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Mixtures of genetically modified wheat lines outperform monocultures

AUTHORS AND AFFILIATIONS

Simon L. Zeller*, Olena Kalinina, Dan F. B. Flynn, Bernhard Schmid

Institute of Evolutionary Ecology and Environmental Studies, University of Zurich, Switzerland

*Corresponding author: simon.zeller@ieu.uzh.ch

ABSTRACT

Biodiversity research shows that diverse plant communities are more stable and productive than monocultures. Similarly, populations in which genotypes with different pathogen resistance are mixed may have lower pathogen levels and thus higher productivity than genetically uniform populations. We used genetically modified (GM) wheat as a model system to test this prediction, because it allowed us to use genotypes that differed only in the trait pathogen resistance but were otherwise identical. We grew three such genotypes or lines in monocultures or two-line mixtures. Phenotypic measurements were taken at the level of individual plants and of entire plots (population level). We found that resistance to mildew increased with both GM-richness (0, 1 or 2 *Pm3* transgenes with different resistance specificities per plot) and GM-concentration (0, 50 or 100% of all plants in a plot with a *Pm3* transgene). Plots with two transgenes had 34.6% less mildew infection and as a consequence 7.3% higher seed yield than plots with one transgene. We conclude that combining genetic modification with mixed cropping techniques could be a promising approach to increase sustainability and productivity in agricultural systems, as the fitness cost of stacking transgenes within individuals may thus be avoided.

KEY WORDS

Biodiversity, *Blumeria graminis*, Green Revolution, multilines, *Pm3a*, *Pm3b*, population level, resistance genes, transgene stacking, *Triticum aestivum*, yield.

INTRODUCTION

Since the mid-20th century, the Green Revolution allowed agricultural yields to increase continuously, for example in bread wheat in Europe from about 1.5 t in 1950 to 7 t of grain per ha in 1996, but since then wheat yields have stagnated (Brisson et al. 2010). Fertilizer, pesticides and new crop varieties contributed to the dramatic increases in yields (Conway 1997). However, the impact of this development on the environment has also been considerable and unfortunately often negative (Tilman et al. 2001). Organic farming, on the other hand, has allowed a reduction of the input of agrochemicals but only at the cost of reduced yields (Maeder et al. 2002).

Genetic engineering may hold solutions to this problem. For example, crop plants with introduced resistance traits may help to reduce pesticide use while maintaining or even increasing yields (Borlaug 2000). Some of these genetically modified (GM) crops have been so successful that they are currently planted on large areas (James 2009). This leads to a high selection pressure on the pests to overcome the resistance by evolution of new genotypes (Tabashnik et al. 2009, Powles 2010), which in turn may reduce the advantages of GM crops. Efforts are being made to slow down the evolution of such new pest genotypes. Besides refuge strategies, the combination of several GM traits within a single plant, also known as pyramiding or stacking, has been promoted (Bravo and Soberon 2008). However, the sustainability of this approach might be compromised, as "super-pests" may evolve that overcome such multiple resistance, particularly if single-transgene and multiple-transgene crops are planted in close proximity (Zhao et al. 2005). Another problem, which to date has rarely been addressed, are potentially increased defense costs that multiple resistances impose on an individual plant (Kalinina et al. 2011).

Here we suggest that one solution to these problems could be using mixtures of lines with different but complementary resistance traits, i.e. stacking genes at the population rather than the

45 individual plant level. In addition to increasing resistance at the population level, such a strategy
46 should allow the different pathogen strains to survive in low numbers on some plants, thus
47 reducing the selection pressure on the pathogen to overcome plant resistance.

48 Ecological theory and results of recent biodiversity experiments suggest this line of
49 argumentation. In grassland biodiversity experiments, productivity generally increases with
50 diversity (Tilman et al. 1996, Hector et al. 1999, Roscher et al. 2005). Such increased productivity
51 of total biomass in grasslands with plant diversity has some analogs with increased yield in
52 agricultural systems. One of the reasons for increased yield with plant diversity in agricultural
53 systems is reduced pathogen susceptibility (Zhu et al. 2000). For example, wheat lines susceptible
54 to mildew have lower levels of infection if they are surrounded by resistant lines (Kalinina et al.
55 2011). Particular pathogens are less likely to become dominant in a diverse system when their
56 particular hosts all occur at low abundance (Keesing et al. 2006). Only generalist pathogens would
57 be able to thrive in diverse systems of hosts, and such generalists may be less efficient in
58 overcoming the defense of a particular host due to trade-offs among the different adaptations
59 needed to overcome the defenses of a diverse set of hosts (Woolhouse et al. 2001).

60 While ecologists are currently investigating the mechanisms by which species-rich plant
61 communities have lower pathogen abundance and higher yields (Maron et al. 2011), agronomists
62 came across similar phenomena some time ago, albeit at the between-variety, within-species level.
63 Mixtures of several varieties of the same crop species can have higher yields than monocultures of
64 single varieties (Browning and Frey 1969, Wolfe 1985). However, diversity strategies have rarely
65 been used so far for technical reasons, such as uniformity requirements for varieties and seed
66 material and harvesting efficiency (Smithson and Lenne 1996). In part these technical difficulties
67 may be overcome with better harvesting technology. Another and probably easier solution would
68 be to produce plants by genetic engineering that only differ in the resistance traits of interest.

