Triploid forage grass hybrids *Festuca apennina* × *F. pratensis* display extraordinary heterosis for yield characteristics

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Abstract Hybrid vigor (or heterosis) is the over-performance of a hybrid over its parents in a specific trait or a set of traits. As such, hybrid breeding serves as a tool to efficiently trigger gains in breeding programs. Moreover, hybrids of genetically distant landraces, varieties or even species may become evolutionary successful. In Swiss alpine swards, we observed frequent prevalence of triploid hybrids of *Festuca pratensis* × *F. apennina* with outstanding competitiveness relative to their parental species in the sites of sympatric occurrence. Observations of these highly vigorous hybrids prompted the study on their heterosis across various environmental conditions. Phenotypic observations during 3 years at four locations at different altitudes (from 200 to 1850 m a.s.l.) have shown significant heterosis for dry biomass production at all sites during the first and second year, and at the mid- and high altitude sites also in the third

year. At mid-altitude (1000 m a.s.l.), heterosis increased steadily and reached a maximum of + 508% for annual yield (+ 626% for a single cut) in the third year. This is by far the highest value of heterosis ever reported for annual dry matter yield of a forage grass. Further utilization of triploid hybrids in forage grass breeding is hampered by their sterility. Hence there is a need for vegetative propagation. However, artificial chromosome doubling of triploids to create fertile hexaploids, or seeking ways to propagate them vegetatively at an industrial scale might overcome this limitation.

Keywords Heterosis · Triploid hybrid · Forage · *Festuca* · Alpine sward

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Introduction

Heterosis, or hybrid vigor, is a genetic phenomenon where F1 hybrids outperform their parents. Arguably, the practical utilization of heterosis in plant breeding created the greatest revolution in agriculture in the 20th century. Apart from dramatic yield improvements in a wide range of crops, hybrid breeding provides a better control of crop genetics, and is of considerable benefit to breeding and seed companies, if not always the growers (Fehr 1987; Lippman and Zamir 2007; Casler and van Santen 2010).

The levels of heterotic effects are different in different crops. Generally, heterosis is higher in cross-pollinating than in self-pollinating species. When inbred lines of cross-pollinating species such as rye or maize are used to make hybrids, heterosis can reach almost 300% over the mid-parent (MP) value but considerably less when the performance of the parental populations (from which the inbred lines were developed) is used as the benchmark. In self-pollinating species, no more than 20% MP heterosis can be expected (Becker 1992). The best-known examples of employing heterosis in practice are maize and pearl millet. In maize, Carena (2005) found an average + 20% MP heterosis in various environments, with up to the average of + 48% in subtropical locations of Mexico, Columbia and Thailand (Reif et al. 2003). In pearl millet, grain productivity increased in India from 305 kg ha⁻¹ in years 1951–1955 to 998 kg ha⁻¹ in years 2008–2012 due to the implementation of hybrid breeding, crop management and agricultural input use (Yadav et al. 2000). In West African pearl millet, grain yield showed an average MP heterosis of + 16.7%, ranging from – 26 to +73% for individual hybrids. Similarly, up to + 81% BP (better-parent) heterosis was found in hybrids of African landraces grown for 2 years at two locations in Niger (Ouendeba et al. 1993).

Much debate concerns the proper selection of parents for crossings in hybrid breeding. The results are inconsistent. Posselt (2010) demonstrated a significant positive correlation between forage yield of perennial ryegrass hybrids and the geographical distance between their parental populations. On the other hand, genetic relationship between parents used for F1 hybrid production was not significantly correlated with heterosis for total herbage yield in hybrids of Italian ryegrasses (Wang et al. 2016). Similarly, geographical distance between two parents presumably reflecting genetic relationship does not always correlate with heterosis. For example, in pearl millet, inter-country crosses had about similar grain yield as intra-country crosses (Pucher et al. 2016). There are probably some prerequisites for positive correlation including high trait heritability. For that, absence of such correlation might be expected for total herbage yield with its low heritability. Moreover, heterosis in the same hybrid or hybrid population seems to differ under various environmental conditions. Pucher et al. (2016) found that heterosis varied in pearl millet from

+ 4% at Samako (Mali) to + 47.3% at Bambey (Senegal). Breese (1969) found a trend for heterosis to be higher in poorer environmental conditions compared to high yielding sites. In line with this, slightly higher heterosis was observed in *Lolium perenne* L. hybrids under low-N compared to high-N growing conditions (Gaue et al. 2003).

