	04 Late	05 Inter	05 Late	06 Inter	06 Late	07 Inter	07 Late	08 Inter	08 Late	Mean Inter	Mean Late
Belgium	1	2	1	3	0	1	0	0	0	0	0.4	1.2
Germany	1	3	1	2	1	4	2	5	6	8	2.2	4.4
Denmark	1	0	1	1	0	3	1	4	2	2	1.0	2.0
France	0	2	1	4	2	6	2	5	2	5	1.4	4.4
Ireland	9	6	7	6	8	6	6	2	7	4	7.4	4.8
N. Ireland	4	6	4	6	6	6	8	6	5	4	5.4	5.6
Netherlands	12	10	13	7	11	3	10	6	6	6	10.4	6.4
UK	2	3	2	4	2	4	1	4	0	4	1.4	3.8

Table 43.3 Yield of inter perennial ryegrass varieties in Irish NL/RL evaluation trials 2004–2010

Sowing Year	2004		2005		2006		2007		2008		
Harvest Year	2005	2006	2006	2007	2007	2008	2008	2009	2009	2010	Mean
Origin	Intermediate varieties, average annual yield T DM/ha										
Belgium	14.9	13.3	13.6	13.9							13.9
Germany	15.8	14.0	15.6	15.5	17.7	15.0	15.8	15.4	14.6	13.2	15.3
Denmark	16.0	13.3	15.5	14.6			16.6	16.2	15.1	13.2	15.1
France			15.1	15.1	16.3	14.6	14.8	14.2	13.9	12.6	14.6
Ireland	15.8	13.7	14.9	15.0	17.2	15.4	15.7	15.4	14.6	13.2	15.1
N. Ireland	15.7	13.5	14.8	15.0	17.1	15.4	15.7	15.5	14.8	13.5	15.1
Netherlands	15.4	13.6	14.5	14.6	16.6	14.9	15.2	14.7	14.4	13.1	14.7
UK	15.4	13.5	14.8	14.7	17.0	15.2	15.2	14.7			15.1
Mean	15.6	13.5	14.9	14.8	17.0	15.1	15.6	15.2	14.6	13.1	14.9

43.3 Results and Discussion

43.3.1 Number of Varieties

The number of varieties tested from each country varied from a small number of Belgian varieties (on average 0.5–1 variety per annum) to between six and ten each year from the Netherlands (Table 43.2). The number of Dutch varieties tested has been declining in recent years.

43.3.2 Yield of Varieties

Annual yields from all sites for each harvest year are presented for Intermediate varieties in Table 43.3, and for Late varieties in Table 43.4.

Table 43.4 Yield of late perennial ryegrass varieties in Irish NL/RL evaluation trials 2004–2010

Sowing Year	2004		2005		2006		2007		2008		
Harvest Year	2005	2006	2006	2007	2007	2008	2008	2009	2009	2010	Mean
Origin	Late varieties, average annual yield T DM/ha										
Belgium	16.3	13.3	13.9	13.8	15.2	13.7					14.3
Germany	16.1	13.5	14.6	14.8	16.9	15.1	14.0	14.1	14.5	13.2	14.7
Denmark			13.9	14.0	16.3	14.7	14.0	14.1	14.1	12.9	14.2
France	16.0	13.5	13.8	14.0	15.7	14.3	13.5	13.6	14.1	12.6	14.1
Ireland	16.0	13.4	14.3	14.7	16.4	15.2	14.1	14.5	14.8	13.4	14.7
N. Ireland	15.8	13.3	13.9	14.5	16.4	15.0	14.2	14.3	14.4	13.2	14.5
Netherlands	15.7	13.1	13.9	14.0	16.1	14.5	13.9	14.0	14.0	12.9	14.2
UK	16.3	13.4	14.3	14.7	16.9	15.0	14.6	14.6	15.3	14.1	14.9
Mean	16.0	13.4	14.1	14.3	16.2	14.7	14.1	14.2	14.4	13.2	14.5

Overall, Intermediate varieties out yielded Late varieties by an average of 0.4 T DM/ha. The lowest yields occurred in 2006 and 2010 from 2nd year harvests (13.1–13.5 T DM/ha), while the highest yields were recorded from 1st year harvests in 2007 (16.2–17.0 T DM/ha).

Varieties from countries climatically closest to the evaluation sites (i.e. Ireland, Northern Ireland, and UK Wales) on average yielded 0.4 T DM/ha more than north western European material (Germany, Denmark and Netherlands), and 0.6 T DM/ha more than French material.

There are apparent differences in potential total annual DM yield based on origin of seed. However, use of other traits such as seasonality of yield, ruminant digestibility, and persistency are needed to give a full assessment of true forage quality.

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Chapter 44
Yield Dynamics and Quality in White Clover and Perennial Ryegrass in the First cut of the Establishment Year

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Abstract A field trial was carried out in the Vojvodina province, Serbia, during 2009 and 2010 in rainfed conditions. The study assessed the dynamics of yield formation, changes in morphological characteristics, as well as the nutritive values of white clover and perennial ryegrass grown as monocultures. Five varieties of white clover (Chieftain, Susi, Aran, Avoca and Rivendale) and eight of perennial ryegrasses (Cashel, Shandon, Magician, Greengold, Glenstal, Millenium, Sarsfield and Glencar), developed at and provided by Teagasc, Ireland, have been used for this research. After four measurements a significant differences in examined parameters with white clover and perennial ryegrass varieties were registered. In all studied parameters the highest values were recorded in the fourth measurement. The stolon length ranged from 1.4 cm (Avoca) to 4.1 cm (Chieftain). Susi had the highest number of stolons (2.1), and Rivendale had the lowest (0.9) but also the highest number of leaves per stolon (3.7). The leaf length ranged from 6.1 cm (Avoca) to 19.3 cm (Aran). Concerning ryegrass, the number of lateral shoots ranged from 2.8 (Millenium) to 4.8 (Magician). The highest number of leaves per shoot was in Cashel (4.8), and the lowest was reported for Millenium and Greengold (3.3). The average shoot height to the first leaf varied between 3.2 cm (Glenstal) and 4.4 cm (Cashel). The highest yield of white clover was achieved by Rivendale (1.19 t ha^{-1}), while the highest yield of perennial ryegrass were recorded by Millennium (8.26 t ha^{-1}) and Magician (8.03 t ha^{-1}). The average crude protein content was higher in white clover (22.8 %) than in perennial ryegrass (12.8 %), while the crude fibre content of white clover and perennial ryegrass were 18.1 and 34.7 %, respectively. These parameters led to a lower digestibility in perennial ryegrass, reflected through the monitored parameters of NDF and ADF.

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44.1 Introduction

Two legumes, alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.), and permanent grasslands have the greatest importance for the forage production in the agroecological conditions of Serbia. However, significant characteristics such as yield and particularly quality in white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) have not been studied to a sufficient extent.

White clover is a perennial type of legume, widely prevalent. It is a very resourceful plant, although with somewhat lower yields than grass, but it is an important component in forage mixtures for improving quality (Turkington 1989; Collins and Rhodes 1989). It is a weak competitor for light and nutrients in comparison to most grass companions in binary (Soussana et al. 1995) or complex legume-grass mixtures (Nyfeler et al. 2009; Annicchiarico and Proietti 2010). It is usually grown with one or more grass companions, the most common being perennial ryegrass (Hill and Michaelson-Yates 1987). When grown in mixtures, white clover and ryegrass provide pastures of high productivity and quality (Lucero et al. 1999). One of the frequent problems occurring with grass-legume mixtures is the timely determination of the physiological maturity for the first cut in the establishment year.

The objective of this study is to determine the potential of white clover and perennial ryegrass in the conditions of the Serbian province of Vojvodina. Additionally, it aimed at defining the growth dynamics and the yields formation in perennial ryegrass and white clover grown separately in the first cut of the establishment year by monitoring their forage yields, forage yield components and forage dry matter quality.

44.2 Materials and Methods

A field experiment was conducted under the rain-fed conditions during 2009 and 2010 at the Experimental Field of the Institute of Field and Vegetable Crops at Rimski Šančevi, Serbia (45° 20′ N and 19° 51′ E and 84 m a.s.l). This research included five varieties of white clover (Chieftain, Susi, Aran, Avoca and Rivendale) and eight varieties of perennial ryegrass (Cashel, Shandon, Magician, Greengold, Glenstal, Millenium, Sarsfield and Glencar). All these varieties have been developed at Teagasc, Ireland, except the white clover Rivendale, bred by DLF Trifolium, Denmark.

Two monofactorial trials were set up as random block systems in three replications. In both years and in each trial, varieties were sown in April with a plot size of 5 m^2. The seeding rate for perennial ryegrass was 25 kg ha^{-1} and for white clover 10 kg ha^{-1}.

Both species were sown separately as pure stands. During the growing period, all the usual agronomic measures for both crops were applied, specifically manual weed control. Samples of 20 plants were taken from each plot every 20 days during the development of the first cut in both years, namely on May 15th, June 5th, June 25th and July 15th. In white clover, stolon length (cm), number of stolons, petiole length (cm), number of leaves per stolon, total number of leaves and dynamics of formation of dry matter yield (t ha^{-1}) were measured. In ryegrass, number of lateral shoots, number of leaves per shoot and shoot height (cm) and dry matter yield (t ha^{-1}) were measured. In the first cut, that is, in its fourth measurement stages, quality parameters were analysed according to Van Soest (1991) such as the content of crude protein (CP), crude fibre (CFb), as well as neutral detergent fibres (NDF) and acid detergent fibres (ADF).

The results were statistically processed by using ANOVA (analysis of variance) and a Fisher's LSD test to detect significant differences between varieties for all mean values at $P = 0.05$. The results were presented and discussed as a 2 year average.

44.3 Results and Discussion

After four measurements, there were significant differences in the examined parameters in white clover and perennial ryegrass varieties. Generally, in all studied parameters the highest values were recorded in the last, namely fourth, measurement, being the objective of the following discussion.

Table 44.1 Dynamics and changes in morphological characteristics and dry matter (DM) yield of white clover varieties

Variety	Stolon length	Stolon number	Leaf length	Leaf number	DM yield	Stolon length	Stolon number	Leaf length	Leaf number	DM yield
Sample Date	May 15th					June 5th				
Chieftain	0.8a	0.9a	4.1a	1.3a	0.44a	1.7a	1.0a	5.8a	1.3b	0.63a
Susi	0.6b	0.7b	2.8b	1.5a	0.10d	1.3b	0.9a	6.4a	1.8a	0.26c
Aran	0.4c	1.0a	1.8c	1.3a	0.35b	0.6d	1.0a	3.7b	1.7a	0.51b
Avoca	0.1e	0.3c	1.1d	0.6c	0.09d	0.3e	0.6b	1.5c	1.1c	0.15d
Rivendale	0.2d	0.3c	1.0d	1.0b	0.23c	0.8c	0.5c	2.0bc	1.3b	0.31c
2009	0.5a	0.6a	2.0b	1.2a	0.26a	1.0a	0.8a	2.8b	1.5a	0.38a
2010	0.4a	0.6a	2.3a	1.0b	0.23b	0.9b	0.7a	4.9a	1.4b	0.36a
Average	0.4	0.6	2.2	1.1	0.24	0.9	0.8	3.9	1.4	0.37
Sample Date	June 25th					July 15th				
Chieftain	2.2a	1.0b	5.5a	1.8bc	0.91a	4.1a	1.8ab	10.8b	2.8bc	1.11a
Susi	1.5c	1.6a	5.8a	2.6a	0.47c	3.6b	2.1a	10.0b	2.9b	0.43d
Aran	1.4d	0.9bc	5.3a	1.9b	0.74b	3.5b	1.5b	19.3a	2.5c	0.97b
Avoca	0.6e	1.0b	1.9b	1.5c	0.32d	1.4c	1.3bc	6.1c	3.4a	0.54c
Rivendale	1.7b	0.8c	2.1b	1.7bc	0.52c	2.1d	0.9c	10.1b	3.7a	1.19a
2009	1.5a	1.1a	3.6b	2.0a	0.57a	3.1a	1.6a	8.8b	3.3a	0.92a
2010	1.4b	1.0b	4.6a	1.8b	0.61a	2.8b	1.4a	13.7a	2.9b	0.78b
Average	1.5	1.0	4.1	1.9	0.59	2.9	1.5	11.3	3.1	0.85

*LSD followed by different letters are significantly different ($P \leq 0.05$)

Table 44.2 Dynamics and changes in morphological characteristics and DM yield of perennial ryegrass varieties

Variety	Number of lateral shoots	Number of leaves per shoot	Shoot height to the 1st leaf	DM yield	Number of lateral shoots	Number of leaves per shoot	Shoot height to the 1st leaf	DM yield
Sample Date	May 15th				June 5th			
Cashel	2.4a	3.4a	2.5a	4.24b	2.8a	3.9a	3.4a	4.77b
Shandon	2.1b	2.9bc	1.7c	3.95b	2.5b	3.9a	2.9b	5.18b
Magician	2.1b	2.8bc	2.0b	4.61ab	1.9c	3.4bc	2.8bc	5.09b
Greengold	1.8c	1.9e	2.0b	4.17b	2.0c	3.1cd	2.3d	4.81b
Glenstal	1.9bc	3.0b	1.8bc	4.41b	2.0c	3.6b	2.3d	4.94b
Millennium	2.5a	2.4d	2.0b	6.83a	2.6ab	3.0d	2.4d	6.92a
Sarsfield	2.6a	2.6c	1.5d	4.00b	2.6ab	3.3c	2.7c	4.47b
Glencar	1.7c	2.1de	1.4d	4.17b	2.1c	3.4bc	2.0e	4.35b
2009	2.3a	3.0a	2.0a	4.84a	2.5a	3.6a	2.8a	5.41a
2010	2.0b	2.6b	1.7b	4.26b	2.2b	3.3b	2.4b	4.72b
Average	2.2	2.8	1.9	4.55	2.3	3.4	2.6	5.07
Sample Date	June 25th				July 15th			
Cashel	3.6a	4.4a	4.2a	5.91bc	3.9c	4.8a	4.4a	6.32bc
Shandon	3.7a	4.0b	4.1a	7.12ab	4.1c	4.2b	4.3a	7.08bc
Magician	3.5a	3.6c	3.2c	6.77b	4.8a	3.8c	3.7b	8.03a
Greengold	2.6c	3.2d	3.2c	5.34bc	3.0e	3.3d	3.5bc	6.19c
Glenstal	2.8bc	3.8bc	3.7b	7.45a	3.2de	4.2b	3.2c	7.88ab
Millennium	2.7bc	3.2d	3.5b	7.85a	2.8f	3.3d	3.3c	8.26a
Sarsfield	3.0b	3.4cd	2.8d	5.31c	4.4b	3.6d	3.5bc	6.73bc
Glencar	2.6c	3.7bc	3.2c	6.13bc	3.4d	3.9c	3.6b	7.15ab
2009	3.2a	3.9a	3.7a	6.87a	3.9a	4.1a	3.9a	7.57a
2010	2.9b	3.4b	3.3b	6.10b	3.5b	3.6b	3.5b	6.84b
Average	3.0	3.7	3.5	6.48	3.7	3.9	3.7	7.21

*LSD followed by different letters are significantly different ($P \leq 0.05$)

The stolon length ranged from 1.4 cm in Avoca to 4.1 cm in Chieftain. Susi had the highest number of stolons 2.1, while the lowest was in Rivendale with 0.9. The values of leaf length ranged from 6.1 cm (Avoca) to 19.3 cm (Aran). The highest number of leaves per stolon (3.7) and dry matter yield (1.19 t ha^{-1}) were produced by Rivendale (Table 44.1).