Fields with mixed lines would then still have uniform phenology and harvest traits and could be easily harvested.

We experimentally compared wheat (*Triticum aestivum* L.) plots consisting of single lines with mixed plots. The lines differed only in their resistance to powdery mildew (*Blumeria graminis* f.sp. *tritici* (DC.) Speer), which was possible due to the introduction of a single gene using gene technology. One non-transgenic control line and two transgenic (GM) lines of spring wheat variety Bobwhite were used in the experiment. Mildew infection, plant production and seed yield were assessed at the level of the individual plants and the plot to test their response to increasing GM-richness (0, 1, or 2 GM lines) and GM-concentration (0, 50, 100% of individuals from GM lines) of the plots. Our hypotheses are as follows:

- H1: If plot-level transgene diversity reduces powdery mildew infection more efficiently than transgene monocultures, both higher GM-concentration and especially higher GM-richness will reduce powdery mildew infection.
- H2: Such reductions in powdery mildew will increase seed yield at the plot level.
- H3: If the underlying mechanism for the transgene diversity effect is mediated by the density of plants, then the effect of diversity will be significant for plant performance at the plot level rather than at the individual level, since results from individual plants will not be effective predictors of plot-level responses.
- H4: In contrast, if the mechanism is for individual plants to have reduced risk of infection as transgene diversity increases, then the effect of diversity will be significant at the level of individual plant performance.

MATERIALS AND METHODS

Genetically modified wheat

We used two transgenic wheat lines, derived from different transformation events of Bobwhite SH 98 26 and carrying transgenes *Pm3a* or *Pm3b*, and the control line Bobwhite SH 98 26 (Peter et al. 2010, von Burg et al. 2010, Zeller et al. 2010, Brunner et al. 2011). These transgenes confer different race-specific resistances to powdery mildew and were cloned from hexaploid wheat (Yahiaoui et al. 2004, Srichumpa et al. 2005). *Pm3a* and *Pm3b* were originally isolated from the wheat varieties Asosan and Chul, respectively. Two lines carrying one of the two genes each were generated by biolistic transformation of spring wheat variety Bobwhite SH 98 26 (Pellegrineschi et al. 2002). The generation and selection of line *Pm3b*#1 has been described in detail before (Zeller et al. 2010, Brunner et al. 2011). Similar protocols were used to generate the line *Pm3a*#1 (S. Brunner, personal communication). For simplicity, these two lines will be named *Pm3a* and *Pm3b*, respectively, throughout this paper. The *Pm3a* and *Pm3b* genes were cloned under the control of the *Zea mays* L. (maize) ubiquitin promoter (Christensen and Quail 1996) and transformants were selected on mannose-containing media using the phosphomannose isomerase (PMI)-coding gene as selectable marker (Reed et al. 2001). Southern hybridization analysis (Southern 2006) showed that *Pm3a* carried two and *Pm3b* one copy of the corresponding *Pm3* transgene. The seeds used in this study were obtained from GM lines that had passed through four (*Pm3*a) or five (*Pm3b*) generations of sexual reproduction.

The expression level of the *Pm3a* and *Pm3b* transgenes in the two GM lines was assessed by qRT-PCR using RNA isolated from leaves collected during the field trial in 2009. *Pm3a* was 6–45 times and *Pm3b* 11–130 times more highly expressed in the GM lines than in wheat line Chul which harbors the *Pm3b* gene naturally (Brunner et al. 2011 and S. Brunner personal communication).

115

116 *Field experiment*

117 The field experiment took place at an agricultural research station in Zurich-Reckenholz,
118 Switzerland, from March–July 2009. Four replicate blocks, each with six 3 x 1.08 m plots, were
119 sown with *Pm3a, Pm3b* and Bobwhite SH 98 26 monocultures and the three 1:1 mixtures
120 *Pm3a*/Bobwhite, *Pm3b*/Bobwhite and *Pm3a*/*Pm3b*. In each plot, 400 seeds were sown in six rows
121 with a distance of 17.8 cm between rows using an Oyjord plot drill system (Wintersteiger AG,
122 Ried, Austria). The experimental plots were alternated with triticale plots in a chessboard-like
123 design to eliminate possible neighbor effects. To allow uniform infection by powdery mildew,
124 single rows of the susceptible winter wheat variety Kanzler were planted on both sides of each
125 plot. Powdery mildew infection occurred naturally and evenly throughout the experiment.

