Biological control of the invasive maize pest *Diabrotica virgifera virgifera* by the entomopathogenic fungus *Metarhizium anisopliae*

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Preface

> **"**.....*Wir träumen von phantastischen außerirdischen Welten. Millionen Lichtjahre entfernt. Dabei haben wir noch nicht einmal begonnen, die Welt zu entdecken, die sich direkt vor unseren Füßen ausbreitet: Galaxien des Kleinen, ein Mikrokosmos in Zentimetermaßstab, in dem Grasbüschel zu undurchdringlichen Wäldern, Tautropfen zu riesigen Ballons werden, ein Tag zu einem halben Leben. Die Welt der Insekten.....***"**
> *(aus: Claude Nuridsany & Marie Perennou (1997): "Mikrokosmos - Das Volk in den Gräsern", Scherz Verlag.*

This thesis has been submitted to the University of Natural Resources and Applied Life Sciences, Boku, Vienna; in partial fulfilment of the requirements for the degree of Dr. nat. techn.
The thesis consists of an introductory chapter and additional five scientific papers.
The introductory chapter gives background information on the entomopathogenic fungus *Metarhizium anisopliae*, the maize pest insect *Diabrotica virgifera virgifera* as well as on control options and the step-by-step approach followed in this thesis. The scientific papers represent the work of the PhD during three years, of partial laboratory work at the research station ART Agroscope Reckenholz-Tänikon, Switzerland, and fieldwork in maize fields in Hodmezòvasarhely, Hungary, during summer seasons. Paper 1 was published in the journal "BioControl", paper 2 in the journal "Journal of Applied Entomology", and paper 3 and paper 4 have not yet been submitted for publications, while paper 5 has been submitted to the journal "BioControl".

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Während meiner Feldversuche in Hodmezòvasarhely entdeckte ich eine tiefe Zuneigung zu diesem Land und den Menschen. Es war eine wundervolle Zeit für mich; ich hatte die Chance eine Menge neuer Freunde und liebenswerter Menschen kennenzulernen - lieber Stefan, Illdiko und Kinder, Ibolya, Erszibeth, Ori, Gabor - ich schätze diese Freundschaften sehr und bin froh über die Zeit, die ich dort verbringen durfte.

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Last, but not least vielen lieben Dank an Christian Schweizer, der mit mir über 3 Jahre lang das Büro teilen durfte (musste). Er begleitete mich vom Anfang (2003 als seine Praktikantin) an mit. Ich möchte mich für all die interessanten, fachlich relevanten, aber auch persönlichen Gespräche bedanken, er war in manchen Zeiten eine wichtige Unterstützung für mich.

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Contents

Summary

This thesis followed a step by step approach in the development of a biological control agent.

As a first step naturally occurring pathogens from several pest populations were collected from larvae, pupae and adults of *Diabrotica virgifera virgifera* in maize fields with special emphasis on the occurrence of the entomopathogenic fungi species *Beauveria* spp. and *Metarhizium anisopliae*. Additionally, the entomoparasitic nematode species *Heterohabditis bacteriophora* and *Steinernema feltiae* could be isolated from infested larval stages of the pest insect.

In the next step all isolated strains of *M. anisopliae* and *Beauveria* spp. together with standard strains used against different pest insects like *Melolontha melolontha*, *Phyllopertha horticola*, *Cydia pomonella*, *Amphimallon solstitiale*, *Leptinotarsa decemlineata* and others, were evaluated for pathogenicity and virulence against larvae and adults of *D. v. virgifera* in bioassays. Screening for virulent fungal strains revealed that isolates of *M. anisopliae* caused significantly higher mortalities when compared to *B. brongniartii* and *B. bassiana* isolates and especially isolates obtained from *D. v. virgifera* samples were more virulent than those from other hosts.

Based on these bioassays and on mass production capability we selected the two most promising *M. anisopliae* strains for future investigations. These strains were formulated as so called fungal colonized barley kernels (FCBK) for inoculations into the soil to target soil-inhibiting stages and as spore suspension to target adults on maize plants.

Field trials during the summer seasons 2006 and 2007 were carried out in maize fields in South-East Hungary, where natural populations of the pest species were present. After application of *M. anisopliae* against larvae of *D. v. virgifera*, the persistence of the fungal strains was determined with standard methods and verified with molecular markers. Highest densities of the fungal biological control agent were found at the time when *D. v. virgifera* were in the larval stage but even a long term persistence of at least fifteen months could be recorded. In the treated areas adult emergence was significantly reduced as compared to the control; efficacies between 31% and 43% were obtained with these two selected strains.

Molecular markers were used for the identification of fungal strains isolated from soil samples and to check whether the applied strains were responsible for the increase of the fungus densities. Molecular markers, like simple sequence repeats (SSR) are able to distinguish between strains within a species. Altogether, 76 fungal isolates from soil samples from treated and untreated areas of an experimental Hungarian maize field were characterized. It could be proofed that SSR are valuable tools to discriminate between fungal strains and thus also to assess the potential for competition and non-target effects within and among fungal species. Based on this technique we

can conclude that the application of fungus colonized barley kernels into the soil was responsible for the high fungal densities measured after applications. Further, we found that several different genotypes of *M. anisopliae* occur within a small scale area and were able to coexist, also after application.

Strategies for adult control were developed by the application of spore suspensions on beetles feeding on maize plants. Depending on the fungal strain we recorded mortalities of about 35% after fungus applications. The persistence of the spores on maize leaves and on the soil surface was evaluated as well. Spores on leaves did not survive more than three days, but spores applied on leaves drifted obviously to the soil surface where increased fungus densities were found.

Finally, we compared the efficacy of an entomopathogenic fungus strain, a nematode species and chemical insecticides to control *D. v. virgifera* in maize fields. This part was carried out with our partner group working on entomoparasitic nematodes (S. Toepfer, CABI Delemont).

The nematode and the two insecticides reduced *D. v. virgifera* by 65 % and the fungus by 31 % on average over fields and years. Results showed that biological control agents like nematodes and the corresponding application technique could already reach control efficacies comparable to the commonly used insecticides, or have the potential like the fungus to reach such efficacies after further development of the application techniques. This encourages the commercialization and use of biological control agents against *D. v. virgifera* larvae.

Zusammenfassung (german language)

In dieser Arbeit wurde schrittweise die Eignung des insektentötenden Pilzes *Metarhizium anisopliae* gegen den Maisschädling *Diabrotica virgifera virgifera* geprüft. Im ersten Schritt wurden natürlich verpilzte Insekten in Schädlingspopulationen in Ungarn, Serbien, Rumänien und Italien gesucht; es wurden neun Pilzstämme von natürlicherweise verpilzten Larven, Puppen und Adulten isoliert. Acht Isolate gehörten zur Pilzart *M. anisopliae* und eine Larve verpilzte an *Beauveria* sp. Diese Isolate wurden im Labor kultiviert und vermehrt. Die Pathogenität und Virulenz dieser Isolate und jene von „Standardisolaten" aus der Kollektion der ART Agroscope Reckenholz (isoliert von Maikäfer *Melolontha melolontha*, Gartenlaubkäfer *Phyllopertha horticola*, Junikäfer *Amphimallon solstitiale*, Kartoffelkäfer *Leptinotarsa decemlineata* und Apfelwickler *Cydia pomonella*) wurden in Biotests an Larven und Adulten getestet. *Metarhizium anisopliae* Stämme zeigten eine bessere Wirkung (Mortalität und Infektion) als *Beauveria* spp. Stämme. Weiters zeigten Stämme, abisoliert von *D. v. virgifera* höhere Mortalitäten als Stämme von anderen Wirten. Aufgrund dieser Virulenzdaten und Daten über die Eignung zur Massenvermehrung auf Medien wurden zwei *M. anisopliae* Isolate zur Anwendung in Feldversuchen ausgewählt (*M. anisopliae* Isolat „2277" und „Bipesco 5").

In Feldversuchen in Ungarn wurde die Wirksamkeit auf das Schadinsekt erhoben, einerseits auf die sich im Boden entwickelnden Stadien durch Formulierung und Einbringung des Pilzes als „Pilzgerste" und andererseits auf das adulte Stadium durch Ausbringung von Sporensuspension. Die Ausbringung der Pilzgerste erfolgte zusammen mit der Maisaussaat in etwa 10-12 cm Bodentiefe und bewirkte eine signifikante Reduktion der bodenbewohnenden Entwicklungsstadien. Auch die Ausbringung von Sporensuspension zeigte eine signifikante Reduktion der Adulten von etwa 35%.

Für den Erfolg einer Ausbringung eines „biological control agents" ist neben der Wirkung auf das Schadinsekt auch die Etablierung und Persistenz des Pilzes im Habitat des Schädlings wichtig. Der Pilz *M. anisopliae* konnte für mindestens 15 Monate im Boden nachgewiesen werden, die höchsten Pilzdichten wurden 2 Monate nach der Applikation, d.h. Mitte Juni erreicht. Da zu diesem Zeitpunkt die Larvenentwicklung von *D. v. virgifera* erfolgt, sind die Infektionschancen optimal. Der Nachweis der Persistenz wurde durch Isolationen auf Selektivmedium erbracht und durch molekularbiologische Untersuchungen abgesichert. Abschliessend wurden in Zusammenarbeit mit den Projektpartnern der Gruppe „biological control with entomoparasitic nematodes" alle in Feldversuchen getesteten Bekämpfungsvarianten (entomopathogene Pilz und entomoparasitische Nematoden sowie Insektizide) zusammengefasst, miteinander verglichen und diskutiert.

Outline of the thesis

The success of a biological control agent against insect pests strongly depends on the efficacy as well as on the augmentation and persistence of highly virulent fungus strains in the environment. Within the KTI Project: “Development of biological products for sustainable control of the Western Corn Rootworm, *Diabrotica v. virgifera*, an invasive maize pest in Europe”; Project Nr: 7487.1 LSPP-LS, basic knowledge of the efficacy of *Metarhizium anisopliae* strains against different life stages of the pest insect were carried out by bioassays in the laboratory and field trials in maize fields. In addition the establishment and persistence of the applied fungal inoculum in maize fields were investigated.

The thesis is divided into the two parts “Introduction chapters” and “Scientific papers”. The first part includes introduction chapters into the topic with descriptions of the biology and ecology of the host and the pathogen as well as biological control options. The second part deals with scientific papers, which describe in detail research studies during the last three years.

The first research paper deals with the natural occurrence of pathogens in *Diabrotica v. virgifera* populations in Hungary. Pathogens were obtained by collecting and rearing both the soil inhabiting stages (larvae, pupa) and the adults in fields. Entomopathogenic fungi were also isolated directly from soil samples from maize fields by using two standard methods (*Galleria* Bait Method and Selective Medium Method). In the second paper the pathogenicity and virulence of the collected strains where tested against the larval and adult stage of the pest insect. The dipping method in suspension was used as bioassay for larvae and adults with the aim to select the most virulent strains for further investigations.

The scientific paper three shows first results of the establishment and persistence of fungal inocula after applications in maize fields by using cultural and molecular biological methods.

Paper 4 deals with the impact of a fungus application on the soil inhabiting and the adult stage of *Diabrotica v. virgifera*.

In paper 5 a comparison between the application of biological control agents (entomopathogenic fungus and nematodes) and chemical control methods is provided. This study was carried out together with the partner group from CABI Delémont (Switzerland) working on entomoparasitic nematodes.

A general conclusion as well as future research activities are discussed in chapter 5.

The whole study was separated into five steps:

1. Field surveys of naturally occurring enemies in *D. v. virgifera* populations in Europe.
2. Selection of isolates under controlled laboratory conditions based on their virulence against different insect stages, cultivation and mass production capacity.
3. Field applications and studies on establishment and persistence of the biological control agent in the environment by cultural and molecular techniques.
4. Impact of the applied fungal strains on soil inhabiting and adult stages of *D. v. virgifera.*
5. Comparison of different control agents (nematodes, fungi and soil insecticides).

Field surveys were done in maize fields in Hungary, Serbia, Romania and Italy in the years 2005 and 2006. We focussed mainly on the occurrence of entomopathogenic fungi and tested some isolates in bioassays against adults and larval stages of the pest insect. After selection and cultivation of two strains first field trials were carried out under the climatic conditions of Hungary in the years 2006 and 2007. The introduction of the pathogen into the habitat of the pest and its persistence is one of the most important and critical factors for a control success.

Two strategies were carried out:

1) *Metarhizium anisopliae* was cultivated on sterilized barley kernels. These fungus colonised barley kernels (FCBK) were tilled with standard drill machines into the soil where the fungus could sporulate and disseminate in the habitat of soil living stages of the pest. The aim was to establish an efficient spore concentration in the area were larvae occur.
2) The application of *M. anisopliae* spore suspension on maize leaves. *M. anisopliae* spores can be applied by standard equipment or by special maize sprayers in the time of flowering and peak occurrence of adult *D. v. virgifera*. The spores should be applied when most adults are hatched and are still in the field before the eggs are laid.

1. Introduction

Biological control

Biological control is defined as “the use of living organisms and viruses to suppress the population density or impact of specific pest organisms, making it less abundant or less damaging than it would otherwise be “(Eilenberg et al. 2001). The living organisms consist of three groups: the predators, the parasitoids and the pathogens. Predator’s ackively search for and kill their hosts. Parasitoides feed on their hosts and therefore kill them. The pathogens (viruses, bacteria, fungi, protozoa, nematodes) infect their hosts and kill them.

Within the last decades a higher awareness of environmentally friendly produced food established, which leadled to an increase of the number of organic farmers in Europe. They manage their farms, following the EU regulation 2092/91 for organic agriculture. Additionally a large number of pest insects got adapted to chemical insecticides and developed resistances in whole insect populations. All this factors revealed a “new thinking” and initiated the search for alternative control strategies to produce eco-friendly food in a suistainable agriculture.

Different control strategies (Eilenberg et. al. 2001), depending on the biocontrol agent, the target pest and the circumstances can be applied:

- **Classical biological control**

The intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control.

- **Inoculation biological control**

The release of living organisms as a biological control agent with the expectation that it will multiply and control the pest for an extended period.

- **Inundation biological control**

The use of living organisms to control pests when control is achieved exclusively by the released organisms themselves.

- **Conservation biological control**

Modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests.

For the application of entomopathogenic fungi all four-control strategies (Eilenberg et al. 2001) can be used. The conservation biological control should always be considered. Its goal is to favour beneficial organisms and to suppress pest species mainly by measures inside or outside the crop. This strategy can be applied alone or in combination with other control strategies. The control of *D. v. virgifera* as an introduced pest is basically a case for a classical biological control, but due to registration constraints the use of locally existing fungal natural enemies is the better option. The best strategy to control *D. v. virgifera* is a combination of inundation biological control and inoculation biological control. So, firstly the fungus is applied like a chemical pesticide (inundation strategy) and enrich the soil with a necessary high amount of infective virulent spores, which results in direct control of those pest individuals which are hit by the applied inoculum, while further individuals will be killed by spores produced and multiplied on infected individuals (autodissemination or inoculation strategy).

2. The Western Corn Rootworm *Diabrotica virgifera virgifera*

2.1 Occurrence and Introduction to Europe

The genus *Diabrotica* belongs to the order Coleoptera, family Chrysomelidae. The species *Diabrotica virgifera virgifera* was described by LeConte in 1868 (Chiang, 1973).

The genus *Diabrotica* includes approximately 338 species (Wilcox, 1972) of which 10 species are recognized as pest insects (Krysan and Miller, 1986). The species *Diabrotica virgifera* is together with *D. barberi* and *D. undecimpunctata howardi* the most serious maize pest in North America, causing damages of about 1000 million US dollars per year. Yields of corn are decreased by about 10-13% (Apple et al. 1977), but *Diabrotica* species also occur on cucurbits, lucerne, clover, rape, soybean and sunflowers. It is assumed that this invasive species evolved together with the host plant in the subtropics of Mexico and Central America (Branson and Krysan, 1981). In the USA two subspecies of *D. virgifera* are present: *D. v. virgifera* LeConte (Western Corn Rootworm) and *D. v. zeae* Krysan and Smith (Mexican corn rootworm) (Krysan et al. 1980). *D. v. virgifera* is nowadays distributed from the Midwestern to eastern and south-eastern USA and north to Ontario and more adapted to temperate climates with a diapause during winter time; *D. v. zeae* is distributed mainly from Texas and Oklahoma to Panama and is more adapted to warm climates without diapause during cold seasons.

We are concentrating on the pest species *D. v. virgifera*, which was first recognized as a pest in the southern USA in 1912 (Steffey et al. 1999) and invaded from the 1950s on into new areas favoured by the practise of continuous maize growing without crop rotation. The first introduction into Europe was observed near the airport of Belgrade in 1992 (Baca, 1994). Within 10 years, this pest species spread over large parts of central- and south-eastern Europe. *D. v. virgifera* was first recorded in Austria in 2002 (Cate, 2002). In Switzerland, populations have established south of the Alps while only few beetles have been captured in northern Switzerland and no population has yet established there. In Germany, beetles were found in Bavaria and Baden-Württemberg in 2007 although the numbers of captured beetles indicate that the first ones may have arrived already before (Project Partners, final KTI Report, 2008).

The pathways for the introduction of *D. v. virgifera* into Europe could either be through infested soil, through maize plants or simply through adults. It is assumed that airplanes are responsible for the first introduction because the first discovery site was close to the Belgrade airport in former Yugoslavia (Edwards et al. 1999).

Molecular biological studies based on micro-satellites as genetic markers identified populations of five outbreak sites: Belgrade/Serbia (1992), Paris/France (date outbreak 2002 and 2004), Alsace/France (2003), Venetia/Italy (2000) and the north-eastern Po valley/Italy (2003). Comparisons with populations from North America showed that the three outbreaks near Belgrade, Paris and Venetia resulted from independent introductions from North America. The outbreaks in the Po valley and in Alsace were due to beetles originating from Serbia and Paris respectively.

Keys for the identifications of larvae and adults of *Diabrotica* species are given by Christensen (1943). Awareness should be taken on the correct identification of larvae, as they can be confused with other soil-dwelling pests like wireworms, which are found at the same time on maize roots. Some other adult chrysomelids like *Acalymma vittatum* (Fabricius), *Cryptocephalus decemmaculata* Linnaeus, *Cryptocephalus morarei* Linnaeus show some resemblance in size, shape and colour, but these species do not occur in maize fields, so confusing is rather unlikely. In Europe *D. v. virgifera* is defined as quarantine pest (“ *a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled”*, EPPO, 2004). Procedures for protective measures against the introduction and spread of *D. v. virgifera* in the community are given by the European Union (Commission decision of 11 August 2006 amending Decision 2003/766/EG on emergency measures to prevent the spread within the Community of *Diabrotica virgifera* Le Conte, notified under document number C (2006) 3582). The total affected area in Europe now covers 310.000 km^2 but economic damage occurs mainly in central and south-eastern Europe as well as in northern Italy where the pest is already present for more than 10 years. Generally, there is a time lag between the first

observation of the beetle and the occurrence of economical damage of at least five to six years (Project Partner, final KTI Report, 2008). Costs arising from control measures against the Western Corn Rootworm (WCR) and from damages in Europe are estimated to be about 300 million Euros and this Figure will increase with the spread of the pest to new countries. It is becoming evident that the WCR significantly affects the maize cropping systems in Europe.

2.2 Biology and damage in maize fields

Diabrotica v. virgifera is a univoltine insect species and its feeding is largely restricted to maize, its primary host plant. The suitable host range of the larvae was tested under laboratory conditions and under field conditions in Romania (Breitenbach et al. 2006).

