

Extraordinary Heterosis Found In A Forage Grass Hybrid, *Festuca apennina* × *F. pratensis*

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Research article

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Abstract

Background: Heterosis (or hybrid vigor) 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.

Results: Observations of these highly vigorous hybrids prompted the study on their heterosis across various environmental conditions. Phenotypic observations during three years at four locations at different altitudes (from 200 m a.s.l. 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 mass yield of a forage grass. **Conclusions:** Further utilization of triploid hybrids in forage grass breeding is hampered by their sterility hence 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.

Background

Heterosis, or hybrid vigor, is a genetic phenomenon where F_1 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 [1].

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 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 [2]. The best known examples of employing heterosis in practice are maize and pearl millet. In maize, Carena [3] found an average + 20% MP heterosis in various environments, with up to the average of + 48% in subtropical locations of Mexico, Columbia and Thailand [4]. 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 [5]. 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 two years at two locations in Niger [6].

Much debate concerns the proper selection of parents for mating in hybrid breeding. The results are inconsistent. Posselt [7] 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 [8]. Similarly, geographical distance between two parents presumably reflecting genetic relationship does not always correlate with heterosis. In pearl millet, inter-country crosses had about similar grain yield as intra-country crosses [9]. There are probably some prerequisites for positive correlation including high trait heritability. For that, absence of such correlation might be expectable 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. [9] found that heterosis varied in pearl millet from + 4% at Samako (Mali) to + 47.3% at Bambey (Senegal). Breese found a trend for heterosis to be higher in poorer environmental conditions compared to high yielding sites [10]. In line with this, slightly higher heterosis was observed in *Lolium perenne* L. hybrids under low-N compared to high-N growing conditions [11].

In forage grasses, improvement of dry matter yield and quality with sustainable seed yield has been the main breeding target over 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 [12, 13]. Here, the employment 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* [14]. On the other hand, only meager heterosis was observed in intergeneric *Festuca* × *Lolium* hybrids [15]. The level of hybrid vigor appears to be related to the genetic distance separating the parents [16]. 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 how 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 [17]. Triploid hybrids were the only cytotype found in several locations indicating their high competitiveness. Besides presumed heterosis, clonal propagation is probably the main trigger of their abundance. We found that they can disperse to the distance of at least 14 m by their rhizomes [18]. Visual observation of these vigorous plants indicates their potential in forage grass breeding.

High frequency of such hybrids at a fairly narrow altitude range 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.

Results

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

Biomass production: forage dry mass yield and vigor

The dry mass yield (DMY) and 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 to 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. Heterosis was the highest at Fruehbuehl and increased steeply from the planting year 2017 (A0, Fig. 1) to the first full harvest year 2018 (H1, Fig. 2) and further to the second full harvest year 2019 (H2, Fig. 3). 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*. The mid-parent heterosis was always positive at Reckenholz (440 m) but clearly lower than that at the 1000 and 1850 m sites, ranking from + 10 to + 92% for DMY and from + 0.98 to + 2.64 for vigor scores. At Olomouc (200 m), heterosis was higher than at Reckenholz in A0 and H1, with a maximum of + 106% in H1 for cross A and + 109% in A0 for cross B. However, it declined completely in H2, and even reached negative values for cross A.

The two independent measures of biomass production used, the dry mass 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. A1 in annex). Overall, one-unit vigor score on the 1 to 9 scale corresponded to an 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.

Heterosis and survival over time

Both the mid-parent (MP) and the better-parent (BP) heterosis for DMY gradually increased throughout the experiment at the two 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 low-altitude sites differed in the MP heterosis. It 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.

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, summer of 2018 (H1) was the period of the most rapid loss of Fape plants. Diploid Fp survived the 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. Both Fp and Fape experienced some mortality during H2, resulting in about 80% survival at the end of the experiment. At Maran (1850 m a.s.l.), very little plant death occurred and only Fp plants were affected.

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 three 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 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 times even more) a positive heterosis for susceptibility (or negative heterosis for resistance). In cross B, the response was intermediate in the hybrids, and in two cases they were not 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 (Additional file 1).

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 almost as low as for Fp. At both high-altitude sites, the hybrids left winter with the least damage, 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 introduced the mid-parent (MP) heterosis as a difference between the trait performance of a hybrid and the mean of the two parental lines [19]. 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 [20]. 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 [21–23]. 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 [24]). 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.

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 [24] 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 [24] 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% [8, 25].

Interestingly, the highest reported heterosis values so far were observed in an interspecific hybrid, *L. perenne* × *L. multiflorum* [24]. 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 [15]. Humphreys et al. report yields similar to, at best 10% higher than the respective *Lolium* parent for the most successful *Festulolium* candidates [26]. Our triploid Fape × 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 [27]. Several approaches are followed, such as developing doubled haploids [28], cytoplasmic male sterility (cms) in hybrid production [29, 30], using the S-Z-self-incompatibility system to develop inbred lines [31] as well as optimizing the construction of self-incompatible hybrids [32]. 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* [33].