126 All seeds were treated with the fungicide Jockey (167g l^{-1} Fluquinconazole, 34 g l^{-1}
127 Prochloraz; Omya Agro AG, Safenwil, Switzerland) before sowing. The amount of mineralized
128 nitrogen, determined at the end of February in the top 100 cm of the soil, was 35.1 and 47.6 kg N
129 ha^{-1} in blocks 1/2 and 3/4, respectively. Nitrogen fertilizer was applied the day before sowing (40
130 kg N ha^{-1} in blocks 1/2, 30 kg N ha^{-1} in blocks 3/4) and again 30 kg N ha^{-1} ("Ammonsalpeter
131 27.5", Lonza, Visp, Switzerland) at the phenological stage 22–29 (Zadoks et al. 1974). The natural
132 field soil provided the plants with sufficient phosphorous, potassium and magnesium (75, 182 and
133 213 mg kg^{-1}). All plots were sprayed with the herbicide cocktail Concert SX (40%
134 Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen, Switzerland) and
135 Starane super (120 g l^{-1} Bromoxynil, 120 g l^{-1} Ioxynil, 100 g l^{-1} Fluroxypyr-metilheptil-ester;
136 Omya Agro AG, Safenwil, Switzerland) at the beginning of May. All plots were treated twice with
137 the insecticide Karate Zeon (100 g l^{-1} Lambda-Cyhalothrin; Syngenta Agro AG, Dielsdorf,
138 Switzerland) against the wheat stem fly (*Chlorops pumilionis* Bjerk.) at the beginning of May and

2 weeks later. Due to weed infestation the whole trial was sprayed with Puma Extra (69 g l^{-1} Fenoxaprop-P-ethyl, 75 g l^{-1} Mefenpyr-Diethyl; Omya Agro AG, Safenwil, Switzerland).

In each plot, ten individual plants were marked shortly after germination. These individuals were distributed evenly over the 3 m plot length and randomly among the four inner rows. This allowed us to obtain a representative sample of the entire plot while excluding edge effects.

Response variables

To address the hypotheses that plant response at the plot level (H3) is the most indicative of infection rates in response to transgene diversity or that the response of individual plants (H4) can be an effective proxy of such plot-level responses, we measured six phenotypic traits on individual plants and five traits on entire plots. Individual plants were assessed for the degree of powdery mildew infection (Eyal et al. 1987) 44, 59 and 78 days after germination. Based on these time points, the Area Under Disease Progress Curve (AUDPC) was calculated (Jeger and Viljanen-Rollinson 2001). Furthermore, phenological stage (Zadoks et al. 1974) and height were assessed 59 and 78 days, respectively, after germination for each plant. The Zadoks scale allows classifying individual cereal plants or entire plots into development stages reaching from 1 (start of germination) to 99 (ripening complete). At the end of the growing season, height was recorded again and then all individual plants were cut at ground level and separated into vegetative and reproductive parts (spikes). Vegetative and reproductive parts were dried at 80 and 25 C°, respectively, and weighed. The reproductive parts were threshed to obtain seeds and determine total seed mass per plant, here referred to as individual seed production. Finally, the seed mass of the individual plants was divided by the number of seeds and multiplied by one thousand to calculate the thousand seed weight (TSW).

Two non-destructive measurements were conducted at the plot level. Leaf Area Index (LAI) was measured on the western side of each plot 25 and 35 days after germination (LAI 2000 Plant Canopy Analyser, LI-COR Biosciences; Lincoln, USA). It consisted of two measurements close to an inner row and one between the rows as well as a control measurement above the canopy. To assess differences in flowering time, the percent of plants with flowering spikes in each plot was determined 64 days after germination. At this time, all plots had flowering spikes. A subplot of 50 x 72.2 cm was harvested in the same place were the LAI was measured in each plot. These subplots were placed 50 cm from the western edge of the plot and excluded the two outer rows. The harvested material was separated into vegetative and reproductive parts to determine biomass, seed yield and thousand seed weight at plot level. The latter was determined on a sample of 1,000 seeds.

Data analysis

We analyzed the data of individual plants and plots separately by mixed-model analysis of variance using the REML (Restricted Maximum Likelihood) method. We used the statistical software GenStat (VSN International Ldt.). The critical significance level was 0.05 in all analyses. However, we also present and discuss some results which were marginally significant at the 0.1 level (Peto et al. 1976, Toft and Shea 1983). The results of the mixed-model analyses are summarized in tables for all variables (see Appendix A and B). Residual plots were examined to identify outliers and to check if the assumptions of normality and homoscedasticity were fulfilled. For the six diversity treatments (three monocultures and three mixtures), two linear but non-orthogonal contrasts were made to test for effects of increasing GM-richness (0 for monoculture Bobwhite control, 1 for each of the two GM monocultures and the mixtures of each GM with Bobwhite control, 2 for the mixture of the two GM) or increasing GM-concentration (0% for

monoculture Bobwhite, 50% for each of the 2 mixtures of one GM and Bobwhite control, 100% for the two GM monocultures and the mixture of the two GM). Since these two contrasts were partly confounded with each other, their fitting sequence was swapped in two alternative statistical models. For GM-richness, which was the focus of our study, the different sequences can be interpreted as follows: when GM-richness is fitted first, confounding effects of GM-concentration are ignored; when GM-richness is fitted second, it measures the difference between richness levels corrected for increasing GM-concentration. Predicted means and standard errors from the REML-output were used to draw figures.