As for ryegrass (Table 44.2), the highest number of lateral shoots was recorded in Magician (4.8), while the lowest one was in Millennium (2.8). The number of leaves per shoot ranged from 3.3 in Greengold and Millenium to 4.8 in Cashel. Glenstal had the lowest values of shoot height to the first leaf (3.2 cm), while the highest values were in Cashel (4.4 cm). Despite the fact that it did not have high values of some of the monitored parameters, Millenium (Table 44.2) had the highest dry matter yield (8.26 t ha^{-1}), while the lowest yield was measured in Greengold (6.19 t ha^{-1}).

The quality parameters differed within varieties. The average crude protein content was higher in white clover (22.8 %) than in perennial ryegrass (12.8 %), while the crude fibre content of white clover and perennial ryegrass were 18.11 and 34.66 %, respectively. The average values of NDF and ADF in white clover varieties were 38.20 and 32.24 %. This is supported by Ayres et al. (1998). In perennial ryegrass varieties, they were 60.67 and 39.28 %. These parameters indicate a lower digestibility in perennial ryegrass (Table 44.3).

Table 44.3 Quality parameters of white clover and perennial ryegrass varieties

Variety	Crude protein	Crude fibre	NDF	ADF
White clover				
Chieftain	16.00c	15.49c	47.63a	42.23a
Susi	23.16b	16.94b	36.12b	31.08b
Aran	22.10b	25.70a	37.10b	29.57bc
Avoca	24.88a	16.52bc	34.67b	28.45c
Rivendale	24.04ab	15.90bc	35.51b	29.86bc
2009	22.23b	17.24b	36.32b	31.02b
2010	23.37a	18.98a	40.09a	33.46a
Average	22.80	18.11	38.20	32.24
Perennial ryegrass				
Cashel	11.03bc	33.09b	63.30ab	40.95ab
Shandon	10.25cd	37.92a	65.11a	42.07a
Magician	22.76a	37.39a	57.86bc	38.22b
Greengold	11.79b	36.44a	58.90bc	38.47b
Glenstal	10.20cd	33.49b	61.21abc	41.47ab
Millennium	9.75d	27.74c	60.34abc	40.26ab
Sarsfield	10.67cd	34.17b	56.40c	34.21c
Glencar	11.02bc	37.04a	62.23abc	38.58b
2009	11.53b	33.13b	57.82b	37.33b
2010	12.84a	36.19a	63.51a	41.22a
Average	12.80	34.66	60.67	39.28

*LSD followed by different letters are significantly different ($P \leq 0.05$)

44.4 Conclusion

In all studied parameters, the highest values were recorded in the last, fourth, measurement. There are significant differences in morphological properties, dry matter yield and quality between tested varieties of white clover and perennial ryegrass. The white clover variety Rivendale and the perennial ryegrass variety Millennium produced the highest forage yields and had the best forage quality.

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Chapter 45
Influence of Plant Growth Promoting Rhizobacteria on Alfalfa, *Medicago sativa* L. yield by Inoculation of a Preceding Italian Ryegrass, *Lolium multiflorum* Lam

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Abstract This study was conducted to test the hypothesis that plant growth promoting rhizobacteria (PGPR) can promote the growth of Italian ryegrass, *Lolium multiflorum* Lam. as well as the growth and nodulation of subsequent alfalfa, *Medicago sativa* L. In a pot experiment, the influence of PGPR on yield and nitrogen content of Italian ryegrass and alfalfa was studied with the aim to improve their growth and provide effective alfalfa nitrogen fixation under unfavourable soil conditions. Plants were inoculated with seven strains belonging to *Sinorhizobium meliloti, Azotobacter* spp. and *Enterobacter* sp. A beneficial effect on yield and N-assimilation in Italian ryegrass was obtained due to the inoculation of the plants with *Azotobacter vinelandii* and some *Sinorhizobium meliloti* strains. In addition, Italian ryegrass seed inoculation with particular rhizobial strains the year before alfalfa growing provided abundant nodulation and better growth of alfalfa.

Keywords Italian ryegrass · Alfalfa · Inoculation · Yield · PGPR · *Sinorhizobium*

45.1 Introduction

In recent years, there has been a growing interest in using bacterial inoculants (biofertilizers) because of the ability of particular rhizobacteria (PGPR) to promote growth and yield in great number of plants including legumes and cereals through different mechanisms, i.e. increasing nitrogen (N) uptake (biological N fixation-BNF), synthesis of phytohormones (auxin, cytokinin), minerals solubilization and iron chelation

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(Antoun and Prévost 2005; Mehboob et al. 2009; Goos et al. 2001; Egamberdiyeva et al. 2004). These biofertilizers represent an alternative to plant growth enhancement by chemical fertilizers. BNF is promoted by both free living soil bacteria (*Azotobacter, Enterobacter, Bacillus*) and by symbiotic association of mostly nodule bacteria (rhizobia) and legumes. *Azotobacter* species are aerobic heterotrophic soil bacteria. Yields of rice, cotton and wheat in many investigations were increased significantly with applications of *Azotobacter* due to its ability to fix N_2 (Kennedy et al. 2004). Enterobacter is a free-living nitrogen fixer that is distributed to the soil matrix via animal feces. These N_2-fixing microbes grow in humid environments on leaf surfaces or in leaf sheaths (phyllosphere), the soil, and root surfaces (Paul and Clark 1996).

Alfalfa (*Medicago sativa* L.) is one of the most important leguminous forage crops with the ability of BNF with specific rhizobia (*Sinorhizobium meliloti).* This association belongs to the most significant agricultural systems for BNF (on average 250 kg N ha^{-1}). The presence, density and effectiveness of natural populations of *S. meliloti* vary in the soil and depend on the soil type and land use regime (Delić-Vukmir et al. 1994). Low number of *S. meliloti* ($\leq 10^2$ g^{-1} of soil) may be caused by bad soil characteristics or the absence of a host plant for a long period of time. Artificial inoculation of legumes with N fixing rhizobia is always applied on soils with no recent cultivation of alfalfa as well as on soils with low fertility with the aim to increase the number and effectiveness of rhizobia in the soil. The possibility for increasing the number of *B. japonicum* before growing soybean for the first time by inoculation of the previous crops was shown (Goos et al. 2001).

Italian ryegrass, *Lolium multiflorum* Lam. is one of the best forage grasses in Serbia, producing high-quality forage from early spring to late summer Simić et al. 2009). It is valuable in crop rotations with row crops (e.g. soybean, maize, sunflower, sugar beet) for maintaining soil structure and health. Italian ryegrass is well-adapted to high rainfall but can be grown where a minimum of about 500 mm rainfall occurs during the growing season. It also can be used as emergency forage after winterkill of alfalfa. It establishes quickly and produces a lot of forage in a short period of time.

The object of this study was to evaluate the possible effects of a preceding ryegrass inoculation with S. *meliloti* strains and free-living rhizobacteria on the number of applied rhizobia in the soil as well as on the yield and N content of ryegrass as the preceding crop and of alfalfa as the subsequent crop.

45.2 Material and Methods

Azotobacter vinelandi strain Av, *Azotobacter chroococum* strain AoNDD, *Enterobacter* sp.strain E1 and four effective *S. meliloti* strains 218, L3Si, 4148ss and 207, from the Collection of the Institute of Soil Science were used for the inoculation of Italian ryegrass and alfalfa. The effects of these strains on the yield and N-content of Italian ryegrass cultivar K-29 t and alfalfa cultivar K-28 (Institute for Forage Crops, Kruševac) were examined in a pot experiment under greenhouse conditions, using

non-sterile soil with no history of cultivation of these crops. The soil used had the following characteristics: pH (in H_2O) 7.4, 900 mg N kg^{-1}, 21 mg kg^{-1} available P.

The first part of the experiment was set up in 2009 with Italian ryegrass. The experiment consisted of seven inoculated treatments and two control treatments without inoculation and without (Ø) or with (NØ) mineral nitrogen fertilization (27 mg N kg^{-1}) in five completely randomized replicates. *Enterobacter*, *Sinorhizobium* and *Azotobacter* strains were cultivated on appropriate medium for 24, 48 and 72 h, respectively. Italian ryegrass seeds were surface-sterilized with a 0.1 % $HgCl_2$ solution. Five seeds per pot were planted and inoculated with 2 ml $plant^{-1}$ of the liquid culture of the single strains containing > 10^9 cells per ml. The pots were kept in a greenhouse conditions for six weeks.

The second part of the experiment consisted of the determination of the number of *S. meliloti* strains in the pots after cutting the Italian ryegrass by the Plant infection count method (Vincent 1970). The most probably number (MPN) of *S. meliloti* strains applied in the rhizobial inoculated treatments was compared with the soil before sowing.

The third part of the experiment was conducted in 2010 with alfalfa growing in the pots with the soil where the Italian ryegrass had grown after removal of the roots of the Italian ryegrass. Alfalfa was inoculated with the same strains applied for the inoculation of Italian ryegrass. In addition, the yield and the N-content of the subsequent crop were compared with alfalfa grown without the preceding crop and inoculated with the same rhizobial strains. The pots were kept in semi controlled conditions for six weeks. Roots of alfalfa were carefully removed from the pots, washed free of soil and the number of nodules was recorded.

In the first and third part of the experiment shoot height of both species was measured. Plant shoots were 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 the CNS analyser (Vario model EL III (ELEMENTAR Analysensysteme GmbH, Hanau, Germany) and it was used to calculate total N content in mg per pot. The data were statistically processed by the LSD and Duncan test using the statistical program SPSS 10.0. Correlation coefficients were calculated to study the associative relations among the measured traits.

45.3 Results and Discussion

In our investigation Italian ryegrass as crop preceding alfalfa was inoculated with four single effective rhizobial strains, one *Enterobacter* strain as well as two single strains of *Azotobacter* spp. The height of the ryegrass plants was 26.57–29.82 cm without significant differences among treatments. The highest average values of ryegrass shoot dry weight (SDW) were obtained in the inoculated treatments with *A. vinelandi* strain Av, *S. meliloti* strain 207 and *A. chroococcum* strain Ao NDD (Table 45.1). These results indicated that shoot dry weight (SDW) was significantly influenced by inoculation with these strains in respect to the other inoculated treatments and

Table 45.1 Effect of bacterial inoculants on plant parameters of Italian ryegrass as preceding crop and alfalfa as subsequent crop

Treatments	Bacterial strains		Italian ryegrass		Alfalfa		
			2009		2010		
			SDW mg per pot	Total N content mg per pot	SDW mg per pot	Total N content mg per pot	Number of nodules per plant
Inoculation of preceding and of subsequent crop	Free-living nitrogen fixers	Av	752.90[a]	31.80[a]	3,729.33[b,c,e]	120.84[b,c]	10.44[b,c]
		E1	622.95[bc]	27.40[b]	3,466.33[b,c,e]	107.45[b,c]	18.20[b]
		Ao NDD	658.15[a,b]	24.50[bcd]	3,655.33[b,c,e]	102.72[c]	18.64[b]
	S. meliloti	L3Si	651.35[b,c]	27.40[b]	4,939.33[a]	172.39[a]	11.33[b,c]
		4148	637.45[b,c]	23.35[cd]	3,087.30[c]	100.31[c]	26.74[a]
		207	701.20[a]	26.35[bc]	3,540.33[b,c]	107.63[bc]	27.84[a]
		218	568.85[c]	21.30[d]	4,216.30[a,b]	121.84[b,c]	17.08[b,c]
	Controls[f]	$Ø_1$	584.65[c]	22.75[cd]	2,180. 00[d]	47.45[d]	11.73[b,c]
		NØ	785.40[a]	34.85[a]	5,397.21[a]	178.10[a]	7.16[c]
Alfalfa common inoculation	*S. meliloti*	L3Si	/	/	4,042.62[b]	137.04[b]	8.00[c]
		4148	/	/	2,900.00[c]	94.25[c]	15.00[b,c]
		207	/	/	3,321.67[b,c]	96.33[c]	16.00[b,c]
		218	/	/	3,530.00[b,c]	97.66[c]	7.00[c]
	Control[f]	$Ø_2$	/	/	2,550.00[c,d]	49.00[d]	12[b,c]
		LSD 0.05	87.55	3.6	896.71	22.26	7.02

[a–d]Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test at the 5 % level ($p \leq 0.05$)
[e]*S. meliloti* strain 207 was applied for inoculation of subsequent crop
[f]Uninoculated controls: NØ-with N and $Ø_{1,2}$-without N

control Ø. There were no significant differences between these treatments and the control treatment with N fertilization (NØ) which pointed out the N fixating and PGPR ability of these strains.

Rhizobia are known to produce growth promoting substances in the presence of a rhizosphere which is not the one of a host plant (Kavimandan 1985). Thus, besides the usual N fixing ability of rhizobia that is expressed only with specific legumes, it can be assumed that *S. meliloti* strain 207 has some of PGPR properties which could affect the SDW increase of Italian ryegrass. Similar results have been noted by Kavimandan (1985, 1986) and Avis et al. (2008). They found that wheat seed inoculation with several rhizobial species increased wheat growth and N uptake under N-limiting conditions. The phosphorus content was significantly increased in cotton plants inoculated with *S. meliloti* (Egamberdiyeva et al. 2004).

Among the free living strains applied, the treatment with a *A. vinelandi* strain resulted in the highest value of total N content (31.80 mg per pot^{-1}) in the shoot dry matter which was well correlated with SDW ($r = 0.912$). Yields of rice, cotton and wheat in many investigations were increased significantly after applications of *Azotobacter* due to its ability to fix N_2 (Kennedy et al. 2004). Treatments inoculated with the other bacterial strains realized lower SDW and total N content and there were no significant differences among them and untreated control-Ø.

Table 45.2 Effect of previous crop seed inoculation on *Sinorhizobium meliloti* number in soil the following year

S. meliloti strains		Index of increase
Inoculants	MPN[a] g^{-1} soil	
L3Si	95×10^2	6.8
218	30×10^2	2.1
207	140×10^2	10
$Ø_3^b$	14×10^2	1

[a]*MPN* most probable number, [b]$Ø_3$ (Control) unseeded soil

The number of rhizobial bacteria was determined before sowing and after cutting the ryegrass inoculated with the investigated *S meliloti* strains. In the soil before sowing the number of autochthonous *S. meliloti* was low (14×10^2 g^{-1} soil) (Table 45.2). The rhizobial number determined after cutting the ryegrass increased by two to ten times depending on the rhizobial strain applied which is in agreement with results of some authors obtained in experiments with other crops (Diatloff 1969; Domit et al. 1990; Goos et al. 2001). This inoculation of Italian ryegrass as crop preceding alfalfa had an influence on the increase of the number of *S. meliloti* and together with the inoculation of the subsequent alfalfa lead to a double inoculation of alfalfa.

In 2010 alfalfa was grown in the pots after the removal of annual ryegrass. All alfalfa plants as a subsequent crop were inoculated with the same rhizobial strains applied for the inoculation of the ryegrass. The rhizobial strain 207 was applied in the pots where ryegrass was inoculated with the free-living bacteria. This is a double inoculation in the same pots with the same strains: seed inoculation of ryegrass as preceding crop and seed inoculation of alfalfa as subsequent crop. For comparison with this double inoculation new treatments were set up in the new pots with alfalfa commonly inoculated without preceding crop. The height of alfalfa was not significantly affected neither by common nor double inoculation. The height of the plants varied between 44.73 and 54.09 cm without significant differences among treatments (data not shown). The double inoculation of alfalfa by the strains L3Si and 218 gave the highest SDW, 4,939.33 and 4,216.30 mg per pot, respectively (Table 45.1). Among alfalfa treatments without preceding crop, strain L3Si had also the highest effectiveness expressed by SDW (4,042 mg per pot) and total N content (137.04 mg per pot), however significantly lower than in the double inoculated treatment.

