Original scientific paper
DOI: /10.5513/JCEA01/20.2.2005
Journal of Central European Agriculture, 2019, 20(2), p.678-685

Mortality of the Western corn rootworm, *Diabrotica virgifera virgifera larvae caused by entomopathogenic fungi*

Mortalita lariev kukuričiara koreňového, *Diabrotica virgifera virgifera*, spôsobená entomopatogénnymi hubami

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ABSTRACT

Mortality of the Western corn rootworm (WCR) larvae caused by entomopathogenic fungi *Beauveria bassiana*, *Beauveria brongniartii*, *Metarhizium anisopliae* was investigated in a laboratory. Larvae were treated with a spore suspension (concentration of 2 x 10^7 conidia/ml) by immersion. Only one strain of entomopathogenic fungi, a strain of B. *brongniartii*, significantly influenced mortality of WCR larvae after 7 days. Average mortality of the larvae was 17.63%. After 14 days, five strains of *B. bassiana*, two strains of *B. brongniartii* and one strain of *M. anisopliae* significantly influenced mortality of WCR larvae, with average mortality ranging from 25.83% to 60.57%. After 21 days from exposure of the larvae to spores, four strains of *B. bassiana*, two strains of *B. brongniartii* and one strain of *M. anisopliae* significantly influenced mortality of the WCR larvae with average mortality ranging from 62.5% to 86.6%. Results confirmed that interaction is possible between fungal strains and insect of a various geographical provenance. More than half of the tested fungal strains significantly influenced the mortality of WCR larvae after 14 and 21 days. It was found that differences between strains were more important than differences between fungal species.

Keywords: biological control, Hypocreales, pathogenicity, western corn rootworm

ABSTRAKT

V laboratórnych podmienkach bola skúmaná mortalia lariev kukuričiara koreňového (WCR), Diabrotica virgifera virgifera, spôsobená entomopatogénnymi hubami *Beauveria bassiana*, *Beauveria brongniartii*, *Metarhizium anisopliae*. Larvy sa ošetrili ponorením do spórovej suspenzie (koncentrácia 2 x 107 konídií/ml). Iba jeden kmeň *B. brongniartii* významne ovplyvnil úmrtnosť lariev WCR po 7 dňoch. Priemerná úmrtnosť lariev bola 17,63%. Po 14 dňoch výrazne ovplyvnilo mortalitu lariev WCR päť kmeňov *B. bassiana*, dva kmeňe *B. brongniartii* a jeden kmeň *M. anisopliae*, s priemernou úmrtnosťou v rozmedzí od 25,83% do 60,57%. Po 21 dňoch od vystavenia lariev spóram štyri kmene *B. bassiana*, dva kmene *B. brongniartii* a jeden kmeň *M. anisopliae* významne ovplyvnili mortalitu lariev WCR s priemernou úmrtnosťou v rozpätí od 62,5% do 86,6%. Výsledky potvrdzujú, že je možná interakcia medzi kmeňmi húb a hmyzom rôzneho zemepisného pôvodu. Viac ako polovica testovaných kmeňov húb významne ovplyvnila úmrtnosť lariev WCR po 14 a 21 dňoch. Zistili sme, že rozdiely medzi kmeňmi sú dôležitejšie ako rozdiely medzi druhmi húb.

Kľúčové slová: biologická ochrana, Hypocreales, kukuričiar koreňový, patogenita

INTRODUCTION

The Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (*Coleoptera: Chrysomelidae*), is a no-native introduced pest of maize in Europe and is considered one of the major pests of maize in Slovakia (Cagáň, 2006). Larval injury makes maize plants susceptible to lodging (Sutter et al., 1990), larval feeding may reduce amount of water and nutrients supplied to developing plants and alters nutrient content of the grain (Kahler et al., 1985), thereby reduce grain yield (Turpin et al., 1972). Larval feeding can also reduce plant vegetative biomass production (Chiang, 1973) and decrease plant height (Urias-Lopez et al., 2000). Feeding by adults can cause the damage on leaves, flowers, pollen and silk by characteristic injuries (Trusca, 2013).