Since several of the measured traits were correlated with each other, we also performed a multivariate analysis of variance (MANOVA) to test for the overall significance of treatment effects. For the individual plant data the six traits, AUDPC, phenological state, plant height, biomass, seed mass and TSW, were included in the MANOVA. For the plot data the five traits, LAI, flowering time, biomass, seed mass and TSW, were included in the MANOVA.

To directly compare mixtures with monocultures of wheat lines, a deviation or D-value (Loreau 1998) was calculated separately for each plot containing a line mixture in each block. For this calculation, the mean of the two monocultures was first subtracted from the mixture and the resulting value then divided by the mean of the two monocultures. A D-value greater than 0 indicates, for example, that the yield of a mixture is higher than what would be expected from the mean of the monocultures. The opposite would be true for a negative D-value. We calculated D-values for powdery mildew infection, plot biomass, seed yield and TSW. Original data were used to calculate D-values and to draw Figures 3 and 4.

To investigate mechanisms that might explain the observed treatment effects in one response variable, we tested the other, earlier-measured response variables as covariates. Powdery

mildew infection had the best explanatory power for variation in the other traits and thus results of REML models with this covariate are also presented.

RESULTS

Individual level responses

The multivariate analysis for the individual plant data showed highly significant effects of the diversity treatment ($P<0.001$, Appendix A). These were also reflected in significant GM-richness or GM-concentration contrasts ($P=0.002$ for each if fitted first) and significant differences between plots containing either *Pm3a* or *Pm3b* ($P=0.001$). Following the finding of significant effects overall for transgene diversity on multivariate plant responses, each response was then analyzed individually.

Powdery mildew infection as measured by AUDPC at the individual plant level decreased with increasing GM-richness and GM-concentration of plots (Figure 1A; $P<0.001$; see Appendix A). Both contrasts were highly significant if fitted first (GM-richness: $P<0.001$; GM-concentration: $P<0.001$) or second (GM-richness: $P=0.038$; GM-concentration: $P=0.031$) in the statistical model. Plots containing two GM lines had 65.1% and plots containing one GM line had 31.7% lower mildew infection than non-transgenic control plots. Plots with 50% GM plants had 31.7% and plots with 100% GM plants had 52.8% lower mildew infection than plots without GM plants. No significant difference between the two GM lines *Pm3a* and *Pm3b* was detected ($P=0.141$). All mixtures were less infected by mildew than expected from the means of the monocultures. D-values were –0.072, –0.144 and –0.345 for the mixtures BW/*Pm3a*, BW/*Pm3b* and *Pm3a*/*Pm3b*, respectively. This means that plants in plots with BW/*Pm3a* had 0.3%, plots with

231 BW/*Pm3b* 20.7% and plots with both GM lines had 34.6% less powdery mildew than expected
232 from the corresponding monoculture means.
233 The phenological development of GM plants measured 59 days after germination was on
234 average not significantly different from that of control plants (Figure 1B and Appendix A).
235 However, *Pm3b* developed significantly faster than *Pm3a* (difference = 2.2 points on Zadoks
236 Scale, P<0.001). This means that an introduced transgene can influence the phenological
237 development of a plant.
238 Individual plants in Bobwhite control plots were significantly shorter than in plots
239 harbo~~u~~ring GM plants (Figure 1C; difference = 3.8cm; P=0.014). Plant height increased with GM-
240 richness and GM-concentration (sum of the two contrasts significant at P=0.013). However, the
241 individual contrasts were only significant if fitted first in the statistical model (GM-richness:
242 P=0.013; GM-concentration: P=0.013).
243 *Pm3a* had significantly more biomass than *Pm3b* (Figure 1D; difference= 0.55 g/plant;
244 P=0.036). There was a trend towards higher biomass with increased GM-richness (P=0.099) but
245 GM-concentration did not influence the biomass of individual plants. *Pm3a* had a marginally
246 higher individual seed production than *Pm3b* (P=0.055) and GM-richness marginally increased
247 individual seed production as well (P=0.092). *Pm3a* had significantly more (data not shown,
248 P=0.003) but lighter seeds than *Pm3b* (Figure 1F, difference = 5.4 g TSW; P=0.003). TSW
249 increased with either GM-richness or GM-concentration if the corresponding contrast was fitted
250 first in the statistical model (GM-richness: P=0.023; GM-concentration: P=0.047) but not if it was
251 fitted second.
252

253 *Plot-level responses*

254 In the multivariate analysis with the plot level data the diversity treatment effects were also highly
255 significant (P=0.002, Appendix B). GM-concentration was significant if fitted first or second
256 (P=0.021 and P=0.005). GM-richness, however, was only significant if fitted second, i.e. after
257 GM-concentration (P=0.020), indicating that after correction for increasing GM-concentration,
258 plots with two GM lines differed from plots with only one GM line. Furthermore, plots containing
259 *Pm3a* differed significantly from plots containing *Pm3b* (P<0.001).