In forage grasses, development of cultivars with improved dry matter yield and quality, and at the same time acceptable seed yield potentials, have been the main breeding targets over the last decades. However, in such major crops as Italian and perennial ryegrasses *Lolium multiflorum* Lam. and *L. perenne* L. only marginal improvements of the dry matter yield have been observed (Redfearn et al. 2005; Wilkins and Lovatt 2011). Here, the deployment of intervarietal or interspecific hybrids with high hybrid vigor could be highly beneficial. The highest heterosis observed in forage grasses to date (+ 70%) was found in the interspecific hybrid *L. perenne* × *L. multiflorum* (Lein 1998). On the other hand, only meager heterosis was observed in intergeneric *Festuca* × *Lolium* hybrids (Ghesquiere et al. 2016). The level of hybrid vigor appears to be related to the genetic distance separating the parents (East 1936). Such distance between different genera would reasonably be expected to be greater than among more closely related taxa. In forage grasses, there are also other attributes speaking in favor of distant hybrids, such as complementarity of their agronomic and end-use profiles. This complementarity is the main driving force behind quite successful breeding of intergeneric hybrids of forage grasses of the *Festuca*–*Lolium* complex, collectively referred to as Festulolium.

One way to maintain high level of heterosis without a risk of inbreeding depression in subsequent generations is a change in the reproductive behavior from sexual to vegetative propagation. Some sterile interspecific hybrids with high heterosis may switch to asexual reproduction, such as apomixis or rhizomes, and become highly competitive to their parental species. Vegetatively reproducing triploid hybrids of diploid *Festuca pratensis* Huds. and *F. apennina* De Not. are abundant at altitudes between 1100 and 1900 m above sea level (a.s.l.) in Swiss Alps (Kopecký et al. 2016). Triploid hybrids were the only cytotype found in several locations indicating their high competitiveness. Besides presumed heterosis, clonal propagation is probably the main reason for

their abundance. We found that they can disperse to a distance of at least 14 m by their rhizomes (Kopecký et al. 2018). Visual observation of these vigorous plants indicates their potential in forage grass breeding.

High frequency of such hybrids at a narrow altitude range (between 1100 and 1900 m a.s.l.) in the Swiss Alps prompted this study, to estimate heterosis in triploid *F. pratensis* × *F. apennina* hybrids under different climatic conditions. Special attention is paid to the effect of altitude and the effect of heterosis on the high abundance of this hybrid in Swiss swards and potential for future breeding. In the course of this study an astonishing level of heterosis was observed, far surpassing published data so far.

Materials and methods

Plant material

Pairwise crosses between genotypes of tetraploid *Festuca apennina* (*Fape*) and diploid *F. pratensis* (*Fp*) were carried out in 2016 by the detached stem approach as described in Boller et al. (2018). Seeds were collected from each of the parental plants separately. Progenies were checked for ploidy by flow cytometry and triploids were assumed to be hybrids; diploids and tetraploids were assumed to have originated from self-pollination and were discarded. Hybrid origin of a sub-set of triploids was later confirmed by genomic *in situ* hybridization (see below). Two crosses (A and B) were selected for the study, with the highest number of triploid progeny. The *Fape* parents are part of the collection described in Kopecký et al. (2016). For cross A with 5 triploid progenies, the *Fape* parent originated from Moléson (Ct. Vaud, Switzerland), for cross B with 13 triploid progenies, from Stoos (Ct. Schwyz, Switzerland). Formal identification of the samples was undertaken by the authors. The *Fp* parents were two different elite genotypes of the Agroscope breeding program, with strong focus on disease resistance.

Half-sib families obtained from seed from the 4 open pollinated parental plants represented the parents. The two *Fape* parents had flowered among 14 genotypes selected from a collection of *Fape* ecotypes. The two *Fp* parents had flowered in the

same polycross with 6 other elite genotypes of the Agroscope breeding program. Half-sib progenies of the parental lines were used instead of the parental plants *per se* to compare plants of similar age and grown from seeds for all three groups.

Flow cytometry

The ploidy levels were estimated according to Dolezel et al. (2007) in nuclear suspensions prepared from 50 mg of leaf tissue, with *F. pratensis* cv. Kolumbus used as the reference standard having $2C = 6.49$ pg (Kopecký et al. 2010). Samples were analyzed using a CyFlow Space flow cytometer (Sysmex Partec GmbH., Görlitz, Germany) equipped with a UV led diode array. At least 5000 events were acquired per sample and only measurements with the coefficient of variation for G0/G1 peaks < 2.0% were accepted.

Genomic in situ hybridization (GISH)

GISH was done a subset of 20 plants according to Kopecký et al. (2016). A probe from the total genomic DNA of *F. pratensis* was labeled using the Dig-Nick Translation Mix (Roche); blocking DNA made by shearing total genomic DNA of *F. glaucescens*. The probe/block mix was applied to metaphase chromosome spreads and the probe hybridization signals were detected by the anti-DIG-FITC conjugate.

Meteorological conditions and field experiments

Eighteen triploid hybrids and 20 genotypes each of diploid *Fp* and tetraploid *Fape*, randomly selected from the half-sib families corresponding to the parents of the hybrids were propagated clonally in the greenhouse. At the time of planting, ramets had two to four tillers. The four best plantlets per genotype were chosen. For several genotypes, not enough ramets survived, resulting in some missing individuals in the experiments.