There were no significant differences between the SDW and N content of alfalfa obtained in treatments inoculated with strain 207 after the preceding crop inoculated with free living bacteria and commonly inoculated alfalfa with strain 207. According to our results it can be assumed that SDW of alfalfa was not influenced by the inoculation with strains of free living rhizobacteria. The benefit of free-living N fixing bacteria concerns cereals as ryegrass, while their benefit for legumes is negligible (Rai and Gaur 1988). Double rhizobial inoculation increased significantly the SDW of alfalfa significantly in comparison with treatments without preceding crop. These results indicated that, in a soil with low rhizobial density where alfalfa was not grown for a long time and with low available N, the rhizobial inoculation of ryegrass with

effective rhizobial strains can increase yield and N content of alfalfa as a subsequent crop. The total N content in the shoots of alfalfa was highly correlated with SDW ($r = 0.966$) while there was no correlation between nodule number on one side and SDW and total N content ($r = -0.277$) on the other side. Both double and common inoculations with L3Si, 218, 207 and 4148ss significantly increased the total N content of alfalfa with respect to the untreated controls. However, only plants double inoculated with L3Si had the highest total N content (172.39 mg per pot) in comparison with plants commonly inoculated (Table 45.1). It can be assumed that the rhizosphere of the preceding crop had influence on the rizobial number increase. There was effect of the increased number of *S. meliloti* on nodule number after inoculation of Italian ryegrass in some alfalfa treatments (strains 4148 and 218). The increase of SDW and total N content of alfalfa double inoculated by highly effective strains L3Si, 218 was likely caused by the increased *S. meliloti* number after the inoculation of the ryegrass as a preceding crop.

45.4 Conclusion

Our results indicate that Italian ryegrass yield and N assimilation was increased by inoculation with *A. vinelandi* strain Av and *S. meliloti* strain 207. Nevertheless, Italian ryegrass seed inoculation with *S. meliloti* strains led to an increased number of S. *meliloti* in a soil with a low density of this species. The Italian ryegrass seed inoculation with *S. meliloti* strains L3Si and 218 provided an abundant rhizobial number in the soil the year before alfalfa growing. This leads together with a common inoculation of alfalfa to a better yield of alfalfa. This double inoculation of alfalfa should be applied in soils without or poor in *S. meliloti*.

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Chapter 46
Optimal Plant Type of Pea for Mixed Cropping with Cereals

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Abstract Pea (*Pisum sativum* L.) in mixed stand (MS) with one small-grain cereal or in pure stand (PS) is gaining interest for silage production. Breeding programmes, however, target essentially the grain crop, selecting semi-dwarf germplasm. The aim of this study was to assess the impact of pea plant stature on biomass production and competitive ability against cereals. Three semi-leafless pea genotypes with a comparable phenology but contrasting plant stature, i.e. (i) one breeding line ('1/15b') lacking dwarfing genes, (ii) one semi-dwarf line with moderate stature (cv. 'Attika') and (iii) one semi-dwarf line with short stature (cv. 'Spirale'), were grown in PS and in binary mixtures with barley (cv. 'Cometa') or triticale (cv. 'Amarillo') in a Mediterranean environment. Harvest and dry matter yield assessment occurred at waxy stage of the pea grain. Seed densities for pure stands (100 seeds/m^2 for pea; 320 seeds/m^2 for cereals) were halved for mixtures. The pea genotypes '1/15b', 'Attika' and 'Spirale' ranked in this order for plant height and pea yield in PS or MS, total (pea + cereal) yield in MS, and pea proportion in MS, indicating the strict association of pea stature with biomass yield and competitive ability. Pea yield and proportion were lower and with smaller genotypic differences in MS with triticale than in MS with barley. The only drawback of the pea '1/15b' was its greater susceptibility to lodging, which emerged as a problem only in PS.

Keywords Dwarfing gene · Competitive ability · Forage · Intercropping · Mixtures · Plant stature

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46.1 Introduction

Field pea (*Pisum sativum* L.) is largely grown in European crop-livestock systems as a high-protein feed crop, owing to its high grain yield potential and the value of its straw as a forage (Carrouée et al. 2003). In addition, pea is gaining interest as a crop for silage production in mixed stand (MS) with one small-grain cereal (such as triticale, barley or wheat) or in pure stand (PS). Silage produced from pea PS has higher protein content but tends to display lower yield per unit area and poorer silage fermentation and quality than that from pea-cereal MS. However, pea silage with satisfactory quality can be obtained by adding an inoculant (*Lactobacillus plantarum*) and/or by ensiling material whose dry matter content has overcome 30 % through delayed harvest and a wilting period (Borreani et al. 2009). Mixed cropping of pea with cereals tends to maximize the crop forage yield and silage quality but usually implies a marked competitive disadvantage for the pea component (Hauggaard-Nielsen and Jensen 2001; Lithourgidis et al. 2011), especially in fertile soils and/or under moderate nitrogen fertilization (Corre-Hellou et al. 2006). In northern Italy the pea proportion in MS may drop below 20 % when adopting a semi-dwarf pea cultivar and component seed rates that are halved relative to seed rates used in pure stands (Tomasoni et al. 2006). The pea competitive disadvantage may be limited either by increasing largely the pea proportion in the seed mixture (Lithourgidis et al. 2011), or by selecting pea varieties with better ability to compete for light and nutrients (Hauggaard-Nielsen and Jensen 2001).

Pea breeding programmes target essentially the grain crop, selecting varieties with dwarfing genes to increase the crop harvest index and standing ability (Ranalli 1995). Additional selected traits for improving the standing ability are semi-leaflessness (i.e. the modification of leaflets in tendrils) and stem stiffness (Stelling 1989; Ranalli 1995). The semi-dwarf plant type may be less suited to silage production in PS or MS than a tall type, as it may imply lower pea biomass production and competitiveness against cereals. Research on wheat has highlighted that tall germplasm tends to display greater biomass production (Annicchiarico et al. 2005) and better competitive ability against weeds (Vandeleur and Gill 2004) than semi-dwarf material. In general, extensive shoot elongation and early growth are the main traits associated with inter-specific competitive ability of crop species (Lemerle et al. 2001). In a study comparing six leafed pea cultivars in a binary mixture with barley, a tall indeterminate cultivar and a very short cultivar were the best- and worst-competing genotypes, respectively (Hauggaard-Nielsen and Jensen 2001). Pea competitive ability against weeds was associated with taller stature in most (Mc Donald 2003; Annicchiarico and Filippi 2007) but not all studies (Townley-Smith and Wright 1994). Leaf type showed negligible influence on pea competitive ability (Mc Donald 2003; Townley-Smith and Wright 1994), supporting the general interest of leaflessness because of its positive effect on the standing ability.

The impact of pea stature on the suitability for silage production should also consider the fact that semi-dwarf cultivars may differ for plant height, owing to the several different dwarfing genes and their possibly different effect (Huyghe 1998).

This study aimed to produce information on the optimal pea ideotype for silage production, by comparing three semi-leafless genotypes with a comparable phenology but contrasting plant stature for biomass production in PS and MS and pea competitive ability in MS with barley or triticale. One genotype lacked dwarfing genes, whereas two were semi-dwarf with moderate or short stature.

46.2 Materials and Methods

The tall, semi-leafless plant type was represented by the advanced line '1/15b', selected by CRA-FLC from a cross between the cultivars 'Santana' (semi-dwarf, early flowering and with good standing ability) and 'Forrimax' (tall and late flowering). The progenies from this cross underwent selection for early flowering, standing ability and winter survival under field conditions in Lodi (Po Valley) for three seasons starting from the F_3 generation. The cultivars 'Attika' and 'Spirale', both featuring early flowering, good standing ability and wide adaptation to Italian environments (Annicchiarico and Iannucci 2008), represented the semi-dwarf germplasm with moderate and short stature, respectively. 'Attika' was the tallest of 46 recent semi-dwarf European and Australian varieties tested by Annicchiarico et al. (2003) in climatically-contrasting Italian sites, whereas 'Spirale' displayed high grain yield and harvest index associated with short stature in the same evaluation. The cereal companions in pea-cereal binary mixtures were the varieties 'Cometa' of barley (*Hordeum vulgare* L.) and 'Amarillo' of triticale (× *Triticosecale* Wittmack), which ranked among the best-yielding cultivars of the respective species in the Italian network of autumn-sown variety trials for 2010.

Two trials were performed in different fields of the Mediterranean site of Sanluri (Sardinia) in 2011. One included the PS of barley and pea genotypes, and pea-barley binary mixtures; the other included the PS and MS of triticale and pea genotypes. Both trials were designed as a split-plot with four replications, holding pea cropping condition (PS or MS) on main plots and pea genotypes on subplots. The cereal PS was added to MS subplots. Seed densities for PS were those recommended, i.e., 100 seeds/m^2 for pea and 320 seeds/m^2 for cereals, halving them and mixing the seed for MS. Plot size was 4.0 × 1.5 m, with rows spaced 0.18 m apart. The autumn sowing was delayed until January 10, owing to unfavourable climatic conditions. Harvests took place between May 18 and May 23 at waxy stage of the pea grain, assessing forage dry matter (DM) yield of pea and cereal genotypes in PS and MS. Forage DM content of pea and cereals were assessed on a fresh herbage sample of at least 3 kg per plot. This sample was also used for assessing the pea proportion on total DM in MS, which estimated the pea competitive ability. Onset of flowering, plant height at crop harvest, and pea susceptibility to lodging at crop harvest (expressed on a visual 5-level scale ranging from 1 = erect to 5 = extensively lodged) were also recorded. The rainfall amount from January 1 to May 15 was 224 mm, slightly higher than the long-term value for the site (195 mm).

46.3 Results and Discussion

The delayed autumn sowing retarded the reproductive development of both cereals and reduced their DM yield in PS and their competitive ability in MS relative to an ordinary cropping season.

The differences in plant stature among pea genotypes were larger between tall and semi-dwarf material than between semi-dwarf genotypes, but 'Attika' was distinctly taller than 'Spirale' in all PS or MS conditions (Table 46.1). Only the pea genotype lacking dwarfing genes displayed taller stature than any cereal companion in MS. The pea genotypes '1/15b', 'Attika' and 'Spirale' ranked in this order not only for plant height but also for pea DM yield in PS or MS, total (pea + cereal) DM yield in MS, and pea proportion in MS (Table 46.1). The respective values of these genotypes averaged across the two experiments were 8.25, 6.86 and 5.52 t ha for pea DM yield in PS; 5.01, 3.04 and 2.00 t ha for pea DM yield in MS; 7.91, 7.07 and 6.91 t ha for total DM yield of mixtures; and 0.61, 0.43 and 0.29 for pea proportion in MS. These results highlighted the positive association of pea stature with pea forage yield in PS or MS and with pea competitive ability in MS. They also suggested that better competitive ability of the less competitive species, i.e. pea, may have a positive effect also on total yield of the mixture, in agreement with prior observations on legume-grass binary mixtures including genotypes of a poorly-competing species such as white clover in association with highly-competing grass species or varieties (Annicchiarico and Piano 1994).

The two cereals showed similar DM yield in PS, but triticale displayed greater competitive ability against pea than barley associated with its taller stature (Table 46.1). The tall pea line '1/15b' was at competitive disadvantage only with triticale on the basis of pea proportion data (Table 46.1). In general, pea yield and pea proportion were lower and with smaller genotypic differences in MS with triticale than in MS with barley (Table 46.1).

PS or MS including the pea genotype '1/15b' also tended to outperform the cereal PS (Table 46.1). The only drawback of the tall genotype was its greater susceptibility to lodging relative to semi-dwarf material, which emerged as a problem only in PS (where its lodging susceptibility score averaged nearly 4.5 compared with 2.9 for 'Attika' and 1.9 for 'Spirale'). Thus, the semi-leafless trait and the selection for standing ability could not provide this genotype with sufficient standing ability in PS in this test environment.

On the whole, our results indicate that the tall semi-leafless pea plant type is superior to the semi-dwarf semi-leafless plant type for silage production in MS with cereals. This indication is likely to be reinforced under timely autumn sowing, which is expected to increase the cereal competitive ability. The seed production of the tall pea type in PS, however, may suffer of losses due to insufficient tolerance to lodging (whereas its seed production in MS is hindered by current regulations for producing certified seed). The semi-dwarf pea type with maximized plant stature is preferable to the tall plant type for silage production in PS, owing to its better standing ability.

Table 46.1 Pea plant height at crop harvest, forage dry matter (DM) yield of the crop and its components, and pea proportion on total DM, for three pea genotypes with contrasting plant stature grown in pure stand (PS) and mixed stand (MS) with barley or triticale in Sardinia

Experiment[a]	Pea height (cm)[bc]	Total DM yield (t/ha)[b]	Pea DM yield (t/ha)[b]	Cereal DM yield (t/ha)[b]	Pea proportion[b]
With barley					
PS, barley	–	5.93 de	–	5.93 a	–
PS, pea '1/15b'	118.1 a	8.83 ab	8.83 a	–	–
PS, pea 'Attika'	72.3 c	7.22 cd	7.22 b	–	–
PS, pea 'Spirale'	54.4 d	5.62 e	5.62 c	–	–
MS, barley + pea '1/15b'	103.2 b	9.23 a	7.05 b	2.18 c	0.76 a
MS, barley + pea 'Attika'	66.5 c	7.79 bc	3.63 d	4.16 b	0.46 b
MS, barley + pea 'Spirale'	49.7 d	7.25 cd	2.23 d	5.02 ab	0.31 c
With triticale					
PS, triticale	–	5.94 bc	–	5.94 a	–
PS, pea '1/15b'	118.5 a	7.67 a	7.67 a	–	–
PS, pea 'Attika'	69.4 b	6.50 b	6.50 b	–	–
PS, pea 'Spirale'	48.9 c	5.42 c	5.42 c	–	–
MS, triticale + pea '1/15b'	108.5 b	6.59 b	2.96 d	3.63 c	0.45 a
MS, triticale + pea 'Attika'	61.4 b	6.35 bc	2.46 d	3.89 c	0.39 a
MS, triticale + pea 'Spirale'	45.5 d	6.57 b	1.78 e	4.79 b	0.27 b

[a]Pea genotypes are: line '1/15b', tall; 'Attika', tall within semi-dwarf type; 'Spirale', short within semi-dwarf type (all semi-leafless). Cereal genotypes are 'Cometa' for barley and 'Amarillo' for triticale

[b]Column means within experiment with different letters differ at $P < 0.05$ according to Duncan's test

[c]Mean cereal plant height at mixed stand harvest: 83.2 cm for barley, 97.1 cm for triticale

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Chapter 47
Dry Matter Recovery and Aerobic Stability of Maize Whole-Crop, Cob and Stover Silages—Harvest Date and Cultivar Effects

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Abstract Forage maize (*Zea mays* L.) has the potential to produce high yields of excellent quality feed for ruminants. However, for regions with a cool overcast climate, improvements in maize silage production systems are required to reduce variation in yield and quality. Ensiling can reduce yield through dry matter (DM) losses and may alter feed quality. This study investigated the effects of harvest date on the DM recovery and aerobic stability of whole-crop, cob and stover silages produced from contrasting cultivars of maize. Six cultivars of forage maize, four of which were categorised as conventional (Tassilo, FAO 190; Beethoven, FAO 200; Andante FAO 200 and Nescio, FAO 230) and two categorised as high biomass (Atletico, FAO 280 and KXA 7211, FAO 260) were sown in 72 m^2 plots under plastic mulch on 7 May 2008. Within each of three replicate blocks, harvest date (16 September, 7 October and 28 October) constituted the main plots and cultivar the sub plots within a split-plot design. Samples of whole crop, stover and cob from each plot were precision chopped and 6 kg of each were ensiled in laboratory silos for 130 days at 15 °C. After opening, sub-samples were subjected to chemical and microbial analyses, while aerobic stability and deterioration were estimated by measuring silage temperature during 8 days exposure to air. The rate of DM recovery of ensiled whole-crop, cob and stover was unaffected ($P > 0.05$) by harvest date or cultivar. No overall effects of harvest date or cultivar were observed on the aerobic stability or aerobic deterioration of whole-crop or stover silages. Cob silages harvested on 16 September underwent more ($P < 0.05$) aerobic deterioration than cob silages harvested at later dates. Cob silages produced from Nescio underwent less ($P < 0.05$) aerobic deterioration than for Tassilo, Beethoven and Andante.