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 [34, 35] or even a varietas of *F. pratensis* [36]. 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* [17]. 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 [18]. 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 [37].

The triploid Fape × Fp hybrids are almost completely sterile. This can be considered an advantage in nature because it guarantees long term stability of F₁ hybridity. Advanced generations of Festulolium hybrids show much less heterosis than would be expected from their F₁ hybrids (Marc Ghesquiere, personal communication). Since our Fape × Fp hybrids can efficiently propagate vegetatively through rhizomes [18], 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 [18]. 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.) (Fig. 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 (Additional file 2), 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* [38, 39]. 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 F₁ plants survived until the second cut, a significant loss of plants occurred in July and August when the drought was 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 (Additional file 1). This correlation was also highly significant for F₁, but low and insignificant for the largely resistant F₂. Over all genotypes in the study, *Xanthomonas* susceptibility was also highly significantly and negatively correlated with subsequent yield, reflecting the higher susceptibility of F₁ and, to a lesser extent, the hybrids, compared to resistant F₂. Resistance to *Xanthomonas* appears to be controlled by few major genes [40]. The two elite F₂ 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 F₁ plant suggests heterozygosity for resistance genes in the F₂ parents. The response of triploid to the dry conditions at Olomouc was more gradual. Similar numbers of F₂ plants and the hybrids survived the drought in H1 (Fig. 5), but yields of the hybrids relative to F₂ (the better parent) declined significantly during H1 (2018) (see Fig. 4). Attrition of the hybrids continued in H2 and yield decreased further. Among the F₁ × F₂ hybrids, a higher drought tolerance of the F₂ parent was apparently not sufficient to adequately reduce the high water requirement of the F₁ parent. Probably, the difficult soil conditions at Olomouc contributed to the poor performance of F₁ and the hybrids after the drought of summer 2018.

At low altitude, F₁ left winter with apparent damage (brown leaves). This dormancy response can be viewed as a survival mechanism, however it results in slow spring growth and lower first cut yields, compared to the winter green F₂ with hardly any signs of winter damage. The hybrids were intermediate between F₁ and F₂. However, at the highest altitude (Maran), F₁ and the hybrids showed significantly less true winter damage by snow mold caused by *Microdochium nivale*) than F₂. A peculiar situation was observed at Fruehbuehl. Here, the hybrids showed negative heterosis for winter damage (positive for tolerance to winter conditions). Apparently, hybrids were less winter dormant than F₁ and less susceptible to snow mold than F₂ 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 F₁ and resistant F₂, not significantly more susceptible than F₂ 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 F₂ 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 [41]. Two major and a number of minor QTLs for rust resistance in a segregating pseudo-testcross population of Italian ryegrass were identified [42]. Schubiger and Boller demonstrated the prevalence of several independent race-specific major genes, each of which showed

dominant, Mendelian segregation in cross progenies of perennial ryegrass [43]. 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 × 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.

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 [44]. Seed was 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 confirmed by the genomic in situ hybridization. Two crosses (A and B) were selected for the study, with the highest number of triploid progeny. The Fape parents originated from the collection described in [17]. For cross A with 5 triploid progenies, the Fape parent originated from Moléson (Ct. Vaud), for cross B with 13 triploid progenies, from Stoos (Ct. Schwyz). No permission was necessary to collect plant samples for non-commercial research activities. Formal identification of the samples was undertaken by authors (B. Boller and D. Kopecký). The Fp parents were two different elite genotypes of the Agroscope breeding program, with strong focus on disease resistance. Field studies were conducted in accordance with local legislations (no permissions needed at Agroscope, Switzerland and IEB, Czech Republic).

Half-sib families obtained from seed from the 4 open pollinated parental plants represented the parents. The two Fape parents had flowered among 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 seed for all three groups.

Flow cytometry

The ploidy levels were estimated according to [45] 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 [46]. Samples were analyzed using a CyFlow Space flow cytometer (Sysmex Partec GmbH., Görlitz, Germany) equipped with a UV led diode array. At least 5,000 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 [17]. 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.

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 above sea level (a.s.l.) were chosen (Table 1, Additional file 2). The sites differed in temperature, precipitation and soil properties. In general, temperature during the growing period (April to October) decreased with altitude by about 0.5 °C per 100 m. Precipitation was the lowest at the lowest site (Olomouc), and the highest at the 1000 m a.s.l. site, Fruehbuehl. The highest altitude site, Maran (1850 m a.s.l.), was slightly 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 (Additional file 2). With just 180 mm of precipitation from March to October, 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 a change between periods of anaerobic conditions due to too much water and physical stress during periods of low water availability.