One of the protection strategies to reduce WCR damage is an application of entomopathogenic fungi in the control of the WCR larvae and adults. No significant effects on non-target animal compositions were detected for the entomopathogenic nematode, the entomopathogenic fungus *Metarhizium anisopliae* (Metschn.) Sorokīn, tefluthrin or clothianidin. However, clothianidin treatments tended to reduce densities of the beneficial arthropod taxa Coccinellidae, Hymenoptera, Araneae and particularly Staphylinidae and Chrysopidae, whilst tefluthrin tended to reduce the Coleopteran families. No such trends were apparent for the two biological control agent treatments. When summing up the Coleoptera found in the treatments, an average of 3.62 specimens were found per cage in the control, 3.69 in the nematode treatment, 3.15 in the fungus treatment, 1.89 in the tefluthrin treatment and 1.43 specimens in the clothianidin treatment (Babendreier et al., 2015).

The efficacy of the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. was tested as a control agent for adults of the WCR in walk-in field cages by Mulock and Chandler (2000). Mulock et al. (2001a) found negative effect of the entomopathogenic fungus on the reproductive potential of female survivors, egg viability and total egg production within cohorts of the WCR adults. In the next experiment, Mulock et al. (2001b) demonstrated the potential impact of infected beetles as a source of secondary inoculum. The percentage of the WCR adults infected with a granular formulation of indigenous *B. bassiana* strain at the time of their emergence from soil was quantified by Bruck and Lewis (2001) and Bruck et al. (2002) in Iowa, USA. Levels of *B. bassiana* natural infection were low and ranged from 0 to 0.7%; 0 to 3.2% in 1999 and 2000, respectively.

In the southeast of the Austrian province of Styria, field study using a blend of entomopathogens (nematodes and *Metarhizium brunneum* Petch) in conjunction with chemical insecticides was carried out to determine to which extent they affect WCR survival, maize root damages and grain yield. *M. brunneum* was applied at a dose of 50 kg barley kernels per hectare, corresponding to an application rate of more than 5 x 10^{11} colony forming units (CFUs) per hectare. Although an integrated pest management approach, i.e. the combination of biological control agents and chemical pesticides resulted in the lowest number of WCR adults, a systematic crop rotation must be applied as emergency measure to significantly reduce rootworm populations in already heavily infested areas (Rauch et al., 2017). It was also stated that for biological control strategies, the attractiveness needs to be increased by phagostimuli to extend contact between larvae and the entomopathogenic fungus growing out of these formulations (Vemmer et al., 2016).

Management efforts are mainly directed towards to the larvae. Several pathogenicity trials with *B. bassiana* were performed against larvae of different species of insects. Efficacy of *B. bassiana* strains was evaluated against 3-day-old larvae of *Epilachna varivestis* (Mulsant) by Garcia-Gutierrez et al. (1999) and the virulence of *B. bassiana* isolates was compared against 2nd-instar nymphs of the pentatomid *Nezara viridula* (L.) in the laboratory by Shimaxu et al. (1994). *M. anisopliae* spores applied on maize leaves to control the WCR were able to survive for no longer than 3 days after application, whereas on the soil surface noticeable increase of fungus densities was found after treatments and a long-term persistence of at least 15 months could be recorded in the soil (Pilz et al., 2011).

Although, the most of the studies were done with WCR adults, the larvae cause the majority of damage and higher economical loss on maize. Little is known about the impact of entomopathogenic fungi *B. bassiana*, *B. brongniartii* (Sacc.) Petch and *M. anisopliae* on the mortality of the WCR larvae.

In this study it was determined and quantified the effect of native entomopathogenic fungi on the WCR and select fungal strains with the highest efficacy on the pest mortality.

MATERIALS AND METHODS

Source of Western corn rootworm larvae for laboratory trial

Larvae of the WCR were obtained from the laboratory rearing at Department of Plant Protection, Slovak University of Agriculture in Nitra. In summer, the WCR adults were captured in the maize field and placed into cages in the laboratory. Containers with sifted and moistened soil were placed in cages for the deposition of the eggs. After a week, containers with eggs were exposed to a two-week prediapause period at 25 °C and then placed into a fridge. Eggs were stored in laying containers in a fridge for twenty-five weeks at 5 °C. After this period the soil with eggs from laying containers was transferred into rearing pots. Maize seeds were added to these pots and young maize plants were the source of the food for emerged WCR larvae. If necessary, another maize seeds were put to the soil until the WCR larvae did not develop to the second instar. Then larvae were obtained from soil by sieving.

Source of pathogen

Local isolates of *B. bassiana*, *B. brongniartii* and *M. anisopliae* were obtained from soil of the maize fields attacked by the WCR in the previous year (SR = locality Mojmírovce, Slovakia, 48°11'56.26"N, 18°2'18.52"E, MA = locality Nitra, Slovakia, 48°19'18.15"N, 18°9'2.18"E, CR = locality Týnec, Czech Republic, 48°46'55.29"N, 16°59'56.43"E). The Galleria bait method and the dodine selective medium (SM) plating method were used to isolate the fungi (Medo and Cagáň, 2011).