260 The LAI measured at the beginning of the growing season (25 days after germination)
261 decreased with increasing GM-concentration (Figure 2A and Appendix B; GM-concentration:
262 P=0.01 if fitted first and P=0.028 if fitted second). However, this effect disappeared 35 days after
263 germination. On day 64 after germination, plots with high GM-concentration had fewer flowering
264 spikes than plots with low GM-concentration (Figure 2B; P=0.005). Fitted after GM-
265 concentration, GM-richness also affected the number of flowering spikes (P=0.012). Furthermore,
266 plots with *Pm3a* had significantly fewer flowering spikes than plots with *Pm3b* (P<0.001). This
267 result is consistent with the individual plant data, where *Pm3a* was shown to develop more slowly
268 than *Pm3b*.

269 The aboveground biomass in the plots did not differ statistically significantly among the six
270 diversity treatments (Figure 2C). However, a positive D-value of 0.062 indicated that the GM-GM
271 mixture tended to have higher biomass than expected from the mean of the two GM monocultures.
272 Clearer differences were found for seed yield (Figure 2D). Plots with high GM-richness had higher
273 yield than plots with low GM-richness (P=0.04). In numerical values plots with two GM lines had
274 a 16.7% higher seed yield than control lines whereas plots with only one GM line only had a 5.4%
275 higher seed yield than control lines. A positive D-value of 0.073 indicated that the GM-GM
276 mixture performed 7.3% better than expected from the mean of the two GM monocultures. Since

277 the mixture was also producing a higher seed yield than the better GM monoculture, there was
278 evidence for transgressive overyielding (Schmid et al. 2008).
279 The TSW increased significantly with GM-richness (Figure 2E, P=0.006). Seeds from plots
280 with two GM lines were 11.9% heavier than seeds from control plots, whereas seeds from plots
281 with only one GM line were only 5.6% heavier than seeds from control plots. This was also
282 reflected in positive D-values for all mixtures. Similar to the individual plant data, seeds from
283 plots containing *Pm3b* were significantly heavier than seeds from plots containing *Pm3a*
284 (P=0.016).
285
286 *Analyses with covariate mildew infection*
287 To assess the influence of the mildew infection on other measured traits we repeated the analysis
288 with AUDPC as covariate. On the individual plant level, plant height and TSW were affected
289 significantly (plant height: P=0.001; TSW: P=0.002) by AUDPC. The inclusion of the covariate
290 fully explained the effects of GM-richness and -concentration on plant height and TSW. Thus the
291 two contrasts were no longer significant if fitted after the covariate. However, the differences
292 between lines *Pm3a* and *Pm3b* persisted.
293 At the plot level, biomass, seed yield and TSW were significantly influenced by the
294 covariate. Whereas the covariate did not remove the significance of the remaining effects on plot
295 biomass, it did explain the GM-richness and -concentration effects on seed yield and TSW at plot
296 level, which both were no longer significant if fitted after the covariate. However, the differences
297 between plots containing line *Pm3a* vs. *Pm3b* remained significant. Overall, these results suggest
298 that the reduced mildew infection found in plots with high GM-richness or GM-concentration had
299 a positive influence on plant height, seed yield and TWS.
300

DISCUSSION

Mixing GM lines reduces mildew infection (H1) and increases yield (H2)

This study demonstrates that genetically modified (GM) wheat plants perform differently when grown in mixtures with other GM lines or control lines than when grown in single-line monocultures. The performance of individual plants and of entire plots generally increased with the number of GM lines (GM-richness, ranging from 0–1–2) or with the proportion of GM plants (GM-concentration, ranging from 0–50–100%) in a plot. Thus, powdery mildew resistance increased with GM-concentration, indicating that the transgene worked as expected. Furthermore, mildew resistance also increased with GM-richness. This was probably due to the fact that the two GM lines harbored transgenes that were effective against different races of powdery mildew and thus they could complement each other in mixture and provide resistance against a wider spectrum of pathogens than if the same lines were grown in single-line mixtures. This indicates that a diversity of resistance transgenes can have a beneficial effect at the plot level, avoiding the need to stack these genes in each single plant, potentially leading to higher fitness costs (Kalinina et al. 2011). If in mixtures a certain proportion of individual plants are resistant against a specific pathogen they can reduce the spread of infection (Browning and Frey 1969, Schmid 1994). Not only mixtures of two GM lines, but also mixtures of a GM line with a control line were less infected with powdery mildew than expected from the means of the two monocultures. In this case as well, the non-resistant plants of the control line may have profited from the protection by neighboring resistant GM plants.