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 to October) mm	2017 (A0)	385	699	1147	1026
	2018 (H1)	147	480	761	761
	2019 (H2)	329	752	1040	1014
	1981–2010	N/A	701	1063	912
mean air temperature (April to October) °C	2017 (A0)	15.57	15.23	12.04	8.64
	2018 (H1)	17.8	16.71	13.21	9.99
	2019 (H2)	16.22	15.10	12.16	8.94
	1981–2010	N/A	14.30	11.00	7.77
soil characteristics	soil type	Gleyic Fluvisol	Gleyic Cambisol (Clayic)	loamy Dystric Cambisol	loamy Eutric Cambisol*

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

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 to 3 times in A0, 3 to 4 times in H1, and 2 to 3 times in H2. Early in spring and after each cut, except the last one 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 mass 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 mass 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 mass of remaining plants was estimated from the fresh weight and the average dry matter content of the oven-dried plants.

Data were analyzed using the GLM procedure of SAS. 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 PDIFF option 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.

List Of Abbreviations

A0 planting year (2017)

BP better parent

DMY dry mass yield

Fape Festuca apennina

Fp Festuca pratensis

H1 first full harvest year (2018)

H2 second full harvest year (2019)

MP mid-parent

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

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.

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Figures

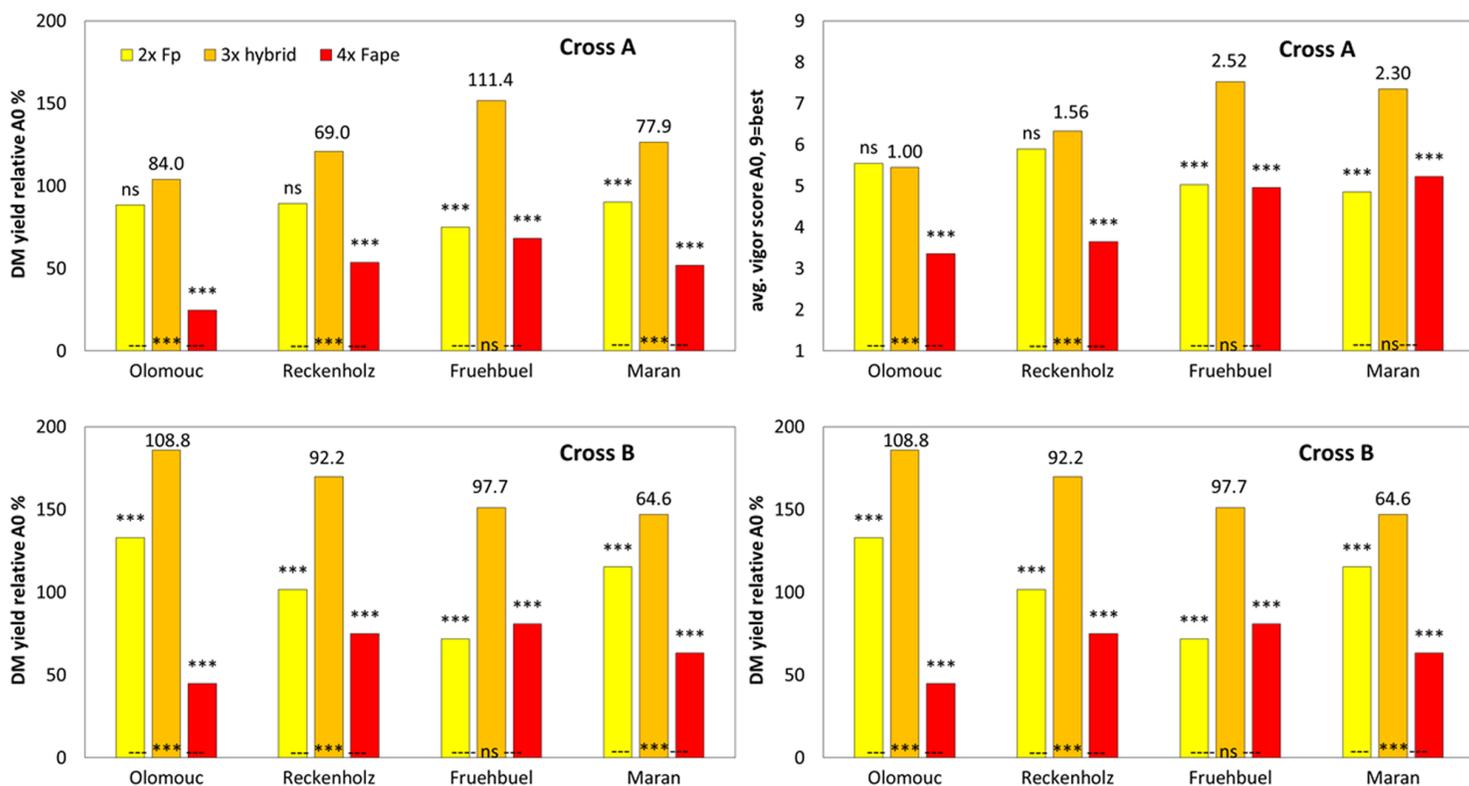


Figure 1

Relative dry mass (DM) yield and average vigor score of 2x Fp, 4x Fape and their triploid (3x) hybrids in the planting year (A0, 2017) at four experimental sites. Figures above 3x 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.