In Europe adult beetles swarm from June till September and feed mainly on maize leaves, silks, pollen and young kernels, although pollen is preferred. After flowering of maize they tend to remain in maize fields and feed on foliage. A small number moves to other crops, like sunflowers, soybean, lucerne, amaranths, ambrosia and others. After mating female deposit their eggs into the soil, mainly of maize fields, at a depth of about 10 - 35cm, most of them in the 15 cm layer (Baca et al. 1995). Eggs overwinter in the soil in a diapause state. The larvae hatch at the beginning to late May of the following year and develop through three larval instars, which live in the soil near maize roots. First instar larvae have to find maize roots within 24 hours, otherwise they starve and die. They cause the main damage by feeding and destroying the root system of the plant, thereby impeding the absorption of water and nutrients. This damage is referred to as “root pruning” (dark channels within the root) and scales are developed for evaluation of the degree of symptoms [Node Injury Scale (Oleson et al. 2005), IOWA scale (Hills and Peters, 1971)]. Later instars also feed on the brace roots, which may cause plant lodging. Each larval instar needs about 10 days to develop. The larvae occur between May and early August with the peak from May to June. When mature the larva moves away from the roots and pupates in the soil. The pupal instar lasts about one week. Adults are found from the middle of June till October (Toepfer and Kuhlmann, 2006) with a peak in the second half of July and during August, depending on climatic conditions and area. The males occur first and a short time afterwards first females are seen in the field.

Adults mostly feed on tassel, silks and leaves before, during and after flowering and can occasionally cause yield losses due to intensive silk feeding which may interfere with pollination and seed set.

Adults copulate and after maturation feeding females lay their eggs into the soil mostly near the places where they feed. The adult beetles die in autumn and the eggs are the only hibernating stage.
To monitor and detect the pest insect pheromone traps (PAL trap), “modified funnel traps” (VARs) or MCA traps (p-methoxy- cinnamaldehyde) are used (EPPO, 2004). They are placed in maize fields or nearby and should be checked regularly. The distance between two traps should not be closer than 20 m and they should be placed at points of entry, airports, and transhipment locations.
If *D. v. virgifera* was monitored in an area, special eradication methods described in the EU regulation 2003/766/EG have to be carried out. These include the definition of an “infestation zone” and “security zone” around the infested area. These zones could be surrounded by an additional buffer zone. It’s obligatory to inform all other EU countries and the EU commission of this new infested area and to follow the rules, given in the regulation.

2.3 Control strategies

Several control strategies have been tested during the last years, like the effect of different cropping systems on the attractiveness to the beetles (Baca et al. 2006a), effects of crop residues on plant lodging caused by larval feeding (Baca et al. 2006b), management by various sowing dates of maize (Furlan et al. 2006), screenings of maize varieties resistant against this pest (Moeser and Vidal, 2006), cultural control by crop rotation and chemical control options with soil insecticides (Levine and Oloumi-Sadeghi, 1991; Wilde et al. 2004; Wilde and Roozeboom, 2006; Rozen and Ester, 2007) as well as using transgenic maize hybrids (Ward et al. 2005; Steiner and Garcia-Alonso, 2006). Most of them have disadvantages such as high economic costs; potentially negative environmental effects on non- target organisms or the environment or may cause resistance problems. One of the most promising control options is the crop rotation although studies in the USA have shown that *D. v. virgifera* may also be able to evolve resistance against this cultural control practice (Tollefson, 1988; Tollefson and Prasifka, 2006). Nevertheless, crop rotation can be a powerful tool to combat *Diabrotica* infestations. However, crop rotation is not or hardly an option for many farmers for a number of economic reasons (e.g. seed producers, farmers producing bio-energy, farmers that have specialized equipment only or farmers in areas favourable for maize growing). In all these cases, crop rotation would reduce the farmer’s income because the substitute crops grown are less valuable.
In this project, we aimed to develop effective biological control agents against this pest insect. Different aspects and agents were taken into consideration for effective control, like a classical

biological control with the parasitoid species *Celatoria compressa* (Diptera: Tachinidae) (which is naturally attacking *Diabrotica* species in Mexico) (Kuhlmann and van der Burgt, 1998; Gamez-Virues and Eben, 2005), the efficacy of predators, especially spiders in maize fields (Meissle, pers. comm.) or the inundative release of entomoparasitic nematodes (Kuhlmann and van der Burgt, 1998; Toepfer et al. 2008) and fungi. In Europe surveys in invaded areas focusing on the natural occurrence of nematodes, entomopathogenic fungi and parasitoids in *D. v. virgifera* populations were carried out in the year 2000 (Toepfer and Kulmann, 2004). This report concluded that no effective indigenous natural enemies are attacking any life stages of *D. v. virgifera*. Only the entomopathogenic fungus species *Beauveria bassiana* Vuill. and *Metarhizium anisopliae* Sorokin were found attacking larvae at an extremely low level (< 1%). Based on that survey we focussed our study on the biological control of the Western Corn Rootworm (WCR) on entomopathogenic fungi. Entomopathogenic fungi are an attractive alternative for the control of insect pests, mainly because they are safer for plants, animals and the environment than conventional pesticides (Khetan, 2001). Furthermore, such fungi have also been evaluated as biological control agents for more than 200 economically important insect species (Ferron, 1981; Tanada and Kaya, 1993; Maurer et al. 1997; Burges, 1998; Butt, 2002). Entomopathogenic fungi have been used successfully in classical, augmentation, and conservation strategies (Gottel et al. 1990; Inglis et al. 2001; Pell et al. 2001). Several hyphocreales species are commercially available in different formulations that can be used as inundadive and inoculative pest control products (Shah and Goettel, 1999). Promising results obtained with entomopathogenic fungi in the control of *Diabrotica* species are already reported from North and South America (Campbell et al. 1985; Silva-Werneck et al. 1995; Krueger and Roberts, 1997; Bruck and Lewis, 2001; 2002; Martin and Schroder, 2000; Mulock and Chandler 2000; 2001; Consolo et al. 2003).

3. The green muscardine fungus *Metarhizium anisopliae var. anisopliae*

3.1 Entomopathogenic fungi

Entomopathogenic fungi can be found throughout the fungal kingdom. The most potent species belong to the order Hypocreales. This order has been recognised since the 19th century to cause natural epizootics in insect populations (Bassi, 1837). Early attempts focused on the inoculation of *Metarhizium anisopliae* against the wheat cockchafer *Anisoplia austriaca* (Metschnikoff, 1879) and the use of *Beauveria brongniartii* against the European cockchafer *Melolontha melolontha* (LeMould, 1893). Approximately 750 species of fungi have been documented to infect insects (Hajek, 1997). Most of the entomopathogenous Hypocreales are soil-borne pathogens and *M. anisopliae* could be isolated from soils all over the world (Zimmermann, 2007).

During the last century many studies have been carried out to evaluate the potential of entomopathogenic fungi for the biological control of insect pests. Today, products based on the following fungi are commercially available: *Lecanicillium lecanii*, *Metarhizium anisopliae*, *Beauveria bassiana*, *Beauveria brongniartii*, *Paecilomyces fumosoroseus*, *Lagenidium giganteum* (Butt, 2002).

3.2. Classification of the genus Metarhizium

The species *Metarhizium anisopliae* was found first on infected larvae of the wheat cockchafer *Anisoplia austriaca* near Odessa (Ukraine) by Metschnikoff (1879). He described the species as *Entomophthora anisopliae*. Later, Sorokin (1883) transferred the species to the new genus *Metarhizium*. The first revision of the genus *Metarhizium* was conducted by Tulloch (1976). Steinhaus (1949) and Müller – Kögler (1965) presented reviews of the history of detection, first investigations and use for biological control.

The genus *Metarhizium* comprises five species: *M. anisopliae*, *M. flavoviride*, *M. album*, *M. brunneum* and *M. guizhouense* (Driver et al. 2000). The species *M. anisopliae* can be separated into the three subspecies *M. a. anisopliae, M. a. acridum und M. a. lepidiotae* (Table 1). A teleomorph state of *M. anisopliae* has not yet been discovered, but it seems likely that the species belongs to the genus *Cordyceps*. Probably, most strains of *M. anisopliae* have lost the capability of reproducing sexually (Humber, 2007).

The haploid filamentous fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin is now attributed to the order Hypocreales; family Clavicipitaceae (Table 1). The genus *Metarhizium* has been isolated from more than 200 insect species; most of them belong to the order Coleoptera (Samson et al. 1988). This reflects the broad host spectrum, but restricted host specificity on strain level is discussed (Ferron, 1978; Enkerli et al. 2005; Pilz et al. 2007).

Figure 1: Habitus of *M. anisopliae* on a *Tenebrio molitor* larva (Photo: Rudolf Wegensteiner, Boku, Vienna)

Table 1: Classification of *Metarhizium* (after USDA-ARS Collection of Entompathogenic Fungal Cultures (ARSEF); http://arsef.fpsnl.cornell.edu/)

	Taxon	
Kingdom	Fungi	
Division	Ascomycota	
Class	Ascomycetes	
Order	Hypocreales	
Family	Clavicipitaceae	
Genus	*Metarhizium*	
Species	*M. anisopliae*	
	M. album	
	M. brunneum	
	M. flavoviride	
	M. guizhouense	
Subspecies		*M.a. anisopliae*
		M. a. acridum
		M. a. lepidiotae

3.3 Metarhizium anisopliae: Infection and disease development

The life cycle of *M. anisopliae* is described in detail by Müller-Kögler (1965), Ferron (1978; 1981), Samson (1981) and Samson et al. (1988). In figure 2 each step of the infection process is drawn.

The infection can be divided into a parasitic and a saprophytic phase: The parasitic phase starts with the germination of the conidia and the formation of an appressorium on the host cuticle, which usually takes place within the first 20 hours after contact with the host insect (Zimmermann, 2007). With the help of enzymes and pressure the germination tube penetrates through the host integument and reaches the nutrient rich haemolymph. Submerse spores or hyphal bodies are formed, which propagate rapidly and colonise the tissues of the host. The host dies probably due to a combination of lack of nutrition and oxygen, a collapse of organs or through the action of fungal toxins. Most isolates of *M. anisopliae* produce cyclide peptides, so called destruxines. In insects the destruxines A, E, B, A2 and desmetholdestruxin B are formed. Different strains of the fungus produce different amounts of these destruxines. The function of the destruxines for the pathogenesis is not clear (Ferron, 1981; Kershaw et al. 1999).

The saprophytic phase starts after the death of the host insect. Hyphae colonise the whole insect body and penetrate mainly through the thin cuticle parts like intersegmental membranes to the surface of the insect. The fungus overgrows the surface of the cadaver with a white mycelial layer, which forms the conidiophores. The conidia are produced on top of the conidiophores (Fig. 1). Since they are usually green the colour of the mycosed insects turns from white to green giving the fungus the common name “green muscardine”. The colour of the spores varies from olivaceous to yellow green or to dark green. Conidiophores that arise from the vegetative hyphae branch irregularly (usually 2-3 branches at each node). Conidia are ordered in chains, are single-celled and thin walled. The size of the conidia differs between subspecies. The conidia of *M. anisopliae* var. *anisopliae* are about 3.5 – 9 µm long, and they are on average 5-8 µm long and 1.5 – 3.5 µm wide. The life cycle is completed with the infection of new hosts.

Generally, germination and successful infection depends on a number of factors, which are linked with the fungus, the host and the environment.

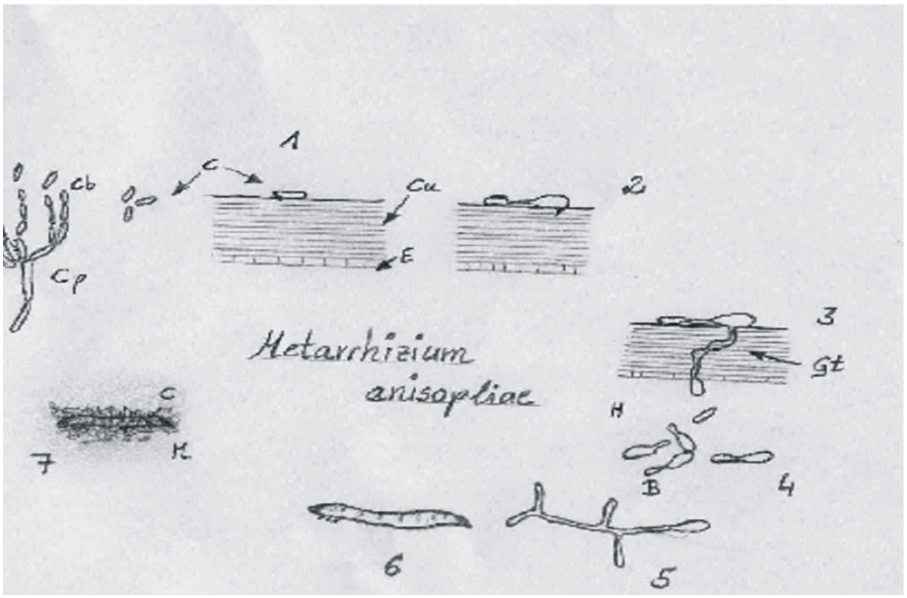

Figure 2: Schematic drawing of the life cycle of *Metarhizium anisopliae*. 1. Adhesion of conidia (C) on the cuticle (Cu). 2. Germination of the conidia on the surface of the cuticle and formation of an appressorium. 3. Penetration of the germination tube (Gt) through cuticle and epidermis (E) into the haemocoel (H) of the insect. 4. Production and multiplication of blastospores (B) in the haemocoel. 5. Germination of blastospores and colonisation of the insect body 6. Mummified insect cadaver filled with mycelium of *M. anisopliae* 7. Outgrowth of hyphal strands or synnemata (Sn) 8. Growth of conidiophores (Cp) with conidia building cells (Cb) forming sterigmata (illustration by S. Keller, modified)

3.4 Ecology of Metarhizium anisopliae

M. anisopliae is a soil-borne fungus and consists of numerous genotypes with a worldwide distribution from the arctic to the tropics. No reports on *M. anisopliae* as an air-borne fungus are known (Zimmermann, 2007). Methods to study distribution and density are established. Selective medium with antibiotics and fungicides to suppress saprophytic and concurrent organisms are used to determine densities expressed as colony forming units per gram (CFU/g) soil (Zimmermann, 1993; Strasser et al. 1996; Inglis et al. 2001). For "fungal collections" or qualitative soil analysis the "*Galleria* Bait Method" is often used (Zimmermann, 1993). The larvae of the wax moth *G. mellonella*, which are very sensitive for fungal infections, are exposed to soil samples and used as bait insects.

Transmission of fungal inoculum into new areas could take place by infected individuals. Butt et al. (1998) describe the transmission of fungal inoculum by honeybees in field trials to infect the pollen beetle *Meligethes aeneus*. Spores of entomopathogenic fungi could be transferred also by earthworms (Hozzank et al. 2003), soil arthropods like collembolans, acari and small dipterous and coleopterous larvae (Zimmermann and Bode, 1983), wind and water (Inyang, 2000; Zimmermann,

2007). There is little evidence in the literature indicating that parasitoids act as direct vectors, however Fransen and van Lenteren (1993) (in Furlong and Pell, 2005) reported that a small proportion of parasitoids infected healthy nymphs of whiteflies via fungal elements attached to their ovipositors.

Numerous biotic and abiotic factors influence the ability of entomopathogenic fungi to infect their target hosts and to persist in the environment. These include antagonistic enemies, behaviour, physiological condition and age of the host; pathogen vigour and age; sunlight, desiccation, temperature, humidity and inoculum thresholds, presence of pesticides (Butt, 2002). All these aspects are discussed in paper 3 and 4 of the thesis. Lacey and Goettel (1995) describe the successful use of entomopathogenic fungi as microbial control agents as a combination of the use of the right propagule, formulated in an optimum fashion and applied at the right time to a susceptible host.

The establishment and persistence of *M. anisopliae* is a major factor for the control success of the agent. This and the ability to remain active in the environment can be defined as environmental competence (Jackson, 1997). The soil appears to be a favourable medium for application of fungal inoculum, however, it is a highly complex medium, varying in mineral composition, texture and structure and displays an extremely competitive environment (Keller and Zimmermann 1989; Jackson, 1999). A detailed experiment with description and discussion of the persistence of *M. anisopliae* spores in the soil, on the soil surface and on maize leaves for *D. v. virgifera* control is described in paper 3 of this thesis.

Since not only pest insects can be attacked by this fungus, the evaluation of non- target effects and the establishment of a risk assessment is needed when fungal strains are applied. As *M. anisopliae* is known to infect a broad range of insects of different orders, special focus has to be given on strain level. Most of the listed host insects of *M. anisopliae* belong to the Coleoptera and especially to soil-dwelling insects. Certain strains and genotypes are more restricted (Ferron et al, 1972). Jaronski et al. (1998) reported that isolates are even more specific under field conditions compared to laboratory studies. Strains of the entomopathogenic fungus vary in virulence, persistence, host range and other criteria, like temperature optima, UV- resistance, growth and sporulation ability.

Isolates often show a high level of specificity to the species from which they were isolated (Klein and Jackson, 1992). This hypothesis is supported by molecular studies. Distinct pathotypes within species can often be determined and defined with genetic markers (Maurer et al. 1997). It is suggested, that the interaction between the insect pest and the pathogen is part of a long process of coevolution. In this context entomopathogenic fungal strains used for microbiological control have at least a temporary advantage in this releationship.

Within the last years risk assessment studies of non-target organisms gained in importance. Most of these studies have been carried out in laboratories exposing insects to a defined fungal inoculum (physiological susceptibility) (Goettel et al. 1990; Furlong and Pell, 2005). However, for field applications the ecological susceptibility is important and has to be determined for qualitative conclusions. Furlong and Pell (2005) discussed the interactions between entomopathogenic fungi and naturally occurring arthropod enemies, while Zimmermann (2007) reviewed in detail the safety of *M. anisopliae* for biological control of pests with the aim to summarise all relevant safety data, which are necessary for the commercialisation and registration process. He concluded that on the basis of the present knowledge, *M. anisopliae* is considered to be safe with minimal risks to vertebrates, humans and the environment. Possible side effects of *M. anisopliae* on non- target organisms under laboratory and field conditions are also summarised by Goettel et al. (1990), van Lenteren et al. (2003) and Vestergaard et al. (2003).

During this project side effects on non – targets after a fungal application were evaluated in field experiments in Hungary and summarized and discussed by Babendreier et al. (in prep.).

During the last years *M. anisopliae* isolates have been characterized by various molecular techniques in addition to morphiological identifications. Randomly Amplified Polymorphic DNA (RAPD) and polymerase chain reaction (PCR markers) were used to study the genetic diversity and releationships among *M. anisopliae* isolates, indicating a high genetic diversity (Fegan et al. 1993; Tigano-Milani et al. 1995; Enkerli et al. 2005).

In paper 3 the identification of *M. anisopliae* strains by simple sequence repeats (SSR, microsatellite loci) is described. The persistence of the fungus applied as fungus colonised barley kernels in the soil was evaluated and microsatellite markers were used for identifications and to distinguish between different re-isolated strains of *M. anisopliae*.

4. Preliminary studies in the laboratory

Beside the labour intensive and time consuming field studies small experiments were conducted during winter times in the laboratory of the research station Agroscope Reckenholz –Tänikon ART. The aims of these experiments were improvements of the cultivation and mass production system for the selected fungal isolates. These experiments are not published yet and no manuscripts are in preparation.

4.1 Spore production of Beauveria spp. and Metarhizium anisopliae in different liquid media

The quantities of submerse spores produced by *M. anisopliae* and *Beauveria brongniartii* was evaluated in three different liquid media ("corn steep", "skimmed milk" and "complete medium").

Recipe of the liquid mediums (Corn steep and Skimmed milk):

Solution 1: 100 ml tap water, 4 g of **X,** 0.452 g KH2 PO4, 0.76 g Na2HPO4

Solution 2: 100 ml tap water, 6 g saccharose

X was either corn steep (Roquette, Lestrem) or skimmed milk (DifcoTM, France).

The two separately autoclaved liquids were mixed together at about 60°C and stirred.

Recipe of the complete medium:

100ml deionised water, 0.036 g KH_2PO_4, 0.16 g Na_2HPO_4 ($2H_2O$), 0.1 g KCL, 0.06g $MgSO_4x7H_2O$, 0.07 g NH_4NO_3, 0.5 g yeast extract (0.5%), 1 g Glucose (1%).