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47.1 Introduction

Forage maize (*Zea mays* L.) silage has the potential to support high animal performance in ruminant production systems compared to more conventional conserved forages in Ireland and Britain (Fitzgerald and Murphy 1999; Phipps et al. 2000; Keady et al. 2007). The limitations often imposed by relatively low solar radiation and temperatures in Ireland, which often result in sub-optimal yields and feed quality, need to be overcome for maize to be considered a sustainable and economically viable alternative forage. Previous studies in Ireland have focused on the effects which crop management factors, such as cultivar selection, the use of plastic mulch, seeding rate and sowing date (Keane 2002; Keane et al. 2003; Farrell and Gilliland 2011) have on the yield and nutritive value of whole-crop maize previous at time of harvest. However, the subsequent ensilage of forages can alter their nutritional value and may incur qualitative and quantitative losses through the production of effluent or gas during the fermentation process (McDonald et al. 1991). In addition, substantial losses due to poor aerobic stability and aerobic deterioration can occur when silage chemical components are respired by aerobic organisms. These can decrease the amount of dry matter (DM) available, produce undesirable by-products which place an animals health at risk, such as mycotoxins (Muck 2010).

The objectives of this study were to determine the effects of cultivar and harvest date on the DM recovery and on the aerobic stability of whole-crop, cob and stover silages.

47.2 Materials and Methods

The experiment had a split-plot design, with three main plots (date of harvest) and six sub plots (maize cultivar) in each of three replicate blocks. Of the six cultivars of maize, four conventional cultivars were representative of what is sown by commercial livestock farmers for silage production in Ireland (Tassilo FAO 190; Beethoven, FAO 200; Andante, FAO 200; Nescio, FAO 230) and two were categorised as high biomass cultivars (Atletico, FAO 280; KXA 7211, FAO 260). The three harvest dates of 16 September, 7 October and 28 October represented an early, normal and late harvest, respectively, for a midland site in Ireland. On each harvest date, whole-crop, cob and stover samples were manually harvested and subsequently precision-chopped. A 6 kg sub-sample of each chopped sample was ensiled in a laboratory silo (O'Kiely and Wilson 1991) for 130 days at approximately 15 °C. Following the ensilage period, effluent (if any) was collected and weighed. Silage was weighed, aseptically mixed and sub-sampled for chemical analyses. Aerobic stability was determined by placing 3.6 kg of each silage into a polystyrene box (2.5 cm thick; 59 cm × 39 cm × 22 cm) lined with polythene and loosely covered with a polystyrene lid. A thermocouple was placed in the centre of each silage sample. The temperature of the silage was recorded on an hourly basis over 8 day period by a data logger (SQ ELTEK 80T;

Eurolec Instrumentation Ltd, Dundalk, Ireland). Reference temperatures were obtained from containers of water stored beside the boxes of silage. The indices of aerobic stability and deterioration were expressed as (i) hours elapsed until the temperature rose more than 2 °C above the reference temperature and (ii) accumulated temperature rise during 120 h exposure to air. Chemical analyses were as described by McEniry et al. (2006). Data were analysed as a split-plot design using a model that accounted for harvest date as the main plot, cultivar as the sub plot, and their interactions, using the PROC GLM procedure of the SAS statistical program (SAS 2002). Treatment contrasts were made using the Fischer least significant differences test.

47.3 Results and Discussion

No effect of harvest date or cultivar ($P > 0.05$) was observed on the DM recovery of ensiled whole-crop, cob or stover (Table 47.1). However, the mean (SD) whole-crop DM recovery value of 908 g (45.4 g) silage DM/ kg DM ensiled was lower than reported by Johnson et al. (2002; 940–1000 g silage DM/kg DM ensiled). The additional losses in the present study likely resulted from the relatively low herbage DM content leading to effluent production and a more extensive fermentation resulting in increased CO_2 production.

The values for the indices of aerobic stability (135–192 h for silage temperature to increase more than 2 °C above ambient temperature) and aerobic deterioration (1–9 °C accumulated temperature rise during 120 h exposure to air) in the present study indicate that these whole-crop silages were quite aerobically stable. This limited aerobic activity reflected a relatively high content of acetic acid in the present study. Acetic acid is inhibitory to yeast, the primary organisms initiating aerobic deterioration (Muck 2010), and yeast numbers were generally low in these whole-crop silages. No effect of harvest date or cultivar ($P > 0.05$) were observed on the aerobic stability or deterioration of whole-crop or stover silages as all these silages had high acetic acid concentrations and low yeast numbers, and were thus aerobically stable. Cob silages from crops harvested on the 16 September had a more extensive deterioration after exposure to air than silages from crops harvested at later dates.

In conclusion, later harvesting resulted in improved DM recovery and aerobic deterioration of cob silages, while whole-crop and stover silages were unaffected by harvest date. The high biomass crops did not confer a disadvantage in DM recovery or aerobic stability of whole-crop, cob or stover silage when compared to the conventional cultivars. The DM recovery and aerobic deterioration values for whole-crop maize silages were generally intermittent between the values for the two individually ensiled components of the crop.

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Table 47.1 Silage fermentation characteristics, DM recovery and aerobic stability and deterioration of whole-crop, cob and stover silages

	Harvest	16 September						7 October						28 October						SEM[f]			Significance		
	Cultivar[a]	T	B	An	N	At	K	T	B	An	N	At	K	T	B	An	N	At	K	H	C	H × C	H	C	H × C
Whole-crop	TFP[b]	185	181	220	171	182	201	112	95	134	128	202	176	76	65	76	76	148	102	6.2	8.5	14.8	***	***	*
	AA[b]	33	41	36	30	44	38	39	31	37	28	48	45	35	28	43	27	60	34	3.1	4.5	7.8		*	
	pH	3.6	3.7	3.6	3.5	3.7	3.6	3.9	3.6	3.7	3.8	3.6	3.6	4.3	4.2	4.1	4.4	4.2	4	0.09	0.08	0.13	*		
	DMR[c]	858	863	859	866	805	923	979	921	924	949	962	938	944	895	948	927	874	919	21.8	19.5	33.8			
	ITR > 2 °C[d]	190	181	172	192	105	175	134	158	144	167	192	135	162	138	192	192	136	192	11.2	18.1	31.3			
	ACT 120 h[e]	3	2	4	2	4	4	9	2	2	1	1	1	3	5	1	2	1	1	1.5	1.4	2.4			
Cob	TFP[b]	61	67	114	80	156	160	66	61	75	55	216	99	47	37	66	43	73	88	11.4	12.6	21.9		***	
	AA[b]	11	12	31	17	55	46	15	9	25	11	57	34	16	14	18	10	33	23	3.4	4.9	8.4		***	
	pH	3.7	3.7	3.8	3.9	4.1	4	3.7	3.7	3.7	3.7	3.7	3.8	4	4.1	4.2	4.2	3.8	3.9	0.04	0.05	0.8	**		**
	DMR[c]	921	908	858	872	837	844	988	925	900	967	983	934	934	983	975	974	948	976	20.7	21.7	37.6			
	ITR > 2 °C[d]	48	41	98	173	101	113	83	117	77	126	192	167	192	192	126	192	133	128	21.3	16.4	24.3			*
	ACT 120 h[e]	44	46	26	1	31	20	18	9	15	7	1	1	2	1	1	2	1	3	3.3	3.7	6.4	*	*	*
Stover	TFP[b]	173	173	155	137	167	155	156	107	165	120	135	144	89	88	121	66	145	83	3.6	7.0	12.2	***	**	*
	AA[b]	68	72	62	66	72	62	72	61	75	63	57	70	49	36	59	41	64	47	3.7	2.1	3.6	*	*	***
	pH	4.2	4.1	4.5	4.2	4.4	4.4	4.2	4.4	4.5	4.3	3.8	4	4.4	4.3	4.3	4.6	4.4	4.4	0.06	0.06	0.1			**
	DMR[c]	855	847	875	873	812	872	943	956	953	920	903	930	903	937	883	866	889	892	15.1	22.5	38.9			
	ITR > 2 °C[d]	162	147	192	192	170	192	192	192	192	192	192	192	167	192	143	173	143	192	10.5	12.7	22.0			
	ACT 120 h[e]	6	9	2	2	3	1	1	3	2	1	1	2	2	0	2	2	1	1	1.0	1.2	2.1			

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

H harvest date, *T* Tassilo, *B* Beethoven, *An* Andante, *N* Nescio, *At* Atletico, *K* KXA 7211, *TFP* total fermentation products, *AA* acetic acid

[a]Cultivar *C*

[b]Gram per kilogram DM

[c]Gram silage DM/kg DM ensiled

[d]Interval (h) until temperature rises more than 2 °C above ambient temperature (ITR > 2 °C; index of aerobic stability)

[e]Accumulated temperature rise (°C) during 120 h exposure to air (ACT, index of aerobic deterioration)

[f]Standard error of the mean

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Chapter 48
Performance of Forage Soya Bean (*Glycine max*) Cultivars in the Northern Balkans

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Abstract Soya bean (*Glycine max* (L.) Merr.) is the most important grain legume crop in many West Balkan Countries. A programme on the alternative uses of soya bean such as forage, biomass or green manure has been recently launched in the Institute of Field and Vegetable Crops, the Faculty of Agriculture in Novi Sad and the Maize Research Institute Zemun Polje in Belgrade. A small-plot trial has been carried out in Novi Sad and Zemun Polje during 2009 and 2010 with four US forage soya bean cultivars. In both years and at both locations, all four cultivars were sown in late April, with a target sowing density of 75 viable seeds m^{-2}, and cut in the stages of full flowering or first pods development, mostly in the second half of July. In a 2-year average, the cultivar Donegal had the highest yields of both green forage (63.9 t ha^{-1}) and forage dry matter (15.1 t ha^{-1}). In single years, the highest yields were recorded in the cultivar Donegal, in Novi Sad in 2010, with 82.4 t ha^{-1} of green forage and 18.4 t ha^{-1} of forage dry matter.

Keywords Forage DM yield · Green forage yield · Serbia · Soya bean

48.1 Introduction

Pea (*Pisum sativum* L.) and common vetch (*Vicia sativa* L.) are the most traditional and widely cultivated annual forage legumes in Serbia and other Balkan countries. Both have autumn and spring sown forms and are often grown in mixtures with small grains such as oat (*Avena sativa* L.) and triticale (× Triticosecale spp.). Annual

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forage legumes may be used as green forage, forage dry matter, forage meal, silage or haylage (Ćupina et al. 2011). They are also considered valuable green manure crops in organic farming and sustainable agriculture (Ćupina et al. 2004).

Soya bean (*Glycine max* (L.) Merr.) is the most important annual legume crop in Serbia, with an average harvested area of 150,000 ha (Mikić et al. 2009). It is cultivated mostly for the production of grain rich in protein and oil. Soya bean crop may also be a source of quality forage during the summer, when other annual forage brassicas or legumes are already cut (Bilgili et al. 2003; Devine et al. 2010).

The first forage soya bean cultivars in USA introduced from Asia were not adapted to the new conditions. At the same time, soya bean breeding programmes for grain produced highly adapted cultivars with enhanced disease, insect, and nematode resistances. Both these and traditional hay-type cultivars were used in the USDA-ARS forage soya bean breeding programme by conventional methods and remarkably tall and lodging resistant lines were developed (Devine et al. 2007).

This research aims at assessing the potential of soya bean for forage production in climatic regions of Europe similar to that of Serbia and northern Balkans, by testing the performance of some of the commercial US forage soya bean cultivars developed at USDA-ARS and widely used throughout USA.

48.2 Materials and Methods

A small-plot trial was carried in 2009 and 2010 at Rimski Šančevi near Novi Sad and Zemun Polje near Belgrade, including four US forage soya bean cultivars, namely Derry (Devine et al. 1998a), Donegal (Devine and Hatley 1998), Tara (Devine and McMurtrey 2004) and Tyrone (Devine et al. 1998b).

In both years and at both locations, the trial was established in the last week of April. All cultivars were sown at a target density of 75 viable seeds m^{-2} (Acikgoz et al. 2007) and at a row spacing of 20 cm (Seiter et al. 2004). The dominant weather conditions during the trial period at Rimski Šančevi and Zemun Polje are given in Table 48.1.

Each cultivar was cut in full bloom, as an optimal stage and a balance between yield and quality in most annual forage legumes (Mihailović et al. 2009). In both years and at both locations, this was in mid- to late August. Plant samples taken immediately before cutting were used to record the main forage yield components, which were plant height (cm), number of internodes (per plant) and number of photosynthetic active leaves (per plant). Green forage yield (t ha^{-1}) was based on the green forage yield per plot, measured immediately after cutting. Forage dry matter yield (t ha^{-1}) was calculated on the basis of the ratio between the green mass of forage samples of 1 kg before and after the drying at the controlled room temperature until the confirmed constant mass.

The results were processed by analysis of variance (ANOVA) using the LSD test, as well as the significance of genotypes, environments and genotype × environment

Table 48.1 Monthly average temperatures and precipitation at Rimski Šančevi and Zemun Polje during the trial

Location	Year	April	May	June	July	Average
Average monthly temperature (°C)						
Rimski Šančevi	2009	15	18	20	23	19
	2010	13	17	20	23	18
	Long-term	11	17	20	21	17
Zemun Polje	2009	16	20	21	24	20
	2010	13	18	20	24	19
	Long-term	13	18	22	23	19
Monthly precipitation sum (mm)						
Rimski Šančevi	2009	2	47	123	57	229
	2010	71	95	174	98	438
	Long-term	47	59	85	70	261
Zemun Polje	2009	7	27	72	31	137
	2010	65	89	155	103	412
	Long-term	43	41	76	56	216

(G × E) interactions (Steel and Torrie 1960) for forage dry matter yield, as the most important agronomic characteristic.

48.3 Results and Discussion

As for the average monthly temperature, both soya bean growing seasons were mostly warmer in comparison to the long-term averages, especially at Rimski Šančevi. The season of 2009 was generally warmer than 2010, especially during April and July. Regarding the monthly precipitation sums, at both locations, the season of 2009 was drier than the long-term averages, while the season of 2010 was extremely wetter, with a specific emphasis in June, with doubled long-term values at both locations. Since the extremity of both years, a specific analysis of the interaction between cultivars and locations was purposely left out for the next seasons and more data.

There were significant differences between the average values of all three forage yield components among the four tested soya bean cultivars (Table 48.2).

The average plant height varied from 141 cm in Tyrone and 142 cm Derry to 161 cm Donegal (Table 48.2). The smallest plant height (138 cm) was both Derry and Tyrone at Zemun Polje. The greatest plant height (166 cm) was in the cultivar Donegal at Rimski Šančevi. This was lower than that recorded in performance trials at Orange, Virginia, USA (Darmosarkoro et al. 2001), where Tyrone reached a height of 180 cm.

Donegal had the highest average values of both number of internodes (29 per plant) and number of active leaves (25 per plant). Tyrone had the smallest average number of both internodes (24 per plant) and active leaves (21 per plant).

Table 48.2 Two-year average of forage yield components of four soya bean cultivars at Rimski Šančevi and Zemun Polje

Cultivar	Location	Plant height (cm)	Number of internodes (per plant)	Number of leaves (per plant)
Derry	Rimski Šančevi	146	26	22
	Zemun Polje	138	29	24
	Average	142	28	23
Donegal	Rimski Šančevi	166	27	23
	Zemun Polje	155	30	26
	Average	161	29	25
Tara	Rimski Šančevi	152	29	25
	Zemun Polje	159	25	22
	Average	156	27	24
Tyrone	Rimski Šančevi	144	24	22
	Zemun Polje	138	23	20
	Average	141	24	21
$LSD_{0.05}$		6	3	2
$LSD_{0.01}$		8	4	3

Donegal had the highest average values of fresh yield (63.9 t ha^{-1}) and forage dry matter (15.1 t ha^{-1}), while the cultivar had the lowest average forage yields, with 44.9 t ha^{-1} of green forage and 10.9 t ha^{-1} of forage dry matter (Table 48.3).