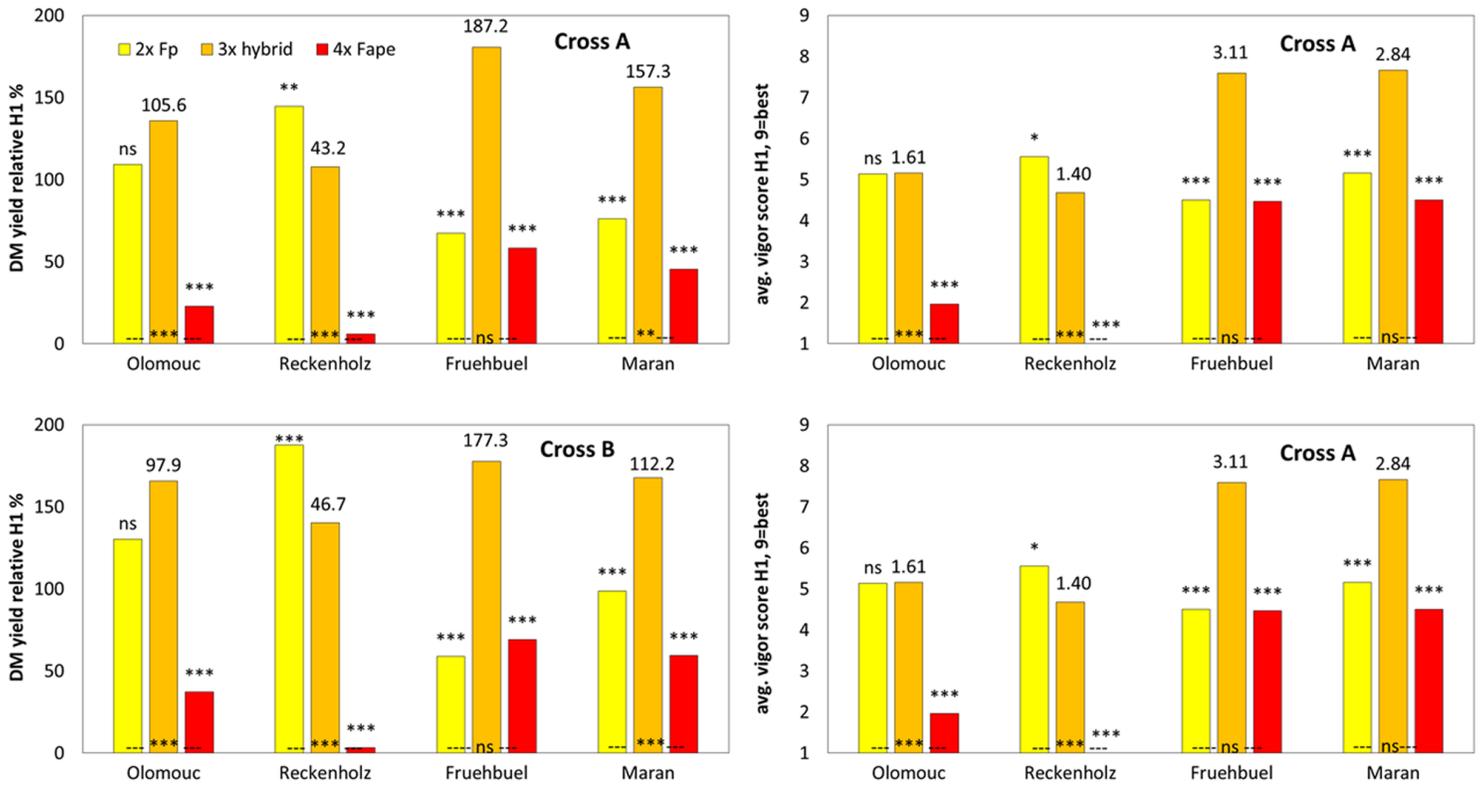


Figure 2

Relative dry mass (DM) yield and average vigor score of 2x Fp, 4x Fape and their triploid (3x) hybrids in the first full harvest year (H1, 2018) at four experimental sites. For explanation of annotations see Figure 1.