Four sites at altitudes ranging from 200 to 1850 m a.s.l. were chosen (Table 1, ESM 1). The sites differed in temperature, precipitation and soil properties. In general, temperature during the growing period (April–October) decreased with altitude by about 0.5 °C per 100 m. Precipitation was the lowest at lowest site (Olomouc), and the highest at the 1000 m

Table 1 Characteristics of the four experimental sites used for the field study

Locality		Olomouc	Reckenholz	Fruehbuehl	Maran
Town		Olomouc–Holice	Zürich	Walchwil	Arosa
Country (canton)		Czech Republic	Switzerland (ZH)	Switzerland (ZG)	Switzerland (GR)
Co-ordinates	Latitude	49 34.416 N	47 25.734 N	47 6.847 N	46 47.589 N
	Longitude	17 17.097 E	8 31.479 E	8 32.452 E	9 41.252 E
Altitude m.a.s.l		200	440	1000	1850
Precipitation (April–October) mm	2017 (A0)	385	699	1413	1026
	2018 (H1)	147	480	896	761
	2019 (H2)	329	752	1417	1014
	1981–2010	N/A	701	1329	912
Mean air temperature (April–October) °C	2017 (A0)	15.57	15.23	12.55	8.64
	2018 (H1)	17.8	16.71	13.79	9.99
	2019 (H2)	16.22	15.10	12.48	8.94
	1981–2010	N/A	14.30	11.47	7.77
Soil characteristics		Soil type	Gleyic fluvisol	Gleyic cambisol (clayic)	Loamy dystic cambisol
				Loamy dystic cambisol	Loamy eutric cambisol ^a

^aThe soil at Maran is very shallow, with a serpentinite subsoil. The topsoil has been incremented with earth deposits

a.s.l. site, Fruehbuehl. The highest altitude site, Maran (1850 m a.s.l.), was clearly dryer than Fruehbuehl. Years 2017 (A0) and 2019 (H2) were about 1 °C warmer than the 1981–2010 mean and with average precipitation. Year 2018 was clearly warmer and much dryer than the average at all sites (ESM 1). With just 180 mm of precipitation from March to October, the summer 2018 at Olomouc had a prolonged period of drought. Soils at the two high altitude sites, Fruehbuehl and Maran, were less heavy and better drained than the ones at the low altitude sites, Reckenholz and Olomouc. Those two were gleyic, indicating there are periods of anaerobic conditions due to too much water and periods of physical stress caused by low water availability.

Field experiments were planted in a completely randomized block design. The experimental unit was a spaced plant with a distance of 50 cm between rows and 30 cm between plants within a row. At each site, four replications (blocks) were used with separate randomization of the 58 genotypes in each block. Trials were planted between April and June 2017 and maintained for three vegetation periods: the establishment year A0 (2017) and the first (H1, 2018) and

second (H2, 2019) full harvest years. All plants were cut 2–3 times in A0, 3–4 times in H1, and 2–3 times in H2. Early in spring and after each cut, except the last cut of a year, 50 units (kg/ha) of nitrogen were applied as ammonium nitrate.

The individual plant vigor was scored near the end of each growth cycle on a 1 (no growth) to 9 (maximum vigor) scale. Winter damage was rated at the beginning of the vegetation season in H1. Susceptibility to crown rust and bacterial wilt were rated on a 1 (no symptoms) to 9 (fully affected) scale when diseases occurred. The dry matter yield (DMY) was determined at one cut in A0, and at two cuts in H1 and H2. Herbage was clipped with a sickle, supported by a 3 cm high, V-shaped wooden template, after raising all tillers manually to the vertical. This resulted in stubble heights of about 5 cm. At the three Swiss sites, the dry matter of all plants was weighed after oven-drying at 105 °C for 24 h. At Olomouc, the fresh weight of each harvested plant was weighed at the field, and at least half the samples were oven-dried and weighed again. Then, dry matter of remaining plants was estimated from the fresh weight and the average dry matter content of the oven-dried plants.

Statistical analyses

Data were analyzed using the GLM procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). DMY and the average vigor were evaluated for each experimental site separately. For comparisons among sites, DMY was first converted to relative DMY by dividing the absolute DMY of each plant by the average DMY of all plants harvested at a particular site, multiplied by 100%. Least squares means delivered by the GLM procedure were used to report the mean response of all hybrid and parental-species genotypes belonging to a cross (A or B). Significance of differences between these means was estimated by contrasts using the PDIF option provided within the GLS procedure of SAS, considering genotypes within a particular progeny as a random effect. In order to evaluate differential growth response of genotypes to altitude, average yield over the two low (Olomouc and Reckenholz) and the two high (Fruehbuehl and Maran) altitude sites was calculated in a GLM analysis over all sites. Heterosis was calculated for each cross separately on the basis of least squares means over all genotypes per cross. Least squares means of the 5 half-sib progeny genotypes of the respective *F. apennina* and *F. pratensis* parents were used as a proxy to estimate performance of parents. Mid-parent (MP) heterosis (%) for yield (Y) was calculated as $Y(\text{triploid hybrid}) / (Y(\text{Fape parent}) / 2) \times 100 - 100$. Better parent (BP) heterosis was calculated as $Y(\text{triploid hybrid}) / Y(\text{higher yielding parent}) \times 100 - 100$.