The highest green forage yields at individual locations and years were measured in the cultivar Donegal at Rimski Šančevi in 2010 (82.4 t ha^{-1}) and at Zemun Polje in the same year (75.5 t ha^{-1}), due to a particularly rainy and moderately warm growing season at both locations. The same cultivar also had the highest forage dry matter yields, also in 2010, with 18.4 t ha^{-1} at Rimski Šančevi and 17.9 t ha^{-1} Zemun Polje.

It should be noted that the cultivar Donegal had as excellent 2-year performance in the conditions of Serbia as it had in the trial carried out at the Royal Agricultural College at Cirencester, UK (Koivisto et al. 2003), where it produced an average yield of 10.0 t ha^{-1} of forage dry matter.

If compared the traditional Balkan annual forage legumes, the four tested forage soya bean cultivars proved at least as equally suitable for forage production as forage pea or common vetch. On average, the autumn sown forage pea cultivars of Serbian origin may produce nearly 48 t ha^{-1} of green forage and more than 10 t ha^{-1} of forage dry matter (Mihailović et al. 2004), while the average yields of the Serbian common vetch cultivars may surpass 39 t ha^{-1} of green forage and 10 t ha^{-1} of forage dry matter (Mihailović et al. 2005).

The stands of all four cultivars, despite their great height, remained erect until cutting and without a single plant lodged. Also, by some still non-clarified reason, some cultivars such as Tara suffered from a reduced number of plants during their growing period, resulting in smaller number of plants per plot and an excessive growth of the above ground parts. Such cultivars, despite the fact that both forage soya bean growing seasons were extreme in comparison to the average, may be

Table 48.3 Forage yields of four soya bean cultivars at Rimski Šančevi and Zemun Polje in 2009 and 2010

Cultivar	Location	Year	Green forage yield (t ha^{-1})	Forage dry matter yield (t ha^{-1})
Derry	Rimski Šančevi	2009	28.4	7.1
		2010	73.4	17.6
		Average	50.9	12.4
	Zemun Polje	2009	31.0	7.4
		2010	64.4	15.6
		Average	47.7	11.5
	Mean		49.3	11.9
Donegal	Rimski Šančevi	2009	49.6	12.4
		2010	82.4	18.4
		Average	66.0	15.4
	Zemun Polje	2009	48.2	11.6
		2010	75.5	17.9
		Average	61.9	14.8
	Mean		63.9	15.1
Tara	Rimski Šančevi	2009	39.1	9.8
		2010	63.2	14.0
		Average	51.2	11.9
	Zemun Polje	2009	52.0	12.5
		2010	60.2	13.7
		Average	56.1	13.1
	Mean		53.6	12.5
Tyrone	Rimski Šančevi	2009	51.5	12.9
		2010	40.2	9.5
		Average	45.9	11.2
	Zemun Polje	2009	44.9	10.8
		2010	43.0	10.2
		Average	44.0	10.5
	Mean		44.9	10.9
$LSD_{0.05}$			13.45	2.91
$LSD_{0.01}$			18.96	4.03

considered not suitable for both cultivation in the conditions of Serbia and use in developing novel genetic variability targeting similar regions.

There were significant differences at the level of 0.05 (*) between genotypes ($F = 20.16^*$), environments ($F = 51.84^*$) and genotype × environments ($F = 2.51^*$). As could be seen from the difference in the ranking of the cultivars in two individual locations, a significant genotype × environment interaction indicated the linear function of the additive environment effects (Mather and Jinks 1982).

48.4 Conclusions

The preliminary performance of forage soya bean cultivars of US origin in the conditions of Serbia opens the possibility of cultivating this crop as a valuable source of forage during the summer. A possible ideotype of the forage soya bean cultivar

for this region could be one with stable forage yields, instead of broad variation in the overall performance between individual growing seasons. The research will be continued with emphasis upon forage quality, especially the content of crude protein and crude fibre fractions, as well as on the reliable seed production in order to secure the potential commercialisation of the newly developed forage soya bean cultivars and their introduction to the Serbian market and fields.

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Chapter 49
Effects of Trinexapac-Ethyl (Moddus) on Seed Yields and Its Quality of Eleven Temperate Grass Species

R. Macháč

Abstract In small plot field trials conducted for 2 years the effects of plant growth regulator (PGR) trinexapac-ethyl in selected grass species were studied. Eleven temperate grass species were used in trials: Perennial ryegrass, annual ryegrass, meadow fescue, red fescue, Kentucky bluegrass, timothy, cocksfoot, loloid and festucoid type of festulolium, yellow oat grass and tall oat grass. Evaluations were made total seed yield, thousand seed weight, germination, germination energy and number of seeds per unit. Grasses treated by PGR achieved of higher seed yield especially in consequence of higher number of seeds. The qualities of seeds (TSW, germination) were comparable to untreated control.

Keywords Grasses · PGR · Trinexapac-ethyl · Seed yield

49.1 Introduction

The grass seed production has a long history in the Czech Republic, however, the production and seed yields fall under average of EU. Main cause of low yields is non-keeping of basic agronomical practices and low inputs. To achieve high seed yields it is necessary to supply sufficient amounts of nitrogen which has significantly effect on the photosynthesis and thereby total productivity of plant. However, increasing of nitrogen supply increase also growth and prolongation of stems that are more inclinable to lodging. Lodging has been identified as one of the most important factors reducing grass seed yield. Losses due to lodging have been estimated to be as great as 60 % (Rolston et al. 1997). Lodged stems are exposed to higher competitive for light and nutrients. Developing seeds may abort or fall due to less effective photosynthesis and decreasing of assimilate supply. Lodged stands are as well more predisposed to diseases. Last but not least is complication of harvest and increasing of seed losses due to no-cutting of lodged stems, increasing moisture of thrashed material etc. In the Czech Republic only chlormequat-chloride (CCC) was

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registered for stem length shortening and reduction of lodging in last years. CCC works on the beginning of gibberellin biosynthesis by inhibiting of kaurene synthesis (precursor of gibberellins). Plant growth regulator trinexapac-ethyl is widely used on grass seed crops abroad (Chastain et al. 2003; Haldrup 2007; Rijckaert 2007, etc.). Rademacher (2000) claims that trinexapac-ethyl is inhibiting the activity of enzyme 3-β hydroxylase that the transforms inactive gibberellins form GA_{20} on highly active forms GA_1 and GA_4. In consequence this inactivation the stem growth is reduced and stalk wall is stronger. Finally, the plant height is lower and the crop is less susceptible to lodging. Main topic of our research was to verify the influence of trinexapac-ethyl on seed yield of eleven temperate grass species and its quality in Central Europe conditions.

49.2 Materials and Methods

Field small-plot trials were conducted at Grassland Research Station at Zubri (Northeast Moravia, 360 m a.s.l., cambisol, average air temperature 7.5 °C, precipitation 864 mm) for 2 years (2007–2008). The trials were conducted with eleven grass species: perennial ryegrass (*Lolium perenne* L.) cv. Olaf, annual ryegrass (*Lolium multiflorum* Lam. ssp. multiflorum) cv. Jivet, meadow fescue (*Festuca pratensis* Huds.) cv. Roznovska, red fescue (*F. rubra* L.) cv. Ta-gera, timothy (*Phleum pratense* L.) cv. Sobol, loloid type of *Festulolium* cv. Lofa, festucoid type of *Festulolium* cv. Hykor, cocksfoot (*Dactylis glomerata* L.) cv. Dana, Kentucky bluegrass (*Poa pratensis* L.) cv. Slezanka, tall oat grass (*Arrhenatherum elatius* (L.) Beauv. ex J. S. et K. B. Presl) cv. Roznovsky and yellow oat grass (*Trisetum flavescens* (L.) P. Beauv.) cv. Roznovsky. The plot size was 10 m^2. Each trial was arranged in a randomized complete block design (with another seven pesticide treatments) with four replications. Two tested treatments with trinexapac-ethyl (TE) were applied: single application of dose 0.2 l TE ha^{-1} at GS 31–32 and split application two times 0.1 l TE ha^{-1}, first application at GS 29 and second application at GS 32.

All treatments were performed with wheelbarrow sprayer driven by compressed air (Lurmark 01F80 nozzles, a pressure 0.25 MPa, spraying volume 300 l ha^{-1}). The standard treatment (MCPA + clopyralid + fluroxypyr) was used for weed control. Fertilizers application: autumn 45 kg N, 20 kg P_2O_5 and 60 kg K_2O per ha, spring—only nitrogen at dose depending on grass species 80–110 kg ha^{-1}. The trials plots were combined directly with plot combine Wintersteiger Elite. Harvested seed was dried and subsequently cleaned by laboratory cleaner Westrup-Kamas for seed yield determination. Seed quality (TSW, germination) and number of seed per inflorescence were analyzed in the lab of GRS Zubri. The results were analyzed by ANOVA and Tukey's post hoc test on significance level 95 % (Statistica 8.0). Due to insertion to the randomized blocks with pesticide treatments the number of degree of freedom (27) was satisfactory for statistical analyses.

Table 49.1 The effect of trinexapac-ethyl on seed yield selected grass species

Species	Treatment	Seed yield					
		2007		2008		Average	
		Kilogram per hectare	Tukey	Kilogram per hectare	Tukey	Kilogram per hectare	Relative
Perennial ryegrass	Untreated	934	d	555	e	744	100
	TE 200	1230	ab	708	a	969	130
	TE 2×100	1238	ab	696	abc	967	130
Annual ryegrass	Untreated	1748	a	1655	c	1702	100
	TE 200	1793	a	1843	ab	1818	107
	TE 2×100	1860	a	1758	a	1809	106
Meadow fescue	Untreated	719	de	356	ab	538	100
	TE 200	765	cd	378	ab	572	106
	TE 2×100	801	bcd	521	a	661	123
Red fescue	Untreated	902	ab	1195	bc	1048	100
	TE 200	995	a	1349	a	1172	112
	TE 2×100	983	a	1227	ab	1105	105
Kentucky blue grass	Untreated	346	bcd	517	b	432	100
	TE 200	471	a	676	a	574	133
	TE 2×100	414	ab	629	ab	521	121
Timothy	Untreated	835	a	640	ab	737	100
	TE 200	859	a	707	a	783	106
	TE 2×100	903	a	729	a	816	111
Cocksfoot	Untreated	838	abcd	626	ab	732	100
	TE 200	800	abcd	763	a	781	107
	TE 2×100	862	abc	6945	ab	778	106
Festulolium Lofa	Untreated	894	bcdef	735	bc	814	100
	TE 200	1055	abc	767	ab	911	111
	TE 2×100	998	abcde	877	a	938	115
Festulolium Hykor	Untreated	938	a	952	abc	945	100
	TE 200	937	a	1099	a	1018	107
	TE 2×100	951	a	1148	a	1049	111
Tall oat grass	Untreated	517	bc	367	c	442	100
	TE 200	672	a	506	ab	589	133
	TE 2×100	549	b	514	a	532	120
Yellow oat grass	Untreated	267	c	208	a	237	100
	TE 200	285	a	231	a	258	109
	TE 2×100	303	b	239	ab	271	114

TE trinexapac-ethyl

49.3 Results and Discussion

The positive effect of trinexapac-ethyl on seed yield was recorded in larger or smaller rate on all of selected grass species. However, there was insignificant decreasing of seed yield of some species in 2007. With regard to dry weather in growing seasons (especially in 2007) the grasses only very few lodged. It is possible to suppose, that if grasses would more lodged the difference in seed yield between untreated plots and plots treated by TE were to be higher. The effect of TE application on seed yield of selected grass species is shown in the Table 49.1. Thousand seed weight (TSW)

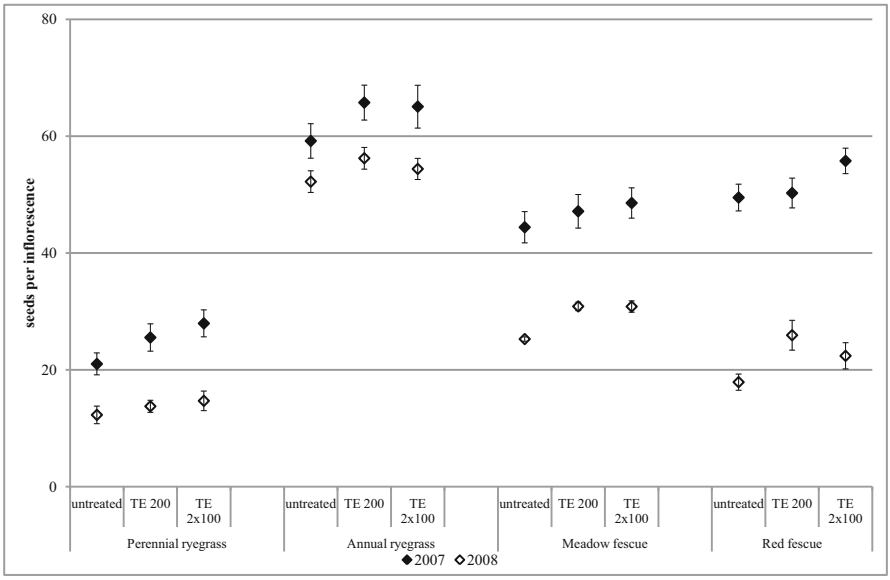

Fig. 49.1 Effect of trinexapac-ethyl on number of seed in inflorescence of *perennial ryegrass*, *annual ryegrass*, *meadow fescue* and *red fescue* including confidence intervals ($p = 0.05$)

was at most grass species insignificant lower on treatments when the TE was applied in comparison with untreated plots. Only in timothy in year 2007 and in yellow oat-grass in both trial years were observed significantly higher TSW on plots treated with TE (data not shown). Minimal and also insignificant differences between treatments were recorded in germination and germination energy (data not shown).

Significant differences were recorded in the number of seed in inflorescence (*see* Fig. 49.1). At the most grass species the number of the seed was higher at treated grasses; however, at the some species then number of the seed was lower in consequence with higher number of fertile stems. Positive influence of trinexapac-ethyl application on increase of number of perennial ryegrass seed was observed by Silberstein et al. (2002), who found out that TE increases number of created ripe seed, while the potential number of seed was not affected. Application TE so has positively effect on Floret site utilization (FSU). According to Young et al. (2007) the nitrogen rate increasing has significant effect on increasing of both, actual and potential seed number, but insignificant effect on FSU in perennial ryegrass. Reduction in plant height and lodging was recorded on all the treated plots.

Application of trinexapac-ethyl on cool season grass seed crops can significant increases seed yields mainly due to number of seed increasing. There is slight variation in the increasing of seed yield between grass species, but average increasing of seed yield is about 12–15 %. Based on trials the PGR Moddus (trinexapac-ethyl 250 g l^{-1}) in rate 0.8 l ha^{-1} preferably applied at growth stage GS 31–32 or 0.4 l ha^{-1} applied two times (GS 29 and GS 32) has been allowed for minor use in seed crops of each grass species under study in the Czech Republic.

