

Plant diversity affects behavior of generalist root herbivores, reduces crop damage, and enhances crop yield

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Abstract. Soil-dwelling pests inflict considerable economic damage in agriculture but are hard to control. A promising strategy to reduce pest pressure on crops is to increase the plant diversity in agroecosystems. This approach, however, demands a sound understanding of species' interactions, which is widely lacking for subterranean herbivore–plant systems. Here, we examine the effects of plant diversification on wireworms, the soil-dwelling larvae of click beetles that threaten crops worldwide. We conducted a field experiment employing plant diversification by adding either wheat or a mix of six associated plants (grasses, legumes, and forbs) between rows of maize to protect it from *Agriotes* wireworms. Wireworm feeding behavior, dispersal between crop and associated plants, as well as maize damage and yield were examined. The former was assessed combining molecular gut content and stable isotope analysis. The pests were strongly attracted by the associated plants in August, when the crop was most vulnerable, whereas in September, shortly before harvest, this effect occurred only in the plant mix. In maize monoculture, the larvae stayed in the principal crop throughout the season. Larval $\delta^{13}\text{C}$ signatures revealed that maize feeding was reduced up to sevenfold in wireworms of the vegetationally diversified treatments compared to those of the maize monoculture. These findings were confirmed by molecular analysis, which additionally showed a dietary preference of wireworms for specific plants in the associated plant mix. Compared to the monoculture, maize damage was reduced by 38% and 55% in the wheat and plant mix treatment, which translated into a yield increase of 30% and 38%, respectively. The present findings demonstrate that increasing the plant diversity in agroecosystems provides an effective insurance against soil pests. The underlying mechanisms are the diversion of the pest from the principle crop and a changed feeding behavior. The deployment of diverse mixes of associated plants, tailored to the specific preferences of the soil herbivores, provides a promising strategy for managing subterranean pests while maintaining crop yield.

Key words: *Agriotes*; maize; molecular gut content analysis; multiple trap cropping; pest control; stable isotope analysis; wireworms.

INTRODUCTION

Agricultural plant production is among the most challenging issues of this century in light of the continuously growing human demand for food. Increasing arable productivity, while reducing the negative impacts of crop production on the environment (“sustainable intensification” [Royal Society 2009]), has become the key strategy to meet this challenge (Godfray et al. 2010). Crop plants, however, are constantly threatened by agricultural pests, with animals devouring an estimated 8–15% of the crop production worldwide despite the current crop protection practices (Oerke 2006). To date, pest control relies on the extensive use of

synthetic pesticides. For a sustainable intensification of agricultural plant production, however, alternative control strategies are needed. Particularly challenging is the control of soil-dwelling pests, because they are difficult to access. Even though these animals are mostly “out of sight,” their feeding pressure on belowground parts of crops is of great economic importance (Hunter 2001, Blossey and Hunt-Joshi 2003, Blackshaw and Kerry 2008). The fact that plant tolerance to root feeders is lower than to defoliating aboveground herbivores (Zvereva and Kozlov 2012) explains why soil pests can be highly devastating. For example, in Europe, the potential average annual economic losses have been estimated at 472 million Euros for the western corn root worm, *Diabrotica virgifera virgifera*, whose juveniles attack maize roots (Wesseler and Fall 2010). Similarly, wireworms, the soil-dwelling larvae of click beetles, inflicted crop losses between 5% and 25% in North America and the UK (Parker et al. 1990, Jansson and Seal 1994) and the damage caused by these pests led to

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price reductions of more than 12% for potato growers in Switzerland (Keiser et al. 2012).

Increasing the plant diversity within and around arable fields has been suggested as an alternative to pesticide-based pest control typically found in intensively farmed monocultures. Although recent literature compilations and meta-analyses on this topic confirm the potential of increased plant diversity for pest regulation and arable production (Poveda et al. 2008, Letourneau et al. 2011), it is unknown if plant diversification per se reduces the incidence of pests (Ratnadass et al. 2012). Quite contrary, greater plant diversity can be highly attractive to generalist herbivores and diversification may have no or even an enhancing effect on pest densities (Mediene et al. 2011). Moreover, diversification schemes often interfere with arable productivity (Poveda et al. 2008, Letourneau et al. 2011). In fact, there are only a few studies that assess the relationship between pest control and crop yield (Poveda et al. 2008). Hence, there is a pressing need to improve our understanding of the mechanisms involved in plant diversification schemes to design successful cropping systems that will be adopted by farmers (Letourneau et al. 2011, Ratnadass et al. 2012).

So far, plant diversification schemes have mainly been employed to control aboveground insect pests (e.g., lygus bugs, stem borers, pollen beetle), including the successful implementation of long-term management solutions, e.g., the use of alfalfa as a trap crop for lygus bugs in cotton (Godfrey and Leigh 1994) and a push-pull strategy to control stem borers in maize (Khan et al. 2001). The conditions for soil-dwelling herbivores differ greatly from those of their aboveground counterparts. For example, they are more restricted in their movement and in the perception of plant signals. This potentially hampers the capacity of soil pests to locate non-crop plants offered in diversified cropping systems. Hence, it is questionable whether the mechanisms that affect aboveground pests are the same for subterranean ones. To date, knowledge on this is poor, primarily because examining pest behavior belowground is fraught with difficulties. For example, it is challenging to assess how plant diversity is exploited by root feeding pests because the food remains in the gut content are usually not microscopically discernible (Johnson et al. 2007). Fortunately, recent advances in technology, such as molecular approaches and stable isotope analysis, open new possibilities to examine the food choice of root feeders under natural conditions. These approaches provide a new means to unravel the mechanisms in subterranean plant–herbivore systems and to move from observational to predictive results. Moreover, the highly specific molecular techniques allow assessing which plants in diversification schemes are functionally important (Wallinger et al. 2012). Based on this knowledge plant diversification schemes could be shaped for the “right kind” of diversity, which reduces pest pressure and increases crop yield (Poveda et al. 2008).