Besides the resistance to powdery mildew, we assessed a number of phenotypic traits correlated with performance. Individual plants grew taller and produced larger seeds in plots with increased GM-richness or -concentration. However, at the plot level we recorded a lower leaf area index at the beginning of the growing season and a later flowering time in plots with high GM-

concentration. This could indicate costs of resistance (Bergelson and Purrington 1996). Nevertheless, seed size and seed yield increased with GM-richness: one of the two plots with a GM/control line mixture (*Pm3b*/BW) increased its yield by 3.8% compared the mean of single monocultures. Because the seed yield of the mixture of the two GM lines was even higher than that of the better single-GM line monoculture (yield of *Pm3b*/*Pm3a* mixture was 6.5% higher than in *Pm3b*), this can be considered as one of the rare cases of transgressive overyielding (Trenbath and Harper 1974, Harper 1977, Vandermeer 1989) in which two parts of a system improve their performance by interacting with each other. Using mildew infection as a covariate in the statistical analysis explained most of the differences in performance between plots with different GM-richness or -concentration, indicating that overall it was indeed the increased mildew resistance that caused the positive effects of GM-richness and -concentration on performance.

Differences among GM lines

Our experiment allowed us to test whether the introduction of different alleles of a *Pm3* transgene also affected plant performance. This was indeed the case. Even though the trait directly linked to the transgene, mildew resistance, was similar in both tested lines, we found that the phenological state and the start of flowering differed strongly between the two GM lines. Although at plot level biomass and seed yield did not differ, individual *Pm3a* plants had higher biomass and marginally higher individual seed production than *Pm3b*. The TSW analysis revealed that *Pm3a* had generally smaller seeds than *Pm3b*. It appears that the slower development of *Pm3a* allowed the individual plants to stay longer in the vegetative phase, develop more biomass and produce more but smaller seeds. Since both GM lines had similar mildew resistance, it is not likely that the performance differences described above were caused directly by the powdery mildew infections or allelic differences between the two lines. Since the lines differed both in the identity of the allele and the

transformation event, it is conceivable that their different performance was due to effects related to the latter, e.g. different gene expression levels as a consequence of different location of the insertion site (Cubas et al. 1999, Filipecki and Malepszy 2006). Such expression differences were for example observed in a previous study using multiple transformation events with a single *Pm3* allele (Zeller et al. 2010).

Individual plant or plot-level effects of diversity? (H3, H4)

An understanding of the mechanism by which diversity affects plant yields at the plot or field level requires an assessment of effects on individual plants, and not only assessment of plot-level performance. For example, determining that higher yields in more diverse plots result from increases in plant density rather than increases in individual plant yield requires measurements of yield at both the individual and plot levels. Our analyses allow us to distinguish between a density effect of transgene diversity (H3) and an effect of transgene diversity on individual plant performance (H4). The similarity of results of statistical analyses at the individual plant and plot level support H4 (Appendix A and B). Differences in phenological development and TSW among the two GM lines were found with both methods. GM-richness and GM-concentration showed similar trends for biomass, seed yield and TSW. Only the significantly increased seed yield due to increased GM-richness at the plot level would not have been predicted by the results from individual plants. The explanation might lie in the density dependence of seed yield. Individual plants can and should be used for all traits like plant height, phenological development, TSW and seed set. However, for correct estimates of biomass and seed yield, the crop density or number of tillers would have to be included in the extrapolation from individual plant to the whole plot.

Generally, assessment of individual plants proved to be useful in testing the performance of genetically modified wheat lines. This method might be labor intensive but there are also several

advantages: only a few plants need to be removed from each plot. This means that the experimental plots stay intact and can be used for other purposes. Furthermore, individual plants can be handled and stored much easier than bulky harvest bags. An important caveat, which must be considered in each case, is a potential confounding of plant density with treatment effects.

Conclusions and applied aspects

Our study demonstrated that mixing wheat lines that differed only in their resistance to different strains of powdery mildew reduced plant susceptibility to this pathogen. This led to an increased performance of these mixtures and even to transgressive overyielding. Not only mixtures of two GM lines compared to monocultures of one GM line, but also mixtures of one GM and one control line compared to monocultures of GM and control lines showed increased mildew resistance and in most cases also higher performance. One could therefore argue that mixing closely related plant lines could increase agricultural output. Ecological research indicates that productivity increases with diversity in most cases that have been experimentally investigated (Tilman et al. 1996, Hector et al. 1999, Roscher et al. 2005, Marquard et al. 2009). However, these results have not been translated into agricultural practice, in part because mixtures of different varieties are difficult to harvest. Gene technology might provide us with very similar plant lines that differ only in their resistance genes. Such mixtures could therefore be harvested without change of practice. We have only assessed mixtures of two lines, either two GM lines or mixtures of one GM and one control line. According to ecological theory, mixtures of more than two lines should lead to even better results. In the future, results of such mixture experiments should be compared to lines that have several resistance genes stacked within the same plant. It may be that costs of resistance would accumulate in such plants, thus potentially diminishing the synergistic benefit of transgene mixtures at the plot level, but further study would be needed to evaluate this hypothesis.