Conidia of two fungal isolates ("2277", "714") of *M. anisopliae* and two fungal isolates of *B. brongniartii* ("Gelbschale1", "Carabid4") were transferred with a loop to 100 ml of each liquid medium. The spore suspension was shaken for six days at 22°C (± 2°C) on a longitudinal shaker to produce submerse spores (detailed description of material and methods in paper 3, section *Origin of fungus isolates and production of fungus colonized barley kernels (FCBK) in the lab*).

Samples of the submerse culture of each fungal strain and each medium were diluted and the spores counted in a haemocytometer to calculate spores produced per ml liquid medium.

Results showed that *B. brongniartii* strains produced on average 6.2 x 10^7 sp/ml, while *M. anisopliae* strains produced on average more than 100 times less spores (3.6 x 10^5 sp/ml) in liquid media.

For mass production of blastospores of *Beauveria* the "complete medium" showed highest densities with an average of 1.35 x 10^8 sp/ml, while the "skimmed milk" yielded an average of 2.95 x 10^7 sp/ml and even lower spore quantities of about 2.19 x 10^7 sp/ml were produced in "corn steep" medium.

In contrast, *M. anisopliae* strains showed opposite results in submerse spore production and in all variants less spores than *Beauveria*: It produced most submerse spores in the "corn steep" medium with an average of 9.25 x 10^5 sp/ml followed by the medium "skimmed milk" with about 1.05 x 10^5 sp/ml. Surprisingly the lowest spore production was found in contrast to *B. brongniartii* in the "complete medium" with an average of 4.65 x 10^4 sp/ml.

4.2 Germination rates of spores of Metarhizium anisoplaie strains stored at 4°C

As fungal isolates have to be stored sometimes due to unexpected factors (like unfavourable conditions for applications in the field) germination rates of *M. anisopliae* isolates (strain Bipesco 5 and strain 2277), stored on medium plates at 4°C for one year compared to fresh isolates stored on medium plates for one week in 4°C, were evaluated.

Spores of one year and of one week old cultures were suspended in 0.05% Tween80 to a final concentration of 1 x 10^6 sp/ml. 20 µl of the spore suspension were transferred with a pipette on slides, covered with a layer of Sabourod-2% Glucose-Agar (Fluka, Buchs). The slides were incubated in a humid chamber at 25°C. For each variant 3 replicates (slides) were done. After an incubation of 24 and 48 hours the germination rates of a total of 100 spores per slide were determined.

Germination rates after 24 hours incubation of isolates stored for one week ranged from 15% to 84% with an average of 54% germination. After 48 hours incubation an average of 75% of the spores germinated with lowest rates of 50% and highest of 100% germination (Figure 3).

Germination rates after 24 hours incubation of isolates stored for one year ranged from 0% to 2%. After 48 hours incubation the same situation could be observed with the highest germination rate of only 3%, but in most samples no spores germinated any more (Figure 4). Therefore, long time storage of *M. anisopliae* isolates on medium plates at 4 °C should be avoided.

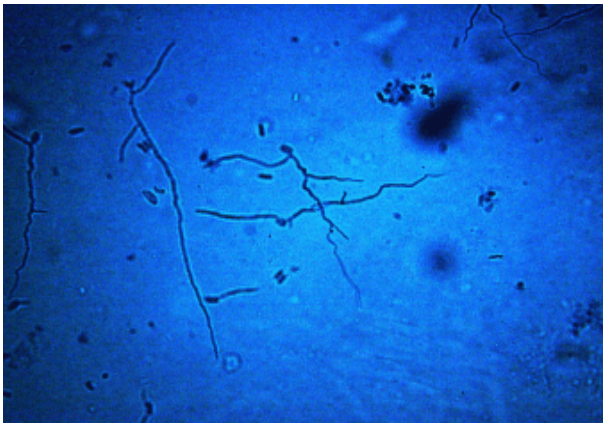

Figure 3: *M. anisopliae* spores, stored on medium plates at 4°C for one week. Germination tubes after 24 hours on Sabourod-2% Glucose-Agar

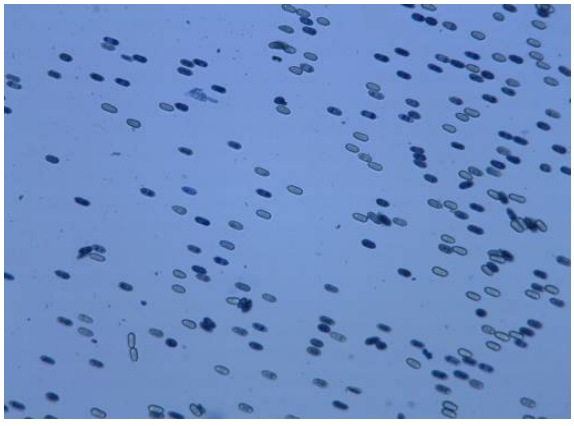

Figure 4: *M. anisopliae* spores, stored on medium plates at 4°C for one year. No germination could be observed after 48 hours.

4.3 Fungal density (CFU/g soil) in the soil at 12°C and 22°C after application of fungal colonized barley kernels

Fungus colonized barley kernels (FCBK) were applied into the soil, when fungal mycelium was covering the kernels and no sporulation was visible yet. The quantity of spores produced on FCBK in the soil was evaluated in the laboratory at two different temperatures (12°C and 22°C) and after two time intervals (one and three months after application).

One FCBK was either placed on 10 g of sterile soil or on 10 g of native (not sterile) soil and incubated either at 12°C or 22°C for one month or three months. Sterilized and native soil without a FCBK was used as control. Each variant was repeated six times. After incubation fungus densities were checked quantitatively by plating soil suspensions on selective medium (Strasser et al. 1996). The soil was shaken with 100 ml of 0.01% Tween 80 for 3 h at 100 rpm on a longitudinal shaker. Then, 100 µl of the soil suspension was plated on the selective medium and incubated at 22°C (±2°C) in the dark for ten days. Colony forming units (CFUs) per Petri dish were counted and the fungus density per g fresh soil was calculated. The results are shown in Figure 5. No fungus was found in the sterile control. Just 50 CFU/g soil were found once in the native control 1 month after incubation at 22°C. FCBK incubated for one month at 22°C showed highest spore densities in sterile soil (average: 2.6×10^4 CFU/g soil), while in native soil lower, but similar densities were found at 12°C and 22°C (1.4×10^4 and 1.1×10^4 respectively). Three months after incubation significantly increased densities of about 4.8×10^4 CFU/g soil at 22°C and 3.8×10^4 CFU/g soil at 12°C were found in native soil, while in sterile soil just $1 – 8 \times 10^3$ CFU/g soil were found.

It seems that 3 months after application of FCBK into native soil highest colony forming units are reached. Other soil organisms inhibit entomopathogenic fungi very easy and this could be one reason for the relatively long time span to establish in naitive environements.

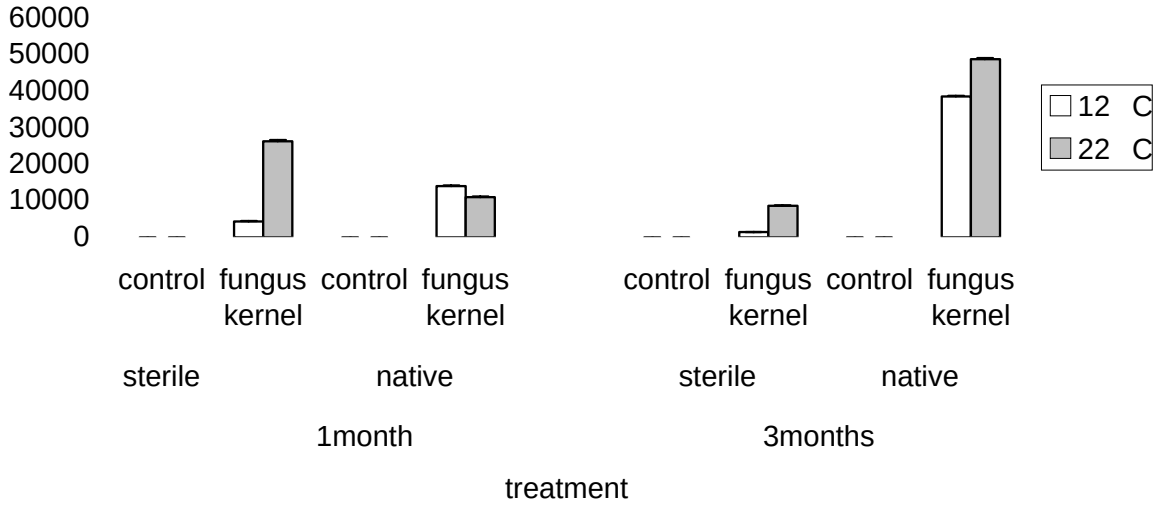

Figure 5: Colony forming units (CFU)/g soil after incubation of one fungus colonized barley kernel for one or three months in sterile or native soil at 12°C or 22°C.

4.4 How many spores are produced by one gram of FCBK?

This experiment is in relation to the former one. The physiological production capacity of one gram FCBK was evaluated. FCBK were transferred to moist filter paper for sporulation. The spores from fully sporulating kernels were washed off by stirring them in sterile water containing 0.05% Tween 80. The spores were counted in a haemocytometer and the spores per fungus kernel or g FCBK calculated. One fungus kernel produced on average 5.36×10^7 spores (minimum: 2.7×10^7; maximum 1×10^8 spores). On average one gram of sporulating FCBK produced about 7.15×10^8 spores with a minimum of 3.8×10^8 spores/g and a maximum of 1×10^9 spores/g FCBK.

4.5 Bioassays with the soil drenching method

In addition to the scientific paper 2 another type of bioassay with larvae of *D. v. virgifera* was conducted by drenching the soil with *M. anisopliae* spores (two strains: “Bipesco 5” and “2277”). We soaked 50 ml of sieved sand-soil mixtures with either 10^5, 10^6, 10^7 or 10^8 *M. anisopliae* spores/g soil to a final water content of 10%. The control group was drenched with the same amount of sterile water. Ten second to third instar larvae of *D. v. virgifera* were placed in the drenched soil together with a germinated maize seed as food. Twenty-five replicates were carried out for each fungal strain and concentration. The whole set up was repeated twice. After incubation of one week at 22°C (± 2°C) and 55% r.h. we searched for the larvae by crumbling the soil. Numbers of alive and dead larvae were counted and efficacy rates calculated in relation to the untreated control by using the formula of Abott (1925).

The results showed that highest efficacies were obtained with the highest spore concentration (Figure 6). Isolate 2277 showed a very low efficacy of about 2% at concentrations of 10^5 till 10^7, while the highest concentration of 10^8 spores/g soil resulted in 66% efficacy. Bipesco 5 showed an increased efficacy with increased spore concentrations (i. e. 3%- 49% efficacy).

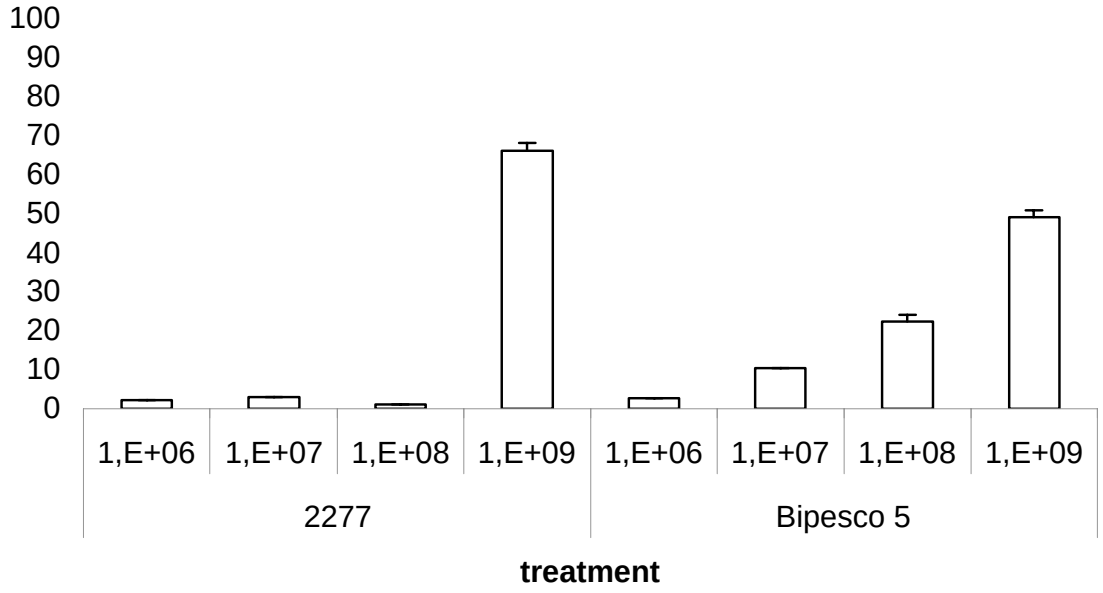

Figure 6: Efficacy (%) of the *M. anisopliae* strains 2277 and Bipesco 5. Bioassay with larvae *of D. v. virgifera* by drenching soils with different concentrations of *M. anisopliae* spores.

5. General conclusion and outlook

In this study the potential of entomopathogenic fungi for the biological control of the western corn rootworm *Diabrotica v. virgifera* in Europe was evaluated. Lacey & Goettel (1995) describe the successful use of entomopathogenic fungi as microbial control agents as a combination of the use of the right propagule formulated in an optimum fashion and applied at the right time to a susceptible host. One of the greatest challenges in the development of fungi as microbial control agents is to clarify the constraints and to develop ways to overcome them. In this thesis, we established the principles for the microbial control of this pest and discussed the further steps to improve the method. We investigated four major topics:

1) Natural occurrence of insect pathogenic fungi in the pest populations

The aim of the survey was to find strains, highly virulent and best adapted to the host and to soil and climatic conditions. We found very few naturally occurring entomopathogenic fungi (EPF) on larvae as well as on adult beetles. However, in comparison to the survey of Toepfer and Kuhlmann (2004) we found mainly the entomopathogenic species *Metarhizium anisopliae* and only occasionally *Beauveria* spp. In addition to that we found entomoparasitic nematodes on larvae too. This survey was very labour intensive and time consuming; nevertheless it is the basic step to search for antagonists. In 2005 we found eight strains of *Metarhizium anisopliae* and one strain of *Beauveria* spp. attacking *D. v. virgifera*.

During the last three years, more strains of *Metarhizium anisopliae* and *Beauveria* spp. were found, sometimes on field-collected adults or in rearing cages. These strains were isolated as well and stored in the laboratory. However, time and capacity did not allow to screen these additionally found strains. Furthermore, in winter 2005 /2006 we had to select the strains to produce the inoculum for the field applications in the following years. All isolates are stored and available for further biocontrol activities. Ideally, they should be tested for virulence against the target and for growth characteristics in mass production systems.

2) Selection of entomopathogenic fungi for the control of *Diabrotica virgifera virgifera*

The selection of virulent strains is a prerequisite for an efficient control of insects by fungal pathogens. For this screening, a standard bioassay method used in the laboratory of ART Reckenholz was used. We dipped the larvae and adults into a defined spore suspension and determined mortality and infection rates after a defined time span. Larvae were reared by our project partner CABI Delémont and we used 2nd to young 3rd instar larvae. The rearing of larvae in a sand-soil mixture or on maize seeds was successfully done by CABI Delémont, while the rearing to the adult stage prooved to be complicated. Therefore, the bioassays with adults were carried out with field-collected adults in Hungary.

Furthermore, these bioassays were conducted under controlled and optimal temperatures, humidity and light conditions for the entomopathogenic fungi. Adult *D. v. virgifera* were more susceptible to entomopathogenic fungi than larvae. It seems that larvae are more adapted to soil conditions and to soil borne pathogens. These findings were proved by a "soil drenching"bioassay. Only high spore concentrations of 1 x 10^8 sp/g soil caused infections. Such high concentrations will not be reached after application of fungus colonized barley kernels in the field.

It is a big challenge to incorporate concentrations of fungal propagules into the soil which are high enough to control the pest population. Improvements may be achieved by research on the following topics: Virulence under fluctuating temperatures and humidities, development of media which increase virulence and spore yield, search for more virulent fungal starins and improvement of long-term storage with special attention to genetic stability.

3) Persistence of *Metarhizium anisopliae* in the soil, on the soil surface and on maize leaves

The formulation of the fungus as "fungus colonized barley kernels" (FCBK) is shown to be a simple strategy to apply the fungus into deeper soil layers (where the soil dwelling stages of *D. v. virgifera* live) with simple standard equipment. The costs for farmers are reduced when FCBK are applied in one step together with sowing. This is also optimal for the development of the fungus because of the sufficient soil moisture, protection from high temperatures and

avoidance of UV light. Furthermore, we found that the application of the product at sowing resulted in highest spore densities exactly at the time when *D. v. virgifera* larvae develop in the soil. Still after 15 months, relatively high fungus densities were found which bears the potential to reduce the pest insect in subsequent years. Further experiments should be done to prove or deny the hypothesis that fungal products with a high persistence are able to control *D. v. virgifera* over years.

For the persistence trials we used molecular markers, which were able to distinguish between different strains of *M. anisopliae*. This prooved to be a valuable tool to monitor the applied fungal strains. Molecular tools are expensive and their use is time consuming. Time can be safed by direct isolation of fungal DNA from soil samples, a method that is under development (Schwarzenbach et al. 2007; J. Enkerli, pers. communication).

We investigated the interactions between the applied strain and the native population of *Metarhizium anisopliae* in soils (paper 3) with respect to competition or replacements of the native population. Based on a relatively small sample set we did not find any impact. Further research with more isolates obtained from soil samples before and after inoculation of a fungal strain would strengthen these results.

The optimal growth and sporulation of the fungus on the barley kernels is a crucial requirement to introduce enough infective propagules into the soil. The quality of the product has to be checked before the application into the soil. We determined the fungal density by plating soil samples on medium plates and counting colony forming units (CFU) (Strasser et al. 1996). In comparison to the “Galleria bait method“(Zimmermann, 1986) a quantitative result could be given. However, not any method is able to distinguish between the “fungal stages” living in the soil, if spores or mycelium are detected on the medium. The soil represents a habitat with high humidity that is usually optimal for fungal growth. Kessler (2005) reports, that soil moisture is important for fungal growth, but as long as the kernels are placed in a soil depth between 5 to 10 cm and the application slits are closed, the moisture conditions for the development of the fungus are sufficient.

The application of a spore suspension against adult *D. v. virgifera* could be an alternative control method. First semi-field trials on maize plants showed promising results (paper 4). Therefore, the formulation of fungal spores on plants should be a main task to prolong persistence and infectivity on leaves. Many of the constraints could be overcome through formulation and application strategies (Zimmermann, 1994). Formulations in oil rather than water can increase virulence under low humidity (Bateman et al. 1993) and prolong contact

with the host insect; also, additions of UV protectants can increase field persistence (Inglis et al. 1995).

The ecological fitness of a pathogen and its efficacy as biological control agent depends also on its ability to establish and persist in the ecosystem of the pest insect under fluctuating, often sub-optimal environmental conditions. For the next steps experiments to evaluate the virulence of strains under sub-optimal temperatures or humidities are suggested.

4) Field trials with the entomopathogenic fungus *Metarhizium anisopliae* for Western Corn Rootworm control

First applications of FCBK in Hungarian maize fields reduced the emergence of *D. v. virgifera* by 43%. We applied about 50 kg FCBK/ha, relating to 4-7 x 10^{13} sp/ha. This concentration is considered to be too low for the infection of the small, short- living *D. v. virgifera* larvae. According to Ferron (1981) an initial concentration of 10^{16}-10^{17} spores/ha is needed for small insects. Further trials with increased amounts of FCBK and therefore increased infection pressure is recommended.