Here, we investigated if maize (*Zea mays* L.), one of the most important crops worldwide (FAOSTAT 2010), can be protected via enhanced plant diversity from *Agriotes* wireworms, which are one of the major pests attacking maize belowground. These soil-dwelling click beetle larvae (Coleoptera: Elateridae) are abundant generalist herbivores, feeding on a wide range of arable crops and non-crop plants (Hill 1987, Traugott et al. 2008). Their generalist feeding behavior has been exploited to lure wireworms away from crops (Vernon et al. 2000, Landl and Glauning 2011). However, no study has yet examined in detail how increased plant diversity affects the behavior of wireworms and other soil pests under field conditions.

Within our field experiment, which corresponds to standard maize cultivation practices, we compared the efficacy of a single associated plant species to multiple associated plants comprised of grasses, legumes, and forbs. As single associated plant species, wheat (*Triticum aestivum* L.) was used, since it is known to be effective in luring *Agriotes* larvae away from the main crop in strawberry fields (Vernon et al. 2000). The associated plants were grown between maize rows to protect the crop during germination and as seedling, when it is most vulnerable to wireworm attack. To examine the effects on feeding behavior, we determined the plant consumption of the wireworms using an approach combining molecular gut content and stable isotope analysis. While the DNA-based analysis provides highly specific “snapshots” of the recently consumed plant material (Staudacher et al. 2011b), isotopic analysis reflects the wireworms’ long-term diet (Traugott et al. 2007). To account for seasonal dynamics in wireworm abundance and crop development, both insect behavior and plant performance were analyzed at two time points: when maize plants were small (in August; about the six-leaf stage) and before the maize was harvested (in late September).

We hypothesized that two main mechanisms occur: (1) diversion, where the soil pest is attracted to the associated plants and hence lured away from the principal crop, and (2) a changed feeding behavior, where the associated plants are preferentially chosen as a food source, i.e., the principal crop is less often consumed. Furthermore, we proposed a diversity effect where a diverse mix of associated plants provides a more attractive food source compared to a single non-crop species, and therefore has the better attraction and retaining effect. Finally, we expected a damage/yield effect where the pest-inflicted crop damage is significantly reduced by the presence of the associated plants.

MATERIALS AND METHODS

Plant diversification experiment

In 2009, a field experiment was established on a perennial grassland (Tyrol, Austria; 544 m above sea level; 11°38′41″ E, 47°18′50″ N [World Geodetic System, WGS 84]) that harbored high densities of *Agriotes*

larvae. The site was plowed on 9 April and maize was sown on 15 June (row spacing 75 cm, distance between plants within row ~15 cm). The experimental field was divided into 21 plots of 3 × 5 m; each plot comprised four maize rows and three inter-crop strips (Fig. 1). On each side of the experimental field, three rows of maize were left untreated, serving as a buffer zone.

Plots were assigned to three different cropping treatments, each replicated seven times: (1) maize monoculture (MONO), (2) maize associated with wheat (WHEAT, seed density 83 g/m²), and (3) maize combined with a diverse mix of associated plants (MIX). The latter included wheat (seed density 3 g/m²), scarlet runner beans (*Phaseolus coccineus* L.; 27 g/m²), narrow leaf lupine (*Lupinus angustifolius* L.; 67 g/m²), white mustard (*Sinapis alba* L.; 3 g/m²), buckwheat (*Fagopyrum esculentum* Moench; 3 g/m²), and perennial ryegrass (*Lolium perenne* L.; 3 g/m²). Seeds in the WHEAT and in the MIX treatment were sown in the inter-crop strips (~30 cm wide) on the same day as the maize. All plots were regularly hand-weeded to prevent the emergence of plants other than the sown ones. While the wheat naturally withered in September, the associated plants (notably the beans) in the MIX treatment had to be trampled down in mid-August to prevent them from overgrowing the maize.

Wireworm distribution and crop damage

Wireworm sampling took place on 5 and 6 August (“August session”) and from 23 to 28 September (“September session”). In August, four soil samples (20 cm diameter, 10 cm deep) were taken per plot, two from an inner maize row and two from the adjacent inter-crop strip (Fig. 1). In September, the sampling was modified by adding an extra sample per maize row and inter-crop strip and moving the actual sampling site to a different place within the plot. Soil samples were hand-searched for *Agriotes* larvae. The wireworms (mostly fifth to eighth instar larvae) were individually frozen in 1.5-mL reaction tubes upon collection and transferred to –80°C on the same day. A subsample ($n = 72$) of the collected larvae was molecularly identified (Staudacher et al. 2011a) and all of these specimens were *Agriotes obscurus* (L.).

Percentage plant cover of maize and the associated plant species in the diversified treatments was estimated per plot within a 1 × 1 m area (covering the soil sampling area) on 5 August and 23 September.