Furthermore, the evolution of resistant pathogens should be studied. Some studies report that resistances may develop faster if single-gene plants that harbor different resistance genes are planted next to double-gene plants (Zhao et al. 2005). However, it is also possible that the resistance development is slower in mixtures due to the lower pathogen population size (Chin and Wolfe 1984).

The comparison of two GM lines that harbor a different allele of the *Pm3* gene revealed a number of phenotypic changes in performance-related traits which might have been of pleiotropic origin. Several studies report that genetically modified plants might differ in many traits even if they share very similar transgenes (Snow et al. 2005, Filipecki and Malepszy 2006).

Finally we checked whether results obtained from individual plants can help to predict the performance of entire populations. We conclude that such measurements can be very useful for performance tests — especially when information about the variation and interactions within the population are of interest. We conclude that today's agricultural systems might become both more productive and more sustainable with biodiversity strategies such as planting line mixtures.

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547

548 **FIGURE LEGENDS**

549

550 **Figure 1. Effects of GM-richness and GM-concentration on individual wheat plants.** Line
551 means were predicted using REML models. GM-richness consisted of the levels "no GM" "one
552 GM" and "two GM" lines and GM-concentration of 0, 50 and 100% GM plants in a particular
553 plot. A–F are different traits that were measured on individual plants. Asterisks indicate the level
554 of significance for the GM-richness or GM-concentration contrast (*P<0.05; ***P<0.001).

555

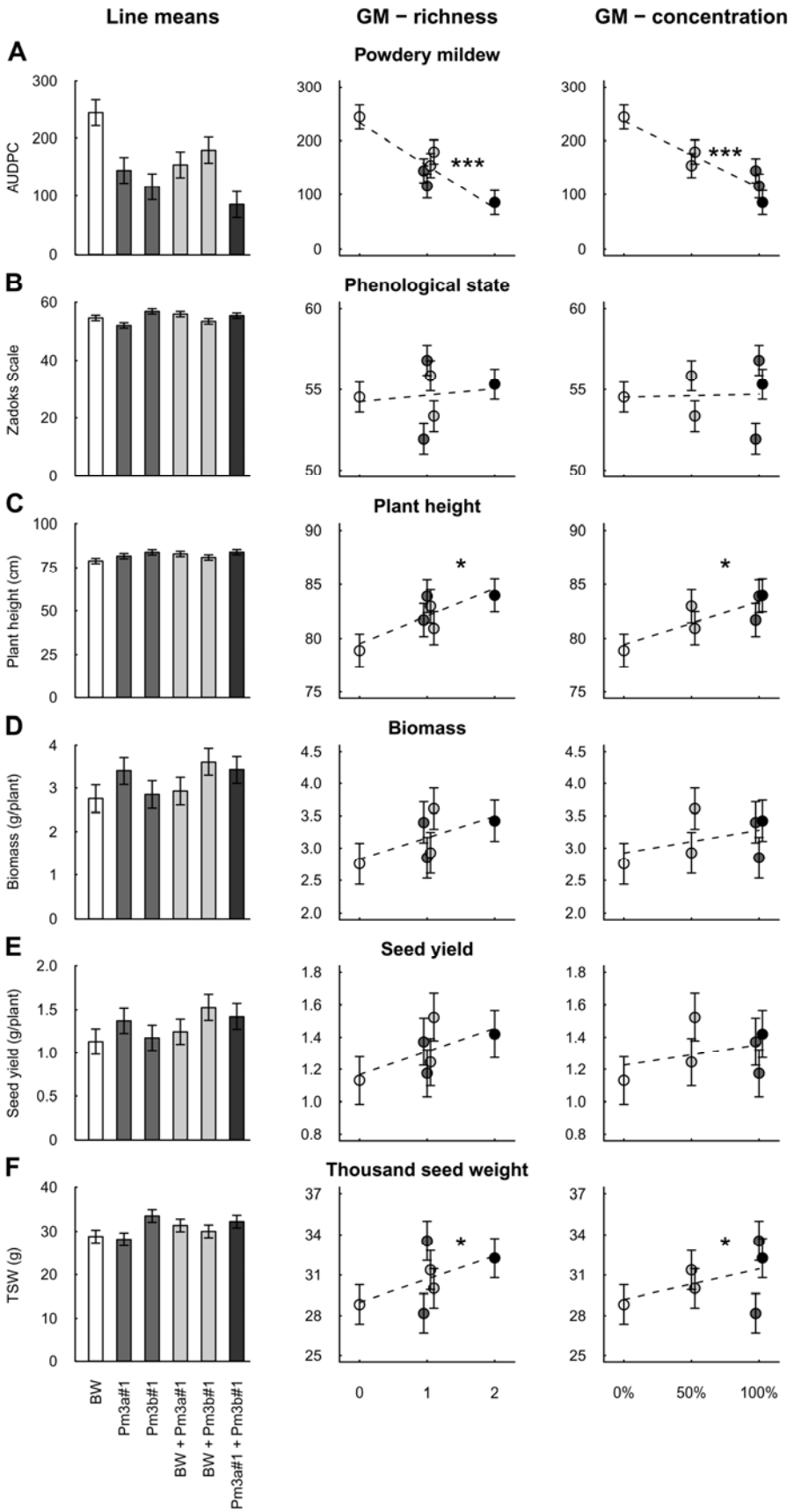
556 **Figure 2. Effects of GM-richness and GM-concentration on wheat at the plot level.** Line
557 means were predicted using REML models. GM-richness consisted of the levels "no GM" "one
558 GM" and "two GM" lines and GM-concentration of 0, 50 and 100% GM plants in a particular
559 plot. A–F are different traits that were measured at the plot level. Asterisk indicate the level of
560 significance for the GM-richness or GM-concentration contrast (*P<0.05; **P<0.01).