Cultivation of fungal strains

Isolated fungal colonies were inoculated on Sabouraud dextrose agar in Petri dishes under permanent lighting at temperature 25 °C. After 21 days the agar surface was covered by aerial mycelium and conidia.

Preparation of conidia suspension

Suspension of conidia was prepared using 21 days old fungal colonies from inoculated agar plates. Mycelium with conidia was harvested by scraping the surface of SDA agar and then transferred into the flask with 75 ml of 0.05% of Tween® 80 solution (Merck). Flasks were hand shaken for about 10 minutes and their content was filtered using a gauze. After filtration, the gauze was rinsed by additional 25 ml of Tween solution. The concentration of conidia in the suspension was measured by hemocytometer under the microscope. The suspension was adjusted to the final concentration of 2×10^7 conidia per ml.

Establishment and pathogenicity bioassay of the laboratory trial

Laboratory trial was conducted in the climatic chamber at the temperature 20 - 21 °C. Larvae obtained from laboratory rearing were dipped into spore suspension of entomopathogenic fungi and control variant solution without fungal spores for 10 seconds. Together 45 WCR larvae were dipped into each suspension. After 48 hours, dead larvae were removed because these larvae probably died due to a damage caused by their separation from soil. Dipped larvae were placed to plastic dishes containing moistened filter paper on the bottom and five germinated maize seeds with roots. New germinated maize seeds with roots were added when necessary (e.g. bad development of some maize plant). Dishes were transferred into lightproof boxes. Each variant was composed of three replications, each with 10 larvae. The trial included the control variant and variants with larvae inoculated with 12 different entomopathogenic fungal strains (fungal species and strains are mentioned in Table

1). Larvae were inspected every day and percentage of dead larvae was determined on the 7th, 14th and 21st day after the exposition to spore suspension. Dead larvae were removed and placed on a moist filtration paper in Petri dish. Development of mycelium and sporulation on the cadaver was considered to be a confirmation of the fungal infection. The mortality data were evaluated using analysis of variance (ANOVA) and tested by LSD multiple range test (P=0.05).

RESULTS

Seven days after inoculation, only the strain of *B. brongniartii* SR5 significantly influenced the mortality of the WCR larvae (Table 1). This strain (SR5) caused 17.63% mortality of WCR larvae. After the same time, mortality of larvae in the control variant (larvae dipped to solution without fungal spores) and in the variant with fungal strain SK115 was 0%. Mortality of larvae in other variants ranged from 3.03 to 16.29% but it was not significantly different from the control variant (LSD test; P=0.05).

Fourteen days after inoculation, mortality of the WCR larvae caused by spore suspensions of entomopathogenic fungi was more apparent (Table 2). The strains SR98, SR104, SR78, SR102, SR99, MA1, CR8 and SR5 had significant impact on the mortality of the WCR larvae with average mortality of 25.83%; 35.24%; 40.16%; 40.53%; 40.57%; 41.31%; 52.38% and 60.57%, respectively. The same strain (SR5) caused the highest mortality of WCR larvae after 7 and 14 days after infection. Average mortality of the WCR larvae caused by strains CR7, 115, CR5 and SR105 achieved 11.62%; 14.29%; 15.78% and 19.84% respectively, and these strains did not have significant impact on the mortality of the WCR larvae (LSD test; P=0.05).

Table 1. Average mortality of the Western corn rootworm larvae (%) caused by entomopathogenic fungi 7 days after inoculation*

Fungal species	Strain	%	S
Control	K	0[a]	0
B. brongniartii	SR115	0[a]	0
B. bassiana	CR7	3.03[ab]	5.248
M. anisopliae	MA1	3.33[ab]	5.774
B. brongniartii	SR105	4.76[ab]	8.25
B. bassiana	CR5	11.62[ab]	10.089
B. bassiana	SR104	12.86[ab]	2.477
B. bassiana	CR8	13.1[ab]	12.543
B. bassiana	SR98	14.17[ab]	5.204
B. bassiana	SR78	14.29[ab]	24.745
B. bassiana	SR99	15.76[ab]	14.122
B. brongniartii	SR102	16.29[ab]	3.453
B. brongniartii	SR5	17.63[b]	4.883

* Larvae were dipped into spore suspensions with the concentration of 2 x 10^7 conidia per ml for 10 seconds. Each variant was composed of three replications, each with 10 larvae.
[a, b] Numbers marked with the same letters are not significantly different (LSD test; P=0.05).