The status of maize plants was recorded prior to the wireworm sampling sessions. On 4 August, all maize plants in the third row of each plot were scored, based on their vitality: plants that were retarded in growth and/or showed withered leaves were classified as “damaged,” in contrast to “vital” plants that showed no signs of damage. Dead or completely missing maize plants formed a third group (“dead”). Numbers of vital, damaged, and dead maize plants per third row were recorded for each plot. On 22 September, the size of

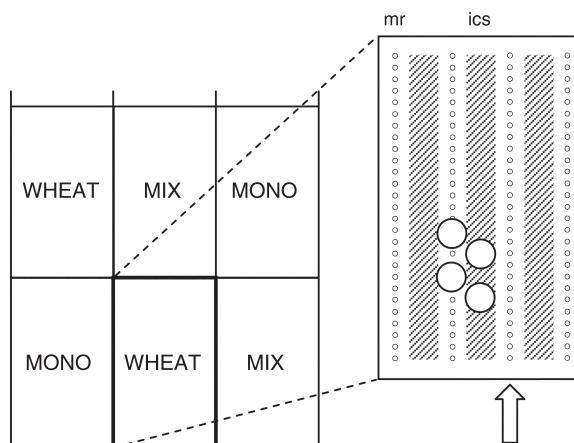


FIG. 1. Setup of the experiment. MONO (maize monoculture), WHEAT (maize associated with wheat), and MIX (maize combined with a mix of six associated species) indicate the treatments to which plots were assigned. Each plot (3 × 5 m) comprised four maize rows (mr) and three inter-crop strips (ics), where the associated plants were planted in the WHEAT and MIX treatments. Soil samples (circles) to collect *Agriotes* larvae were taken from maize rows as well as inter-crop strips (August sampling mode displayed only). The arrow indicates the third maize row, which was used to score the crop.

each maize plant in the third row of each plot was measured (± 5 cm). The total length accumulated by these maize plants per plot was used as a parameter for yield, since the maize was later to be used as forage for livestock (silage).

Percentages of wireworms in maize rows, as well as plant parameters, were tested for significant differences between the cropping treatments using Mann-Whitney *U* tests and one-way ANOVAs (post hoc: Bonferroni), respectively, implemented in PASW Statistics 18 (IBM, Armonk, New York, USA).

Consumption of maize and associated plants

Molecular gut content analysis.—At first, all reaction tubes containing frozen wireworms were checked for regurgitates; if present, the wireworms were transferred to fresh tubes, so that regurgitates and wireworms could be processed and tested separately (see Appendix A: Table A1). Prior to DNA extraction, wireworms were cleaned from any plant material sticking to their body surface by washing each larva in 1 mL of 1–1.5% sodium hypochlorite solution (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 s. Subsequently, the larva was rinsed twice with molecular-grade water (for details, see Wallinger et al. [2013]). Then, the head capsule and the ninth abdominal segment of each wireworm were cut off and retained frozen. The latter were subjected to stable isotope analysis (see *Stable isotope analysis*). The remaining middle part of the larva, including its intestinal tract, was homogenized with glass beads for 1 minute at 5000 rpm using a Precellys 24 Tissue Homogenizer (Bertin Technologies, Montigny-le-Bre-

tonneux, France). For DNA extraction, a CTAB-based protocol described in Juen and Traugott (2005) was employed with the following modifications to increase the DNA yield: the extraction buffer contained 430 μL TES-buffer (0.1 mol/L TRIS, 10 mmol/L EDTA, 2% SDS; pH 8), 10 μL Proteinase K (20 mg/mL), and a spatula tip (diameter 3 mm) of PVP (polyvinylpyrrolidone) and samples were incubated overnight at 58°C. In contrast to the larvae, regurgitates were immediately dissolved in extraction buffer and incubated for 2 h only. All extractions were carried out in a separate pre-PCR laboratory and two extraction negative controls were included in each batch of 30 samples to check for cross-sample contamination.

All wireworm/regurgitate samples were screened for plant DNA applying multiplex PCR assays described in Wallinger et al. (2012). Each sample was tested with a duplex PCR to detect DNA of wheat and maize (duplex "TZ"), while samples stemming from the MIX treatment were additionally tested with another duplex PCR for lupine and buckwheat (duplex "LF") and a multiplex PCR to detect DNA fragments of buckwheat, ryegrass, beans, and mustard (multiplex "FLPS"). Samples that did not amplify in a first run were re-tested in a second PCR. Several positive (highly diluted target DNA) and negative controls (PCR-grade water instead of DNA) were run within each PCR to check for amplification success and DNA carry-over contamination, respectively. Each 15 μL PCR contained 4 μL of DNA extract and 7.5 μL 2 \times TypeIt Mutation Detect PCR Kit (Qiagen, Hilden, Germany) plus extra MgCl_2 (Qiagen) to a final concentration of 4 mmol/L, 0.5 μg bovine serum albumin (BSA), 0.5 μL 5 \times Q-solution (Qiagen), 0.7 μL PCR-grade RNase-free water (Qiagen) and primers in their respective concentrations (see Wallinger et al. 2012). The thermocycling program was 95°C for 5 minutes, 40 cycles of 92°C for 20 s, 53°/54°/56°C (LF/TZ/FLPS) for 90 s, and 70°C for 90 s and finally 70°C for 5 min. PCR products were separated and visualized using QIAxcel (Qiagen; AL320 separation method) and scored with BioCalculator Fast Analysis Software version 3.0 (Qiagen). All samples with signal strength above 0.1 relative fluorescent units for the expected fragment length were deemed to be positive.

For further analysis, the PCR results of wireworms and their corresponding regurgitates were combined, i.e., individuals were counted positive if at least one of the samples (larva or regurgitate) was positive for a specific plant taxon. Likewise, samples that tested positive for buckwheat in both the duplex LF (amplifying a 206 bp fragment) and multiplex FLPS (380 bp) were counted only once.