Results

All examined triploids (ca. 10% of the total number of triploids used in the field study) had 14 chromosomes with the GISH signal of *F. pratensis* and 7 chromosomes without a signal (indicating *F. glaucescens*-like chromosomes originating from *F. apennina*), confirming their hybrid origin. It is assumed with confidence that all triploids used in this study were in fact *F. apennina* $\times$ *F. pratensis* hybrids.

Heterosis and biomass production: forage dry matter yield and vigor

The relative dry matter yield (DMY) and visually assessed vigor of the triploid hybrids exceeded those

of both their parents at both high altitude Swiss sites and in all three experimental years (Figs. 1, 2 and 3). The differences between the triploid hybrids and their parents were highly significant ($p < 0.001$) for both cross directions. The mid-parent heterosis ranged from +65 to +508% in terms of DMY and from + 2.04 to + 4.42 in terms of the mean vigor scores. Both the mid-parent (MP) and the better-parent (BP) heterosis for DMY gradually increased throughout the experiment at both high-altitude sites, with the MP reaching a maximum of + 626% at Fruehbuehl for the last cut of H2 (2019), and + 392% at Maran for the first cut of H2 (Fig. 4). The BP heterosis peaked simultaneously with the MP heterosis at + 468 and + 370%, respectively.

The two lowland sites, Olomouc (200 m a.s.l.) and Reckenholz (440 m a.s.l.), differed dramatically from the two high-altitude sites, but growth responses were similar at both sites. In A0, the hybrids had the highest yield of the three cytotypes. The difference between the hybrids and diploid *Fp* was significant in cross B, but not in cross A. In H1, yields of *Fp* and the hybrids were many times higher than those of tetraploid *Fape* at both sites, but the hybrids did not out-yield *Fp*; DMY of triploids was significantly lower at Reckenholz. In H2, *Fape* stopped growing completely at both low-altitude sites, and the hybrids had significantly lower yields and vigor than *Fp*. MP heterosis fluctuated around 100% at Reckenholz for most of the time, except for a final increase to + 218% in H2 (Fig. 4). Conversely, the MP heterosis declined sharply in Olomouc after reaching a maximum of + 141% in the summer of H1 (2018), becoming negative throughout H2. In A0 and H1, the BP heterosis decreased from low values to zero at both low-altitude sites and became negative in Olomouc from the end of H1 and throughout H2. However, it recovered to about + 50% in Reckenholz in the second half of H2.

The two independent measures of biomass production used, the relative dry matter yield (DMY) and visual scores (vigor), showed very similar responses, even though DMY was measured only for one (A0) or two cuts (H1, H2) while vigor was scored at each growth cycle (up to 5, based on the number of cuts). A comparison of vigor scores and DMY for individual cuts allowed determination of their mutual relationship. DMY increased logarithmically with the vigor scores in each case (Tab. ESM1). Overall, one-unit vigor score on the 1–9 scale corresponded to an

Fig. 1 Relative dry matter (DM) yield and mean vigor score of $2 \times Fp$, $4 \times Fape$ and their triploid ($3 \times$) hybrids in the planting year (A0, 2017) at four experimental sites (Olomouc, Czech Republic; Reckenholz, Switzerland; Fruehbuehl, Switzerland and Maran, Switzerland). Figures above $3 \times$ hybrid columns indicate mid-parent (MP) heterosis of hybrids over

parental species (as % for DM yield and as difference for vigor scores). Statistical annotations above Fp and $Fape$ columns refer to difference to triploid hybrids, and those at the base of columns to difference between Fp and $Fape$ as follows: *** $p < 0.001$, ** $p < 0.01$; * $p < 0.05$; tr $p < 0.10$, ns not significant

increase of 0.436 log units, i.e. with a 1.54-fold increase of DMY. We used the vigor-DMY relationship calculated for each experimental site to estimate DMY at those instances where only vigor scores were taken. These estimated DMY scores were used along with the measured DMY to follow the development of heterosis over time at different locations.

Survival over time

Survival, the inverse of the death rate due to lack of adaptation, was an important component of large differences in the growth response of diploid Fp , tetraploid $Fape$ and their triploid hybrids between low- and high-altitude sites (Fig. 5). At low altitude sites, $Fape$ was clearly not adapted to local conditions and no plant survived to the end of the experiment. At both sites, the summer of 2018 (H1) was the period of the most rapid loss of $Fape$ plants. Diploid Fp survived best at low altitudes, and the hybrids behaved intermediately, but not much worse than Fp . At Fruehbuehl (1000 m a.s.l.) the hybrids were the best adapted with 100% survival to the end of the experiment. On

contrary, several plants of both Fp and $Fape$ died (mostly during H2), resulting in about 80% survival at the end of the experiment. At Maran (1850 m a.s.l.), all triploid hybrids and $Fape$ plants survived and only few Fp plants died.