Root feeding coleopteran larvae, concealed in soil burrows are typical soil dwelling pests. These insects have a close association with the soil and it is not surprising that they are hosts to many soil – borne pathogens. It is almost a contradiction, that soil dwelling pests are characterized by their resistance to generalist entomopathogens (Jackson, 1996). Thus, while there is often a wide range of organisms to choose from, it may be difficult to find agents which are effective for control of soil dwelling pests in the field (Jackson, 1999). Therefore, successful biological agents have in common their high degree of specificity. Isolates often show a high level of specificity to the species from which they were isolated (Klein and Jackson, 1992). A permanent evaluation and selection of new strains as well as quality checks of the applied strains (paper 1) is suggested.

The efficacy of spore suspensions applied against adults could certainly be improved by a suitable formulation. Moreover, the target pest should be contacted directly by the spore suspension. To optimise the efficacy of the spore application we suggest repeated treatments during flight peaks. Timing of applications should be coincidend with beetles swarming. Further, special focus should be given to the application technique. When fungal spores are applied against adults not only adult mortality, but also sub-lethal effects of the fungus on

female *D. v. virgifera* must be considered like reduced fertility, altered egg laying behaviour and reduced viability of the progeny.

An alternative way could be the combination of entomopathogenic fungi (EPF) with the application of entomoparasitic nematodes, i.e. nematodes for a fast action and EPF incorporated into the soil for a long-term control (paper 5). Also, studies combining nematodes or EPF with Bt-maize have been conducted (Meissle, pers. communication).

Very high infestations of *D. v. virgifera* and damages on maize plants are mainly found in maize fields, which are cultivated in monoculture over several years. Normally, organic farmers are not dealing with the problem of *D. v. virgifera* infestations in their maize fields. Nevertheless, they are potential customers for biological products to control this pest insect. Although the application of *M. anisopliae* did not result in a sufficient control of *D. v. virgifera* important information were obtained through this thesis which may help to control other agricultural pests like wireworms and rape blossom beetles with entomopathogenic fungi.

In conclusion, the results of this thesis demonstrated the existence of natural pathogens in *D. v. virgifera* populations in Europe. First results of an application of *Metharzium anisopliae* in field trails against this novel pest insect are given. The success of a biological control agent depends on a number of factors: the selection and permanent evaluation of strains in standardised bioassays, the use of the right initial spore concentration with optimal formulation and application time. The application of fungus colonized barley kernels in spring together with the sowing of maize seeds is suggested as we found the highest fungal density exactly at the time, when the pest insect is in it`s larval stage. For the application of spore suspensions against adults new formulations and adapted application techniques have to be developed. A permanent quality control of the fungal strain is recommended. Mass production methods for high yields of conidia have to be established and smaller granules as alternatives to the relatively big FCBK could be an option.

6. References

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7. Scientific papers

7.1 Natural occurrence of insect pathogenic fungi and insect parasitic nematodes in Diabrotica virgifera virgifera populations (published in BioControl)

7.2 Selection of entomopathogenic fungi for the control of the western corn rootworm Diabrotica virgifera virgifera (published in Journal of Applied Entomology)

7.3 Persistence of the biological control agent Metarhizium anisopliae in the soil, on the soil surface of a maize field and on maize leaves

7.4 Field trials with the entomopathogenic fungi Metarhizium anisopliae for Western Corn Rootworm Diabrotica virgifera virgifera control

7.5 Comparative efficacy assessment of fungi, nematodes and insecticides to control western corn rootworm larvae in maize (submitted to BioControl)

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ORIGINAL PAPER

Natural occurrence of insect pathogenic fungi and insect parasitic nematodes in *Diabrotica virgifera virgifera* populations

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Abstract The Western Corn Rootworm *D. virgifera virgifera* Le Conte (Coleoptera: Chrysomelidae), a serious pest of maize, has been recently introduced into Europe. Several approaches for its control are presently under investigation including microbial agents. In order to get information on the role of naturally occurring pathogens in the regulation of *Diabrotica* populations, we started an investigation in established populations in Hungary, Romania, Serbia, Austria, and Italy in 2005 and 2006. In infested maize fields in Hungary, plants and their root systems were grubbed out and larvae and pupae were collected. Adult *D. v. virgifera* were collected in Hungary, Austria, Romania, Serbia and Italy. Additionally, the occurrence of entomopathogenic fungi in soils of maize fields was determined using *Galleria mellonella* and *Tenebrio molitor* larvae as bait insects. The density of entomopathogenic fungi was obtained by plating soil suspension on selective medium. *Metarhizium anisopliae* and *Beauveria* spp. infections were found in 1.4% of field collected larvae, 0.2% of field collected pupae and 0.05% of field collected adults. Whereas natural infections of *D. v. virgifera* were rarely found, a high density of insect pathogenic fungi was recorded in Hungarian soils. *M. anisopliae* could be detected in every maize field either using the ''bait method'' or a ''selective medium'' method. This is the first report of a natural occurrence of entomoparasitic nematodes (*Heterorhabditis* sp., *Steinernema* sp.) in *Diabrotica v. virgifera* in Europe.

Keywords Chrysomelidae · *Diabrotica virgifera virgifera* · Entomopathogenic fungi · Entomoparasitic nematodes · *Metarhizium anisopliae* · *Beauveria* spp. · *Heterorhabditis* sp. · Western corn root worm

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Introduction

Diabrotica virgifera virgifera Le Conte (Western Corn Rootworm) (Coleoptera, Chrysomelidae), a serious quarantine pest of maize, has been recently introduced into Europe (Kiss et al. 2005). At present, *D. v. virgifera* occurs nearly all over Central Europe and beetles are still migrating further into previously unaffected European regions (Miller et al. 2005). An important alternative control measure providing environmentally friendly and sustainable plant protection is the use of biological control agents (Burges 1981; Lacey and Kaya 2000). A first step for the development of biological control measures is to find strains highly virulent and host specific to the target host insect (Butt et al. 2001). This study aimed to find strains of naturally occurring entomopathogenic fungi (EPF) and nematodes (EPN) in different life stages of *D. v. virgifera* in order to select potential biological control agents.

Materials and methods

Collection and preparation of larvae and pupae

Larvae and pupae of *D. v. virgifera* were collected from a total of 240 maize plants in twelve Hungarian fields (Table 1). In all fields, maize had been grown in monoculture for at least 2 years and all fields hosted natural *D. v. virgifera* populations. Roots with adhesive soil (blocks of 50 cm × 50 cm × 30 cm) of twenty lodging plants per field were collected. Due to the small size of first instars, only second and third instar larvae and pupae were detectable by crumbling the soil. Insects were transferred to moist filter paper in plastic cups (6 cm × 5 cm) closed with a lid. Larvae and pupae from the same plant were kept together in one cup and incubated in the laboratory at room temperature ranging from 20 to 26°C. Dead individuals were separated daily to Petri dishes (Ø 4 cm) with moist filter paper to promote fungal growth. *Metarhizium anisopliae* and *Beauveria* sp. were identified by mycelium growth on the insect and after isolation on media by spore morphology and colour (Samson et al. 1988). The fungi were isolated on selective medium with antibiotics (Strasser et al. 1996) and incubated at 22°C (±2°C) and 70 % r.h. in darkness. All remaining dead larvae were individually placed on a nematode trap (Spears 1968) in Petri dishes. Nematode infection was determined by (a) recording grey colouring of larvae typical for *Steinernema* spp. or red colouring typical for *Heterorhabditis* spp.; (b) observing adult EPNs in the larval body using a stereomicroscope; and/or (c) by capturing the infective juveniles of EPNs that had left the cadaver. Whether the nematodes were entomopathogenic was verified by infecting *Galleria mellonella* larvae with infective juveniles.

Collection and preparation of adult *D. v. virgifera*

In Hungary, adult *D. v. virgifera* were collected at five sites (in "*Kondoros A*", in "*Kondoros B*" and in "*Oroshaza A*" in the year 2005 and in "*Kondoros A*" "*Szekszard*" and "*Szikancs*" in the year 2006). In *"Lovrin"* (Romania), *"Kula"* (Serbia) and *"Como"* (Italy), adult beetles were collected at one site between July and August 2006. Beetles were caught by shaking them from maize leaves and silk

Table 1 Mean number of colony forming units/g fresh soil (CFU/g soil) out of six samples/site and the standard deviations (in brackets), percentage of infected bait insects (*G. mellonella* and *T. molitor* larvae) and collected and with entomopathogenic fungi and entomoparasitic nematodes infected larvae and pupae of *D. v. virgifera* on 20 maize plants per site and on twelve sites in Hungary 2005

Locations in	EPF densities	EPF occurrence		Number		EPF occurrence on *D. v. virgifera*			EPN occurrence on *D. v. virgifera*	
Hungary	Mean number CFU/g soil (SD)	% Infected		Collected *D. v. v.*		% Infected		Species	% Infected	Genus
		G. mellonella	*T. molitor*	Larvae	Pupae	Larvae	Pupae		Larvae	
HodmezovasarhelyA	137 (4.2)	0	16.6	39	4	0	0	–	0	–
HodmezovasarhelyB	3.571 (37.8)	4.1	50	25	0	0	0	–	8	*Heterorhabditis*
KondorosA	620 (4.2)	0	16.6	30	40	0	0	–	0	–
KondorosB	1.591 (56.4)	0	8.3	68	47	0	0	–	1.7	*Heterorhabditis*
Kunagota	6.046 (175.8)	8.3	16.6	112	46	0	0	–	0	–
Bataszek	233 (3.7)	0	0	20	39	0	0	–	0	–
Szekkutas	716 (18.5)	0	8.3	12	8	0	0	–	5	*Heterorhabditis* or *Steinernema*
Gyomendröd	191 (3.9)	0	8.3	50	34	0	0	–	0	–
OroshazaA	846 (18.3)	0	8.3	30	32	6.6	3.1	*M. anisopliae*	0	–
OroshazaB	104 (1.9)	0	33.3	11	12	0	0	–	0	–
Szatymaz	2.454 (36.8)	8.3	8.3	37	6	8.1	0	*M. anisopliae*/ *Beauveria*	0	–
Szeged	2.646 (51.1)	0	25	44	16	2.3	0	*M. anisopliae*	6.6	*Heterorhabditis* or *Steinernema*
Σ				478	284					
Means	1.597	1.7	16.6			1.4	0.2		1.8	

into a funnel with an attached gauze bag. In the laboratory beetles were transferred to rearing cages (300 mm × 300 mm × 500 mm) supplemented with agar as a water source, a food source of artificial diet and maize silks, which were changed weekly. Dead beetles were removed once a week with an aspirator and transferred to small Petri dishes (Ø 4 cm) with moist filter paper and incubated at 22°C (±2°C) and 70% r.h.

In Austria adult beetles were collected with pheromone traps (VAR traps, Csalomon, Hungarian Academy of Sciences, Budapest, Hungary) at four sampling plots at an experimental site of the Austrian Federal Office for Food Safety (AGES, Vienna) in eastern Austria (Deutsch Jahrndorf, Burgenland) during a 6-week period in August and September 2005. One of the sampling plots had been treated with *H. bacteriophora* (nema-green, e-nema GmbH, Raisdorf, Germany) on July 4, 2005, the others served as untreated controls. Adult *D. v. virgifera* were removed once a week from the traps and stored at 4°C until dissection. After 5 min in the deep freezer (–18°C) the presence of nematodes was recorded by dissection of the insect and observation of the haemolymph. Smears were dried, fixed with methanol and then stained with Giemsa solution (Weiser 1977) prior to microscopic inspection at 40–1,000 × magnification to search for the presence of protozoan and other entomopathogens.

Entomopathogenic fungi in soils of Hungarian maize fields

Six soil samples per field were taken with a core borer (diameter 3 cm) to a depth of 25 cm at the same locations where *D. v. virgifera* larvae and pupae had been collected in Hungary (Table 1). Fungal presence was checked qualitatively using bait insects. Two *Galleria mellonella*- and two *Tenebrio molitor*-larvae per sample were put together into plastic cups (5 cm × 6 cm), filled with 60 g sieved soil. After covering the cups with a lid, they were incubated at 22°C (±2°C) in the dark. The first 5 days the cups were turned daily to force larval movement. After 3 weeks incubation, *T. molitor* and *G. mellonella* larvae infected with fungi were counted. Fungal densities were also checked quantitatively by plating soil suspensions on selective medium (Strasser et al. 1996). Twenty g fresh sieved soil was shaken with 100 ml of 0.01% Tween 80 for 3 h at 100 rpm on a longitudinal shaker. Then, 100 µl of the soil suspension was plated on the selective medium and incubated at 22°C (±2°C) in the dark for ten days. Colony forming units per Petri dish were counted and the fungal density per g fresh soil (CFU/g soil) was calculated.

Results

Entomopathogenic fungi on larvae, pupae and adult *D. v. virgifera*

A total of 478 larvae and 284 pupae were found on 240 maize roots (Table 1). Larvae and pupae infected with fungi were found on maize roots at three out of 12 sites; EPF infection rate of all found larvae was 1.4% and of all found pupae 0.2%. In *OroshazaA* 6.6% of collected larvae were infected, whereas in *Szatymaz* and *Szeged*, infection levels of larvae were 8.1% and 2.3%, respectively. One infected pupa was found at *OroshazaA*, which results in 3.1% infection of collected pupae at this site (Table 1). Fungal infections at these locations were caused by *M. anisopliae*, except one larva from *Szatymaz*, which was infected with *Beauveria* spp.

Table 2 Collection sites in Hungary (HU), Romania (RO), Serbia (CS) and Italy (I) and number of collected adult *D. v. virgifera*; infection rates (%) of adults (numbers *n*) by the entomopathogenic fungal species *Metarhizium anisopliae* and *Beauveria* sp.

Collection sites	Number of adults collected	% Infected *D. v. virgifera* (number of adults)	
		M. anisopliae	*Beauveria* sp.
Oroshaza(HU)	33,900	0.003 (1)	–
Kondoros A (HU)	48,550	0.02 (8)	0.008 (4)
Kondoros B (HU)	4,600	–	–
Szeksard (HU)	11,100	0.02 (3)	–
Szikancs (HU)	22,430	–	0.05 (12)
Lovrin (RO)	11,600	0.26 (30)	0.02 (3)
Kula (CS)	9,470	0.02 (2)	0.01(1)
Como (I)	4,900	0.12 (6)	0.06 (3)

Over the 2 years of collections, eight beetles from *KondorosA*, one from *OroshazaA*, and three from *Szekszard* showed infections with *M. anisopliae*, corresponding to infection rates of 0.02%, 0.003% and 0.02%, respectively. In *KondorosA* four beetles and in *Szikancs* 12 beetles were infected with *Beauveria* sp., which results in infection rates of 0.008% and 0.05% (Table 2).

In *Lovrin* 0.26% ($n = 30$), in *Kula* 0.02% ($n = 2$) and in *Como* 0.12 % ($n = 6$) of the beetles were infected by *M. anisopliae*, whereas *Beauveria* spp. have been found on three beetles (0.02%) in *Lovrin*, one beetle (0.01%) in *Kula* and three beetles (0.06%) in *Como* (Table 2).

Entomopathogenic fungi in soils of Hungarian maize fields

The mean fungal density in soil samples of all sites ($n = 12$) was 1,597 (SD: 1.81) colony forming units (CFU) per gram fresh soil (range 104–6,046) and 9.37 % infection (SD: 0.36) for both bait insects together (range 0–29 %). Significantly more *T. molitor* larvae (range 0–50 %; mean: 16.6%; SD: 13.8) were infected than *G. mellonella* larvae (range 0–8.3 %; mean: 1.73 %; SD: 3.3) ($P = 0.01$; *t*-test). The highest number of colony forming units per g soil was determined at *Kunagota* (mean: 6.046; SD: 175.9) while the highest number of infected bait insects was detected at *HodmezovasarhelyB* (4.1% of *G. mellonella* and 50% of *T. molitor*) (Table 1).

Entomopathogenic nematodes in larvae

Of the 478 larvae collected, 1.8% ($n = 9$) were parasitized by EPNs. These nine infected larvae were found in July at four out of 12 locations in Hungary (Table 1). Seven larvae were infected by *Heterorhabditis* spp. and two larvae had been killed either by a *Heterorhabditis* sp. or a *Steinernema* sp. (Table 1).

Entomopathogenic nematodes in adults

In Austria 605 beetles were collected and dissected, 365 beetles in the plot treated with nematodes and 485 beetles in the control plots. Only three beetles (a male and two females) were found with nematodes in the haemolymph, still ensheathed in the

second-juvenile cuticle. The parasitized male beetle was found on September 8 and the two parasitized females were found on September 29. These beetles originated from an experimental plot where nematodes had been applied. Due to the few individuals found, identifications were not possible. No nematodes were found in the 485 beetles collected in the control plots. In no case could any protozoan or other pathogens be detected in smears.

Discussion

This study confirms the results of Toepfer and Kuhlmann (2004) that the natural occurrence of EPFs and EPNs in *D. v. virgifera* populations in Europe is low. Entomopathogenic fungi infections were detected on larvae, one pupa, and on several adult beetles. *M. anisopliae* was more often detected than during the survey by Toepfer and Kuhlmann (2004), who found a higher infection rate of *Beauveria* spp. on adult beetles.

Natural fungal concentrations in Hungarian maize fields were found to be high; we found more than 2,000 cfu/g soil in some of the fields, similar to field surveys in Switzerland where Keller et al. (2003) reported densities of about 1,000 cfu/g in arable soils. Survival of the conidia and augmentation of EPFs in soils are fundamental criteria indicating the quality of a biological control agent (Butt and Goettel 2000; Goettel et al. 2000; Inglis et al. 2001). However, the presence of fungal propagules in the soil was not correlated with a high natural infection of *D. v. virgifera* larvae and pupae by EPFs. This could be due to the relatively low larval and pupae density (about three larvae and one pupa per plant), a reduced host—pathogen compatibility and/or environmental factors (Butt and Goettel 2000). It must be considered that first instar larvae were not collected. However, it is known that this stage suffer from a high mortality (Toepfer and Kuhlmann 2006) which might be due to fungal infections.

This study records for the first time a natural infection of *D. v. virgifera* larvae by EPNs. Most of the EPNs found in *Diabrotica* larvae belonged to the genus *Heterorhabditis*. Griffin et al. (1999), who analysed soil samples from Hungary, found *Heterorhabditis* spp., predominantly *H. bacteriophora*, in sandy soils. Our observations are the first report of EPNs in adult *D. v. virgifera.* Although they originated from a plot previously treated with EPNs, the finding demonstrates that nematodes are able to invade adults as well. EPNs probably entered the beetles before or during their emergence from the soil.

In conclusion, entomopathogenic fungi have been found as natural enemies in *Diabrotica* populations in Hungary, Romania, Serbia and Italy. Entomopathogenic nematodes have been found naturally in larvae in Hungary. The fungal isolates will be screened to investigate their potential as biological agents to control *D. v. virgifera.*

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Selection of entomopathogenic fungi for the control of the western corn rootworm *Diabrotica virgifera virgifera*

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Abstract: The western corn rootworm *Diabrotica virgifera virgifera* Le Conte (Col., Chrysomelidae), a serious pest of maize, has been recently introduced into Europe. Several approaches for its control are presently under investigation including microbial agents. During a field survey in Hungary in 2005, naturally occurring entomopathogenic fungi were found to attack this pest. These novel isolates together with standard isolates were tested for virulence against *D. v. virgifera* larvae and adults. Twenty strains of *Metarhizium anisopliae*, *Beauveria bassiana* and *Beauveria brongniartii* were used in bioassays in the laboratory. Larvae and adults were dipped into a spore suspension with a concentration of 1×10^7 conidia (con.)/ml. They were kept for 14 days at 22°C (±2°C) and 70% relative humidity. The number of infected larvae and adults were counted and infection rates were calculated. Adults were significantly more susceptible to entomopathogenic fungi than larvae. The most virulent isolate infected about 47% of larvae (*M. anisopliae* Ma2277), whereas the infection rate in adults was up to 97% (*M. anisopliae* Ma2275). Isolates of *M. anisopliae* caused significantly higher mortalities than isolates of *B. brongniartii* and *B. bassiana*. Most of the adult beetles were killed within 12 days. Isolates from *D. v. virgifera* were more virulent than those from other hosts.