The proportion of *Agriotes* larvae testing positive for a plant species was expressed as DNA detection rate; these rates were compared between plants using one-sample *t* tests combined with a bootstrapping procedure including 9999 resamples (Spotfire S+ 8.1 for Windows, TIBCO Spotfire, Somerville, Massachusetts, USA). The

tilting confidence interval was set at 95% and, accordingly, non-overlapping intervals indicate significant differences at $P < 0.05$. Additionally, DNA detection rate was linked with plant food supply to estimate selective feeding in the MIX treatment. Supply was derived from aboveground plant cover because it is difficult to obtain species-specific measurements of root biomass in the field. Still, all plant species within this experiment exhibit a medium degree of root penetration in the soil and they are similar in their root formation (Kutschera and Lichtenegger 1992), at least in the 0–15 cm soil layer. This is where 70% of the total root biomass (Bolinder et al. 1997) and the feeding-active wireworms are situated. Based on the relation between supply and demand (DNA detection rate) the Manly's alpha preference index (constant prey population [Manly 1974]) was calculated per plant species. An index value above or below the "1/*m* threshold" (here 0.14; *m*, number of available plant species) indicates a preference or avoidance, respectively.

Stable isotope analysis.—A subsample of the wireworms was subjected to carbon isotopic analysis at the Centre for Stable Isotope Research and Analysis (University of Goettingen, Goettingen, Germany) as described in Traugott et al. (2007) (Appendix A: Table A1). The natural difference in the $\delta^{13}\text{C}$ signatures between C_3 plants ($-28.05\text{‰} \pm 1.35\text{‰}$ [mean \pm SD], $n = 299$ plants) and C_4 plants ($-12.72\text{‰} \pm 1.07\text{‰}$, $n = 53$ plants; data from Traugott et al. [2008]) allows inferring the dietary carbon uptake of wireworms over longer time periods. All associated plant species used in the present experiment are C_3 plants, allowing a precise distinction from the principal crop, maize, which is a C_4 plant. An isotopic threshold, based on data derived from a previous feeding experiment, was used to make an individual assignment (Schallhart et al. 2011): *Agriotes* larvae with $\delta^{13}\text{C}$ signatures exceeding the threshold of -23.3‰ (i.e., $\delta^{13}\text{C}$, mean + 2SD, of C_3 -plant-fed larvae; $n = 35$ larvae) were classified as maize consumers, those with signatures below this threshold as feeding on the associated plants.

In addition, IsoError 1.04 (Phillips and Gregg 2001) was used to calculate the mean proportion of the diet attributable to maize along with its 95% confidence interval. Here, the dietary choice of larval populations (i.e., all larvae retrieved from a treatment) was compared between cropping treatments. Non-overlapping confidence intervals indicate significant differences at $P < 0.05$. As late instar *Agriotes* larvae do not completely equilibrate with the signature of a new diet after a diet switch (i.e., incomplete trophic shift [Traugott et al. 2007]), the mean maize $\delta^{13}\text{C}$ signature (-12.72‰) was corrected by 1–2‰ for the current analysis.

IsoError calculations display the proportion of maize diet of the whole wireworm population. The threshold-based assignment of individuals, however, identifies those specimens that consumed maize and provides a

proxy for their individual maize consumption, which will be higher than the proportion of maize estimated by IsoError for the whole population. Therefore, the proportion of maize in the maize-feeders' diet was recalculated taking the percentage of non-maize-feeders into account.

RESULTS

Wireworm distribution

In total, 454 *Agriotes* larvae were collected (167 August, 287 September; Appendix B: Table B1). The proportion of wireworms collected in maize rows varied significantly between the three cropping treatments (Fig. 2). In August, the majority of the larvae in the MONO treatment was found in the maize rows ($95.2\% \pm 12.6\%$ [mean \pm SD]) compared to the WHEAT ($21.2\% \pm 14.3\%$) and MIX ($28.0\% \pm 18.7\%$) treatment. In September, all larvae retrieved from the MONO treatment were collected in maize rows. In the WHEAT and in the MIX treatments, $81.8\% \pm 14.9\%$ and $49.3\% \pm 27.2\%$ of the wireworms were collected in the maize rows, respectively. At this sampling date, the percentage of wireworms in maize rows was significantly higher in the WHEAT than in the MIX treatment ($U = 4.500$, $P = 0.007$).

Consumption of maize and associated plants

In August, plant DNA detection rates differed considerably between treatments (Table A1): in the MONO treatment, 10.3% of the wireworms tested positive for maize DNA; in the WHEAT treatment, significantly more larvae tested positive for wheat (19%) compared to maize (3.8%). In the MIX treatment, no larva tested positive for maize but 42.4% of the wireworms contained DNA of at least one of the associated plants in their gut content/regurgitate (Fig. 3A). Out of the six plants offered in the plant mix, buckwheat (22%) and wheat (10.2%) were detected most frequently. In accordance with this result, Manly's alpha index values for August showed that these two plant species were preferred by the *Agriotes* larvae (Fig. 3B).

In September, the maize DNA detection rate in the MONO treatment was 17.3%. In the WHEAT treatment, a significantly higher proportion of wireworms contained DNA of maize (20.7%) than of wheat (5.4%). In the MIX treatment, similar proportions of wireworms tested positive for maize (17.6%) and associated plants (19.8%; Fig. 3A). For this time point, Manly's alpha index values showed a dietary preference for beans besides wheat and maize (Fig. 3B).