Genetic variation within species

In order to visualize variation among genotypes within each of Fp , $Fape$ and their hybrids, average DMYS of each genotype at the two low-altitude sites were contrasted against the averages for the two high-altitude sites (Fig. 6). $Fape$ genotypes performed relatively better at higher altitudes, while Fp genotypes performed better at low altitudes. Within both $Fape$ and Fp , there was a positive correlation between the performance of individual genotypes at low and high altitudes, significant for $Fape$ in A0 and H1 and for Fp in all 3 years. The hybrids behaved differently. With an increasing tendency from A0 to H2, there was a high variability among genotypes, from those performing much better at low altitude to those performing much better at high altitude. They showed

Fig. 2 Relative dry matter (DM) yield and mean vigor score of $2 \times Fp$, $4 \times Fape$ and their triploid ($3 \times$) hybrids in the first full harvest year (H1, 2018) at four experimental sites (Olomouc, Czech Republic; Reckenholz, Switzerland; Fruehbuehl, Switzerland and Maran, Switzerland). Figures above $3 \times$ hybrid columns indicate mid-parent (MP) heterosis of

hybrids over parental species (as % for DM yield and as difference for vigor scores). Statistical annotations above *Fp* and *Fape* columns refer to difference to triploid hybrids, and those at the base of columns to difference between *Fp* and *Fape* as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; tr $p < 0.10$, ns not significant

no or even a slightly negative correlation between performance at low and high altitudes. However, with one exception, the hybrids out-yielded all *Fp* and *Fape* plants in H2 at high altitudes and three of them also out-yielded all *Fp* and *Fape* plants at low altitudes. However, the hybrid which performed best at low altitudes was the second poorest at high altitudes in H2. Interestingly, this genotype (no. 117) was among those two derived from a seed harvested on the *Fp* parent. The other one of the two also performed much better at low than at high altitudes. Microscopic inspection revealed that both these hybrids were infected with the endophyte *Neotyphodium uncinatum*, as was their *Fp* mother.

Impact of disease susceptibility

Crown rust, a common disease caused by *Puccinia coronata*, occurred at Reckenholz and Fruehbuehl. *Fape* plants were highly susceptible, while *Fp* were nearly completely resistant (Fig. 7). The response of hybrids was not the same in the two crosses. In cross A, the hybrids were almost as susceptible as *Fape* (at

the first scoring at Reckenholz even more) indicating a positive heterosis for susceptibility. In cross B, the response was intermediate in the hybrids, and only as the second scoring at Reckenholz they were significantly more susceptible than their resistant *Fp* parent.

Bacterial wilt, a devastating disease of *Lolium* and *Festuca* species in lowland Switzerland caused by *Xanthomonas translucens* pv. *graminis*, markedly affected plants at Reckenholz. Similar to crown rust, *Fp* was highly resistant, while *Fape* was highly susceptible. The hybrids behaved intermediately, but exhibited positive heterosis for resistance, especially in the cross B. The hybrids and *Fape* showed a strong negative correlation between susceptibility to *Xanthomonas* and subsequent DMY throughout the remaining period of the experiment (ESM 2).

Winter damage was scored at each location as the proportion of brown (dead) leaves just before the start of spring growth. At both low altitude sites, *Fape* showed much higher winter damage than *Fp*, with the hybrids behaving intermediately. Winter damage of the hybrids at Olomouc was more severe (close to *Fape*) when compared to Reckenholz, where it was

Fig. 3 Relative dry matter (DM) yield and mean vigor score of $2 \times Fp$, $4 \times Fape$ and their triploid ($3 \times$) hybrids in the second full harvest year (H2, 2019) at four experimental sites (Olomouc, Czech Republic; Reckenholz, Switzerland; Fruehbuehl, Switzerland and Maran, Switzerland). Figures above $3 \times$ hybrid columns indicate mid-parent (MP) heterosis of

hybrids over parental species (as % for DM yield and as difference for vigor scores). Statistical annotations above Fp and $Fape$ columns refer to difference to triploid hybrids, and those at the base of columns to difference between Fp and $Fape$ as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; tr $p < 0.10$, ns not significant

Fig. 4 Development of mid-parent (a) and better-parent (b) heterosis of $3 \times$ hybrids over their parents $2 \times Fp$ and $4 \times Fape$ during experimental years A0 (2017), H1 (2018) and

H2 (2019) at four sites (Olomouc, Czech Republic; Reckenholz, Switzerland; Fruehbuehl, Switzerland and Maran, Switzerland)

almost as low as for Fp . At both high-altitude sites, the hybrids were least damaged after winter, significantly less than Fp . For the highest altitude site Maran, $Fape$ showed significantly less winter damage than Fp , but the inverse was true for Fruehbuehl. In terms of winter damage, the two crosses A and B behaved identically.