Key words: *Beauveria bassiana*, *Beauveria brongniartii*, *Diabrotica virgifera virgifera*, *Metarhizium anisopliae*, bioassays, Chrysomelidae, entomopathogenic fungi, virulence

1 Introduction

The western corn rootworm *Diabrotica virgifera virgifera* Le Conte (Col., Chrysomelidae) is a serious quarantine pest of maize recently introduced into Europe (Kiss et al., 2005). The damage (plant lodging) of this chrysomelid beetle, which is caused by the larvae feeding on maize roots, was first observed close to Beograd airport in 1992 (Baca, 1994). In Europe, the larvae of *D. v. virgifera*, which hatch in May and June after a diapause of the eggs during winter, develop through three larval instars and a pupal stage into adults in the second half of July. Adult beetles can occasionally damage maize fields by feeding on pollen, silk, immature kernels and leaves (Chiang, 1973). At present, *D. v. virgifera* occurs nearly all over Central Europe and is still migrating further into previously unaffected European regions (Miller et al., 2005). In North America, control measures using granular insecticides applied to soil, seed treatments with insecticides and crop rotation were partly successful, but failed to solve the problem in the long term because of the development of resistance or adaptation to chemical insecticides and crop rotation (Spencer et al., 2005).

An important alternative control measure providing environmentally friendly and sustainable plant protection is the use of biological control agents such as pathogens, parasitoids or predators (Burges, 1981; Lacey and Kaya, 2000). Entomopathogenic fungi (EPF), such as *Beauveria* spp. and *Metarhizium anisopliae* (Metschnikoff) Sorokin, which are ubiquitous soil-borne pathogens, can attack the host in their soil-living stages (Müller-Kögler, 1965). *M. anisopliae* has a broad host spectrum and has been isolated from more than 200 insect species, mostly of the order Coleoptera (Samson et al., 1988). Successful applications of EPF against different pest insects have been reported by Ferron (1981).

In a previous study, the natural occurrence of EPF and entomoparasitic nematodes in Hungary was investigated (Pilz et al., 2007). During this study we collected nine isolates from larval and adult *D. v. virgifera* and 133 isolates from soils taken in maize fields. In the present study, we screened the virulence of 20 EPF strains against larvae and adults of *D. v. virgifera*

to select the most virulent ones for future investigations as a potential biological control agent.

2 Materials and Methods

2.1 Sources and rearing of *D. v. virgifera* and of EPF

Larvae of a non-diapausing strain of *D. v. virgifera* were obtained from the quarantine laboratory of CABI Delémont (Switzerland). They were reared at 25°C for 14 h in light and 15°C for 10 h in dark and 65% relative humidity (RH) in plastic trays (500 mm × 300 mm × 60 mm) containing a sterilized sand–soil mixture and germinated maize seeds as food source. Instars between 'late L2 and early L3' (identified by their body length) of *D. v. virgifera*, used in the bioassays, were searched in the soil. The experiments were carried out under quarantine conditions at the laboratory of Agroscope Reckenholz-Tänikon Research Station ART, Zürich.

Adult *D. v. virgifera* were collected during mass collections in the south-eastern part of Hungary (Kondoros) in July and August 2006. They were caught by shaking them from maize maize leaves and silks into a funnel with an attached gauze bag. The gauze bag was closed with a band and kept in a cool box during transportation. In the laboratory of the Plant Health Station Hodmezovasarhely, beetles were transferred to rearing cages (300 mm × 300 mm × 500 mm) and kept at 25°C (± 2°C), 40% RH at day light. Beetles were provided with a water source (water-agar), a food source of artificial diet (Sing and Moore, 1985) and maize silks, which were changed weekly. They were reared for 2 weeks in cages before being used in bioassays to avoid natural infections from the field.

The 20 fungal strains used in this study were isolated from different hosts (table 1). Nine of them were isolated from field-collected larvae, a pupa and adults of *D. v. virgifera* in Hungary in 2005, and 11 were isolated from other insect hosts and soils from Hungary, Switzerland, Austria and India (table 1). Each strain was tested against larvae and adult beetles of *D. v. virgifera*, except strain Ma5017, which was only used in bioassays against adults and strain Ma5019, which was just used against larvae. The fungal strains were maintained in Petri dishes with selective medium with antibiotics and fungicides at 22°C (± 2°C) and 70% RH (Strasser et al., 1996). Conidia from 14-day-old sporulating cultures were harvested by washing them off with 0.01% of the wetting agent Tween 80. The spore suspension was filtered through a gauze net in order to obtain individual spores. The spore concentration was determined with a haemocytometer and adjusted to a final concentration of 1×10^7 conidia (con.)/ml and stirred till the experiment was started. Additionally, 100 μl of prepared spore suspension was plated on agar plates and incubated at 22°C (± 2°C) to check the viability of the spores.

2.2 Screening for virulence against *D. v. virgifera* larvae

Batches of 10 larvae were transferred carefully with a brush into a tea sieve (mesh: 1 mm). The tea sieve was closed and they were dipped into the spore suspension for 5 s. Afterwards, larvae were placed individually in 12-well culture plates (diameter 2.5 cm, Semadeni, Ostermundingen, Switzerland, No. 92012), filled with sterilized sand and a 2-day-old germinated maize seed as a food source. The larvae were incubated at 22°C (± 2°C) and 70% RH in darkness for 14 days. Food was changed as needed, or when saprophytic fungi started to grow. When necessary, drops of sterilized water were added to the sand. The larvae were checked daily and numbers of dead larvae were recorded and transferred to small Petri dishes (diameter 4 cm) with moist filter paper to promote fungal growth on the insect.

The control group was dipped into 0.01% Tween 80. Two successive bioassays were conducted: the first with 13 and the

Table 1. *The origin of the entomopathogenic fungus species (*Metarhizium anisopliae *and* Beauveria *spp.) tested against* Diabrotica virgifera virgifera *larvae and adults and the infection rates (%) they caused after incubation of 14 days and 22°C (± 2°C); additionally LT_{50} values are given in days for adults; strains 2273–2281, 2256 and 2258 originated from Hungary; 858–997, 5017, 5019 and 5026 from Switzerland, 2062 from India and Bipesco5 from Austria*

Strain	Sample origin	Fungal species	Infection (%)		
			Larvae	Adults	LT_{50}
Ma2273	*Diabrotica* adult	*M. anisopliae*	40	90	6
Ma2274	*Diabrotica* adult	*M. anisopliae*	37	80	10
Ma2275	*Diabrotica* adult	*M. anisopliae*	27	97	6
Ma2276	*Diabrotica* larva	*M. anisopliae*	37	86	7
Ma2277	*Diabrotica* larva	*M. anisopliae*	47	67	7
Ma2278	*Diabrotica* larva	*M. anisopliae*	43	62	7
Ma2279	*Diabrotica* larva	*M. anisopliae*	37	63	8
Ma2280	*Diabrotica* larva	*M. anisopliae*	27	70	7
Ma2281	*Diabrotica* pupa	*M. anisopliae*	23	66	7
Bbr858	*Melolontha melolontha*	*Beauveria brongniartii*	13	10	> 14
Bbr857	*M. melolontha*	*B. brongniartii*	7	3	> 14
Bba987	*Leptinotarsa decemlineata*	*Beauveria bassiana*	3	10	> 14
Ma997	*Agriotes* larva	*M. anisopliae*	7	46	12
Ma2256	Soil (Hungary)	*M. anisopliae*	0	60	9
Ma2258	Soil (Hungary)	*M. anisopliae*	13	80	11
Ma5026	*Agriotes* adult	*M. anisopliae*	3	32	10
Ma2062	Soil (India)	*M. anisopliae*	23	90	11
MaBipesco5	*Cydia pomonella*	*M. anisopliae*	43	62	12
Ma5017	*Agriotes* larva	*M. anisopliae*	–	46	14
Ma5019	*Phylloperta horticola*	*M. anisopliae*	27	–	–
Check	0.01% Tween 80	–	0	3	> 14

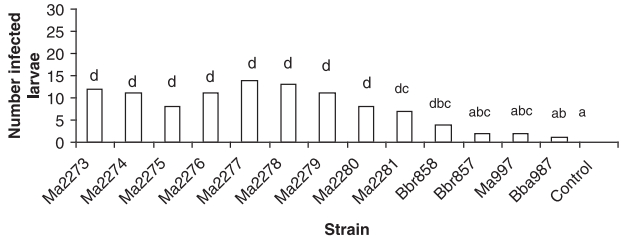

Fig. 1. *Bioassay 1: Number of infected larvae (n = 30), with infection caused by different strains of* Metarhizhium anisopliae *and* Beauveria *spp. in* Diabrotica virgifera virgifera *larvae at a concentration of* 1×10^7 *con./ml (chi-square test). For strain numbers see table 1*

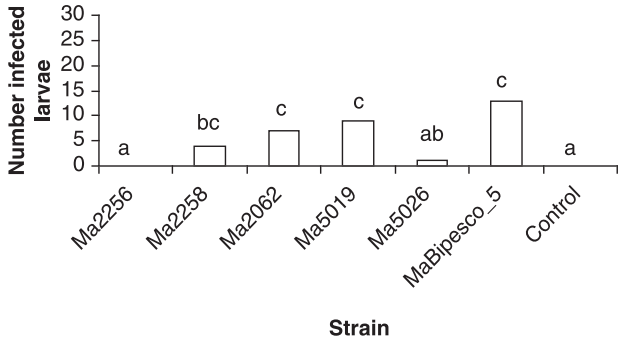

Fig. 2. *Bioassay 2: Number of infected larvae (n = 30), with infection caused by different strains of* Metarhizhium anisopliae *in* Diabrotica virgifera virgifera *larvae at a concentration of* 1×10^7 *con./ml (chi-square test). For strain numbers, see table 1*

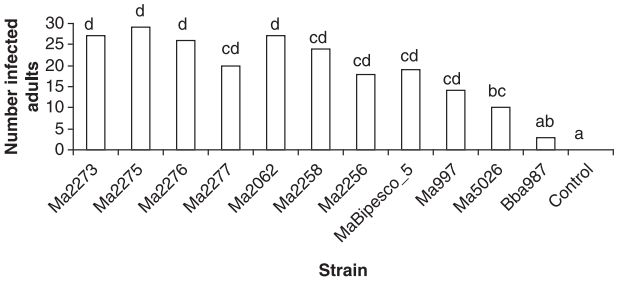

Fig. 3. *Bioassay 1: Number of infected* Diabrotica virgifera virgifera *adults (n = 30), with infection caused by different strains of* Metarhizhium anisopliae *and one strain of* Beauveria bassiana *at a concentration of* 1×10^7 *con./ml (chi-square test). For strain numbers, see table 1*

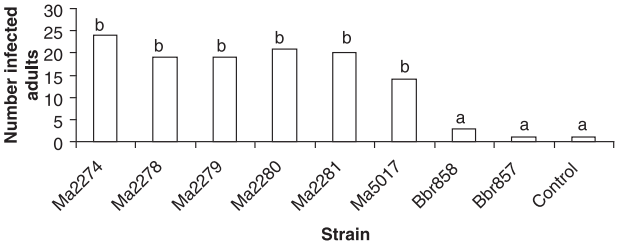

Fig. 4. *Bioassay 2: Number of infected* Diabrotica virgifera virgifera *adults (n = 30), with infection caused by different strains of* Metarhizhium anisopliae *and* Beauveria brongniartii *at a concentration of* 1×10^7 *con./ml (chi-square test). For strain numbers, see table 1*

second with six isolates, each with a control group (figs 1 and 2). Thirty larvae were used for each fungal strain and for the controls. A larva was considered as infected with the fungus, when mycelium and spores developed on the insect.

2.3 Screening for virulence against *D. v. virgifera* adults

Adult beetles were inoculated with the same fungal strains as larvae, except the exchange of the strains Ma5017 and Ma5019. Beetles which have been caught 2 weeks before inoculation were aspirated individually from the rearing cages (300 mm × 300 mm × 500 mm) dipped for 5 s into a spore suspension with a concentration of 1×10^7 con./ml and then transferred individually to a perforated plastic vial (diameter = 4.5 cm, height = 6 cm) with moist filter paper and a piece of corn cob as a food source. They were incubated at 22°C (± 2°C) and 70% RH with a light:dark cycle of 14:10 h for 14 days. Mortality was checked every second day starting on the fourth day after inoculation; on the sixth day fresh food was added. Dead beetles were removed and placed in small Petri dishes (diameter 4 cm) with moist filter paper and incubated at 22°C (± 2°C) and 70% RH. Numbers of dead beetles were recorded and infections by EPFs were identified by the development of mycelia and spores on the insect. Mortality and infection rates on the different observation days were calculated. Two successive bioassays were carried out, the first with 11 and the second with eight isolates, each with a control group. Thirty adults were used for each fungal strain and the control groups (figs 3 and 4).

2.4 Statistical analysis

Differences in virulence of the fungal strains against larvae and adults were analysed with the chi-squared test; calculations were performed using the number of insects (infected/not infected), whereas the results are discussed using the percentages (%) of infections; the average time to death (LT_{50}) was calculated by using the Survival Analysis with the program SPSS, version 11.5 (SPSS, Base 15 for Windows, 2000–2007; SPSS Schweiz AG, Zurich, Switzerland).

3 Results

3.1 Virulence of fungal strains against *D. v. virgifera* larvae

Of the 19 fungal strains tested, 18 (95%) were pathogenic for *D. v. virgifera* larvae and caused infection rates between 3% and 47%. No fungal strain caused a larval infection above 50%. The isolate Ma2277 was the most virulent, infecting 47% ($n = 14$) of the larvae. Most of the *M. anisopliae* strains were significantly more virulent than *Beauveria* strains (figs 1 and 2 show number of infected larvae caused by *M. anisopliae* and *Beauveria* spp. strains). Very low infection rates were caused by strain Ma997, originating from a larval *Agriotes* sp. (Switzerland) in the first bioassay, strain Ma2258 from soil (*Tenebrio*

molitor; Hungary) and strain Ma5026 from an adult *Agriotes* sp. (Switzerland) in the second bioassay. Seven *M. anisopliae* isolates reached more than 30% infections, but just three (Ma2277, Ma2278 and MaBipesco5) were able to infect more than 40% of the larvae. Strain Ma2256, which originated from a soil sample (Hungary), caused no infections.

On average 70% of the larvae died or failed to develop to the pupal stage in the treatments during the 14 days, although most of these were not infected by the fungus. During the first 10 days after inoculation, the natural mortality in the control group was 26% in the first bioassay and 0% in the second bioassay, whereas mortality in the treatments was on average 40% in the first bioassay and 57% in the second bioassay. Afterwards, mortality increased quickly. First signs of infection (mycelium growth and sporulation) were observed on the sixth day after inoculation. Because of the low infection rates, no LT_{50} values could be calculated for larval mortality.

3.2 Virulence of fungal strains against *D. v. virgifera* adults

Nineteen strains of EPF were tested against adult *D. v. virgifera*, 16 strains of *M. anisopliae*, two strains of *Beauveria brongniartii* and a strain of *Beauveria bassiana*. All strains were pathogenic.

In the first bioassay four strains, all *M. anisopliae*, caused more than 80% (infected $n \geq 24$) infections but they did not differ significantly from five other isolates of *M. anisopliae*, which caused infections between 40% and 80% (fig. 3). Strain Ma2275 was the most virulent with 97% ($n = 29$) infected beetles (fig. 3). Among the four most virulent strains three were isolated from *D. v. virgifera* adults and one (Ma2062) from a soil sample in India. Low infection rates were obtained with strain Ma5026 (32% infection) isolated from an adult *Agriotes* sp. and with *B. bassiana* (10% infection) isolated from *Leptinotarsa decemlineata*. No beetles were infected in the control group.

In the second bioassay, the six *M. anisopliae* strains, five originating from *D. v. virgifera* and one from an *Agriotes* larva, infected between 40% and 80% of the beetles and differed significantly from the two *B. brongniartii* isolates, which caused 3% and 10% infections, respectively (fig. 4). A single beetle in the control group succumbed to *M. anisopliae*. It is not clear if the infection occurred during the bioassay or previously in the field.

Signs of infection (growing mycelium and spore development) were first observed on the eighth day after inoculation. All beetles treated with the isolates Ma2273–Ma2281 and with the isolates Ma2062 and Ma2256 died within 14 days, although not all showed symptoms of fungal infection. In the control group, 26% (bioassay 1) and 13% (bioassay 2) of the beetles also died within 14 days.

Strain Ma2275 had the shortest LT_{50} with 5.71 days. Another seven isolates had an average mortality time of up to 8 days and another eight strains were able to kill 50% of beetles within 12 days. No LT_{50} values could be calculated for the *Beauveria* strains, which did not reach 50% mortality during the bioassays (table 1).

4 Discussion

Bioassays conducted with EPF are mostly aimed to find strains highly virulent to the target insect (Holdom and Li, 1996; Inglis et al., 2001). In our bioassays, the tested *M. anisopliae* strains were more virulent against *D. v. virgifera* larvae and adults than the three tested *Beauveria* spp. strains, which is concurrent with the natural occurrence of EPF in *D. v. virgifera* in eastern Europe (Pilz et al., 2007; Toepfer and Kuhlmann, 2006). Moreover, strains which originate from *D. v. virgifera* field populations appear to be more virulent than strains originating from other hosts. This finding is in accordance with previous studies by Hall (1982), Vey et al. (1982), Chandler (1992) and Altre et al. (1999). Samson et al. (1988) report about the broad host range of *M. anisopliae*, therefore, host specificity of each single strain is an important criterion for a potential biological control agent.

However, in contrast to our results, most bioassays against *Diabrotica* spp. showed that *Beauveria* strains were more virulent than *M. anisopliae*, e.g. *D. speciosa* showed 45% mortality when treated with *M. anisopliae* and 70% when treated with *B. bassiana* with a concentration of 10^8 con./ml (Consolo et al., 2003). Additionally, applications against *D. v. virgifera* have been mainly conducted with *B. bassiana* (Campbell et al., 1985; Krueger and Roberts, 1997; Mulock and Chandler, 2000, 2001; Bruck and Lewis, 2001, 2002; Consolo et al., 2003). In contrast to these results, Silva-Werneck et al. (1995) obtained higher infection rates when they treated *D. speciosa* larvae in Brazil with *M. anisopliae* than with *B. bassiana*, but the highest infection rate was 30% with *M. anisopliae* spores at a concentration of 10^8 con./ml.

Infection of *D. v. virgifera* larvae was very low when compared with that of adults. We did not manage to reach 50% larval infectivity at a concentration of 1×10^7 con./ml (standard dose), although more larvae (without symptoms) died in the treatments with EPF than in the control groups. However, in one control group 26% of larvae died, and this can be explained by the use of different batches and so different conditions of larvae. However, the higher mortality in the treatments indicates that the fungus has a greater influence than we obtained through fungal symptoms; this phenomenon is also recognized when white grubs were inoculated with EPF (Keller, 1978). Davidson and Chandler (2005) reported similar results when they infected onion maggots with EPF; they obtained lower mortalities with larvae of *Delia antiqua* than with adults. Reasons for low infection rates of larvae could be their smaller surface or that larvae are less sensitive against fungal infections compared with adults. Insect cuticle acts as a barrier to fungal penetration and its thickness increases with each moult, so differences in the susceptibility of different larval instars to entomopathogenic fungi can be explained by their

cuticular characteristics (Boucias and Pendland, 1991). It has also been shown that the length of the intermoult period depends on environmental conditions and the shorter the period, the less time remains for the fungus to germinate and penetrate (Skrobek, 2001). Wagner et al. (1996) reported that different surface structures of insects resulted in differences in wettability and contaminability. Sites with thinner cuticle, such as intersegmental membranes, are more suitable for penetration than others. Therefore, the distribution of conidia of EPF on larvae could have an effect on mortality and speed of killing (Skrobek, 2001). It was also shown, that insects died soon after inoculation and showed fungal growth on the cuticle 12 days after inoculation, whereas some insects like wireworms and white grubs sometimes need a couple of months to become infected (Schweizer G., pers. comm.). The reasons why adults are significantly more susceptible than larvae are still unknown, but could be explained by the better adaptation of larvae to soil conditions and, therefore, to soil-living entomopathogens than adults are. Moreover Toepfer and Kuhlmann (2006), who constructed a life table of *D. v. virgifera* based on surveys in Hungary, found adults infected with EPF (*M. anisopliae* and *B. bassiana*); other natural enemies targeting different stages of *D. v. virgifera* could not be observed. One adult in the control group was also infected with *M. anisopliae*. We believe that it became infected in the field and that the quarantine time of 2 weeks was too short to recognize the disease.