The isotopic signatures of wireworms collected in August in the WHEAT and MIX treatments indicated that 34.5% and 18.5% of the larvae had consumed maize, respectively. In the MONO treatment, however, 71% of the larvae were assigned to a maize diet (Fig. 4A; Table A1). This was mirrored at the population level (IsoError calculation): we found a significantly higher proportion of the total populations' diet attributable to

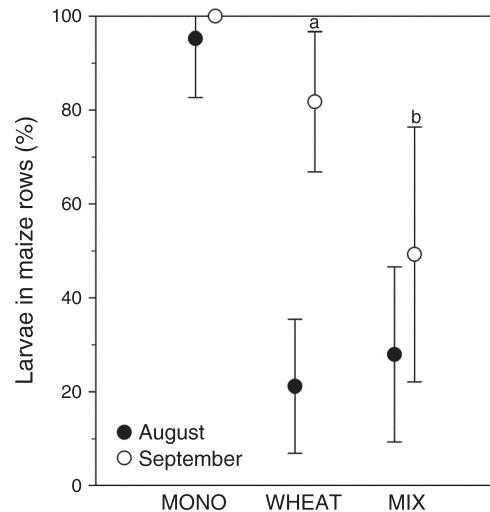


FIG. 2. Percentage (mean \pm SD) of *Agriotes* larvae collected in maize rows in the three different cropping treatments in August and September 2009. Different letters above error bars indicate significantly different percentages between WHEAT and MIX treatments at $P = 0.007$.

maize in the monoculture (54.5%) than in the vegetationally diversified treatments (WHEAT 23.4%; MIX 7.7%; Fig. 4B). Hence, after recalculation, the proportion of maize in the diet of larvae that proved to have fed on maize was 77%, 68%, and 44% in the MONO, WHEAT, and MIX treatment, respectively.

In September, 89.5% of the larvae collected in the MONO treatment were classified as maize feeders. Note, however, that individuals not exceeding the threshold in the maize monoculture most likely were feeding-inactive individuals, as in this treatment no plant species other than maize were available. In the vegetationally diversified treatments, fewer larvae were assigned to a maize diet (WHEAT 81%; MIX 51%; Fig. 4A). Similarly, the proportion of the diet attributable to maize was significantly higher in the MONO (74.1%) than in the WHEAT (56%) treatment; in the latter, the proportion of maize diet, again, was significantly higher than in the MIX treatment (34.8%; Fig. 4B). Consequently, for the maize-feeding larvae only, 82%, 69%, and 69% of the diet was attributable to maize in the MONO, WHEAT, and MIX treatments, respectively.

Crop damage

In August, in the MONO treatment, significantly more damaged and dead maize plants (55.4%) were recorded than in the two treatments in which the plant diversity was increased (WHEAT 33.8%; MIX 24.7%). In the MONO treatment, the number of vital maize plants per row was 14.7 ± 3 (mean \pm SD), thus significantly lower than in the vegetationally diversified treatments (WHEAT 21.9 ± 2.2 ; MIX 24.9 ± 2.3 ; for both comparisons $F_{2,18} = 30.235$, $P < 0.001$).