Discussion

The level of heterosis can be measured in several different ways. Falconer and MacKay (1996) introduced the mid-parent (MP) heterosis as a difference between the trait performance of a hybrid and the

Fig. 5 Mortality of $2 \times Fp$, $4 \times Fape$ and their triploid ($3 \times$) hybrids during experimental years A0 (2017), H1 (2018) and H2 (2019) at four sites (Olomouc, Czech Republic; Reckenholz, Switzerland; Fruehbuehl, Switzerland and Maran, Switzerland)

Fig. 6 Genetic variation for annual dry matter yield among genotypes of $2 \times Fp$, $4 \times Fape$ and their triploid ($3 \times$) hybrids at low (sites Olomouc and Reckenholz) vs. high (sites Fruehbuehl and Maran) altitude in experimental years A0 (a), H1 (b), and H2 (c)

mean of the two parental lines. Occasionally, the better-parent (BP) heterosis is used. However, in outbreeding species, populations are often used for the hybrid production. For that, the panmictic mid-parent (PMP) and the panmictic better-parent (PBP) parameters of heterosis were established to reflect the performance of a hybrid population relative to its two parental populations (Lamkey and Edwards 1999). In this study, we use the MP and BP criteria.

Heterosis in forage grasses is usually studied under space planting and the heterotic effect appears to be

more pronounced under this system than under sward conditions (Foster 1971, 1973; Posselt 1989). However, even when using spaced plants, PMP heterosis for yield is rather modest, from +1 to +48% in *Lolium perenne* compared to −9 to +8% when plots were used (reviewed in Posselt 2003). Here we decided on the spaced-plant experimental design, for several reasons, including convenience and the ability to gather a wider range of observations than that possible in a sward.

Fig. 7 Susceptibility to crown rust (R), bacterial wilt (Xtg) and winter damage (WD) of $2 \times Fp$, $4 \times Fape$ and their triploid ($3 \times$) hybrids in cross A (top) and cross B (bottom). Visual disease scores (1 = healthy, 9 = fully affected) taken at Reckenholz (rust Re1 and Re2, Xtg) and Fruehbuehl (rust Fb), and scores of winter damage at Olomouc (WD Olo), Reckenholz (WD Rec), Fruehbuehl (WD Frb) and Maran (WD Mar). Figures above $3 \times$ hybrid columns represent mid-parent heterosis as difference in scores, with negative values pointing to positive heterosis for resistance. Statistical annotations (see Fig. 1) refer to significance of difference between $3 \times$ hybrids and their parents. All scores were highly significantly ($p < 0.001$) different between $2 \times Fp$ and $4 \times Fape$

Extent of heterosis for dry matter yield

Very large mid-parent (MP) and better-parent (BP) heterosis values for the hybrids of non-inbred *Fp* and *Fape* observed here were far greater than any values reported so far in forage grasses. Posselt (2003) cited 15 studies involving 5 different forage grass species in a range of environments with a maximum MP heterosis for the annual forage yield of + 70% (19% on average), compared to over + 500% in our study. For individual cuts, the maximum MP heterosis reported by Posselt (2003) was + 152%, compared to + 625% here with a comparable experimental design (individual spaced plants). Other studies with sown plots yielded even lower heterosis, usually less than + 20% (Posselt 1993; Wang et al. 2016).

Interestingly, the highest reported heterosis values so far were observed in an interspecific hybrid, *L. perenne* $\times$ *L. multiflorum* (Posselt 2003). Interspecific

hybrids within the *Festuca-Lolium* complex, commonly referred to as *Festulolium*, are of interest mainly to combine stress resilience of *Festuca* species with productivity and quality of *Lolium* species. Heterosis for yield *per se* is rather small (Ghesquiere et al. 2016). Humphreys et al. (2014) report yields similar to, at best 10% higher than the respective *Lolium* parent for the most successful *Festulolium* candidates. Our triploid *Fape* $\times$ *Fp* hybrids provide an example of an interspecific hybrid within the *Festuca-Lolium* complex with a high potential for heterosis for forage yield. This may add a new dimension to the discussion of the potential of interspecific forage grass hybrids. Indeed, exploitation of heterosis is considered a highly promising approach to speed up the supposedly low progress in breeding of forage grasses for yield (Herridge et al. 2019). Several approaches are followed, such as developing doubled haploids (Begheyn et al. 2016), cytoplasmic male sterility (cms) in hybrid production (Islam et al. 2014; Sykes et al. 2017), using the S-Z-self-incompatibility system to develop inbred lines (Do Canto et al. 2016) as well as optimizing the construction of self-incompatible hybrids (Pembleton et al. 2015). However, the use of interspecific hybrids to exploit heterosis for forage yield is not among the prime approaches followed, even though some promising examples exist, such as the energy grass *Miscanthus* 'Giganteus', an interspecific hybrid between *Miscanthus sinensis* and *Miscanthus ogiformis* (Robson et al. 2013).