Toepfer and Kuhlmann (2006), who observed an average natural mortality of 99.7% during one generation of *D. v. virgifera*, recommend that each stage of *D. v. virgifera* would be an equally suitable target for a biological control programme. However, for biological control of field populations of *D. v. virgifera* it would be easier to target the soil-living stages than the swarming adults. For the control of white grubs in Switzerland, a spore concentration of 10^5 colony-forming units per gram soil is efficacious, which is equivalent with about 50 kg of *Beauveria*-colonized barley grains per hectare (Keller, 2004). However, for the control of *D. v. virgifera* different spore concentrations and application times have to be tested. Potential application techniques could be the well-established application of fungus-colonized barley grains into the soil with drill machines, which is used for over 15 years to control larvae of the European cockchafer, *Melolontha melolontha* (Keller, 2004). Although adults of *D. v. virgifera* are distinctly more sensitive than larvae, their control could only be achieved with a high-wheel spray equipment or with an aerial treatment.

In conclusion, *M. anisopliae* strains were more virulent against *D. v. virgifera* larvae and adults than *B. brongniartii* and *B. bassiana*. *M. anisopliae* strains isolated from *D. v. virgifera* showed on average higher infection rates than strains isolated from other hosts. The most virulent strains of *M. anisopliae* infected 97% of adult *D. v. virgifera* and 47% of the larvae. As next step, we need to evaluate the most virulent strains for their suitability as biological control agents.

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*Persistence of the biological control agent *Metarhizium anisopliae* in the soil, on the soil surface of a maize field and on maize leaves

Summary

The entomopathogenic fungus *Metarhizium anisopliae var. anisopliae* (Metsch.) Sorokin (Hypocreales: Clavicipitaceae) is applied in maize fields to control the Western Corn Rootworm *Diabrotica v. virgifera* Le Conte (Coleoptera: Chrysomelidae). Important criteria for a biological control agent are the impact on the target pest, persistence and augmentation of fungal spores in the environment as well as competitiveness of the isolate against soil biota.

This study investigates the establishment and persistence of three strains of *M. anisopliae* after application as so called "fungal colonized barley kernels" (FCBK) into the soil and as a spore suspension (2 x 10^7 CFU/ml) on maize leaves and on the soil surface in 2006 and 2007 at four locations in Hungary, Austria and Switzerland.

The entomopathogenic fungi were able to establish at all locations and a long term persistence of at least fifteen months could be recorded in the soil. The results showed that most colony forming units could be found in the soil at the time when *D. v. virgifera* was in the soil-dwelling stage. Most *M. anisopliae* spores were able to survive on maize leaves for no longer then three days after application. A proportion of spores applied on leaves drifted obviously to the soil surface where increased fungus densities were found.

Additionally, molecular markers were used to identify *M. anisopliae* strains before and after application in the soil of a Hungarian maize field.

Keywords: *Diabrotica virgifera virgifera*, biological control, *Metarhizium anisopliae*, maize, entomopathogenic fungus, fungus product, persistence, soil, leaves

* All listed references in this paper are listed in chapter 6: References

Introduction

Diabrotica virgifera virgifera Le Conte (Western Corn Rootworm) (Coleoptera, Chrysomelidae) is a serious quarantine pest of maize recently introduced into Europe (Kiss, 2005). The damage of this chrysomelid beetle was first observed close to the airport of Beograd in 1992 (Baca, 1994) by finding plant lodging caused by the larvae feeding on the maize roots. The larvae of *D. v. virgifera* hatch after the winter diapause of the eggs develop through three larval instars and a pupal stage into adults between mid and end of July. Adult beetles can occasionally damage maize fields by feeding on pollen, silks, immature kernels and leaves (Chiang, 1973). At present, *D. v. virgifera* occurs almost throughout Central Europe and beetles are still migrating and expanding into new European regions (Miller et al., 2005).

In North America control measures using granular insecticides, seed treatments with insecticides and crop rotation, were partly successful, but failed to solve the problem at long terms because of the development of resistances or adaptation to chemical insecticides and crop rotation (Spencer et al., 2005). An important alternative control measure providing environmentally friendly and sustainable plant protection is the use of biological control agents like pathogens, parasitoids or predators (Susurluk, 2005). Entomopathogenic fungi (EPF) like *Beauveria* spp. (Hypocreales: Cordycipitacea) and *Metarhizium anisopliae* (Metsch.) Sorokin (Hypocreales: Clavicipitaceae), which are ubiquitous soil borne pathogens, have the potential to attack the host in their soil living stages (Müller-Kögler, 1965). *M. anisopliae* has a broad host spectrum; it was isolated from more than 200 insect species, mainly of the order Coleoptera (Samson et al., 1988). Successful applications of EPF against different pest insects have been reported by Ferron et al. (1991), Roberts (1989), Butt and Goettel (2000), Butt et al. (2001), and Butt (2002).

M. anisopliae was found to be naturally present in maize fields, causing infections in larvae and adults of *D. v. virgifera* (Pilz et al., 2008). Bioassays showed that adults of *D. v. virgifera* were more sensitive to spore suspensions than larvae (Pilz et al., 2007). Apart from efficacy against the pest insect establishment and persistence in the environment as well as competitiveness of the isolate against the soil biota are important criteria for a long term control. O'Callaghan (1998) described the importance of colonizing ability and pathogenic properties of a biological control agent for pest control. The aim of this study was to get information about establishment and persistence of *M. anisopliae* spores under field conditions, after application of spores on leaves, on the soil surface and in the soil.

To verify that the application of fungus material was the reason for the increase of the fungus densities, *M. anisopliae* strains isolated from soil samples before and after applications were identified with molecular tools (Enkerli et al., 2005).

Material and Methods

To assess the establishment and persistence of three strains of the biological control agent *M. anisopliae* under field conditions, the fungus was applied as "fungus colonized barley kernels" (FCBK) into the soil (to target the larval stage) and as "spore suspension" on maize leaves (to target the adult stage) in the years 2006 and 2007.

Field sites

Field trials were carried out in four maize fields at four locations (A, B, C, D) in Central Europe in the years 2006 and 2007. The maize fields A and B were located in the south – eastern part of Hungary (Hodmezovasarhely and Orosazha; Csongrad County), C was located in south-east Austria (Doiber, Burgenland County) and D was located in central Switzerland (Thun, Canton Bern).

Maize field A was the main experimental field, where the fungal strains were applied as fungus colonized barley kernels into the soil and as a spore suspension on maize leaves. The application of fungus colonized barley kernels was conducted on plant and field scale level. At that location soil temperature was measured with a soil data logger (HOTDOG DT1, Elpro Switzerland) at a depth of 15cm and the moisture content was determined with tensiometers (ART Agroscope Reckenholz, High flow candle Typ B01M3. Soil moisture Equipment, 1 bar high flow round bottom tapered neck up, 0.875" OD, 0.100" Wall, 2.75" Long, 0.531" Neck x 0.500" L), Tensimeter: Ballmoos AG, Horgen).

76 *M. anisopliae* strains, isolated before and after application of the fungus into the soil were identified by molecular markers (Table 2).

The maize fields at the locations B, C and D were treated at field scale level with the fungal strains formulated as fungus colonized barley kernels.

Table 1: Coordinates, altitude and soil type for each maize field at the locations in Hungary (A, B), Austria (C) and Switzerland (D). For location D the soil type was not defined (n. d.).

Location	A (Hungary)	B (Hungary)	C (Austria)	D (Switzerland)
latitude	46°25`N	46 30 N	46°92`N	46°83N
longitude	20°20È	20 44E	16°12 E	7°55 E
altitude (m asl)	83	92	244	548
soil fractions % (clay /silt/ sand)	clay loam soil (31.5/35.3/29.2)	sandy loam (15/18.6/64.7)	sandy loam (18.6/29.3/50.2)	- n. d.

Table 2: Experiments done at location A in Hungary in 2006 and 2007. Application of fungus colonized barley kernels (FCBK) at a concentration of 50kg/ha (i.e. 133 kernels/row meter or. 4 - 7 x 1013 spores/ha) or spore suspension on plant or field scale level. Number of replicates for each experiment, name of applied strains, time of applications and numbers of fungal isolates identified by molecular analyses in each experiment.

type of experiments at location A (Hungary)	replicates	Fungal strain	concentration	Time of application	Nr. of identified ***M. anisopliae*** isolates
application of FCBK at plant scale	3	2277; Bipesco5	50kg FCBK, i.e. 4 - 7 x 10^{13} sp/ha	April 2006 and 2007	46 isolates
application of FCBK at field scale	2	2277	50 kg FCBK, i.e. 4 - 7 x 10^{13} sp/ha	April 2006	30 isolates
application of spore suspension on leaves and soil surface	2	2277	2 x 10^{7} sp/ml	July 2007	-

Table 3: Application of fungus colonized barley kernels (FCBK) in field scale experiments in maize fields on the three locations in Hungary, (B), Austria (C) and Switzerland (D) in 2007 with number of replicates at each location, name of applied strains and time of application.

Locations	number of replicates	applied strains	application time
B (Hungary)	2	2277	April 2007
C (Austria)	2	2277	April 2007
D (Switzerland)	1	714, Bipesco5	May 2007

All maize fields in Hungary (A and B) were sown with non–treated maize grains of the hybrid variety “Magister” (UFA Semences, Bussigny, Switzerland). In Austria the maize variety “DieSAMANTA Cruiser“(Company: Die Saat, A) treated with Thiamethoxarn was used and in Switzerland grains of the maize hybrid “Aurelia” (Samen Schweizer, Thun, Switzerland) were applied. Individual maize grains were sown every 150 mm in rows, the distance between the rows was 750 – 800 mm. Fields at location A were treated once with 0.16 l per hectare of the herbicide Merlin SC (75% Isoxaflutol, Bayer CropScience, Germany) and at location C once with the herbicide SL 950 (1 l/ha) at maize stage 3 to 5

(Weber and Bleiholder, 1990). At location A an indigenous population of *D. v. virgifera* was detected in former years using pheromone traps. Therefore we expected the occurrence of an indigenous population in these years too. At the other experimental locations no indigenous population of the pest insect has been determined before.

Origin of fungus isolates and production of fungus colonized barley kernels (FCBK) in the lab

The fungal strain 2277 was isolated from a *Diabrotica v. virgifera* larva during field surveys in Hungary in 2005 (Pilz et al., 2008), the strain Bipesco 5 originated from *Cydia pomella*, collected in Austria (Eilenberg et al. 2007) and fungal strain 714 was isolated from *Agriotes* sp. in Switzerland (Mycothek, ART). For application into the soil, strains were formulated as FCBK, which were produced in the laboratory of the Swiss Federal Research Station ART Agroscope Reckenholz-Tänikon one month before field application. After a host passage through *Diabrotica v. virgifera* larvae, the strains were cultivated on selective medium (Strasser et al., 1996) and incubated at 22°C (± 2°C), 55% r.h. in darkness. Fully overgrown plates were washed off with 6 ml 0.05% Tween 80 into 250 ml Erlenmeyer flasks containing 100 ml of a modified liquid medium originally described by Blachère et al. (1973):
Solution 1 contains: 1000 ml tap water, 40 g corn steep, 4.52 g KH_2PO_4, 7.6 g Na_2HPO_4.
Solution 2 contains: 1000 tap water, 60 g sucrose (commercial sugar). The two solutions were mixed in a 1:1 ratio after autoclaving, when the liquids were cooled down to about 60°C.
The Erlenmeyer flasks were shaken for 7 days on a longitudinal shaker (130 rpm, 22°C ± 2°C) to produce submersed spores and mycelium, which were used to inoculate peeled barley kernels.
The production of fungus colonized barley kernels is described in detail by the protocol of Keller (2004). They were transported to the field sites in a cool box.
For quality control 100 kernels, covered by fungal mycelium, per bag were transferred to sand moistened with 10% water (w/v). After 10 days incubation at 22°C and 70% r.h. the percentages of sporulating, not sporulating and contaminated kernels were determined. Only bags in which the fungal mycelium fully covered the kernels and sporulated were used in the field.

Production of spore suspension

Spores of *M. anisopliae* strain 2277 were cultivated in Petri dishes on selective medium at 22°C (± 2°C), 55 % r.h. and darkness. Fully overgrown plates were washed off by pipetting 10 ml of 0.05% Tween 80 onto the plate and by scraping off the conidia with the pipette. The spore suspension was transferred to an Erlenmeyer flask, shaken and filtered through gauze (mesh: 500 µm) to remove mycelium. The spore concentration was determined with a haemocytometer (Thoma chamber) and diluted to a final concentration of 2 x 10^7spores/ml. The spore suspension was shaken before and during the application in the field.

Additionally spore traps constructed of microscopic slides with a layer of 2% water agar were placed on leaves. They were removed after application and placed in humid chambers (90% r.h.) and incubated at 22°C (± 2°C) for 24 h and 48 hours to determine the germination rate. A spore was called "germinated", when the germ tube was half as long as the diameter of the spore. The germination rate after 24 h and 48 h were determined with a light microscope at a magnification of 200 x.

Application of fungal colonized barley kernels (FCBK) in maize fields

a) *Plant Scale experiment:*

Location A: Maize was sown by hand at a distance of 15 cm between seeds and 80 cm between rows on the 20th April 2006 and on the 18th April 2007. At the same time FCBK were applied on a small scale of successive maize plants (7-8 plants) in a row. Three different treatments were carried out: In two treatments FCBK were applied (strain 2277 and strain Bipesco5); whereas the control plants were treated in the same way with sterilized barley kernels. One fungal treatment consisted of eight plants in a row and each treatment was repeated five times. The rows were opened with a rotator to a depth of 15 cm. At first maize seeds were sown and then FCBK were applied in the same still open furrow at a concentration of 50 kg/ha in the treatments "2277" and "Bipesco5". In the treatment "control" the same procedure was done with sterilized barley kernels. The furrow was closed by hand and pressed by walking a couple of times on the soil surface. The whole experiment was repeated three times, once in the year 2006 and twice in the year 2007, each on an area of about 80 m^2.

Before the application soil samples were taken from each treatment to determine the natural density of *M. anisopliae*.

b) *Field Scale Experiment*

These experiments were carried out on a larger scale by using agricultural machines. A maize field at location A was treated in 2006, while fields at the locations B, C and D were treated in the year 2007. Each treatment (fungal strain and control) was repeated two times per location, except location D, where two fungal strains were applied on smaller areas and repeated five times.

Location A: Maize with a distance of 15 cm between seeds and 80 cm between rows was sown on April 21, 2006, using a maize sowing machine with four seed containers (ISARIA, 2.5m). Afterwards the same machine was used again, but instead of maize, FCBK (strain 2277) were filled into the seed containers and were applied by directly driving on the previously sown maize rows at a depth of about 8 - 10 cm and at a concentration of 50 kg/ha. An area of 400 m^2 was treated and another 400 m^2 served as untreated control. Each treatment was repeated twice (two control plots and two treated plots).

Location B: FCBK (strain 2277) were applied together with the sowing of maize on April 25th 2007. A sowing machine (John Deere, MaxEmerge), containing 6 application tanks for maize (distance between rows 80 cm) and additional 6 smaller tanks for granulated fertilizers were used. This allowed the application of fertilizer and maize at the same depth and at the same time. In our experiment we filled FCBK instead of fertilizer into the tanks and applied them together with maize seeds at a concentration of 50 kg/ha on an area of 9200 m^2. Each treatment was repeated twice (two control plots and two treated plots).

Location C: FCBK (strain 2277) were applied together with the sowing of maize on May 1st 2007 on an area of 840 m^2. Therefore, a drill machine (Feldherr, Austria) with two application tanks and two smaller tanks for fertilizers in front was used. Each treatment was repeated twice (two control plots and two treated plots)

Location D: A drill machine (Kuhn, Maxima), which contained four application tanks for maize and two smaller tanks for fertilizers was used and the same method as described for the other locations was applied. On average a concentration of 50 kg/ha of FCBK (strain Bipesco 5 and strain 714) were applied on plots with a size of 6 m x 10 m on May 12th, 2007. Each treatment was repeated five times.

At each location the control plots were treated with sterile barley kernels. In all experiments soil samples were taken before the applications i.e. in April 2006 and 2007 (locations A, B and C) and in May 2007 (Location D).

Establishment and persistence of Metarhizium anisopliae spores in the soil

Location A: In the plant scale experiment 2006 and 2007 soil samples were taken monthly (n = 15 per treatment and date) till July and then again in early November and in April of the following year. In the field scale experiment 2006 soil samples (n = 96 per treatment and date) were taken two and three months after application and again in November of the same year and in April, June, July and November of the following year, covering a time span of 19 months.

Location B, C and D: Soil samples (n = 30 per treatment and date) were taken two and three months after application and then again in November of the same year. At location D soil samples (n = 15 per treatment and date) were taken monthly till July 2007 and then again in November.

Soil samples of 25 cm length and 2 cm diameter were taken with a core borer. A sample consisted of three insertions; the first three cm of the soil surface were discharged and the soil was put in a plastic bag and stored at 4°C (± 2°C) till analysis. Stones and organic material, like plants and roots were removed and the soil was crashed to small fractions. 20 g of each sample was given in an Erlenmeyer flask (250 ml). 100 ml tap water, containing 1.8 g/l of 4 mM tetra-sodiumpyrophosphate ($Na_4P_2O_7$ x $10H_2O$) was added and the soil suspension was shaken on a longitudinal shaker (120 rpm) at room temperature for 3 hours. The next steps were carried out under sterile conditions. Before plating the soil suspension was shaken again for 15 seconds and after 15 seconds sedimentation 100 µl aliquots of the top layer were plated in triplicate with a Drigalski spatula on selective medium (Strasser et al., 1996). The plates were incubated at 22°C (± 2°C), 55% relative humidity and in darkness. After 10 days the colonies /Petri dish were counted and CFU/g fresh soil calculated.

Application of Metarhizium anisopliae spores on maize leaves and on the soil surface

A maize plot of 40 m^2 was used to measure persistence after a spore application of fungal strain 2277 on location A in 2007. No other treatments were carried out at this site, except the spraying of the herbicide Merlin at maize stage 3 -5 (same procedure as described in chapter “field sites”). At the application time maize plants were fully grown, i.e. all leaves and the cobs were developed.

The fungal strain 2277 formulated in 0.05% Tween 80 was applied at a dose corresponding to 10^{13} spores/ha and with a volume corresponding to 500 l/ha. Establishment and persistence

of the spores on maize leaves as well as of the spores which were drifted to the soil surface were measured by sampling leaves and soil from the soil surface. Two replicates per treatment (fungal application and control) were performed and the whole experiment was repeated two times on the 2nd of July and 31st of July 2007.

Application in the field

The spore suspension was applied always in the evening between 6 pm and 7 pm when wind was calm and the intensity of the sunlight was reduced. Before application six spore traps per treatment were fixed on randomly selected maize plants in the axils of the third or fourth leave. Application was done with a back sprayer (Typ F 200 Electra 12V, Fox Motori, Italy; nozzles of the type XR Teejet 110 02 VS) with 3 bars pressure from about 15 cm above the top of the plants by steady walking.

Establishment and persistence of Metarhizium anisopliae spores on maize leaves and on the soil surface

Leave samples were taken before application and one hour, 16, 24, 72 and 168 hours after each application. A sample consisted of three leaves from the same plant, namely the 4th, 5th and 6th leave. Leaves were cut off with a sterilized scissor and transferred into a plastic bag. Four replicates from treated and untreated plants were done at each sampling date. Samples were frozen at -18°C till analysis.