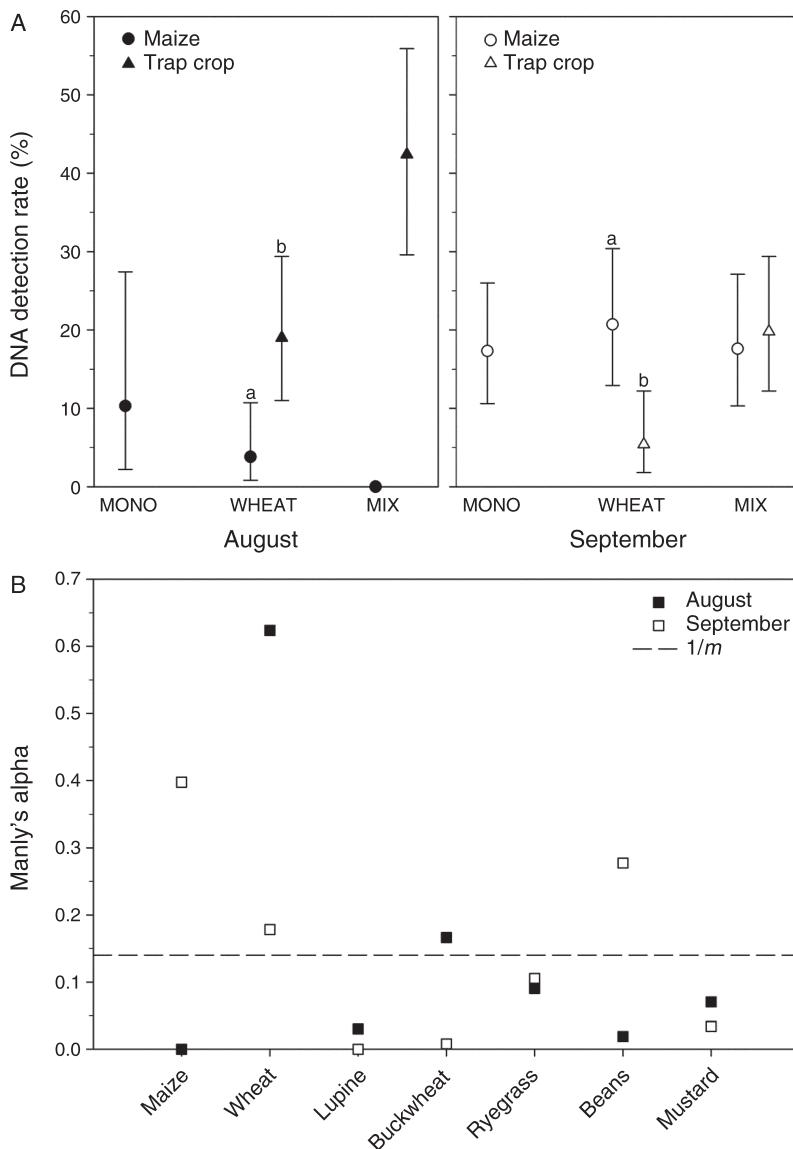


FIG. 3. Molecular gut content analysis of *Agriotes* larvae collected in the three different cropping treatments in August and September 2009. (A) Plant DNA detection rate and 95% tilting confidence interval; different letters above error bars indicate significantly different detection rates within a treatment at $P < 0.05$. (B) Manly's alpha preference index values for the different plants in the MIX treatment. The dashed line displays the "1/m threshold", where m is the number of available plant species. Values exceeding this threshold indicate a preference; those below it indicate an avoidance for the respective plant species.

The proportion of vital plants in the MIX treatment was 75.3%, which fits to the significantly lowest number of damaged plants (1.6 ± 2.1) found there ($F_{2,18} = 21.340$; MIX vs. MONO, $P < 0.001$; MIX vs. WHEAT, $P = 0.049$). Likewise, the number of damaged plants was significantly lower in the WHEAT vs. the MONO treatment ($P = 0.004$; Fig. 5).

As a yield-related parameter, the total length of third-row maize plants was compared between treatments in late September prior to harvest: the vegetationally diversified treatments had a significant higher yield than the maize monoculture ($F_{2,18} = 10.894$). The total length (mean \pm SD) was significantly higher in the WHEAT

(45 ± 2.4 m) compared to the MONO treatment (34.7 ± 8.1 m; $P = 0.008$) and likewise, in the MIX (47.8 ± 4.5 m) compared to the MONO treatment ($P = 0.001$).

DISCUSSION

Our findings demonstrate that non-crop plant diversity provides an effective insurance against wireworms and increases crop yield. Most importantly, our data elucidate a behavioral mechanism for the observed pattern: the pest moves away from the principal crop and accumulates in the associated plants. The importance of this mechanism has also been identified in other experiments that aimed at luring wireworms away from

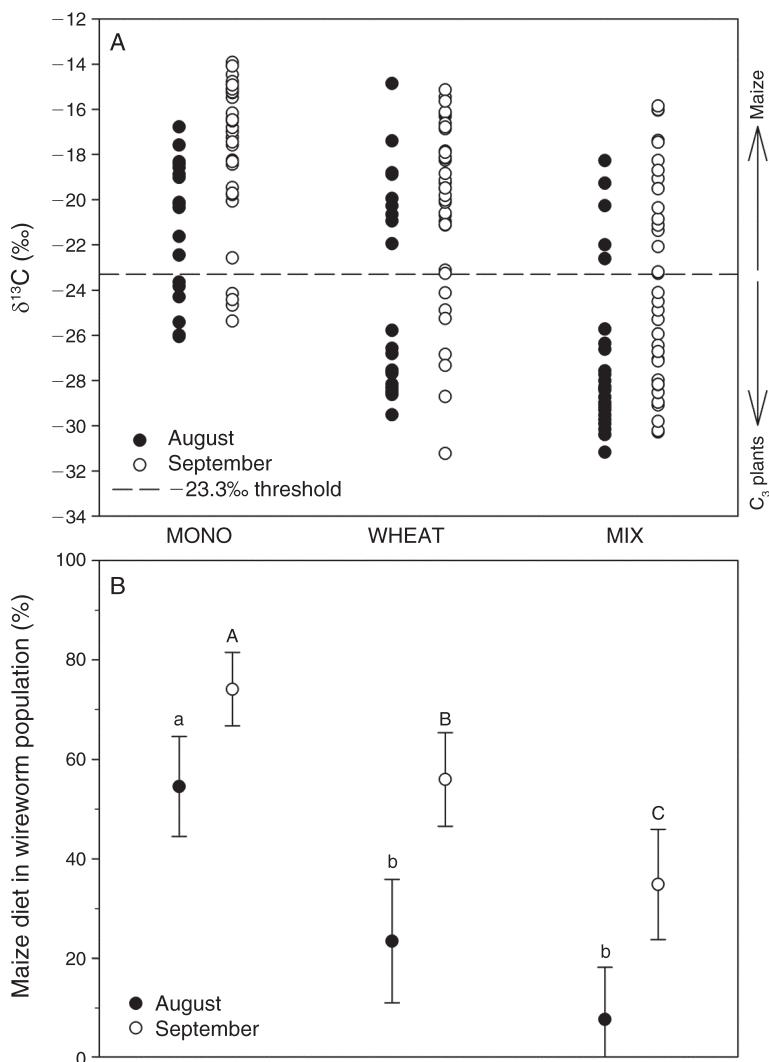


FIG. 4. The $\delta^{13}\text{C}$ signatures of *Agriotes* larvae collected in the three different cropping treatments in August and September 2009. (A) Individual $\delta^{13}\text{C}$ signatures of all wireworms analyzed (each circle represents the signature of one larva); the threshold of -23.3‰ separates wireworms feeding on maize (upper part of panel) from those consuming associated plants (lower part of panel). (B) Percentage of maize diet within the wireworm population (all plots of a treatment; mean and 95% CI); different letters above error bars indicate significantly different means between treatments within one sampling session at $P < 0.05$. The $\delta^{13}\text{C}$ signature of maize was corrected for the incomplete trophic shift by 1‰ and 2‰ in September and August, respectively.

the crops (Miles and Petherbridge 1927, Vernon et al. 2000, Vernon 2005, Landl and Glauning 2011). Beyond that, the combination of molecular gut content and stable isotope analysis revealed that plant diversification caused a clear switch from feeding on maize to the associated plants, diminishing the feeding pressure on the principal crop. In accordance with previous mesocosm experiments where the food choice of *Agriotes* larvae was assessed (Schallhart et al. 2012), our study showed that wireworms actively choose between food plants when several plant species are available within their foraging range. The current results can thus be best explained by the trap crop hypothesis

(Vandermeer 1989), which predicts that the pest is attracted to the associated plants.