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Chapter 50
The Chemical Composition of a Range of Forage Grasses Grown Under Two Nitrogen Fertiliser Inputs and Harvested at Different Stages of Maturity

C. King, J. McEniry, M. Richardson and P. O'Kiely

Abstract Grass species, rate of N fertiliser application and plant maturity at harvest represent three of the most important grassland management factors affecting plant chemical composition. This study investigates the effects of two N fertiliser inputs and five harvesting dates in the primary growth on the yield and chemical composition of five common grass species. Perennial ryegrass, Italian ryegrass, cocksfoot, timothy and tall fescue, were grown in triplicate field plots under two inorganic nitrogen fertiliser inputs (low = 0 kg N ha^{-1}, high = 125 kg N ha^{-1}) and harvested at five sequential dates (fortnightly from 12 May–7 July; Harvests 1–5) in the primary growth in both 2009 and 2010. At each harvest date, herbage was weighed to estimate DM yield and representative samples were used to determine herbage chemical composition. In general for the five grass species investigated, herbage dry matter (DM), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) concentrations increased ($P < 0.001$), while dry matter digestibility (DMD), buffering capacity (BC) and water soluble carbohydrate (WSC) concentration decreased ($P < 0.001$) with advancing harvest date. An exception to this trend was for the cocksfoot where a decrease ($P < 0.001$) in DM concentration was observed from Harvest 4–5. Cocksfoot had the lowest DM yield while no difference ($P > 0.05$) was observed between the other grass species. Although timothy and PRG had a similarly high DMD, of the five grass species timothy had the highest ($P < 0.001$) NDF and ADF concentration. The IRG had the highest ($P < 0.001$) WSC concentration and lowest ($P < 0.001$) BC making it the most suitable species for ensiling.

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50.1 Introduction

Approximately 35 % of the utilised agricultural area in Europe is under permanent grassland. These grasslands contribute substantially to agricultural production systems, while also providing an important resource in terms of biodiversity, carbon sequestration in soils and, in recent years, bioenergy and biorefining (Hopkins and Wilkins 2006). These grasslands are usually managed to enhance the production of herbage and its quality. Three of the main management factors affecting herbage yield and chemical composition are grass species, rate of nitrogen fertiliser application and stage of maturity at harvest (Buxton 1996). For example, grass harvested at an early vegetative growth stage will generally have a higher digestibility, crude protein (CP) and ash content than herbage harvested at a later stage of growth (Buxton and O'Kiely 2003), with the optimal time for harvesting being determined by the particular grassland use. Inorganic nitrogen fertiliser is widely used to improve herbage yields, but can also effect the chemical composition of the herbage (e.g. increased CP with increasing N fertiliser application rate). Although the majority of reseeded temperate grassland in Europe is dominated by perennial ryegrass, different grass species may offer a potential alternative for non-agricultural uses. The objective of this study was to investigate the effects of two N fertiliser inputs and five harvesting dates in the primary growth on the yield and chemical composition of five common grass species

50.2 Materials and Methods

Five common grass species, perennial ryegrass (PRG; *Lolium perenne* L. var. Gandalf), Italian ryegrass (IRG; *Lolium multiflorum* Lam. var. Prospect), cocksfoot (*Dactylis glomerata* L. var. Pizza), timothy (*Phleum pratense* L. var. Erecta) and tall fescue (*Festuca arundinacea* Schreb. var. Fuego) were grown in field plots (20 m^2)at Teagasc, Grange, under two inorganic fertiliser nitrogen inputs (low = 0 kg ha^{-1}, high = 125 kg ha^{-1}; applied as urea (460 g N kg^{-1}) in mid-March when soil temperature > 6 °C) and harvested at five sequential dates (fortnightly from 12 May–7 July; Harvests 1–5; $n = 150$ plots) in the primary growth during both 2009 and 2010 ($n = 300$ samples). At each harvest date, herbage was harvested and weighed using a Haldrup forage plot harvester (J. Haldrup, Løgstor, Denmark) cutting to a 6 cm stubble height. A representative 2 kg sample of each herbage was taken and stored at −18 °C prior to chemical analyses. After harvesting in 2009, the grass plots received 250 kg ha^{-1} of a compound fertiliser (240 g N, 25 g P and 100 g K kg^{-1}). Dry matter (DM) concentration was estimated following drying in a ventilated oven with forced air circulation at 98 °C for 16 h. Replicate samples (200 g) were also dried at 40 °C for 48 h before being milled (Wiley mill; 1 mm screen). Dried, milled samples were used for the determination of DMD, NDF, ADF, ADL, ash, BC, CP and WSC as previously described by Purcell et al. (2011). Data were analysed as a split-split plot design using the Proc MIXED procedure of SAS, Version 9.1.2 (SAS 2004) with

harvest date as the main-plot, nitrogen fertiliser as the sub-plot and grass species as the sub-sub plot, and with year and replicate blocking being accounted for.

50.3 Results and Discussion

On average, with advancing harvest date there was an increase ($P < 0.001$) in DM yield (except from Harvest 4 to Harvest 5 where values did not differ ($P > 0.05$)), together with an increase ($P < 0.001$) in herbage DM concentration and an increase ($P < 0.001$) in the concentrations of NDF, ADF and ADL (Table 50.1. An exception to this trend was for the cocksfoot where a decrease ($P < 0.001$) in DM was observed from Harvest 4–5. As a plant matures the proportion of cell wall components (e.g. cellulose, hemicellulose and lignin) increases, reflecting the general decrease in plant leaf to stem ratio and the increasing cell wall content of the stems in particular (Hatfield 1993). This was accompanied by increased lignification within the cell wall fraction, which resulted in a decrease ($P < 0.001$) in herbage DMD. Furthermore, herbage CP concentration is higher in grass leaves than in stems and decreased ($P < 0.01$) with advancing harvest date in accord with Ballard et al. (1990). The decrease ($P < 0.001$) in BC and WSC concentration with advancing plant maturity can also be attributed to the decrease in the plant leaf to stem ratio and is in Sagreement with Keating and O'Kiely (2000).

On average, of the two nitrogen fertiliser treatments employed in this study, the high N treatment gave a higher ($P < 0.05$) DM yield (Table 50.2). However, of the five grass species only tall fescue had a significantly higher ($P < 0.05$) DM yield with the high N fertiliser treatment. This is in contrast to Reid (1985) who reported that ryegrass and cocksfoot produced the best responses at high rates of N fertiliser application. The increase ($P < 0.001$) in herbage BC and CP concentration following addition of N fertiliser is in agreement with Keady and O'Kiely (1996) who reported that herbage BC is positively correlated to CP concentration. In contrast, herbage DM and WSC concentrations were higher ($P < 0.001$) for the low N treatment. An exception to this trend was for Harvest 4 and 5 where herbage DM concentration did not differ ($P > 0.05$) between the two N fertiliser treatments. Nowakowski (1962) reported that WSC concentration decreased in grasses with increasing levels of N fertilisation due to an accelerated growth rate following application. The increase in BC and decrease in WSC concentration with N fertiliser addition may have negative implications for grass ensilability.

Of the five grass species investigated, on average cocksfoot had the lowest ($P < 0.001$) DM yield (with no difference ($P > 0.05$) observed between the other grass species) and DMD (along with IRG and tall fescue), and the highest BC ($P < 0.001$), ash ($P < 0.001$) and CP concentrations ($P < 0.001$; along with timothy; Table 50.1). Green et al. (1971) reported that all varieties of ryegrass out-yielded other common grass species, which is in contrast to this study where, with the exception of cocksfoot which had the lowest DM yield, no difference ($P > 0.05$) was observed between the grass species. The PRG and timothy grasses had similarly higher ($P < 0.001$) DMD

Table 50.1 Yield (t DM ha^{-1}) and chemical composition (g kg^{-1} DM, unless indicated otherwise in footnotes) of five grass species harvested at five sequential dates in the primary growth (averaged across year and nitrogen fertiliser treatment)

S	H	Variables									
		Yield	DM	DMD	NDF	ADF	ADL	Ash	CP	WSC	BC
PRG	1	5.04	195	827	454	239	7.8	83.9	141	245	522
PRG	2	6.95	189	776	518	287	11.3	81.8	122	196	464
PRG	3	8.20	207	681	588	344	23.7	72.2	93	159	391
PRG	4	12.01	286	666	606	350	25.1	68.1	80	160	305
PRG	5	9.81	296	613	616	372	34.5	67.9	72	148	272
IRG	1	5.46	211	780	453	242	7.3	81.6	126	262	426
IRG	2	7.01	220	746	470	261	11.1	76.2	117	268	371
IRG	3	9.39	252	698	526	313	23.6	67.4	89	224	331
IRG	4	9.79	291	644	556	339	27.2	74.9	86	185	282
IRG	5	8.72	295	623	583	361	32.3	79.2	81	147	277
TF	1	4.93	198	770	496	261	14.4	79.8	146	196	485
TF	2	6.33	190	726	541	301	15.5	82.7	126	157	443
TF	3	9.25	223	681	602	361	25.5	78.7	103	126	407
TF	4	9.69	280	657	617	365	30.3	75.3	89	129	340
TF	5	9.42	280	608	613	366	32.6	79.9	80	127	336
CF	1	4.42	184	771	493	254	14.2	87.4	154	178	511
CF	2	5.50	182	720	558	309	24.0	86.3	136	146	477
CF	3	6.77	224	664	599	347	26.4	84.9	108	113	425
CF	4	9.04	309	579	650	376	33.7	77.4	100	87	340
CF	5	6.95	252	596	635	366	37.1	94.8	95	74	363
TIM	1	4.47	191	821	518	261	11.2	79.8	153	163	493
TIM	2	6.23	178	758	571	321	22.3	82.6	138	101	469
TIM	3	9.37	202	710	651	382	28.4	74.1	107	80	396
TIM	4	10.84	270	662	656	380	36.6	71.3	107	81	329
TIM	5	9.75	309	616	643	389	44.5	65.6	87	101	304
SEM											
Species (S)		0.289	2.6	6.2	4.4	2.0	0.77	0.91	1.5	3.4	3.8
Harvest (H)		0.335	5.6	9.3	4.4	3.6	1.27	0.89	2.1	5.2	4.9
S × H		0.558	8.0	13.8	7.5	5.9	2.11	2.06	3.7	8.0	9.0
Levels of significance											
Species		***	***	***	***	***	***	***	***	***	***
Harvest		***	***	***	***	***	***	**	***	***	***
S × H		*	***	*	***	***	*	***	NS	***	***

PRG perennial ryegrass, *IRG* Italian ryegrass, *TF* Tall fescue, *CF* Cocksfoot, *TIM* timothy, *Harvest1* 12 May, *Harvest2* 26 May, *Harvest3* 9 June, *Harvest4* 23 June, *Harvest5* 7 July, *DM* dry matter (g kg^{-1}), *DMD* dry matter digestibility (g kg^{-1}), *NDF* neutral detergent fibre, *ADF* acid detergent fibre, *ADL* acid detergent lignin, *CP* crude protein, *WSC* water soluble carbohydrates, *BC* buffering capacity (mEq kg^{-1} DM), *SEM* Standard error of the mean, *NS* not significant
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

values compared to the other grasses, despite timothy having the highest ($P < 0.001$) NDF and ADF concentrations and a similarly high ($P > 0.05$) ADL concentration to cocksfoot The IRG had the highest DM ($P < 0.001$) and WSC ($P < 0.001$) concentrations, and the lowest ($P < 0.001$) NDF and ADF concentrations and BC. In general,

Table 50.2 Yield (t DM ha^{-1}) and chemical composition (g kg^{-1} DM, unless indicated otherwise in footnotes) of five grass species grown under two nitrogen fertiliser (N) inputs (averaged across year and harvest date)

S	N	Variables									
		Yield	DM	DMD	NDF	ADF	ADL	Ash	CP	WSC	BC
PRG	Low	7.89	242	723	554	311	18.1	72.1	84	207	366
IRG	Low	8.32	264	698	516	303	18.6	73.9	83	239	313
TF	Low	7.14	242	703	568	327	23.1	77.7	97	164	379
CF	Low	5.95	231	676	579	328	25.6	85.9	104	127	420
TIM	Low	7.60	243	719	612	349	27.2	71.1	102	122	358
PRG	High	8.91	227	703	559	325	22.9	77.4	119	156	422
IRG	High	7.83	243	698	519	304	22.1	77.9	117	195	362
TF	High	8.71	227	674	580	334	24.2	80.9	121	130	423
CF	High	7.12	230	655	595	333	28.6	86.4	133	112	444
TIM	High	8.66	217	709	604	344	30.0	78.2	135	89	418
SEM											
Species (S)		0.289	2.6	6.2	4.4	2.0	0.77	0.91	1.5	3.4	3.8
Nitrogen (N)		0.235	1.2	5.1	3.8	1.1	0.37	0.54	1.0	2.7	2.7
S × N		0.368	3.7	8.2	5.4	2.9	1.13	1.30	2.2	4.6	5.5
Levels of significance											
Species		***	***	***	***	***	***	***	***	***	***
Nitrogen		*	***	NS	NS	*	**	***	***	***	***
S × N		*	*	NS	*	*	NS	NS	NS	***	*

PRG perennial ryegrass, *IRG* Italian ryegrass, *TF* Tall fescue, *CF* Cocksfoot, *TIM* timothy, *DM* dry matter (g kg^{-1}), *DMD* dry matter digestibility (g kg^{-1}), *NDF* neutral detergent fibre, *ADF* acid detergent fibre, *ADL* acid detergent lignin, *CP* crude protein, *WSC* water soluble carbohydrates, *BC* buffering capacity (mEq kg^{-1} DM), *Low N* 0 kg N ha^{-1}, *high N* 125 kg N ha^{-1}, *SEM* Standard error of the mean, *NS* not significant
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

the higher WSC concentration and the lower BC observed for the two ryegrasses may make these grasses more suitable for silage production (Buxton and O'Kiely 2003).

There were no significant interactions ($P > 0.05$) between harvest date, nitrogen fertiliser and grass species for any of the variables measured.

50.4 Conclusions

On average, herbage grown under the high N treatment and harvested at a later date had a higher DM yield. With the exception of cocksfoot which had the lowest DM yield, there was no difference between the other grass species. The later harvested herbage had a higher concentration of NDF and had a lower digestibility. Although timothy and PRG had a similarly high DMD, on average timothy had the highest NDF and ADF concentrations. The IRG had the highest WSC concentration and lowest BC making it the most suitable species for ensiling.

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Chapter 51
NIRS Calibration Strategies for the Botanical Composition of Grass-Clover Mixtures

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Abstract In literature, different calibrations to predict the species composition of grass legumes mixtures or mixtures of different grass species are described. Mostly, these calibrations were developed using so called "artificial samples". These artificial samples are obtained by mixing pure (ground) material of the species for which the calibration is developed in known proportions. The plant material used for these artificial samples may have been grown in mixtures or in pure stands. Calibrations based on artificial samples mostly have very good calibration statistics but fail to predict real validation samples. "Real samples" are obtained by hand separation of species mixtures into the different species followed by recomposition. The advantage of the use of artificial samples relative to real samples is that a lot of calibration samples with a different composition can be obtained with a relative small labour input. We built calibrations to predict the white clover content in grass clover mixtures, based on real and artificial samples with the same composition, and validated them with the same independent samples. Calibrations based on real samples performed far better than calibrations based on artificial samples. The failure of the latter can be explained by the lack of environmental variation in their spectra. We recommend a calibration strategy based on fewer but more diverse hand sorted samples, rather than making a lot of artificial samples that contain relatively little spectral information.

51.1 Introduction

A visual estimation of the composition of multi-species grass swards is a fast but subjective method. Hand sorting of a sample is very precise but time consuming as one needs samples of a representative magnitude. Hand sorting is too much labour demanding in large field experiments. NIRS (Near Infrared Reflectance Spectroscopy) can work both precisely and quickly, but a calibration database is needed.

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Several calibrations to predict the botanical composition of multi-species swards were developed in the past. They differ according to the species involved and by the strategy used to build the calibration.

Coleman et al. (1985) and Petersen et al. (1987) built calibrations for the botanical composition based on so called **artificial samples:** species were grown in single species plots, harvested material was mixed in known proportions, varying between 0–100 % with 5 % increments. These calibrations had good calibration statistics, and were validated, to a certain extent, with independent samples.