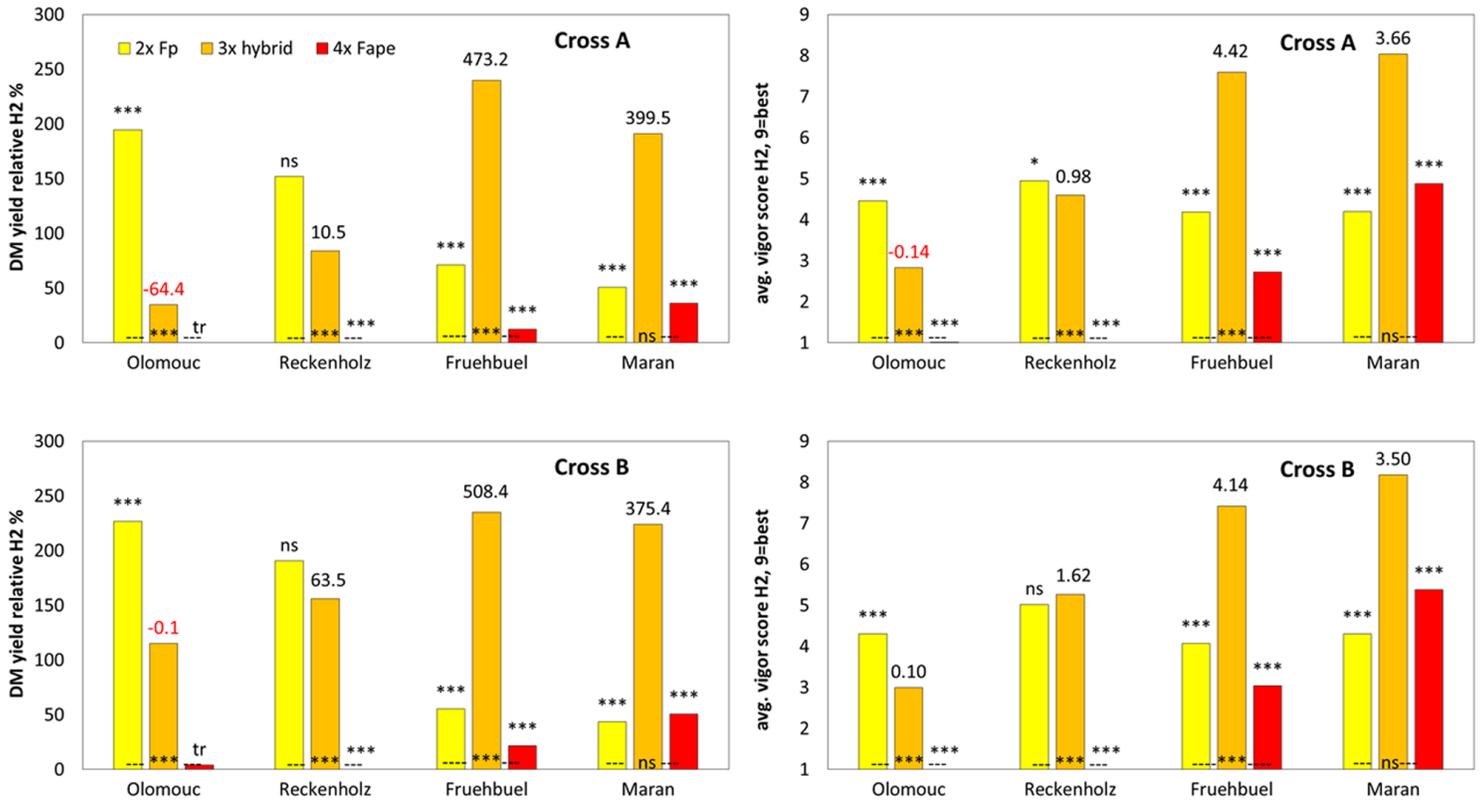


Figure 3

Relative dry mass (DM) yield and average vigor score of 2x Fp, 4x Fape and their triploid (3x) hybrids in the first full harvest year (H2, 2019) at four experimental sites. For explanation of annotations see Figure 1.