Our data do not support hypotheses that propose repellent properties of associated plants (Uvah and Coaker 1984), resource concentration, or natural enemies (Root 1973) as mechanisms for lower pest damage in mixed cropping compared to monoculture. However, the associated plants might have masked (Tahvanainen and Root 1972) or altered (Finch and Collier 2000) the odor of the maize roots and thereby reduced the appearance of the crop to the pests. Although it is unlikely that these mechanisms influenced our experiment, we cannot completely preclude it. Soil-

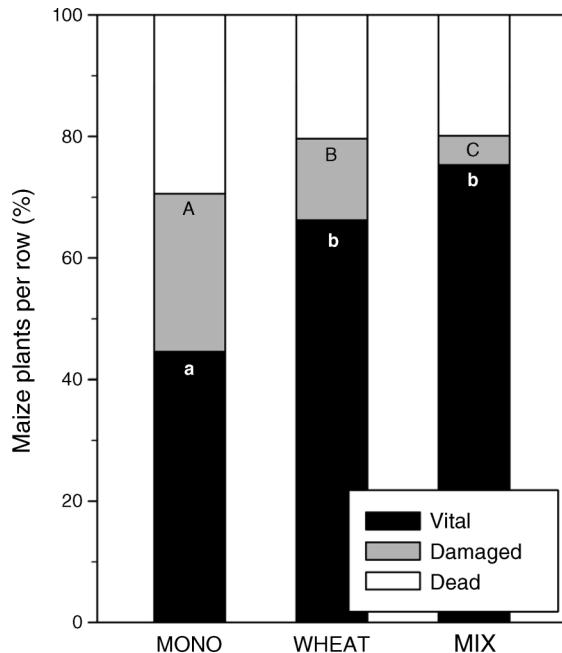


FIG. 5. Percentage of maize plants per row scored seven weeks after the maize was sown. Different letters indicate statistically different means for vital/damaged plants between the three different cropping treatments (vital plants, MONO vs. vegetationally diversified treatments [$P < 0.001$]; damaged plants, MONO vs. WHEAT [$P = 0.004$], MONO vs. MIX [$P < 0.001$], WHEAT vs. MIX [$P = 0.049$]).

dwelling herbivores such as wireworms are attracted by CO_2 released by growing plants (Doane et al. 1975), albeit roots are known to exude a range of other chemicals that attract or repel subterranean herbivores (Johnson and Nielsen 2012). Vernon (2005) showed that wireworm attraction to trap crops is positively correlated to plant density, which might be due to an increased concentration of root exudates and CO_2 .

The mix of associated plants provided a better attraction and retaining effect than the single non-crop plant, which corroborates the multiple trap cropping hypothesis (Shelton and Badenes-Perez 2006). By adding multiple associated plants, season-wide crop protection was achieved, both when maize plants were small and before the harvest of the principal crop. In the single trap crop treatment, on the contrary, seasonal changes in the attractiveness of the associated plant were clearly visible. Here, the wheat naturally withered in September and no longer offered an attractive food source for the wireworms. A late-season remigration from the wheat to the maize as well as an increased maize consumption was observed. The plant mix, however, included species that were attractive as food source at different times of the season causing an extended attraction effect. For example, in August, wheat and buckwheat were highly preferred, whereas in September, beans were favored as a food source. While wireworms preferred specific plant species in the plant

mix, others were rarely consumed. Thus the choice of mix is likely to be important. Schallhart and colleagues (2012) found that the preference and avoidance of wireworms for specific plants was independent of root abundance and plant diversity, although the extent of avoidance and preference for specific plants changed with increasing plant diversity. Altogether diversity, identity and density of associated plants as well as seasonal changes in the plant's attractiveness influence the success of the plant diversification for managing soil pests.

Earlier work on the use of wheat as an inter-crop to reduce wireworm damage on strawberry plants showed strongly reduced levels of crop plant mortality, albeit the wheat had to be planted one week in advance of the main crop to achieve a high level of crop protection (Vernon et al. 2000). In our plant diversification scheme successful crop protection could be achieved when both the principal crop and the associated plants were planted at the same time, which provides a practical advantage.

Molecular gut content analysis represents a powerful tool to determine the herbivores' dietary choice under field conditions and to examine how feeding behavior changes according to plant identity and diversity. Ingested plant material can be detected for at least three days post-feeding, independent of consumer mass and meal size (Staudacher et al. 2011b). The PCR assays employed here are highly specific and sensitive (Wallinger et al. 2012). Note, however, that plant identity can affect post-feeding DNA detection success (Wallinger et al. 2013), which indicates that the current plant DNA detection rates cannot be directly transferred into absolute root consumption rates. Supplementary feeding experiments, where plants are individually labeled with a tracer such as ^{15}N (Schallhart et al. 2012), would be necessary to establish absolute consumption rates. However, these experiments are time consuming and technically challenging, both of which affects their applicability in field experiments. Therefore, the detailed trophic information gathered by molecular gut content analysis provides a good starting point to develop pest-specific plant mixes for an optimized bottom-up control of root feeding insects.