Frozen leave samples were weighted before preparation (mass ranged from 20 – 44 g of fresh leaves). They were cut into small pieces and homogenized for 20 seconds in 200 ml tap water, containing one drop of Tween 80 (Merck, Germany), using a Braun homogenizer MX 32. The leave suspension was filtered through gauze (mesh: 1 mm) into an Erlenmeyer flask to remove larger particles. The suspension was shaken for one hour at 120 rpm and room temperature (25°C ± 2°C) on a longitudinal shaker. Afterwards 100 µl were plated in triplicates on selective medium. After incubation at 22°C (± 2°C), 55 % r. h. for 10 -14 days the colonies were counted and the CFU/g leave were calculated.

Samples of the soil surface were taken at the same sampling times as leave sampling. Therefore, boxes (d: 5 cm, h: 10 cm) were pressed by hand on the soil surface to a depth of about 3 cm, stones and organic material were removed and they were closed with a plastic lid and stored at 4°C till analysis. These samples were analysed in the same way as described for the soil samples.

Analysis of fungal genotypes using simple sequence repeat markers

Molecular analyzes were carried out with 76 *M. anisopliae* isolates. They were randomly chosen from selective medium plates used to analyze fungal densities in soil samples from the plant and field scale experiments at location A (Table 2). Genotypes of isolates present before and after application of FCBK (strain 2277 and strain Bipesco 5) were determined using simple sequence repeat markers (SSR) developed by Enkerli et al. (2005).

Extraction of genomic DNA

M. anisopliae isolates were cultivated in pure cultures on Sabouraud 2% Glucose Agar medium (BioChemika, Sigma-Addrich Chemie GmbH, Buchs, CH). 80 ml of CM (complete medium, Riba et al., 1986) were inoculated with conidia collected from these plates. Liquid suspension cultures were incubated for 3 days on a longitudinal shaker at 120 rpm and 22°C (± 2°C). After three days mycelium was harvested by filtration as described by Enkerli et al. (2001) and frozen at –80°C. Genomic DNA was extracted from lyophilised mycelium by use of the DNeasy plant mini kit (QIAGEN, Hilden, Germany). DNA was quantified by agarose gel electrophoresis using the low-mass DNA ladder (Invitrogen, CH) as a standard and the GelDoc XRS (Bio-Rad Laboratories, Hercules, CA) gel imaging system with Quantity One analysis software (Bio-Rad Laboratories).

The eight *M. anisopliae* SSR loci Ma099, Ma142, Ma145, Ma210, Ma195, Ma307, Ma325 and Ma417 were amplified from genomic DNA according to Enkerli et al. (2005). PCR were performed in reaction volumes of 20 µl containing 20 ng genomic template DNA, 0.2 µM of fluorescently labelled forward primer (FAM, HEX), 0.2 µM of unlabeled reverse primer, 0.6 mg/ml bovine serum albumin (BSA), 200 µM deoxynucleoside triphosphate (dNTP), 3 mM MgCL, 1 x PCR Puffer (QIAGEN), and 0.5 Units/reaction Hot Star TAQ Polymerase (QUIAGEN). Cycling conditions consisted of a 15 min. initial denaturation at 95 °C followed by locus specific cycle numbers of 30 s at 95 °C, 30 s of locus specific annealing temperature and 40 s at 72 °C and a final extension of 10 min. at 72 °C (Table 4).

Table 4: Specific cycle conditions (annealing temperature °C and locus specific cycle numbers) for each SSR locus.

Locus	annealing temperature (°C)	numbers of cycles
Ma099	56	32
Ma142	56	32
Ma210	50	36
Ma325	50	36
Ma145	56	34
Ma307	56	34
Ma417	56	34
Ma195	44	34

The quality of amplification was confirmed by 1.5% agarose gel electrophoresis and ethidium bromide staining (70µl/100ml). Size determination of amplification products was performed on ABI Prism 3130xl genetic analyzer equipped with 36-cm capillaries, POP7 polymer (Applied Biosystems, Foster City, CA) and GeneScan ROX 400 (Applied Biosystems) as an internal size standard. Data were analysed using GenMarker analysis software Version 1.51 (Soft Genetics, State College PH, USA).

Statistical Analysis

Establishment and persistence of fungal spores in the soil, on the soil surface and on maize leaves were analysed using a within-subjects ANOVA (Kinner and Gray, 2000) and comparisons between treatments with the the post hoc LSD test. Single treatments were compared with an unpaired t-test, using the software programmes SPSS, version 11.5 and Microsoft Office Excel 2003.

Results

Establishment and persistence of M. anisopliae spores in the soil

Plant scale experiment:

Location A: Before the application in April 2006 and 2007 the density of *M. anisopliae* was in average 693 CFU/g soil (SD ± 814). After the application of the two strains 2277 and Bipesco 5 formulated as FCBK an increase of the fungal densities in the soil was measured. Highest fungal densities were measured in May, i. e. one month after application. In treatment Bipesco5 significantly higher densities of 14 888 CFU/g soil (SD ± 2.9 x 10^4 CFU/g soil) were measured ($p = 0.001$). Also with strain 2277 higher densities of 6800 CFU/g soil (SD ± 1.7x104 CFU/g soil) were found ($p = 0.287$) but the difference was not significant. Higher fungal densities (CFU/g soil) were observed in the fungal treatments in relation to the control till November ($p =0.08$), when fungal densities decreased again in both fungal treatments. In April of the following year similar densities were measured in all treatments; in the control on average 1627 CFU/g soil (SD ± 1584) and in treatment 2277 in average: 1133 CFU/g soil (SD ± 575) and in the treatment Bipesco5 in average: 1550 CFU/g soil (SD ± 1356).

In the control treatment no significant change in fungal densities between successive sampling times could be measured during the observation period of one year. The maximum density of about 4000 CFU/g soil (SD ± 407) was determined in May. On all other sampling times fungal densities remained below 3700 CFU/g (SD ± 960) soil (Figure 1).

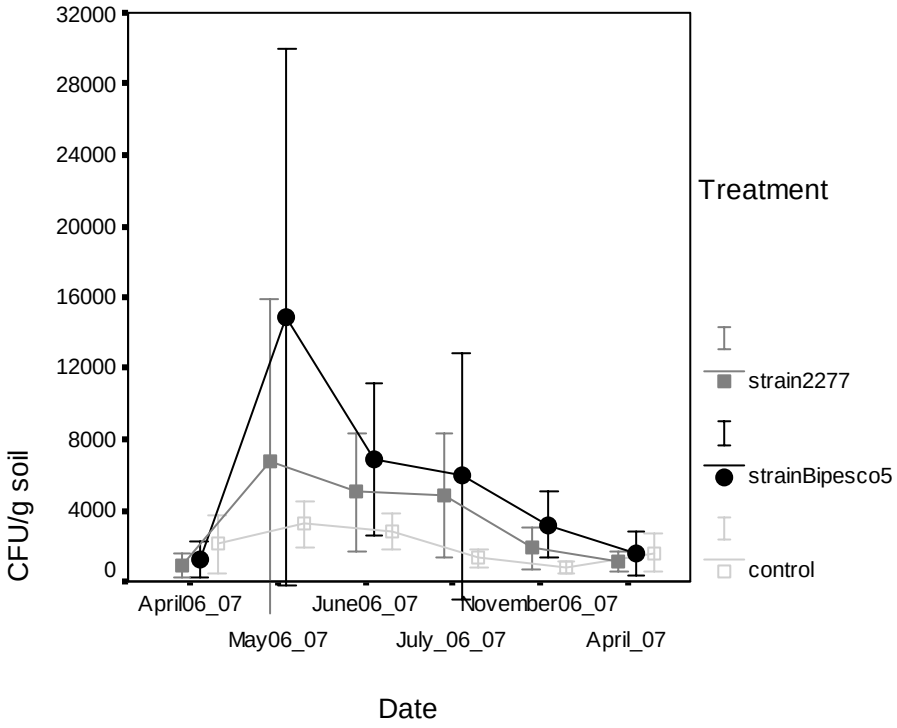

Figure 1: Plant Scale Experiment, location A, in 2006 and 2007. Development of fungus densities treated with strain 2277 and strain Bipesco 5 at 50 kg FCBK/ha. Data of three replicates were pooled (no significant differences between replicates (df= 2, F= 0.453; p = 0.636).

Field scale experiment:

Location A: After application of FCBK in April 2006 strain 2277 was able to establish and persist in the soil (Figure 2). The density increased from initially 5 x 10^2 CFU/g (SD ± 304) in April 2006 to 1 x 10^4 CFU/g (SD ± 3320) in July 2006. Till April 2007 it decreased to the density of the untreated plots (2 x 10^3 CFU/g soil, SD ± 255), but increased again to an average of about 7 x 10^3 CFU/g (SD ± 2739) in July 2007. The densities in the fungal treated plots differed significantly from the untreated ones at all sampling dates (July 2006; November 2006; June2007; July 2007, 0.00 <p> 0.036), except in April 2007 and in November 2007, where a low density of 1.7 x 10^3 CFU/g soil (SD ± 1665) was measured in the fungal treated plots too (p = 1.00).

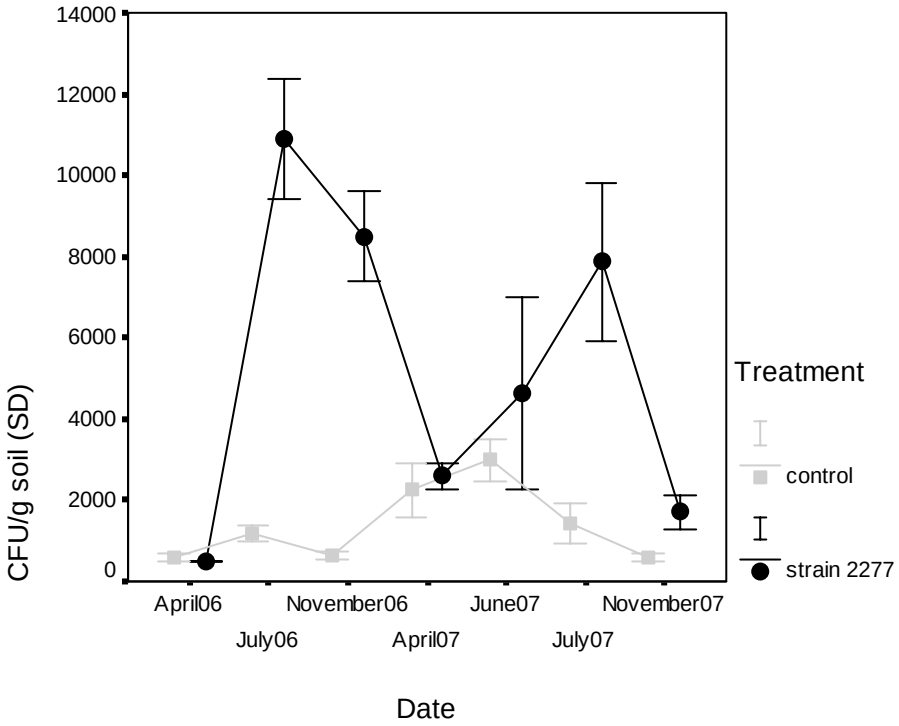

Figure 2: Development of fungus densities (CFU/g soil) in the treated (strain 2277) and untreated (control) area in the field scale experiment, location A (Hungary) in 2006. Samples were taken 3, 7, 12, 14, 15 and 19 months after application (mean and standard deviation). Control: No treatment; strain 2277: Application of fungus colonized barley kernels, strain 2277 at 50 kg/ha.

Location B: The natural fungus density at this field site ranged from 7 x 10^2 to 1.1 x 10^3 CFU/g soil (SD ± 2199 - 3886). After the application of FCBK (50 kg/ha) no increased fungal densities were observed in June 2007, even a decrease in the treated plots to about 482 CFU/g soil (SD ± 780) was measured, while in the control fungal densities of 960 CFU/g soil (SD ± 3757) were recorded. Only in July 2007 a significant increase to a five times higher concentration of 2.2 x 10^3 CFU/g soil (SD ± 6226) was found in the treated area ($p < 0.05$), while in the untreated area the fungus density decreased to densities of 400 CFU/g soil (SD ± 727). Further on, in November 2007 both treated and untreated plots showed low densities of about 1 x 10^2 CFU/g soil (SD ±107-1006) (Figure 3).

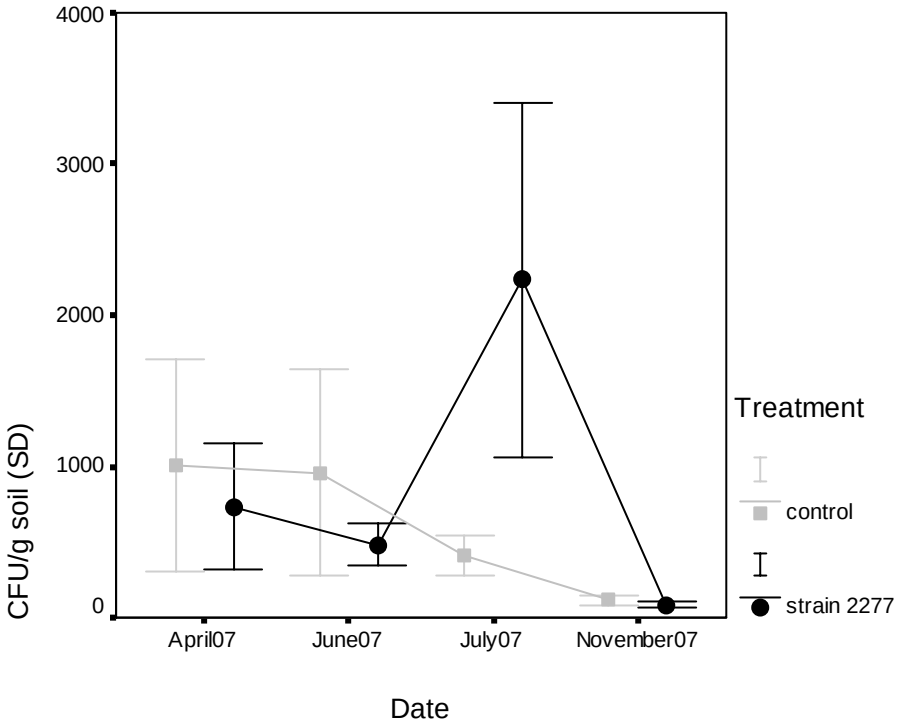

Figure 3: Development of fungus densities (CFU/g soil) in the treated and untreated (control) area at location B (Hungary) in 2007. Samples were taken before and 2, 3 and 7 months after application (mean and standard deviation). Control: No treatment; strain 2277: Application of fungus colonized barley kernels, strain 2277 at 50 kg/ha.

Location C: Natural fungus density at this field site ranged from 1.7 – 3.1 x 10^3 CFU/g soil (SD ± 2100-5800) in April 2007. After the application of FCBK (strain 2277 at 50 kg/ha) the fungal density in the treated area increased significantly to about 5 x 10^3 CFU/g soil (SD ± 5800) in June, while the fungus densities in the untreated area reached about 2 x 10^3 CFU/g soil (SD ± 1359, p = 0.005) (Figure 4). In July 2007 the fungus density in the treated area dropped down to 1.2 x 10^3 CFU/g soil (SD ± 1500) while that in the untreated control remained stable (1.8 x 10^3 CFU/g soil (SD ± 2003)). In November 2007, again, the densities in the two areas did not differ significantly. They were 1 x 10^3 CFU/g soil (SD ± 1350) in the treated area and 5x 10^2 CFU/g soil (SD ± 612) in the untreated area (Figure 4).

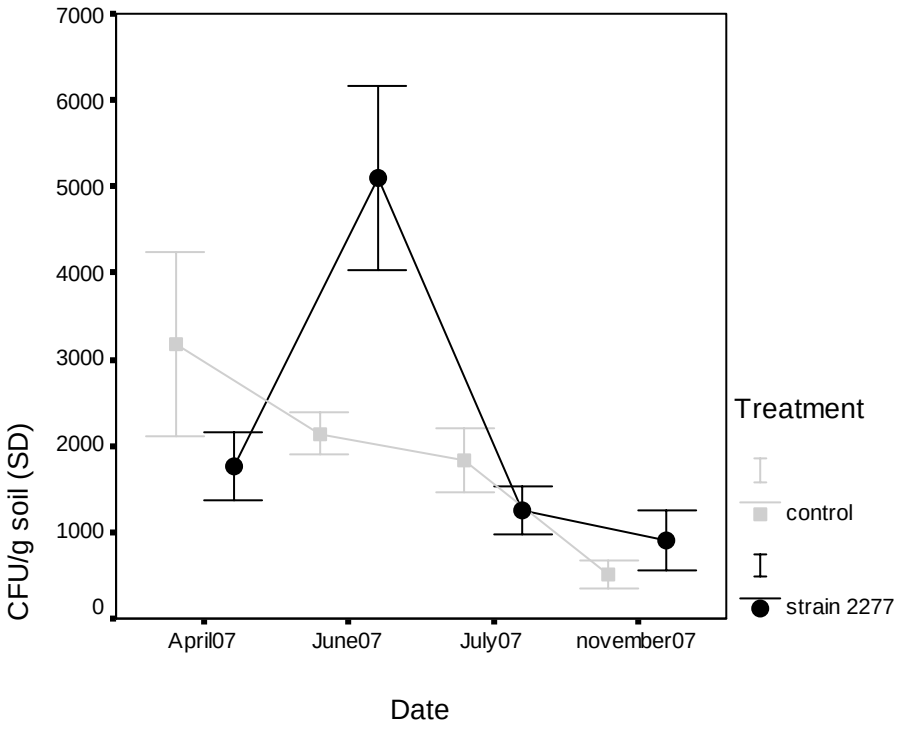

Figure 4: Development of fungus densities (CFU/g soil) in the treated and untreated (control) area at location C (Austria) in 2007. Samples were taken before and 2, 3 and 7 months after application (mean and standard deviation). Control: No treatment; strain 2277: Application of fungus colonized barley kernels, strain 2277 at 50 kg/ha.

Location D: The natural density of *M. anisopliae* in the soil before fungal treatment in May 2007 was low with an average 2.5 x 10^2 CFU/g soil (SD ± 239). After application of FCBK, strain 714 and Bipesco5, the densities for strain 714 increased to 1000 (SD ± 936) and for strain Bipesco 5 to 2100 x 10^3 CFU/g soil (SD ±2891) in the treated plots and to 9 x 10^2 CFU/g (SD ± 788) soil in the untreated plots (June 2007), but without significant differences between treatments and control. In July the densities in the treated plots increased again. Strain 714 differed significantly from the control and amounted 4.3 x 10^3 CFU/g soil (SD ± 5177). Strain Bipesco 5 reached 3 x 10^3 CFU/g soil (SD ± 1526) and did not differ

significantly from the control. However, in November 2007 the densities in the plots treated with this strain further increased and reached on average 7 x 10^3 CFU/g soil (SD ± 6786), which differed significantly from the treatment with strain 714 and the untreated control ($p < 0.05$) (Figure 5).