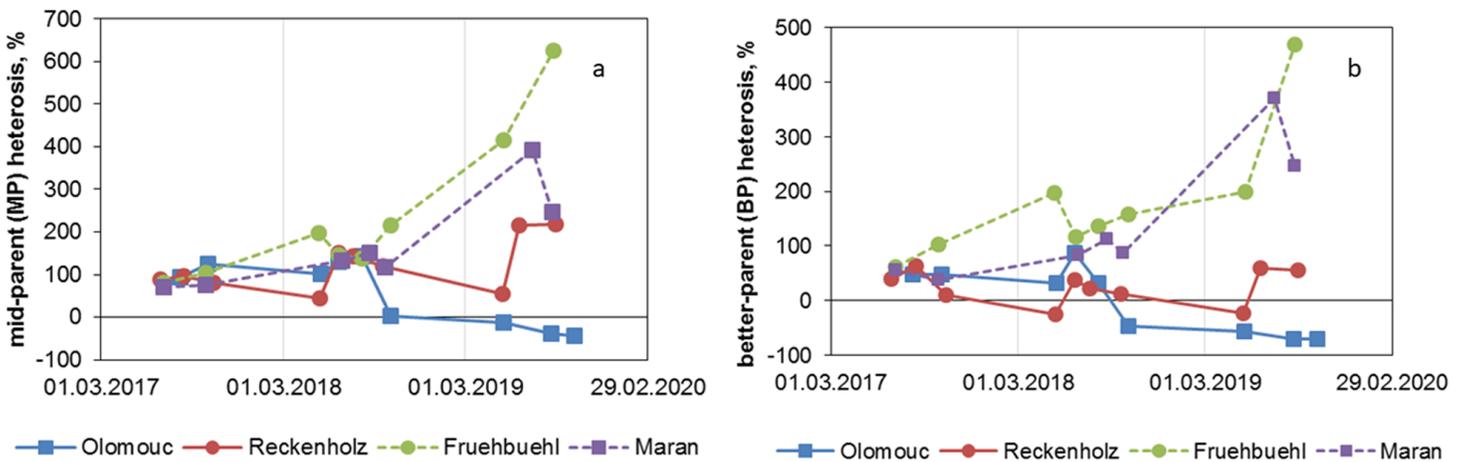


Figure 4

Development of mid-parent (a) and better-parent (b) heterosis of 3x hybrids over their parents 2x Fp and 4x Fape during experimental years A0 (2017), H1 (2018) and H2 (2019) at four sites.

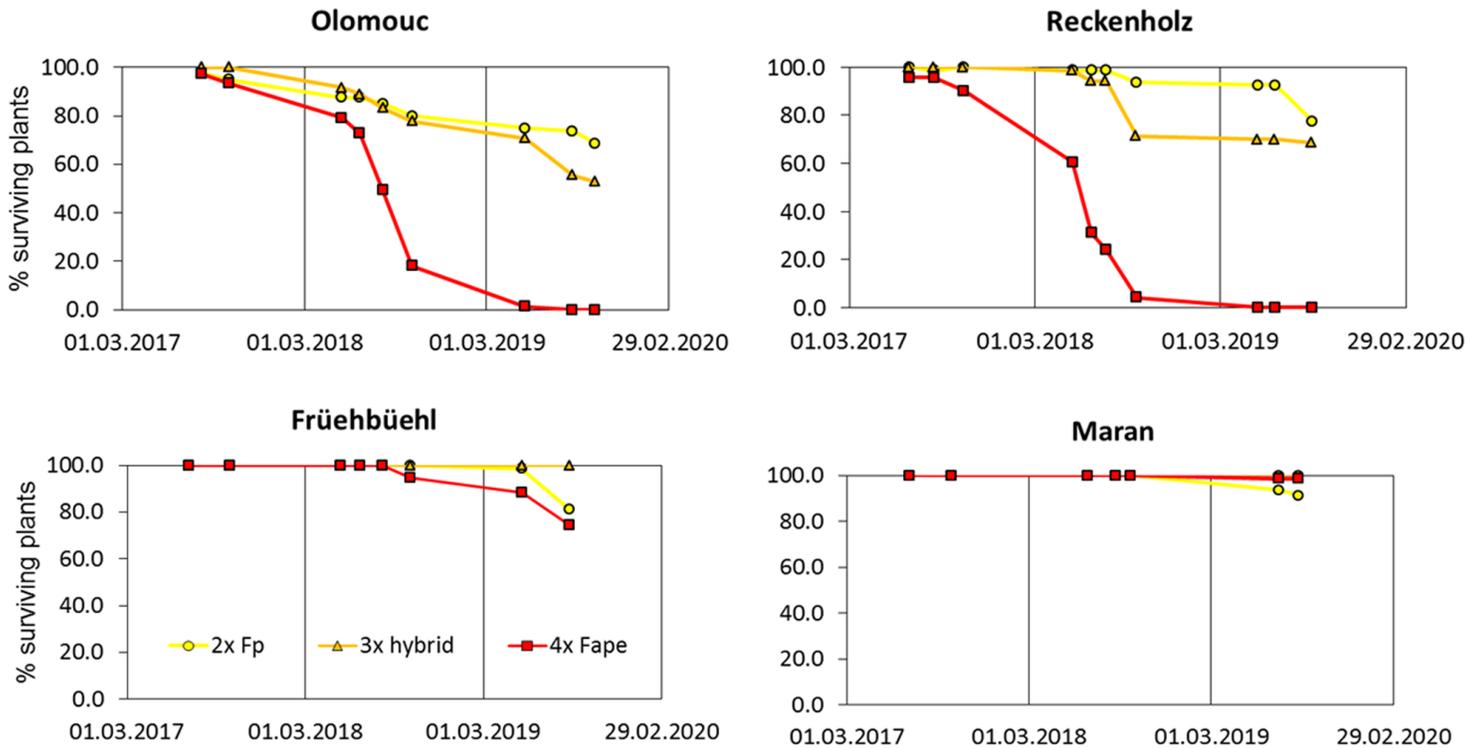


Figure 5

Mortality of 2x Fp, 4x Fape and their triploid (3x) hybrids during experimental years A0 (2017), H1 (2018) and H2 (2019) at four sites.