Combined with stable isotope analysis, the molecular data also allowed narrowing down the period when the wireworms were feeding on maize. The low percentages (<11%) of larvae that contained maize DNA in August indicate that the wireworm population was not heavily feeding on the crop roots within the last few days before sampling. However, the larval $\delta^{13}\text{C}$ signatures show that the insects were feeding on maize during the initial period of the crop development (18–71% of the specimens), i.e., the larvae were feeding on germinating and small maize plants within the first few weeks of the experiment.

The mix of associated plants might also have offered a suitable rhizosphere environment for the wireworms, similar to grassland, the prime habitat of *Agriotes* larvae

(Parker and Howard 2001, Blackshaw and Kerry 2008). After the dense root felt has established (especially compared to maize monoculture), apart from food supply, habitat requirements such as soil moisture were suitable up to late September, making larvae stay in the associated plant mix. Results of a previous study, showing that *Agriotes* larvae rarely disperse between crops as long as the local food supply and other habitat parameters are sufficient (Schallhart et al. 2011), support this idea.

So far, it has been noted that the effects of trap crops on production remain unclear (Poveda et al. 2008). Moreover, no trap crop studies providing yield data were available for inclusion in the meta-analysis of Letourneau et al. (2011) on plant diversification effects on agroecosystems. Our study now fills this gap and provides a first proof that trap cropping prevents yield loss: compared to the maize monoculture, the maize yield was increased by 30% and 38% in the single and multiple trap crop treatments, respectively.

One of the major concerns with increased plant diversity is that the presence of associated plants could increase the resource base for the pests, e.g., by providing alternative food or oviposition sites. Another concern is the competition of the associated plants with the principal crop for water, light, and nutrients, which can retard crop growth and diminish yields (Ratnadass et al. 2012). Although our results clearly show that the latter was not the case in the diversified cropping system, the higher plant diversity increased the available plant food sources of the wireworms and might have attracted females to oviposit. However, *Agriotes* larvae are not food limited in arable fields as usually there are enough plant roots available to these generalist herbivores (Schallhart et al. 2011). Therefore it is unlikely that the introduced non-crop plants increased the larvae's survival. As *Agriotes* females prefer dense grass-clover vegetation as oviposition site (Tischler 1965), it is also unlikely that the current plant diversification schemes increased the egg load in the field and transformed the associated plants from a sink to a source of these soil pests.

Plant diversification alone does not necessarily diminish pest population levels, especially when the pests are attracted to the associated plants. Lethal trap cropping, i.e., insecticidal-assisted trap cropping, combines diverting the pests away from the crop with reducing pest numbers (Muthiah 2003, Dogramaci et al. 2004, Lu et al. 2009) and has also been successfully applied to control *Agriotes* larvae (Vernon 2005). As observed in the present study in plots with the single trap crop wheat, this strategy would prevent late-season remigration of larvae from the associated plants to the principal crop. The effects of the plant mix treatment found here suggest that the combination with insecticidal control would create a prolonged attract-and-kill effect. In the context of sustainable intensification, lethal trap crop-

ping minimizes the amount of soil-applied insecticides and also the area of application.

Regional differences in abiotic and biotic factors can determine the effectiveness of plant diversification for controlling pests (Bender et al. 1999). However, these differences might not be as critical for subterranean pests because the soil environment is more buffered against abiotic extremes. In fact, plant diversification approaches may be well suited for controlling soil-dwelling pests. Root feeders are more restricted in their spatial dispersal abilities (Villani and Wright 1990, Schallhart et al. 2011), which allows us to effectively manipulate their behavior by increasing the plant diversity in the field. Moreover, implementing multiple trap cropping can provide an insurance against unpredictable weather conditions if associated plant species are combined that differ in their abiotic requirements.

The wireworm population at the current study site was dominated by *A. obscurus*, which is widespread in Europe and northern America (Furlan and Tóth 2007, Noronha et al. 2008, Ellis et al. 2009). However, there are at least nine other *Agriotes* species that are of agricultural importance (Staudacher et al. 2013). These species have been shown to differ in their environmental needs and distribution (e.g., Staudacher et al. 2013, Benefer et al. 2012). Hence, it is possible that they are also different in their dietary preferences, which would be of interest for the plant diversification scheme developed here.

Prior to adoption of plant diversification schemes, the performance needs to be tested within the crop cultivation practice as exemplified in our study. Preliminary lab-based trials alone, albeit often showing promising results (Badenes-Perez et al. 2004, Cook et al. 2007, George et al. 2009), might provide contradictory outcomes when transferred to a real-field scenario (see Charleston and Kfir 2000). Moreover, in laboratory and mesocosm experiments, often extremely high infestation rates have been simulated that far exceed the herbivory levels under natural conditions, giving rise to misleading conclusions (Zvereva and Kozlov 2012). On the contrary, our findings demonstrate that the deployment of a diverse mix of associated plants, specifically tailored to the preferences of the focal soil herbivore, is a promising strategy for managing subterranean pests while maintaining crop yield.

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SUPPLEMENTAL MATERIAL

Appendix A

Screening results (molecular gut content and stable isotope analysis) of *Agriotes* larvae collected in the three different cropping treatments in August and September 2009 (*Ecological Archives* A023-060-A1).

Appendix B

Densities of *Agriotes* larvae in maize rows and inter-crop strips in the three different cropping treatments in August and September 2009 (*Ecological Archives* A023-060-A2).