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Semi field trials to evaluate undersowings in maize for management of western corn rootworm larvae

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Abstract

Western corn rootworm larvae (*Diabrotica virgifera virgifera* LeConte) need to feed on maize roots after hatching from overwintering eggs. It was hypothesized that the roots of undersown plants mixed with maize roots disrupt the host finding of the larvae, lowering their survival and subsequently reducing larval densities. Six undersowings (perennial rye grass, Italian ryegrass, a mixture of Italian ryegrass and white clover, white clover, yellow mustard and sunflower) were tested with a standard maize cultivar under semi field conditions. The larval density per plant was determined by extracting the larvae from the root core of the maize plants with a Kempson extraction system at the end of larval development. Contrary to the hypothesis only sunflower caused a significant reduction in larval densities, whereas white clover as an undersowing resulted in a significantly higher larval density than in the control. In conclusion, undersowings generally do not provide an alternative control measure against western corn rootworm larvae. Sunflowers mixed with maize plants indicate a promising option as an additional control measure, but would have to be tested under field conditions to confirm its potential for western corn rootworm management.

Key words: *Diabrotica virgifera virgifera*, cultural control, undersowing, maize, semi field.

Introduction

The Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera Chrysomelidae) is a serious invasive root feeding pest of maize, *Zea mays* in Europe (Ciosi *et al*., 2008). In North America maize production losses and costs for WCR management and control result in more than 1 billion dollars per year (Spencer *et al*., 2009). Independent introductions from North America to Europe (Ciosi *et al*., 2008) resulted in a spread into more than 20 European countries after the first beetles were detected near Belgrade, Serbia in 1992 (EPPO, 2016). WCR is a univoltine species, the eggs overwinter in the soil and the larvae hatch in spring (Krysan, 1986). The three larval instars feed upon the roots during a 3 week period, causing a disruption of water and nutrient uptake (Urias-Lopez *et al*., 2000) and plant lodging at higher larval densities (Spike and Tollefson, 1991). This makes the larvae the most damaging life stage of the beetle (Meinke *et al*., 2009).

In North America the most widely used management option is the use of transgenic cultivars with granular soil insecticides (Huang *et al*., 2011) whereas in Europe rotation of maize to non-maize crops and soil insecticides or seed treatments (Van Rozen and Ester, 2010) have initially been applied. However, recent serious non-target effects of insecticidal seed treatments on bees (Girolami *et al*., 2012; Pistorius *et al*., 2015) further enhanced the interest in previously reported biological control options using entomopathogenic nematodes (Toepfer *et al*., 2010) and cultural control such as crop rotation (Gray *et al*., 2009). The latter is regarded the most effective management strategy, despite the development of rotation tolerant corn rootworm strains in the USA (Miller *et al*., 2009). Crop rotation has a positive agronomic and environmental impact (Vasileiadis *et al*., 2013), but may be difficult to adopt in certain European regions due to specialised cropping systems (Meissle *et al*., 2010). Therefore the evaluation of further cultural control methods is needed to increase management options for farmers.

One potential option is the use of undersowings, a widely used technique that has been proven to reduce plant damage but tends to vary in its effectiveness (Trenbath, 1993). It involves the cultivation of two or more crops on the same field at the same time (Lithourgidis *et al*., 2011), where spatial and temporal arrangement of the undersowings can differ (Capinera *et al*., 1985). In undersowings a major mechanism contributing to lower plant damage is the direct interference in the activity of a pest with olfactory masking (den Belder *et al*., 1999). According to the food source concentration hypothesis (Root, 1973) plant species can have a direct effect on a herbivore to find its host by masking the host finding stimuli of the herbivore or by deterring the pest (Uvah and Coaker, 1984). This can be especially effective against attacking organism with a narrow host range (Trenbath, 1993) such as the WCR larvae (Moeser and Hibbard, 2005). Therefore this study aimed at investigating the use of undersowings as an alternative cultural control option against this invasive pest.

Materials and methods

The undersowings were tested in a series of 6 experiments in microhabitat containers (120 × 80 × 60 cm) simulating a 1 m^2 portions of a maize field (experiments 2 and 3). A 5-mm plastic sheet (PVC CAW, Germany) fitted to the container dimensions was fixed into each container to create a 0.5 m^2 plot size (experiments 1, 4-6). This enabled an easier handling by reducing the soil volume in each experiment. Haplic luvisol was taken from an arable land near Göttingen (51°29'52.88N 9°55'38.26E) for experiments 1-4 and in Göttingen

(51°31'16.21N 9°57'49.30E) for experiments 5 and 6 and homogenized using a soil shredder (Unifix 300, Moeschle, Ortenberg, Germany). Both locations were used as permanent grassland in previous years. Prior to each experiment the soil was passed through a 1 cm mesh sieve to create an even soil structure for all samples and to remove unwanted root material and gravel. For each experiment the soil was mixed with peat soil (Fruhstorfer Erde, Typ P, Hawita Gruppe GmbH, Vechta, Germany) in a ratio of 1:1 and filled up to 40 cm in each container. The containers were randomly distributed in the greenhouse.