561

562

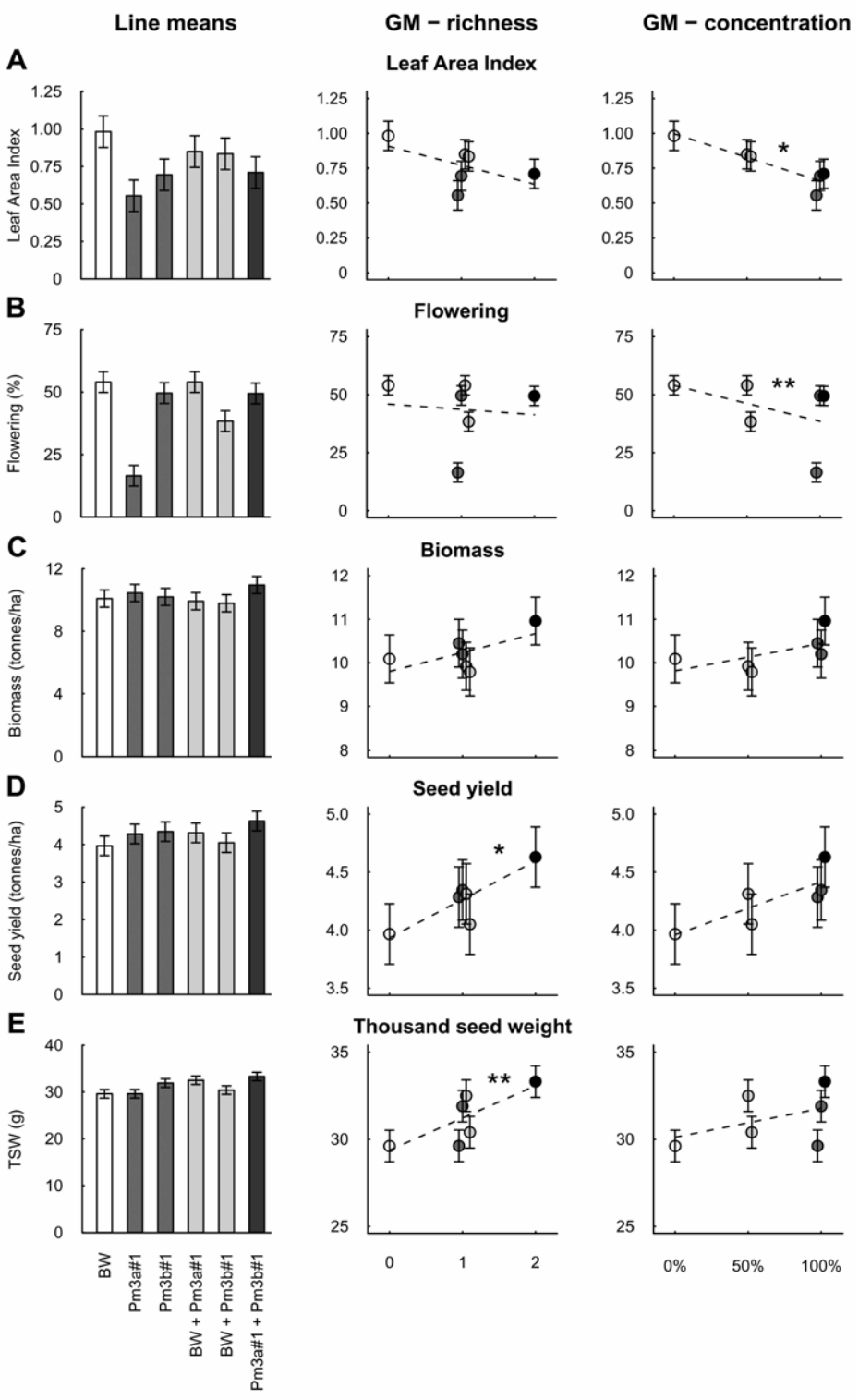
563 **Figure 1**

564



565 **Figure 2**

566



APPENDIX A

Table with summaries of Manova and REML analyses of several traits measured on individual plants level (*Ecological Archives xxxxxxxxxx*).

APPENDIX B

Table with summaries of Manova and REML analyses of several traits measured at the plot level (*Ecological Archives xxxxxxxxxx*).