Table 2. Average mortality (%) of the western corn rootworm larvae (%) caused by entomopathogenic fungi 14 days after inoculation*

Fungal species	Strain	%	S
Control	K	0[a]	0
B. bassiana	CR7	11.62[ab]	10.089
B. brongniartii	SR115	14.29[ab]	14.285
B. bassiana	CR5	15.78[abc]	2.942
B. brongniartii	SR105	19.84[abcd]	21.605
B. bassiana	SR98	25.83[bcd]	10.104
B. bassiana	SR104	35.24[bcde]	19.447
B. bassiana	SR78	40.16[cdef]	14.802
B. brongniartii	SR102	40.53[def]	25.923
B. bassiana	SR99	40.57[def]	11.849
M. anisopliae	MA1	41.31[def]	22.002
B. bassiana	CR8	52.38[ef]	4.122
B. brongniartii	SR5	60.57[f]	4.373

* Larvae were dipped into spore suspensions with the concentration of 2 x 10^7 conidia per ml for 10 seconds. Each variant was composed of three replications, each with 10 larvae.
[a, b, c, d, e, f] Numbers marked with the same letter are not significantly different (LSD test; P=0.05).

Table 3. Average mortality of the Western corn rootworm larvae (%) caused by entomopathogenic fungi 21 days after inoculation*

Fungal species	Strain	%	S
Control	K	32.34[a]	13.836
B. brongniartii	SR105	37.1[ab]	20.238
B. bassiana	CR7	50.66[abc]	32.821
B. brongniartii	SR115	50.79[abc]	19.827
B. bassiana	CR5	55.18[abcd]	16.429
B. bassiana	SR104	56.19[abcd]	15.737
B. bassiana	SR98	62.5[bcde]	23.848
B. bassiana	SR78	65.34[bcde]	20.702
B. bassiana	SR99	73.44[cde]	14.424
B. brongniartii	SR102	79.55[cde]	9.906
B. brongniartii	SR5	82.37[de]	4.883
B. bassiana	CR8	82.74[de]	6.76
M. anisopliae	MA1	86.6[e]	5.804

* Larvae were dipped into spore suspensions with the concentration of 2 x 10^7 conidia per ml for 10 seconds. Each variant was composed of three replications, each with 10 larvae.
[a, b, c, d, e] Numbers marked with the same letter are not significantly different (LSD test; P=0.05).

After 21 days, the mortality of the WCR larvae caused by spore suspensions of entomopathogenic fungi was from 37.1% to 86.6% (Table 3). Strains SK98, SK78, SK99, SK102, SR5, CR8 and MA1 significantly influenced the mortality of the WCR larvae (LSD test; P=0.05). Average mortality caused by these strains achieved 62.5%, 65.34%, 73.44%, 79.55%, 82.37%, 82.74% and 86.6%, respectively (Table 3). On the other hand, the strains SK105, CR7, SK115, CR5 and SK104 did not have significant influence on the mortality of the WCR larvae although the average percentage of the WCR larval mortality was higher in comparison with the control variant.

DISCUSSION

This research confirmed significant effect of some strains of *Beauveria bassiana*, *Beauveria brongniartii* and *Metarhizium anisopliae* collected from the soils in the maize field of Slovakia and Czech Republic on WCR larval mortality. In ten maize fields in Iowa, USA, *B. bassiana* and *M. anisopliae* s.l. were present in 60% ± 6.3% and 55% ± 6.4% of soil samples, respectively and subsequent laboratory bioassays found that some *M. anisopliae* s.l. strains collected from maize fields killed a greater proportion of the WCR larvae than a standard commercial strain (Rudeen et al., 2013). Similarly, the pathogenicity of selected *B. bassiana* strains isolated from *Ips typographus* was significantly higher than that of the mycoinsecticide Boverol®, which was used as a reference strain (Barta et al., 2018).