Cultivars

Maize (cultivar: Ronaldhino, KWS, Einbeck, Germany) was grown in plastic trays at 23 ± 1 °C and transplanted to the containers 7 days after sowing (BBCH 11) (Lancashire *et al*., 1991). Two maize rows were set up with a 60 cm row spacing with each row consisting of 3 in a 0.5 m^2 plot or 7 plants in a 1 m^2 plot each 13-15 cm apart. The plants were watered daily and fertilised once a week with a 2% Hakaphos® Blau solution (Water soluble NPK compound fertilizer, Compo, Münster, Germany).

Six different types of undersowings were tested at sowing rates equivalent to the field (table 1): perennial ryegrass (*Lolium perenne*) (cultivar: Trend., Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Holtsee, Germany); Italian ryegrass (*Lolium multiflorum)* (cultivar: Gisel; Saaten-Union, Hannover, Germany); Italian ryegrass with white clover (*Trifolium repens)* (cultivar mixture: Gras Mineral, Saaten-Union, Hannover); white clover (cultivar: Vysocan); white mustard (*Sinapis alba*) (cultivar: Maxi, Saatzucht Lundsgaard purchased via Saaten Union, Hannover, Germany); sunflower (*Helianthus annuus*) (cultivar: Alisson, Euralis Saaten GmbH, Norderstedt, Germany; seeds were coated with fungicides Celest XL (a.i.: Fludioxonil; 125 ml/ 100 kg seeds) and Apron XL (a.i. Mefenoxan; 282 ml/ 100kg seeds). Grasses and clover were selected as they are commonly used as an undersowing in maize (DSV, 2016) and make a practical application more feasible, whereas mustard and sunflower were selected as additional experimental treatments. Undersowings with *Lolium* spp. only (experiment 1 and 2) was done by sowing the seeds in three rows (between row spacing: 15 cm; row width: 1 cm) in 0.5 cm depth. For the undersowing with sunflowers (experiment 6), the plants were sown in one row 30 cm from the maize row (= half way between the maize rows). In undersowings with white clover (experiment 3 and 4) and mustard (experiment 5), the seeds were sown across the whole plot (table 1). The seeds were spread on the soil surface with a 2 mm sieve and covered with a 1 cm soil layer to avoid desiccation and to ensure that seeds could not be washed off their application area during watering. For sowing across the 1 m^2 plot in experiment 3, the plot was divided into 4 equal sized subplots (30 × 80 cm) to have a more homogenous distribution of the seeds.

Western corn rootworm eggs

WCR eggs from a non - diapausing strain were obtained from the USDA-ARS, North Central Agricultural Research Laboratory, Brookings, North Dakota, USA. This laboratory strain does not show a significant performance difference compared with the wild type strains (Hibbard *et al*., 1999). The eggs were stored in Petri dishes at 8 °C. Hatch tests with egg samples were carried out at 25 °C and 65% relative humidity (RH) and showed first egg hatching after 13 days. About two days prior hatching (day 11 of incubation) eggs were washed from the soil matrix in which they were held with a 250 µm sieve and mixed in a 0.15% agar solution until they were evenly distributed. The egg concentration was determined by counting the number of eggs in 10 µl subsamples. Agar-water-solution was added until a concentration of 100 eggs in a 200 µl agar solution was reached.

The eggs were applied at a soil depth of 7 cm with an Eppendorf pipette. The eggs were either applied in random or uniform distribution across a semi field plot (table 1). For the random distribution a mesh consisting of 40 grids (14 × 16 cm each) was carefully placed on the soil. Each grid was given a number; these numbers were written on a card and a card randomly drawn to determine the point of inoculation. The number of inoculation points in a semi field plot equalled the number of plants in the plot. With a uniform distribution, the eggs were applied 30 cm from each plant halfway between the maize rows. For both inoculation types 120 eggs/ plant were inoculated. At this egg density intraspecific competition can be minimised (Weiss *et al*., 1985). Hatching time and rate were measured by applying 30 eggs on wet filter paper in Petri dishes and placing them in pots with soil near the containers. The larvae started to hatch 2-4 days post inoculation in all experiments.

Due to quarantine regulations in Germany, experiments had to be terminated after a maximum of 21 days after the first larval hatch to avoid adult emergence. A soil cube (ca. 15 × 10 × 10 cm) below the maize stalk was cut out and the larvae were extracted from the sampled soil cubes with a high gradient Kempson extraction system (Kempson *et al*., 1963). In this system the soil cubes were transferred to a box with netting at the bottom (mesh size 0.7 cm) and placed above water - filled containers. Red light bulbs placed over the soil created a heat gradient that forced the larvae to move downwards and to fall into the water. The number of larvae in a soil cube was recorded to determine larval density per plant.