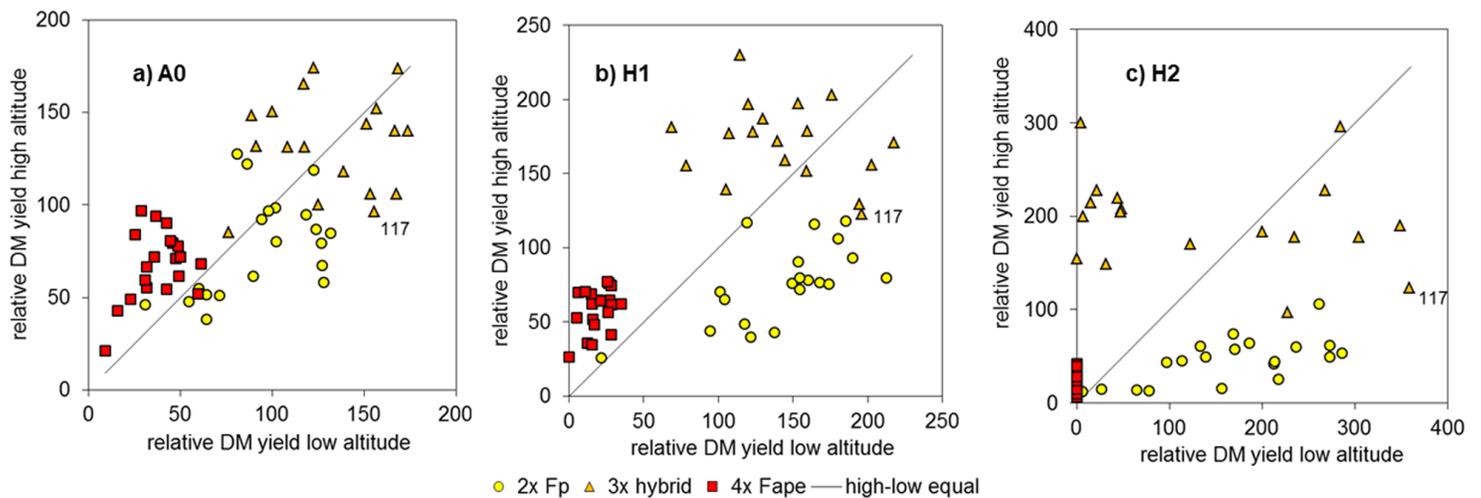


Figure 6

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

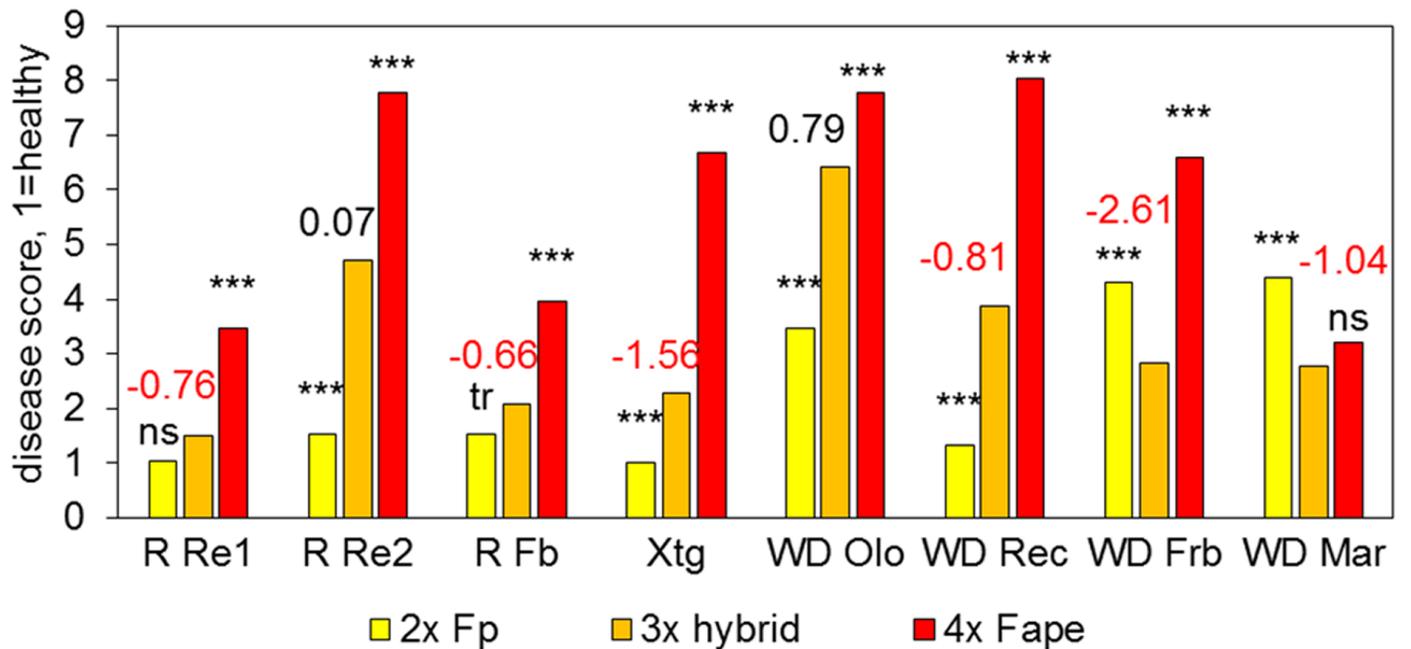
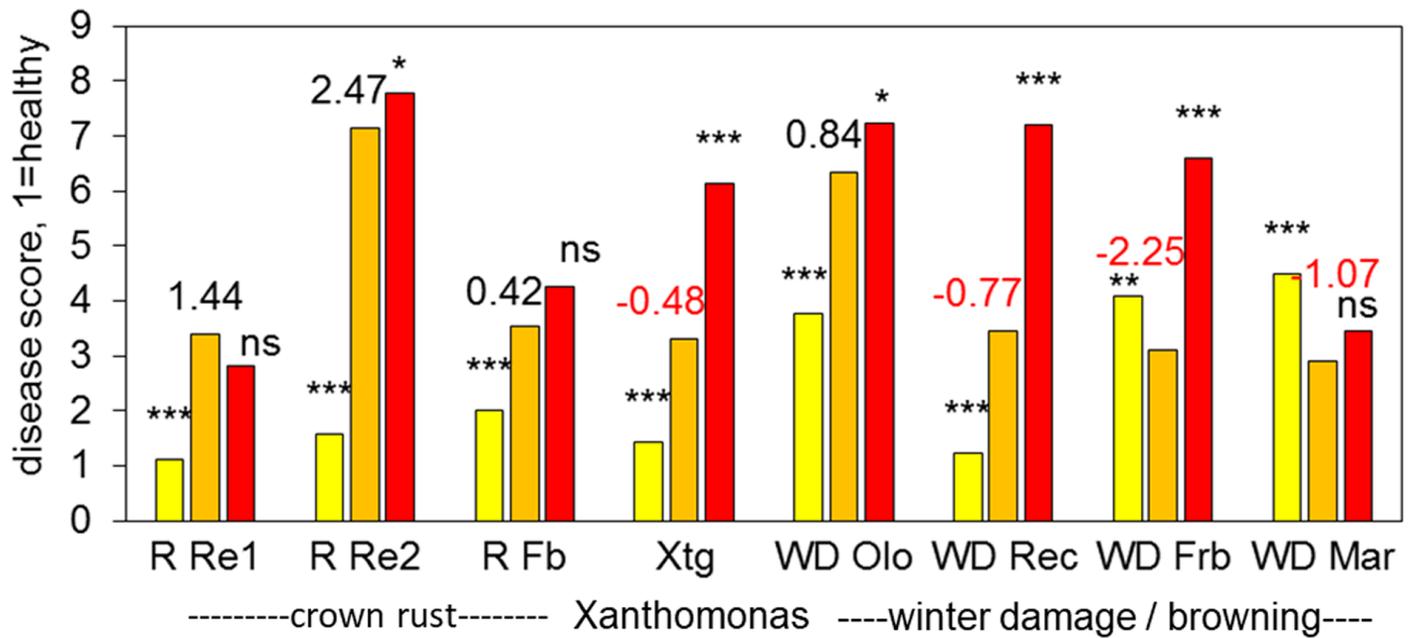


Figure 7

Susceptibility to crown rust (R), bacterial wilt (Xtg) and winter damage (WD) of 2x Fp, 4x Fape and their triploid (3x) hybrids. 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), Fruehlbuehl (WD Frb) and Maran (WD Mar). Figures above 3x 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 3x hybrids and their parents. All scores were highly significantly ($p < 0.001$) different between 2x Fp and 4x Fape.

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