Another way to build calibration samples is by determining the botanical composition of samples harvested in multi-species pastures/trials by separating them and recomposing them afterwards (Shaffer et al. 1990; Wachendorf et al. 1999). Samples obtained in this way are called **real samples**. The advantage of artificial samples compared to real samples is that more samples can be made with less labour.

Pitmann et al. (1991) compared NIRS calibrations based on these two different strategies and concluded that despite the excellent statistics of calibrations based on artificial samples, they were not acceptable for estimating the composition of pasture samples. Our own preliminary research confirmed this finding. In order to understand the failure of calibrations based on artificial samples, we compared similar sets of artificial samples and real samples.

51.2 Material and Methods

Calibrations were built to predict the botanical composition of swards in a trial comparing the performance of white clover (**TR**) with either perennial ryegrass (**LP**) or tall fescue (**FA**) or a mixture of both grass species with white clover. The mixtures differ in the initial proportion of the ryegrass component in the seed mixture (0 %LP, 12.5 %LP, 25 %LP or 100 %LP on a seed number base) and the ploidy level of the ryegrass (diploid or tetraploid). The trial was established in 2009, and the yield and the botanical composition were recorded since 2010. At each harvest, a representative sample of the harvested material was sorted into the different species.

The sorted plant material of the first cut in 2011 was used to build calibrations in two different ways (Fig. 51.1). **"Real samples"** samples were obtained by sorting a sample of the harvested material of each plot. After weighing the different species, they were put together again, dried (16 h at 75 °C) and finally ground (Brabender shear mill, 1 mm sieve). We obtained 25 calibration samples in this way. **"Artificial samples"** were based on plant material obtained from plots that contained a single grass species (FA or LP) and white clover. Pure FA, LP and TR were obtained by hand sorting grass and clover. This plant material was dried and ground. The powder was mixed in proportions that matched the composition that was found in the real samples. Hence, 2 sets of 25 calibrations samples with the same botanical composition were created.

NIRS spectra were collected with a Foss NIRSystems 5000 and WinISI II 1.50 software. The inverse reflectance (log(1/R)) was measured from 1100–2500 nm in

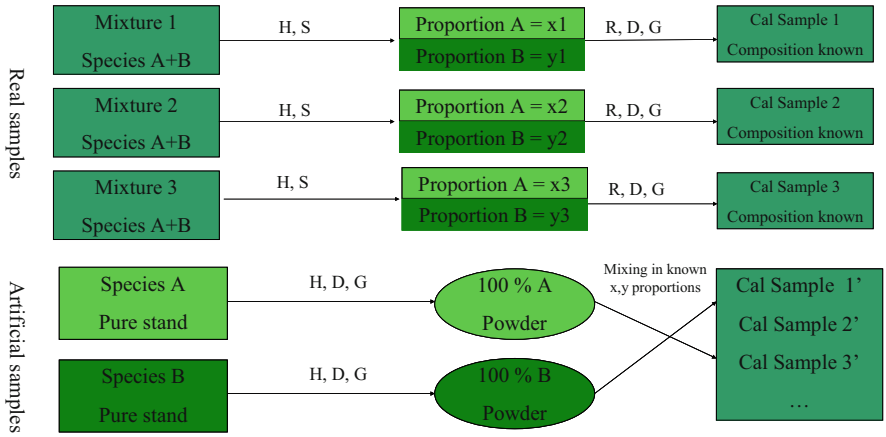

Fig. 51.1 Real calibration samples versus artificial calibration samples. *H* harvesting, *S* sorting, *R* recomposing, *D* drying, *G* grinding

Table 51.1 Species proportion in the calibration samples

Species	Mean (%)	Range (%)	Standard deviation
FA	62.4	28.1–93.9	20.1
LP	33.4	5.7–66.9	17.7
TR	5.2	0–18	5.1

steps of 2 nm. The calibrations were derived using modified partial least squares regression (Shenk and Westerhous 1991). Scatter effects were reduced using first derivate of the spectra and multiplicative scatter correction. The standard errors of calibration and cross validation were calculated.

51.3 Results and Discussion

FA was the main component in the calibration samples. The standard deviations for FA and LP were comparable (Table 51.1). Variation for TR was smaller.

From a statistical point of view, the calibration based on artificial samples was far better than the calibration based on real samples: for all three species, Standard error of calibration (SEC) and cross validation (SECV) were at least three times higher in the real samples (Table 51.2). Despite the excellent calibration statistics, the calibration based on artificial samples failed to predict the composition of real samples. Standard errors of prediction (SEP) were 57.3, 37.8 and 20.1 for the proportion of FA, LP and TR respectively. Inversely, when the calibration based on real samples was used for prediction of the artificial samples, SEP were 13.6, 11.1 and 4.2 for the proportion of FA, LP and TR respectively. Especially for TR, these values are only slightly higher than the SECV found for the calibration, which indicates that

Table 51.2 Calibration statistics of calibrations based on artificial and real samples

Species	Real samples			Artificial samples		
	SEC	SECV	RSQ	SEC	SECV	RSQ
FA	4.7	7.3	0.95	1.46	1.92	0.99
LP	5.0	7.5	0.92	1.55	1.79	0.99
TR	3.3	4.1	0.57	0.62	0.84	0.99

SEC standard error of calibration, *SECV* standard error of cross validation, *RSQ* R squared

the calibration based on real samples works for the prediction of the clover content of artificial samples.

Principal component analysis indicates that although the botanical composition of both types of calibration samples was exactly the same, the spectral information included in the set of artificial samples was smaller than that of the set of real samples. The artificial samples occur as a concentrated cloud, when plotted in the space formed by the first three principal components, whereas the real samples occur as a large cloud around the artificial samples. Surault et al. (2006) observed the same phenomena and suggested that the spectral signature of a grass species may vary when grown in pure stands or in mixtures with other species.

The reason for the difference between the two types of calibration samples probably lies in the lack of environmental variation in the plant material used to build the artificial samples. The material grown in pure stands was harvested in different plots, pooled together, and handled as a single sample when dried, ground and stored before mixing, whereas the material for the real samples was harvested in different plots and samples remained separated per plot from harvest until the spectra were collected. The method of grinding, processing and storing the samples are affecting the NIRS spectra (Shaffer et al. 1990). The material used to build the artificial calibrations is homogenised and variables that affect the NIR spectra are partially lost.

Although good calibrations for determination of white clover content based on artificial samples were described (Chataigner et al. 2010; Locher et al. 2005), our findings suggests there is more advantage in taking the time and the effort to work with real samples, that represent all the variables which would affect the NIR spectra, rather than creating a lot of artificial samples. The results shown here are preliminary, further research is needed to understand exactly why the calibration based on artificial samples fails to predict the botanical composition of real samples.

51.4 Conclusion

In our experiments, calibrations based on artificial samples failed to predict the botanical composition of real samples, which is the aim of building a calibration. Based on our preliminary work, we recommend a calibration strategy based on fewer but more diverse hand sorted samples, rather than making a lot of artificial samples that contain relatively few spectral information.

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Chapter 52
Comparison of LOCAL and GLOBAL Calibration Models to Predict Ryegrass Quality Using Near Infrared Reflectance Spectroscopy

G. A. Burns, T. J. Gilliland, D. Grogan and P. O'Kiely

Abstract GLOBAL calibration models using near infrared reflectance spectroscopy have been successfully developed for determining the quality of forages. The use of LOCAL calibration models has been shown to improve the accuracy of large calibration sets by selecting a representative sub-section from within the complete dataset to form a unique calibration for each individual sample. GLOBAL and LOCAL calibration models ($n = 2{,}076$) were developed to predict four quality attributes (buffering capacity, crude protein, *in vitro* dry matter digestibility and water soluble carbohydrates) using three ryegrass species (perennial, Italian and hybrid ryegrass). The GLOBAL approach produced accurate calibration models with $R^2 > 0.86$. The maximum number of samples was altered in the LOCAL approach (10–250) to assess the optimal number of 'local' samples required. GLOBAL calibrations were more accurate than LOCAL calibrations for all quality traits except buffering capacity which was of similar accuracy. This research recommends the use of GLOBAL calibration models for the assessment of the quality of ryegrasses in the national variety evaluation scheme in Ireland.

52.1 Introduction

Near infrared reflectance spectroscopy (NIRS) is commonly used to assess the quality of forages in breeding programs and national variety evaluation schemes (e.g. Burns et al. 2011). NIRS is a secondary technique that requires calibration models to form predictions of quality traits based on their near infrared spectra. A GLOBAL approach

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Table 52.1 Summary of the reference values of four quality traits for three ryegrass species

	n	Mean	Minimum	Maximum	SD
In vitro dry matter digestibility (g kg^{-1})					
Perennial ryegrass	1836	801	629	890	36.9
Italian ryegrass	137	748	576	870	73.7
Hybrid ryegrass	103	773	587	886	70.7
Water-soluble carbohydrate (g kg^{-1} DM)					
Perennial ryegrass	1836	180	50.4	376	51.9
Italian ryegrass	137	207	92.9	430	74.4
Hybrid ryegrass	103	212	71.6	413	70.4
Crude protein (g kg^{-1} DM)					
Perennial ryegrass	1836	150	57.4	266	36.8
Italian ryegrass	137	128	73.7	267	40.5
Hybrid ryegrass	103	133	71.4	241	43.4
Buffering capacity (mEq kg^{-1} DM)					
Perennial ryegrass	1836	437	215	736	90.8
Italian ryegrass	137	360	188	559	87.5
Hybrid ryegrass	103	368	207	616	83.6

SD standard deviation

is usually employed in which the full calibration set is used to form these calibration models. The calibration set in GLOBAL models should ideally encompass as much variation as the model will face in practice (Berzaghi et al. 2000). However, this can potentially lead to a trade-off between the accuracy and robustness of calibration models (Shenk et al. 1997). Shenk et al. (1997) developed the LOCAL regression algorithm that selects a calibration set of spectrally similar samples from within the full calibration set, to the sample of interest. Berzaghi et al. (2000) showed LOCAL calibration models produced lower standard errors of predictions than a GLOBAL approach that encompassed several for ages. This was attributed to the non-linear relationship between the spectra and quality trait values which can exist in large databases, reducing the accuracy of GLOBAL models. The LOCAL algorithm selects representative sub-sets from within the full database that allow for modeling of non-linear relationships while maintaining heterogeneity in the calibration set (Shenk et al. 1997). The aim of this research was to compare GLOBAL calibration models (Burns et al. 2011) that encompassed three ryegrass species, perennial (*Lolium perenne* L.), Italian (*Lolium multiflorum* Lam.) and hybrid ryegrass (*Lolium boucheanum* Kunth) with LOCAL calibration models for the prediction of four quality traits of ryegrasses in a national variety evaluation scheme.

52.2 Materials and Methods

52.2.1 Field Trials

Field trials were carried out at the Grass and Clover Variety Evaluation Unit at Backweston, Co. Kildare, Ireland (53° 26′ N, 06° 30′ W). Trial management and protocol are described in detail by Grogan and Gilliland (2010). A c. 300 g

Table 52.2 Summary of optimal modified partial least squares regression calibration models for predicting four ryegrass quality traits from Burns et al. (2011)

						Validation		
Quality trait	n	Mean	SD	SEC	R^2	SEP	SEP (%)	R^2
BC (mEq kg^{-1} DM)	1985	428	93.1	20.3	0.952	21.2	4.95	0.953
CP (g kg^{-1} DM)	1941	149	37.1	5.0	0.982	5.3	3.56	0.980
DMD (g kg^{-1})	1986	798	43.1	15.9	0.864	16.0	2.01	0.845
WSC (g kg^{-1} DM)	1941	182	53.0	10.4	0.961	11.4	6.26	0.956

BC buffering capacity, *CP* crude protein, *DMD* In vitro dry matter digestibility, *WSC* water-soluble carbohydrate, *SD* standard deviation, *SEC* standard error of calibration, *SEP* standard error of prediction, *SEP %* standard error of prediction as percentage of the mean

sub-sample was collected from each harvested plot for analysis. Two thousand seventy six ryegrass samples (1,836 perennial, 137 Italian and 103 hybrid ryegrass: Table 52.1) were selected for developing the calibration models. These represented the range of species, ploidy, maturity and development stage over five harvest years occurring at this site. Each sample was oven dried at 80 °C for 16 h and then milled using a Retsch mill (1 mm sieve) prior to NIRS analysis.

52.2.2 NIRS Analysis

Absorbance (log 1/reflectance) was measured on a NIRsystems 6500 or standardized NIRsystems XDS (Foss UK Ltd., Warrington, UK) at 2 nm intervals over the wavelength range 1,100–2,500 nm. Each sample was subsequently analyzed for *in vitro* dry matter digestibility (DMD), water soluble carbohydrate (WSC) concentration, crude protein (CP) concentration and buffering capacity using reference methods (Burns et al. 2011). GLOBAL calibrations were developed using modified partial least square regression as reported by Burns et al. (2011) and the optimal calibration models are summarized in Table 52.2. A standard normal variate and detrend (Barnes et al. 1989) and a 1,4,4,1 curve smoothing technique were applied to the spectra prior to calibration development. The LOCAL calibration technique used a maximum number of spectrally similar samples (k) and these were selected as 10, 25, 50, 100, 150, 200, 250.

52.3 Results and Discussion

52.3.1 GLOBAL Calibration

The GLOBAL equation (Table 52.2) produced results with R^2 greater than 0.95 for WSC concentration, CP concentration and buffering capacity. *In vitro* DMD had a lower R^2 than the other three traits. The lower R^2 for *in vitro* DMD in comparison with other quality traits is similar to other GLOBAL calibrations (Jafari 2003) as this is a chemically diverse assemblage influenced by animal factors that cannot be measured in the spectrum of forage (Coleman and Moore 2003). In addition the relatively low

Table 52.3 Regression statistics of four quality traits using a LOCAL calibration with ($k = 10, 25, 50, 75, 100, 150, 200, 250$) local samples

k	R^2	SEP	SEP(c)
Buffering capacity (mEq kg^{-1} DM)			
10	0.867	34.09	34.08
25	0.917	26.91	26.91
50	0.935	23.80	23.79
100	0.949	21.05	21.05
150	0.952	20.35	20.36
200	0.953	20.35	20.24
250	0.952	20.36	20.36
Crude protein (g kg^{-1} DM)			
10	0.946	8.82	8.82
25	0.960	7.58	7.58
50	0.966	7.04	7.03
100	0.969	6.68	6.67
150	0.970	6.61	6.61
200	0.968	6.77	6.76
250	0.966	7.01	7.00
In vitro dry matter digestibility (g kg^{-1})			
10	0.710	24.82	24.82
25	0.739	23.42	23.43
50	0.759	22.47	22.48
100	0.785	21.26	21.27
150	0.791	20.95	20.95
200	0.792	20.90	20.90
250	0.789	21.09	21.09
Water-soluble carbohydrate (g kg^{-1} DM)			
10	0.893	18.14	18.15
25	0.917	15.99	16.00
50	0.929	14.85	14.84
100	0.934	14.30	14.30
150	0.934	14.28	14.28
200	0.933	14.40	14.41
250	0.931	14.55	14.56

k maximum number of local samples in calibration model, *SEP* standard error of prediction, *SEP*(c) standard error of prediction of calibration

standard deviation of perennial ryegrass, relative to Italian and hybrid ryegrass may indicate a clustering of values closer to the mean. As perennial ryegrass contributes a large proportion of the calibration set (0.88) this may be a limiting factor in the accuracy of the GLOBAL *in vitro* DMD model.

52.3.2 LOCAL Calibration

As the maximum number of samples (k) selected for use in the LOCAL calibration increases a similar pattern emerged for all four quality traits (Table 52.3); initially

there was a sharp increase in R^2 associated with decreasing standard error of prediction. R^2 increases at a decreasing rate between 50–150 samples followed by a plateau in accuracy as k increased further. The selection of the optimal number of samples was based on the criteria of high R^2 and low standard error of prediction. The optimal number of samples for CP and WSC concentration was $k \approx 150$ and for *in vitro* DMD and buffering capacity it was $k \approx 200$ when developing calibration models using the LOCAL algorithm.