Statistical analysis

The number of extracted larvae per plant in the control and an undersowing treatment were tested using a parametric pairwise comparison t - test. In case normality and homogeneity of variances was not met, a non-parametric Mann Whitney U test was used. The mean efficacy of each undersowing was calculated as the reduction in larval density relative to the untreated control [corrected efficacy % = 100 − (larval density in treated plots × 100 / larval density in the control)] and tested with a Kruskal Wallis test followed by post-hoc comparisons of mean ranks of all pairs of groups. All statistical analyses were performed with Statistica (Version 10, StatSoft, Tulsa, OK, USA).

Table 1. Experimental parameters for each undersowing treatment.

Experiment	Undersowing	Area (m^2)	Number of plants/row	Undersowing application		WCR egg inoculation				Number of replicates	
				Sowing pattern and rate	BBCH of maize	Egg application	BBCH of maize	Egg number/ plant	Days of larval development	Control	Undersowing
1	Perennial ryegrass (*Lolium perenne)*	0.5	3	Three rows every 15 cm between maize rows (15 kg/ha)	11	30 cm from each maize row	32/33	120	20	3	4
2	Italian ryegrass (*Lolium multiflorum*)	1	7	Three rows every 15 cm between maize rows (15 kg/ha)	13	Random	32/33	120	17	2	6
3	Italian ryegrass/ white clover (*Trifolium repens/ Lolium perenne*)	1	7	Whole plot (5 kg/ha 5 kg/ha)	13	Random	32/33	120	20	3	5
4	White clover (*Trifolium repens*)	0.5	3	Whole plot (10 kg/ha)	11	30 cm from each maize row	32/33	120	14	4	6
5	White mustard (*Sinapis alba*)	0.5	3	Whole plot (20 kg/ha)	12	30 cm from each maize row	32/33	120	20	4	6
6	Sunflower (*Helianthus annuus*)	0.5	3	One row 30 cm from each maize row (8 seeds/m^2)	1 week prior to sowing of maize	30 cm from each maize row	32/33	120	17	6	10

Table 2. Larval density per plant after 14-21 days of development in the root core of maize plants in control and different types of undersowing plots (±SD) (Pairwise comparison between larval density in control and undersowing plot with t-test or alternatively Mann Whitney U test, when normality and homogeneity of variances was not given; numbers in bold indicate significant differences at $P < 0.05$).

Type of undersowing	Control	Undersowing
Perennial ryegrass *(Lolium perenne)*	2.86 ± 1.03	3.11 ± 1.92
Italian ryegrass (*Lolium multiflorum*)	26.07 ± 0.20	26.15 ± 9.71
Italian ryegrass/white clover (*Lolium perenne / Trifolium repens*)	20.95 ± 2.72	26.51 ± 2.54
White clover (*Trifolium repens)*	**4.75 ± 2.67**	**11.78 ± 3.14**
White mustard (*Sinapis alba*)	6.33 ± 2.56	4.28 ± 1.99
Sunflower (*Helianthus annuus*)	**5.34 ± 3.60**	**2.42 ± 1.27**

Results

Influence of undersowings on larval density

Undersowings with ryegrass had no influence on larval density (Italian ryegrass: M.-W. U test: U = 4.00; P = 0.62; Perennial ryegrass: M.-W. U test: U = 1.00; P = 1.00) (table 2). When white clover only was used as an undersowing, larval density/plant significantly increased (M.-W. U test: U = 0.00; P < 0.05), whereas a mixture of white clover and Italian ryegrass showed no significant increase in larval density (M.-W. U test: U = 1.00; P = 0.07). The use of sunflower or white mustard reduced larval densities; a significant reduction was only found in an undersowing with sunflower (sunflower: t - test: 2.56; P < 0.05; white mustard: M.-W. U test: U = 6.00; P = 0.24).

Corrected efficacy of undersowing expressed as the reduction of larval density

The efficacy for the reduction of larval densities significantly differed between the tested undersowings (Kruskal-Wallis-Test: $H_{5,37}$ = 24.96; P < 0.0001). The use of sunflower and mustard reduced the number of WCR larvae by 54% and 32% compared to the untreated control, respectively. The application of clover alone or in combination with ryegrass enhanced larval density by 26.55% and 147.95%, respectively. Perennial and Italian ryegrass reached a control efficacy of −8.75% and −0.32%, respectively (figure 1).