It is somewhat surprising that our interspecific hybrid between two closely related species of *Festuca* show such a large heterosis for biomass yield. *F. apennina* (*Fape*) is morphologically so similar to *F. pratensis* (*Fp*) that in older literature it was referred to as a *subspecies* (Hegi 1908; Conert 1998) or even a *varietas* of *F. pratensis* (Stebler 1904). However, recent studies clearly show that *F. apennina* is an allotetraploid originating by hybridization of a progenitor of the current *F. pratensis* with a so-far unidentified species related to the modern *F. glaucescens* (Kopecký et al. 2016). One would, nevertheless, expect little heterosis because the *Fp* genome is present in *Fape*. However, *Fp* showed about five times more genetic diversity (estimated by DArT markers) than *Fape*, suggesting that the *Fp* genome present in *Fape* is only distantly related to modern *Fp* (Kopecký et al. 2018). Generally, heterosis increases as the

genetic disparity of the parents or parental populations increases and interspecific hybrids seem to display greater heterosis than intraspecific hybrids (East 1936).

The triploid *Fape* × *Fp* hybrids are almost completely sterile. This can be considered an advantage in nature because it guarantees long term stability of F_1 hybridity. Advanced generations of *Festulolium* hybrids show much less heterosis than would be expected from their F_1 hybrids (Marc Ghesquiere, personal communication). Since our *Fape* × *Fp* hybrids can efficiently propagate vegetatively through rhizomes (Kopecký et al. 2018), heterosis is conserved and can be expected to contribute significantly to their competitiveness in natural grassland. Highly competitive populations of the triploid hybrids were observed at mid-altitude sites (Kopecký et al. 2018). They dominated the swards in several locations and were the unique cytotype of all 54 *Festuca* specimens collected in one location at 1350 m a.s.l. However, sterility of triploid hybrids is an obvious obstacle for their further exploitation in grass breeding and agriculture.

Effect of altitude on relative performance of triploid hybrids and their parents

The performance of triploid hybrids relative to their parents was affected by the altitude of the trial site, and this effect increased over time. In the year A0, the MP heterosis ranged among the sites from + 64 to + 111% without a clear altitudinal pattern (Fig. 1). In H1, it was lower, at about + 45% at Reckenholz (440 m a.s.l.) but increased to about + 180% at Fruehbuehl (1000 m a.s.l.) and about + 140% at Maran (1850 m a.s.l.) (Figure 2). At Olomouc (200 m a.s.l.), the relative performance of hybrids was better than at Reckenholz, but not as good as at Maran. In H2, a clear altitudinal pattern was observed (Fig. 3), with negative values for the MP heterosis at Olomouc, low values at Reckenholz, and an overarching maximum at Fruehbuehl of about + 500%. In H2, the MP heterosis was also high in Maran, with over + 350%. These patterns were highly consistent for both crosses (A and B) investigated. The highest levels of precipitation combined with a deep, well-drained soil at the mid-altitude site, Fruehbuehl (ESM 1), probably contributed to good performance of *Fape* relative to *Fp*, and to the maximum expression of heterosis in the hybrids. The shallow soil at Maran with poorer water

holding capacity and nutrient delivery potential was probably responsible for the poorer relative performance of *Fape*, compared to Fruehbuehl, and the somewhat lesser expression of heterosis. The altitudinal and temporal patterns of the MP heterosis were related to the relative performance of the two parental species, *Fape* and *Fp*. When *Fape* performed very poorly compared to *Fp*, such as at low altitude in H2, the MP heterosis of triploid hybrids was also low or even negative. When *Fape* performed moderately well, higher values for the MP heterosis were observed. In H2, a consistent altitudinal pattern of the relative performance of *Fp* and *Fape* was reached, with performance of *Fape* rising steadily with altitude.

Impact of biotic and abiotic stresses

Gradual dying of *Fape* plants to extinction during the last year of the experiment at low altitudes was responsible for their overall low performance at Olomouc and Reckenholz. At Reckenholz, bacterial wilt was the most damaging factor. Some plants died already during the spring 2018 when the disease was scored, and a dramatic loss of plants occurred after the first cut in H1. Yield losses and lack of regrowth after cutting is a common feature of heavy infestation with *Xanthomonas* (Egli et al. 1975; Egli and Schmidt 1982). Therefore, relatively good *Xanthomonas* resistance of the hybrids contributed to the longer persistence of positive heterosis for yield at the Reckenholz site. At Olomouc, summer drought in 2018 was the likely cause of *Fape* death. While most *Fape* plants survived until the second cut, a significant loss of plants occurred in July and August when the drought and heat were the most severe. The hybrids responded differently to these stresses. At Reckenholz, the hybrids showed considerable variation in the susceptibility to *Xanthomonas*. Resistant plants survived without visible symptoms and kept producing biomass, while the susceptible ones reduced growth, and 25% died during 2018. This produced a very strong negative correlation between the *Xanthomonas* susceptibility and yields of hybrids (ESM 2). This correlation was also highly significant for *Fape*, but low and insignificant for the largely resistant *Fp*. Over all genotypes in the study, *Xanthomonas* susceptibility was also highly significantly and negatively correlated with subsequent yield, reflecting the higher susceptibility of *Fape* and, to a lesser extent, the hybrids,