Using the optimal number of samples, the LOCAL calibration produced a similar level of accuracy to the GLOBAL calibration model for buffering capacity (SEP 20.4 vs. 20.3). However the accuracy of the LOCAL calibration models for *in vitro* DMD, WSC and CP concentration was inferior to their respective GLOBAL models.

Berzaghi et al. (2000) showed the improvement of LOCAL algorithms using a calibration set of forages that encompassed a larger range of feeds (hay, corn silage, haylage, small grain silage and total mixed ration) than for the current calibration set (perennial, Italian and hybrid ryegrass). It is likely that bias existed between these categories of forage and non-linear relationships were present due to the large range in composition which could have potentially limited the performance of the GLOBAL calibration models. Similar levels of bias might not exist between monocultures of three ryegrass species in the present study and the use of a GLOBAL calibration may be more appropriate when most variation is encompassed with little bias between factors in the model.

52.4 Conclusions

Initially increasing the maximum number of samples selected for calibration using the LOCAL algorithm decreased the standard error of prediction for all four quality traits. The decrease in standard error of prediction continued at a decreasing rate as more locally selected samples were used in the calibration set and as more local samples were used the decrease in standard error plateaued.

The GLOBAL calibrations produced more accurate equations for *in vitro* DMD, WSC and CP concentration and were of similar accuracy for buffering capacity. From this set of results the continued use of GLOBAL calibrations is recommended for the accurate and robust analysis of the quality of ryegrasses of the Irish national variety evaluation scheme.

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Chapter 53
Grass for Biogas Production—Anaerobic Methane Production from Five Common Grassland Species at Sequential Stages of Maturity

K. O'Riordan, J. McEniry, T. Woodcock, C. King and P. O'Kiely

Abstract Grassland biomass represents the most significant feedstock resource in Ireland, accounting for approximately 91 % of the 4.3 million hectares of agricultural land. Grass can be an excellent energy crop and may be classified as a high yielding (up to 15 t dry matter ha^{-1} a^{-1}), low input perennial crop. Consequently, grass will be a dominant feedstock for anaerobic digestion (AD) on Irish farms. This study investigated the effects of stage of maturity of five grass species on methane production using dried, milled samples in a small-scale (160 ml), high-throughput batch digestion test. Five common grass species (perennial ryegrass, Italian ryegrass, cocksfoot, timothy and tall fescue) were grown in field plots (with three replicate blocks) under a high nitrogen fertiliser input (125 kg N ha^{-1}) and harvested at five sequential dates (fortnightly from 12 May to 7 July; n = 75 plots) in the primary growth. Of the five grass species investigated, average total CH_4 production was highest ($P < 0.01$) for the perennial ryegrass. On average, the rate of digestion decreased ($P < 0.001$) with increasing plant maturity. Although total CH_4 production decreased numerically with advancing plant maturity, this difference was not significant ($P > 0.05$).

53.1 Introduction

Grassland biomass represents the most significant feedstock resource in Ireland, accounting for approximately 91 % of the 4.3 million hectares of agricultural land. Grass can be an excellent energy crop and may be classified as a high yielding (up to 15 t dry matter ha^{-1} a^{-1}), low input perennial crop. Consequently, grass will be a dominant feedstock for anaerobic digestion (AD) on Irish farms.

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Grasslands are usually managed to enhance the production and quality of herbage. Two of the main management factors affecting herbage chemical composition are grass species and stage of maturity at harvest. The physiology of different grass species can impose differences in adaptability, productivity and chemical composition, which may impact on their methane production potential. However, there is an absence of detailed information on the specific methane yield of different grass species grown under similar management conditions. One study by Seppala et al. (2009) reported that there was no significant difference between the specific methane yields of cocksfoot (318 L CH_4 kg^{-1} volatile solids (VS)), tall fescue (314 L CH_4 kg^{-1} VS) and timothy (311 L CH_4 kg^{-1} VS) grown under boreal conditions.

In general, advancing maturity of grass from the vegetative to the inflorescence growth stage is characterized by an increase in fibre components (Stefanon et al. 1996) and a decrease in digestibility (Ballard et al. 1990). Herbage nutritive value for animal production is generally considered to decline with advancing plant maturity and a delayed harvest can have a significant negative impact on herbage quality. Delaying the harvest date also has an important negative influence on methane production from AD (Prochnow et al. 2009).

This study investigated the effects of stage of maturity of five grass species on methane production using dried, milled samples in a small-scale (160 ml), high-throughput batch digestion test.

53.2 Materials and Methods

Five common grass species, perennial ryegrass (PRG; *Lolium perenne* L. var. Gandalf), Italian ryegrass (IRG; *Lolium multiflorum* Lam. var. Prospect), cocksfoot (*Dactylis glomerata* L. var. Pizza), timothy (*Phleum pratense* L. var. Erecta) and tall fescue (*Festuca arundinacea* Schreb var. Fuego) were grown in field plots (with three replicate blocks) under a high nitrogen fertiliser input (125 kg N ha^{-1}) and harvested at five sequential dates (fortnightly from 12 May to 7 July; Harvests 1–5; $n = 75$) in the primary growth. On each harvest date, the appropriate plots were harvested to a 6 cm stubble height and a representative sample of each herbage was oven dried at 40 °C for 48 h before being milled (Wiley mill, 1 mm screen). Dried, milled samples were used for the determination of neutral detergent fibre (NDF) as described by McEniry et al. (2006).

Replicate samples were also analysed for methane production in 160 ml batch digestion tests, according to VDI guideline 4630 (2006). Briefly, substrate and inoculum were added to the 160 ml incubation bottles at a VS inoculum to substrate ratio of 2:1 and at a final VS concentration of 10 g kg^{-1} (i.e. 0.7 g VS per bottle; 0.47 substrate: 0.23 inoculum). The inoculum (pH = 7.98; 4 g DM kg^{-1}, 2 g VS kg^{-1}) was sourced from a cattle slurry digester at the Agri-Food and Biosciences Institute in Hillsborough, Northern Ireland. Micro- and macro- mineral solutions were added to ensure that nutrient conditions in the bottles were not limiting and sodium hydrogen carbonate was added to act as a buffer system (3.5 g L^{-1}). Water was added to each bottle to adjust the final volume to 70 ml. Six replicate blank (no grass substrate added) and

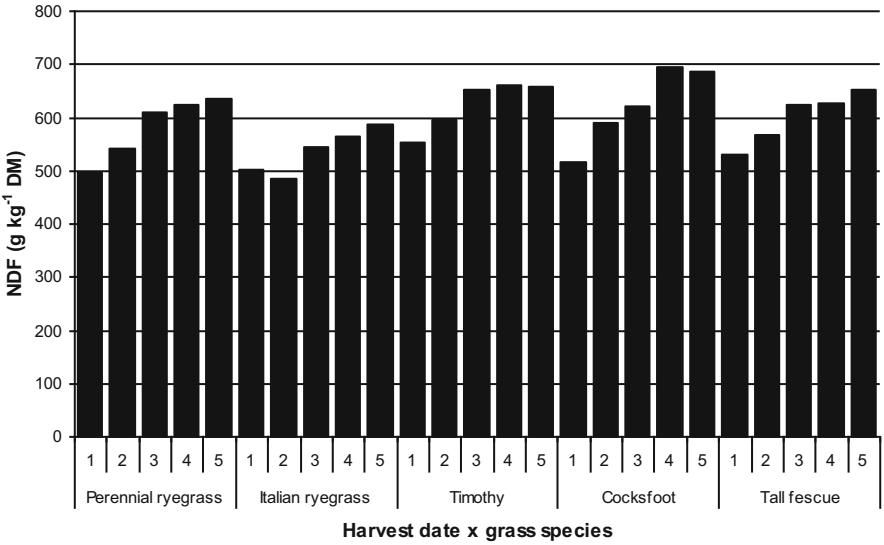

Fig. 53.1 Effect of harvest date and grass species on neutral detergent fibre (NDF) concentration (SEM 8.9; $P < 0.001$)

cellulose (reference sample to assess the biological activity of the inoculum) controls were also included. The final pH in the incubation bottles was adjusted to 7.2 before bottles were flushed with N_2 for 1 min and sealed with butyl rubber stoppers and aluminum crimp seals. Bottles were incubated at 38 °C and hand-mixed daily.

Using a detachable pressure transducer, the gas headspace pressure inside each bottle was recorded after 2, 5, 7, 9, 12, 15 and 19 days incubation. The total amount of gas produced was estimated using the following equation: Gas production (ml) $= (V_h/P_a) \times P_t$; where V_h is the headspace volume (ml), P_a is the atmospheric pressure (hPa) and P_t is the gas headspace pressure (hPa). Following determination of gas volume, a 0.8 ml sample of gas was used to determine CH_4 concentration by gas chromatography (Purcell et al. 2011). Analysis of the results included the following steps: (a) headspace correction for gas values on day 2, (b) subtraction of the volume of gas produced by the inoculum (i.e. blank) from the volume produced in the batch digestion test with substrate and inoculum and (c) normalising the gas volume to standard conditions (i.e. dry gas, 273 K, 1,013 hPa).

Data were analysed as a split-plot design using the Proc MIXED procedure of SAS, Version 9.1.2 (SAS 2004) with harvest date as the main plot and grass species as the sub-plot, and accounting for repeated measures effect of sampling day.

53.3 Results

In general, herbage NDF concentration increased ($P < 0.001$) with advancing plant maturity (Fig. 53.1). Of the five grass species investigated, the NDF concentration was lowest ($P < 0.001$) for the IRG followed by the PRG.

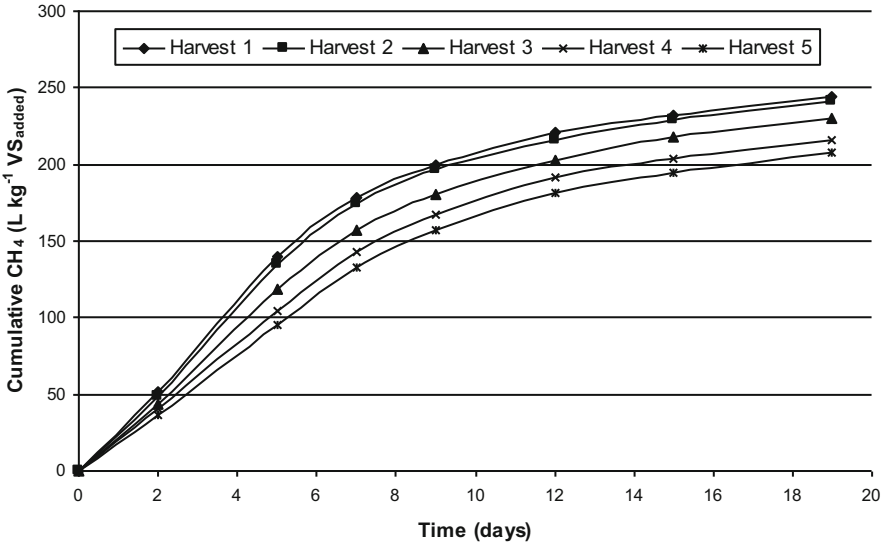

Fig. 53.2 Effect of harvest date on cumulative methane production in batch digestion tests over a 19 day incubation period (averaged across grass species; SEM 4.4; $P < 0.001$)

On average, 0.69 of the CH_4 produced over the 19 day incubation period was produced by day 7 of the batch digestion test (Fig. 53.2). Of the five grass species investigated, average daily CH_4 production ($P < 0.001$; data not shown) and total CH_4 production ($P < 0.01$) over the 19 day incubation period were highest for the PRG, followed by the IRG, timothy, tall fescue and cocksfoot (Fig. 53.3). The specific methane yield varied from 192–259 CH_4 kg^{-1} VS_{added}. The early harvest PRG (Harvest 1–12 May) had the highest specific methane yield (259 L CH_4 kg^{-1} VS_{added}), while similarly high values were observed for the Harvest 2 PRG (257 L CH_4 kg^{-1} VS_{added}) and Harvest 1 IRG (258 L CH_4 kg^{-1} VS_{added}). The lowest specific methane yield (192 L CH_4 kg^{-1} VS_{added}) was recorded for the late harvest cocksfoot (Harvest 5–7 July). On average the apparent rate of digestion decreased ($P < 0.001$) with advancing harvest date (Fig. 53.2). Although total CH_4 production decreased numerically with advancing harvest date, this difference was not significant ($P > 0.5$). This trend of decreasing CH_4 production with advancing plant maturity was similar ($P > 0.05$) across the five grass species investigated.

Cellulose was used as the reference substrate in this study producing 267 L CH_4 kg^{-1} VS_{added}, which represents 0.63 of the maximum theoretical yield following complete degradation.

53.4 Discussion

As a plant matures the proportion of cell wall components (e.g. cellulose, hemicellulose and lignin) increases, while the proportion of cell contents decreases (Stefanon et al. 1996; Ugherughe 1986). These changes particularly reflect the general decrease

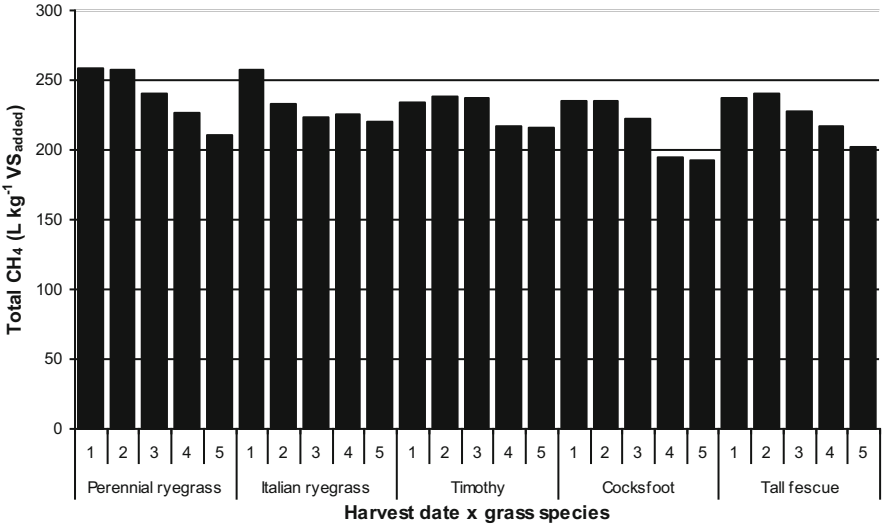

Fig. 53.3 Effect of harvest date and grass species on total CH_4 production (over 19 days incubation; SEM 14.2, $P > 0.5$)

in plant leaf to stem ratio and the increasing cell wall content within the stems. Since this process is accompanied by increased lignification within the cell wall fraction, there is an overall decrease in herbage digestibility resulting in a slower rate of digestion. Seppala et al. (2009), in a study investigating the effect of various harvesting dates and cultivation years on the methane production potential of four grass species, reported that the average specific methane yield of the first harvest of all grasses (345 L CH_4 kg^{-1} VS) was higher than the second harvest (311 L CH_4 kg^{-1} VS).

In temperate grassland regions, PRG is preferred for animal production because of its high digestibility and water soluble carbohydrate content, and reduced fibre concentration. These factors also combined in the current study to produce the highest methane yield in batch digestion tests.

The lower than expected CH_4 yield with the cellulose control suggests either that the activity of the inoculum was sub-optimal or that the duration of the assay was too short. This is further supported by the lower CH_4 yields than that reported for grass samples (range 298–467 L CH_4 kg^{-1} VS; Braun et al. 2010).

53.5 Conclusions

Of the five grass species investigated, PRG may be most desirable as a feedstock for biogas production. The rate of digestion decreased with advancing plant maturity. Although the total CH_4 produced also decreased numerically with advancing plant maturity, this trend did not reach statistical significance.

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