Impact of novel and standard isolates of entomopathogenic fungi to the WCR larvae and adults was examined in the study similar to ours (Pilz et al., 2007). Larvae and adults of the WCR were dipped into a spore suspension and kept for 14 days at 22 °C (± 2 °C). The most virulent isolate infected about 47% of larvae and isolates of *M. anisopliae* caused significantly higher mortalities than isolates of *B. brongniartii* and *B. bassiana*. These results are different from findings achieved in current study to some extent. MA1 strain of *M. anisopliae* caused 41.31% mortality of WCR larvae after 14 days and it caused lower mortality than the most effective strains of *B. bassiana* (CR8) or *B. brongniartii* (SR5) (Table 2). On the other hand, strain of *M. anisopliae* (MA1) caused higher mortality of the larvae than the other strains of *B. bassiana* and *B. brongniartii*.

In the experiment, the greatest mortality of the WCR larvae seven days after infection by the strain of *B. brongniartii* SR5 was 17.63% and the mortality caused by the strain of *M. anisopliae* MA1 was 3.33% (Table 1). On the contrary, isolates of *M. anisopliae* FT8 exhibited 100% cumulative mortality against second instar larvae of Spodoptera exigua 3 days after treatment at 1×10^7 conidia/ml and Paecilomyces fumosoroseus FG340 caused 100% mortality 6 days after treatment at 1×10^4 conidia/ml (Han et al., 2014). Concentration was, in most of the cases tested, a critical parameter that determined the "speed of kill" of the stored-grain pest Sitophilus oryzae for *B. bassiana* and *M. anisopliae*. Conversely, concentration was not that critical for Isaria fumosorosea, and survival was high in some of the combinations tested, even after 14 days from exposure (Kavallieratos et al., 2014). The study also showed higher mortality of target insect caused by *B. bassiana* or *M. anisopliae* 7 or 14 days after infection as it was in the experiments achieved in this study. More authors working with different insect species reported similar results (e.g. Ekesi et al, 2000; Schapovaloff et al., 2014; Erler and Ates, 2015, etc.). Possible reason of this difference may be in diversity in temperatures during insect rearing. Laboratory trial was conducted in the climatic chamber at the temperature 20 – 21 °C, the other experiments used 25 °C or more.

Similar experiments with entomopathogenic fungi confirmed that young larvae of *Polyphylla fullo* (1st and 2nd instars) were more susceptible to infection than older ones (3rd instar) (Erler and Ates, 2015). This explains why in this experiment there were used as young larvae as possible. The first instar of the WCR larvae was too small for manipulation, the third instar larvae would be probably less susceptible to the infection.

A pathogenicity trial with *B. bassiana* on third instar larvae of *Diabrotica speciosa* caused 70% mortality (Consolo et al., 2003). Field studies of the efficacy of soil

application of *M. anisopliae* and *B. bassiana* for the control of the Diabrotica undecimpunctata in maize revealed that high treatment rate of *M. anisopliae* was equal to uninfested control in preventing goosenecked plants and larval feeding on roots (Krueger and Roberts, 1997). In another study the efficiency of the entomopathogenic fungus *M. anisopliae* was even slightly less compared to soil insecticides or nematodes (Pilz et al., 2009). Soil-borne entomopathogens can complement Bt maize by protecting roots from feeding injury from corn rootworm when pest abundance is high, and can decrease root injury to non-Bt maize when rootworm abundance is low (Petzold-Maxwell et al., 2013). It seems that selection of more effective entomopathogenic fungal strains will probably increase their impact on soil insects. The study confirmed that differences between strains were more important as the differences between fungal species.

It was also found that strains native to Europe were infectious to new insect species. Reaction of insect species to strain or species of entomopathogenic fungi does not depend only on the virulence of the entomopathogen. Another factor is the interaction between the fungal spore and the insect epicuticle (Ortiz-Urquiza and Keyhani, 2013) and differences in free fatty acids (FFAs) chemical composition of insects may be responsible for susceptibility or resistance to fungal infection (Gutierrez et al., 2015). Consequently, it was confirmed that such interaction is possible between fungal strains and insect with different geographical origin.

CONCLUSIONS

The research confirmed significant effect of some strains of *Beauveria bassiana*, *Beauveria brongniartii* and *Metarhizium anisopliae* collected from the soil in the maize field on WCR larval mortality. It was found that differences between tested strains were more important as the differences between fungal species. Achieved data showed that spore suspensions of entomopathogenic fungi *B. bassiana*, *B. brongniartii* and *M. anisopliae* can have significant effect on the mortality the WCR larvae developing on maize roots in laboratory conditions. Consequently, it was confirmed that interaction is possible between fungal strains and insect with different geographical origin.

ACKNOWLEDGEMENTS

This study was supported by project of Slovak scientific agency VEGA (Grant No. 1/0849/18).

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