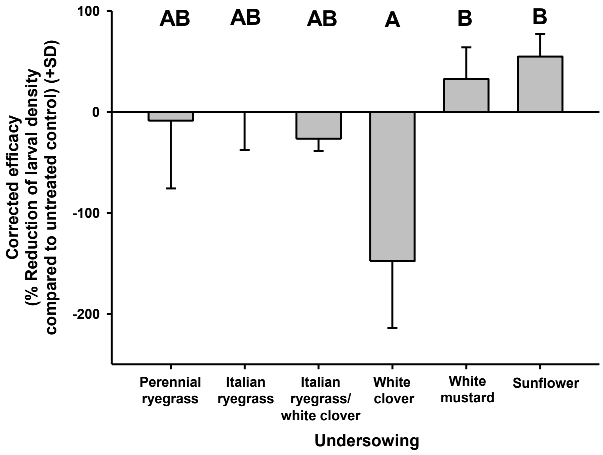

Figure 1. Mean percent reduction of Western corn rootworm larval density in semi field plots. Roots were infested with 120 eggs per plant. Larval density was assessed in the root core 14 - 21 days after first larval hatch. Error bars = SD; letters above bars indicate significant differences after post-hoc comparisons of mean ranks of all pairs of groups (P < 0.05)

Discussion and conclusion

The selection and spatial arrangement of an undersowing play a vital role in the reduction of WCR larvae. The use of grass species (both *Lolium* spp.; table 1) had no effect on larval densities, demonstrating that a diversified root system does not interfere with host location of WCR larvae. This supports the important role of specific orientation cues, next to the CO_2 (Bernklau and Bjostad, 1998), to discriminate host from non-host roots (Bernklau *et al*., 2009). The higher root biomass makes maize more competitive as it creates a spatial advantage over grass roots and makes it more likely for a WCR larva to find a maize root. Grass roots may have also provided additional food resources as WCR larvae can feed on a wider range of monocot host plants other than maize (Branson and Ortman, 1970) including species from the Poaceae family (Breitenbach *et al*., 2005, Moeser and Vidal, 2004). The nutritional value is expected to be lower, but the additional food resources might help to overcome starvation shortly after larval hatch as larval feeding starts close to their point of hatch due to their limited mobility (Bergman *et al*., 1983, Schumann and Vidal, 2012). Larval feeding on grass roots may have therefore reduced root damage, a factor not measured in this study and a potential parameter for future studies.

White clover only or as a mix with ryegrass favored larval survival (table 2). This could be primarily due to the changes in the microclimate of the soil, an effect often observed in cover crops (Zibilske and Makus, 2009). The spread of clover seeds across the whole plot resulted in a dense vegetation cover, potentially increasing the moisture content of the soil by lowering water evaporation from the soil. Neonate larval survival may have been increased as they require adequate levels of soil moisture for survival (Gaylor and Frankie, 1979). Furthermore, berseem clover (*Trifolium alexandrium*) as an intercrop in maize reduces the neutral detergent fiber and acid detergent fiber content in maize roots (Javanmard *et al*., 2009). Changes in plant physiology through clover roots exudates may have therefore improved host plant quality and enhanced WCR larval development.

White mustard and sunflower did result in a reduced larval density, whereas only the use of sunflowers caused a significant reduction. Sunflower as an intercrop has already been proven successful in the reduction of above ground pests, such as diamond back moth in cauliflower (Muthukumar and Sharma, 2009) and thrips in French beans (Nyasani *et al*., 2012), but not yet for a below ground pest. Sunflower roots release substances with known anti-herbivory properties, such as sesquiterpene dehydrocostus lactones (Joel *et al*., 2011, Padilla-Gonzalez *et al*., 2016) into the rhizosphere, which could be deterrent for WCR larvae. Root tissue of mustard and other *Brassicaceae* plants are also associated with biocidal substances (Furlan *et al*., 2010) such as sulphurous volatiles (e.g. methyl sulphide and dimethyl sulphide (Wang *et al*., 2009) and hydrolysed metabolites such as isothiocyanates (Morra and Kirkegaard, 2002). They have been used in IPM as biofumigants against soil pests such as wireworms (Elberson *et al*., 1996) and may also affect WCR larvae (Vaughn *et al*., 2006).

The integration of undersowings against insect pests of field crops is more difficult than other crop types (Risch *et al*., 1983). Direct (e.g. release of root exudates) and indirect (e.g. changes in soil properties) mechanisms determine the success of undersowings in

WCR control. Field studies are needed to confirm the potential of undersowings as it will also allow evaluating WCR density (development to the adult stage), maize root damage and yield. Latter is especially important as the here positively tested sunflowers tend to be more competitive than maize (Nassab *et al*., 2011) and would thus reduce yields. The diversity of natural enemies of WCR larvae (Lundgren *et al*., 2009, Toepfer *et al*., 2009) can also be taken into account as they can contribute to the success of undersowing treatments (Lundgren and Fergen, 2010).

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