compared to resistant *Fp*. Resistance to *Xanthomas* appears to be controlled by few major genes (Studer et al. 2006). The two elite *Fp* plants which were used to produce our triploid hybrids were from the Agroscope breeding program after several cycles of recurrent selection for *Xanthomonas* resistance, and likely carried such genes. The marked segregation among the triploids from crosses with a highly susceptible *Fape* plant suggests heterozygosity for resistance genes in the *Fp* parents. The response of triploid to the dry conditions at Olomouc was more gradual. Similar numbers of *Fp* plants and the hybrids survived the drought in H1 (Fig. 5), but yields of the hybrids relative to *Fp* (the better parent) declined significantly during H1 (2018) (see Fig. 4). Attrition of the hybrids continued in H2 and yield decreased further. Among the *Fape* $\times$ *Fp* hybrids, a higher drought tolerance of the *Fp* parent was apparently not sufficient to adequately reduce the high water requirement of the *Fape* parent. Probably, the difficult soil conditions at Olomouc contributed to the poor performance of *Fape* and the hybrids after the drought of summer 2018.

At low altitude, *Fape* plants were seriously damaged after winter (brown leaves). As such, this dormancy response can express a survival mechanism. On the other hand, it results in slow spring growth and lower first cut yields, compared to the winter green *Fp* with hardly any signs of winter damage. The hybrids were intermediate between *Fape* and *Fp*. However, at the highest altitude (Maran), *Fape* and the hybrids showed significantly less winter damage by snow mold caused by *Microdochium nivale*) than *Fp*. A peculiar situation was observed at Fruehbuehl. Here, the hybrids showed negative heterosis for winter damage. Apparently, hybrids were less winter dormant than *Fape* and less susceptible to snow mold than *Fp* and thus they showed strong heterosis.

The hybrids derived from the two crosses A and B differed markedly in their response to crown rust infection. Hybrids from cross A showed a strong positive heterosis for susceptibility (negative for resistance), while those from cross B were intermediate between susceptible *Fape* and resistant *Fp*, not significantly more susceptible than *Fp* in two out of three scorings, and thus showed negative heterosis for susceptibility (positive for resistance). Indeed, rust susceptibility/resistance was the only case of negative heterosis for one, and positive for the other cross. This

differential response points to a different architecture of the rust resistance in the two parent *Fp* plants. The crown rust resistance in the *Festuca–Lolium* complex is polygenic, as evidenced by a successful genomic prediction model to select for rust resistance in perennial ryegrass (Arojjju et al. 2018). Two major and a number of minor QTLs for rust resistance in a segregating pseudo-testcross population of Italian ryegrass were identified (Studer et al. 2007). Schubiger and Boller (2017) demonstrated the prevalence of several independent race-specific major genes, each of which showed dominant, Mendelian segregation in cross progenies of perennial ryegrass. Likely, the *Fp* parent of cross B possesses some dominant, partly homozygous major resistance genes, while the resistance of the *Fp* parent of cross A relies mostly on minor resistance genes overridden by susceptibility genes of the *Fape* parent.

Conclusions

The triploid *Fape* $\times$ *Fp* hybrid investigated in this study provided by far the highest values for the mid-parent heterosis ever reported for forage crops. This points to a hitherto unexploited potential of heterosis for biomass yield within the economically important *Festuca–Lolium* complex. Maximum levels of heterosis were only observed in a specific environment, at mid-altitude in Switzerland. This environment is squarely half way between the natural ranges of the two parental species: low-altitude (up to about 900 m a.s.l.) for *Fp* and high-altitude (above 1500 m a.s.l.) for *Fape*. Thus, at mid-altitude, factors responsible for the altitudinal limitation of occurrence of the parent species prevail at an intermediate expression and thus provide an ideal environment for the interspecific hybrid. High heterosis of the hybrids at mid-altitude reflects their dominance over the two parent species observed in natural grassland, suggesting that heterosis plays an important role in their evolutionary success in these habitats. At present, practical exploitation of such interspecific hybrids with large heterotic effects in agriculture is hampered by their sterility. Perhaps chromosome doubling in such hybrid will not only restore fertility but also fix the heterotic effects. Alternatively, an industrial-scale vegetative propagation should be explored.

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Author's Contribution B.B. prepared plant material and designed the experiment, D.K. conducted flow cytometry and in situ hybridization analyses, B.B. and D.K. conducted the field phenotyping experiments and wrote the manuscript. Both authors approved the submitted version.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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