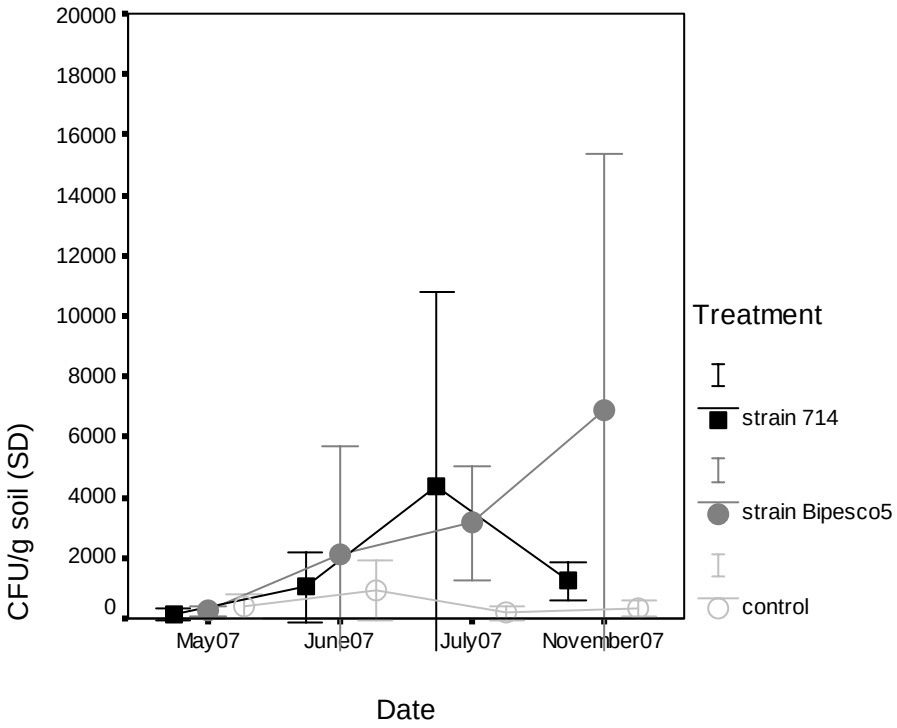

Figure 5: Development of fungus densities CFU/g soil) in the treated area (strain Bipesco 5 and strain 714) and untreated (control) area at location D (Switzerland) in 2007. Samples were taken before and 1, 2 and 6 months after application (mean and standard deviation). Fungi applied as FCBK at 50 kg/ha.

Establishment and persistence of M. anisopliae on maize leaves

No *M. anisopliae* spores were found on maize leaves before application. After the application of spore suspension of strain 2277 the fungal densities significantly increased. They were 1.4 x 10^4 CFU/ g leave (SD ± 13596) one hour and 8 x 10^3 CFU/g leave (SD: 13800) 16 hours after application ($p = 0.013$). However, fungal spores were found in the control area too with 1.6 x 10^3 CFU/g soil (SD ± 2300) one hour after application. Between 16 hours and 24 hours after application the spore densities on treated plants decreased again significantly. The densities were 420 CFU/g leave (SD ± 467) 24 hours and 28 CFU/g leave (SD ± 33) 72 hours after application. After 168 hours, still 180 CFU/g leave (SD ± 466) were found on treated leaves, but no spores were found on the untreated leaves any more (Figure 6). The fungal densities at the latter two sampling dates (72 and 168 hours) differed significantly again in the two treatments (0.026 <p> = 0.044). The fungus densities on treated and untreated plants differed significantly at all samplings except at 24 hours after application.

Within the fungal treatment significant differences in spore densities were found just between sampling time before application and one hour (p = 0.00) as well as 16 hours (p = 0.04) after application. Although fungal spores were found in the control plots no differences in fungal densities between all consecutive sampling times in this treatment could be found (Figure 6). Germination rates of spores on 2% water agar ranged from 30% (SD: ±9) 24 hours to 66% (SD: ±2) 48 hours after application.

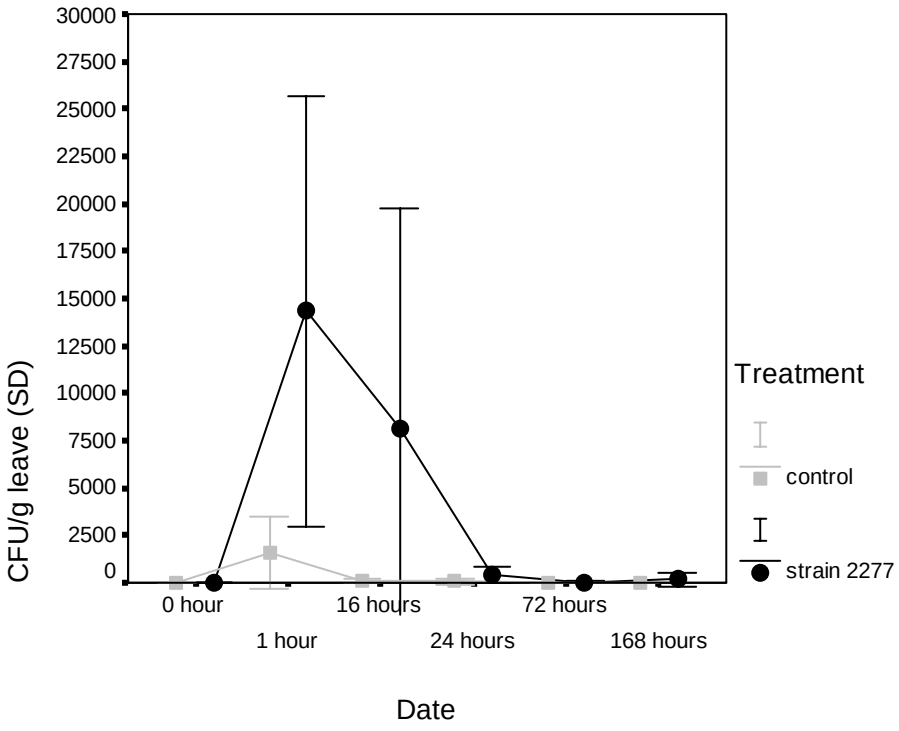

Figure 6: Development of fungus densities (CFU per gram leave) on plants treated with a spore suspension (strain 2277) and on untreated (control) plants (mean and standard deviation). Sampling at time "0 hour" was taken before application. Data of two replicates (on 2nd and 31st July 2007) are pooled (no significant differences between replicates, df: 1, F= 1.47; p = 0.237)

Establishment and persistence of M. anisopliae on the soil surface

Samples taken from the soil surface where maize plants had been treated with spore suspension proved that *M. anisopliae* spores drifted from leaves and subsequently establishment on the soil surface (Figure 7). After the application of a spore suspension of *M. anisopliae* at a concentration of 2 x 10^7 spores/ml on maize leaves higher fungal densities on the soil surface of treated plots could be found during the whole observation period (Figure 7). The natural fungus density before the application was low (1 – 4.2 x 10^2 CFU/g soil; SD ± 125 - ± 240) but increased significantly after the application. After one hour it was 7.7 x 10^3 CFU/g soil (SD ± 9942) in the treated area (p = 0.03) and 1 x 10^3 CFU/g soil (SD ± 1400) in the untreated area. After 16 and 24 hours the fungus density decreased in the treated area to

5.3 x 10^3 CFU/g soil (SD ± 4752) and to 2.2 x 10^3 CFU/g soil (SD ±2133) respectively. At the same times the fungus densities in the untreated area was 2 x 10^2 CFU/g soil (SD ± 160) (p = 0.03) and 60 x 10^1 CFU/g soil (SD± 102) respectively and differed significantly from the treated area. After 168 hours the density in the treated area again increased significantly to 4.7 x 10^3 CFU/g soil (SD ±5632) (p =0.04) compared to the control, where on average 60 CFU/g soil (SD ±53) could be measured (Figure 7).

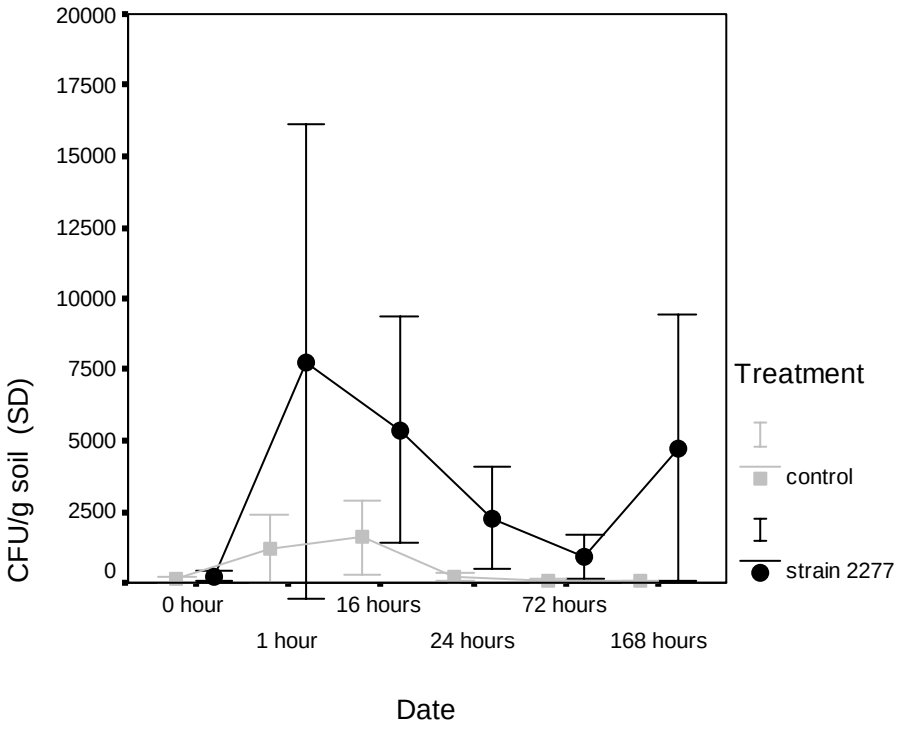

Figure 7: Development of fungus densities (CFU per gram soil surface) in treated (strain 2277) and untreated (control) plots (mean and standard deviation). Sampling at time "0 hour" was taken before application. Data of two replicates are pooled (no significant differences between replicates, df: 1, F= 1.83; p = 0.179).

Analysis of fungal genotypes of M. anisopliae using simple sequence repeat markers

Seventy-six *M. anisopliae* strains, isolated from soil samples at location A of the plant scale experiment 2007 and field scale experiment 2006 (Table 2) were analyzed. Seventeen different genotypes were detected based on 8 SSR loci (Table 5). Genotypes differed at 1 to 6 loci among each other. Genotype A represents the applied strain 2277 and genotype B the applied strain Bipesco 5 (Table 5). In general, the two loci Ma099 and Ma417 showed no different alleles within the 76 *M. anisopliae* isolates, while the highest variability's were determined at locus Ma195 with seven alleles and at loci Ma325 and Ma307 with six different alleles.

Table 5: Genotypes (A-Q) detected among 76 *M. anisopliae* strains, analysed from soil samples at location A (Hungary), based on eight SSR markers (*Ma099-Ma417*).

Genotype	SSR (simple sequence repeats) loci							
	Ma099	***Ma142***	***Ma145***	***Ma210***	***Ma195***	***Ma307***	***Ma325***	***Ma417***
A (2277)	152	129	111	95	149	162	155	261
B (Bip5)	152	106	116	99	121	199	145	261
C	152	129	111	95	112	146	159	261
D	152	129	111	109	106	141	165	261
E	153	116	120	95	106	162	155	261
F	152	129	111	109	106	162	165	261
G	152	129	111	109	106	199	165	261
H	152	129	111	109	106	162	108	261
I	152	129	111	95	119	162	154	261
J	152	129	113	95	131	162	154	261
K	152	129	113	106	112	152	154	261
L	152	129	111	70	112	146	159	261
M	152	129	111	95	106	162	165	261
N	152	129	111	109	106	109	165	261
O	152	129	111	109	112	146	159	261
P	152	106	116	109	121	199	145	261
Q	152	129	111	95	140	162	155	261

Fungal genotypes, detected in the plant scale experiment

All together 46 *M. anisopliae* isolates were genotyped; 21 isolates before and 25 isolates after the application of FCBKs, strain 2277 (genotype A) and strain Bipesco 5 (genotype B).
Before applications four different genotypes (A, C, J and K) were identified. Genotype C was the most dominant with an abundance of 40 - 100% (Table 6). In treatment 2277 genotype C was found exclusively. The applied genotype A was found naturally occurring before the application with a frequency of 40% in the treatment Bipesco5 and with a frequency of 10% in the control area. Genotype B (strain Bipesco 5) was not detected.
After the application of FCBK 25 *M. anisopliae* isolates were analyzed and nine different genotypes could be determined (A, B, C, F, L, M, N, O, P). In treatment 2277 60% of the isolates (n = 5) belonged to the applied genotype A (strain 2277). This genotype was also present in the treatments Bipesco 5 and in the control. Genotype C, which was detected before application to 100% was not found. In treatment Bipesco 5 the frequency remained the same as before the application (40%) and in treatment control it increased from 10% to 25 %. One isolate from treatment 2277 belonged to the applied fungal strain Bipesco 5

(genotype B), but this genotype was not present in the samples collected from treatment Bipesco 5. Six other genotypes could be determined after application, one in treatment 2277 (L) and five in treatment control (F, M, N, C; P) at low frequencies of 5 % – 20% (Table 6).

Table 6: Abundance of genotypes before and after application of FCBK, strain 2277 (genotype A) and strain Bipesco 5 (genotype B) among the analysed number of isolates per treatment, in the plant scale experiment at location A in 2007.

Treatment		CFU/g soil	Nr. of isolates	Abundance of genotypes (%)										
				A	B	C	F	J	K	L	M	N	O	P
before	2277[a]	2150	5			100								
after	2277[b]	300	5	60	20					20				
before	Bipesco5[a]	2000	5	40		40			20					
after	Bipesco5[b]	2650	5	40		60								
before	control[a]	2950	11	10		80		10						
after	Control[b]	2800	15	25		25	15				20	5	5	5

[a] before application of genotype A (2277) or genotype B (Bipesco5)

[b] after application of genotype A (2277) or genotype B (Bipesco5)

Fungal genotypes detected in the field scale experiment

Thirty *M. anisopliae* isolates, randomly selected from soil samples in the field scale experiment, were analysed by simple sequence repeats (SSR). Only genotype A (strain 2277) was applied as FCBK into the soil (Table 2). Out of all isolates nine different genotypes were identified (Table 7).

Sixteen strains isolated before application, eight from the treated (strain 2277) and eight from the untreated (control) area were genotyped. In the treated area genotype A was dominant (50%) and 4 other genotypes were present at low frequencies at 12% (C, D, E and Q). In the untreated area 3 genotypes inclusively the applied genotype were detected with frequencies of 25% - 50% (Table 7).

After application, fourteen isolates, eight from the treated and six from the untreated area were genotyped. The abundance of the applied genotype A increased in the treatment as well as in the control (Table 7). Nine out of 14 analyzed strains or 64% belonged to the applied genotype A (strain 2277). Six or 75% of this genotype resulted from the treated area and three or 50% from the control (Table 7). Genotype C and D, which were detected before the application, were not isolated anymore. An additional genotype I with 25% abundance was detected in the treated area and three genotypes (F, G and H) with frequencies of 16% each were found in the control area (Table 7).

Table 7: Abundance and distribution of genotypes before aand after b application of strain 2277 (genotype A) in the field scale experiment at location A in 2006.

Treatment		CFU/g soil	Nr. of isolates	Abundance of genotypes (%)								
				A	C	D	E	F	G	H	I	Q
before	2277[a]	496	8	50	12	12	12					12
after	2277[b]	10947	8	75							25	
before	control[a]	496	8	25	25	50						
after	control[b]	1160	6	50				16	16	16		

[a] before application of genotype A (2277)

[b] after application of genotype A (2277)

Soil temperature and suction power at location A (Hungary)

At the main experimental field site in Hungary, location A soil temperature as well as suction power were measured during field seasons in the years 2006 and 2007. Measurements started with the application of FCBK into the soil together with maize sowing (April) and ended in August each year, when cages were digged out and the field experiments stopped. Average day temperatures of data, registered every hour were calculated (Figure 8 and 9).

Soil temperatures ranged from 15°C to 28°C with an average of 22°C (SD: 4) during the whole observation period from April 19th to August 9th 2006. In May 2006 lowest temperatures of an average of 20°C (SD: 2.74) and in July 2006 highest temperature of 25°C (SD: 2.21) were measured. Between June 4th and June 6th lowest temperatures of 15°C were found and the maximum temperature with 27 °C was found between July 26th and August 2nd (Figure 8).

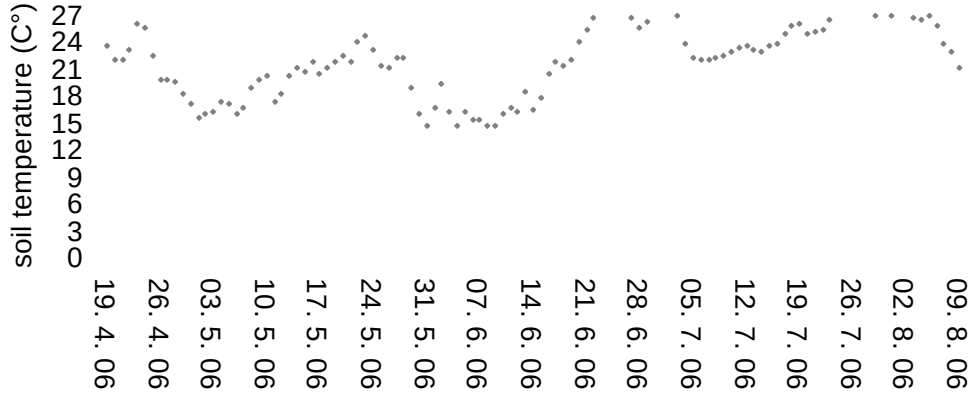

Figure 8: Soil temperatures at a depth of 15 cm during field experiments at location A in Hungary 2006. 19.4.2006: Application of maize grains and FCBK - 9.8.2006: experiments finished

In the year 2007 generally lower soil temperatures were measured with an average of 21°C (SD: 3) within the whole observation period from April 13th to August 16th 2007. Soil temperature values ranged from 14°C to 24 °C, while lowest temperatures with an average of 16°C (SD: 0.88) were found in April 2007 and highest temperatures with an average of 23°C (SD: 1. 82) in July 2007. Minimum temperature was measured on April 20th with 14°C and maximum from July 22nd to July 25th with 26°C (Figure 9).

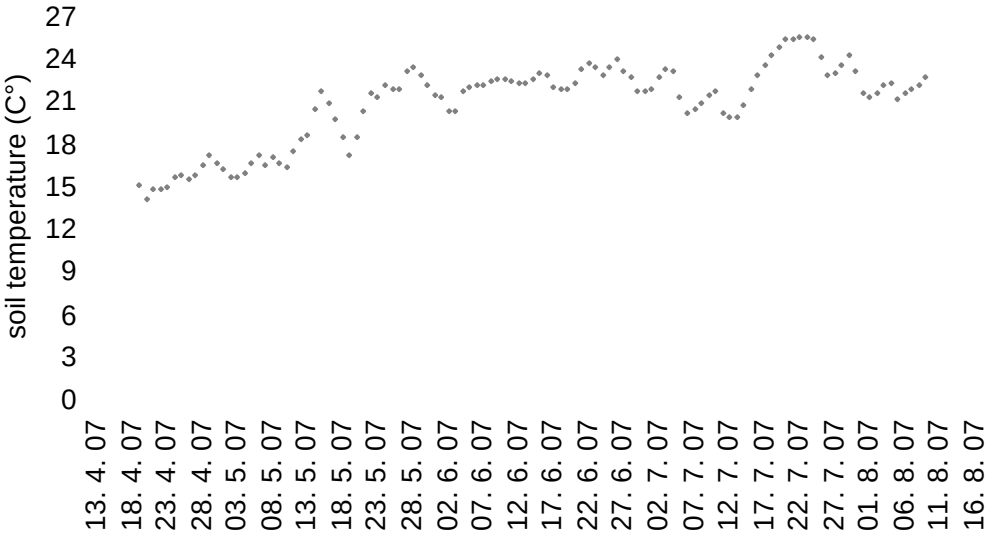

Figure 9: Soil temperatures at a depth of 15 cm during field experiments at location A in Hungary 2007. 13.4.2006: Application of maize grains and FCBK application - 16.8.2006: Experiments finished

Average values of suction power from a total of five tensiometers, digged in at a deepness of 12 to 15cm into the soil are shown in Figure 10 and 11.

During the whole observation period values ranging from -50 hPa up to a maximum of -750 hPa were observed. Between May 22nd and May 29th 2006 as well as between June 20th and June 23rd values about -750 hPa were measured, which displays a critical time for field crops to be shortened of available water; i.e. fine pores of soil are filled with water. The data showed that the soil dried out quickly after rainfalls on 19th June and 5th of July 2006 (Figure 10).

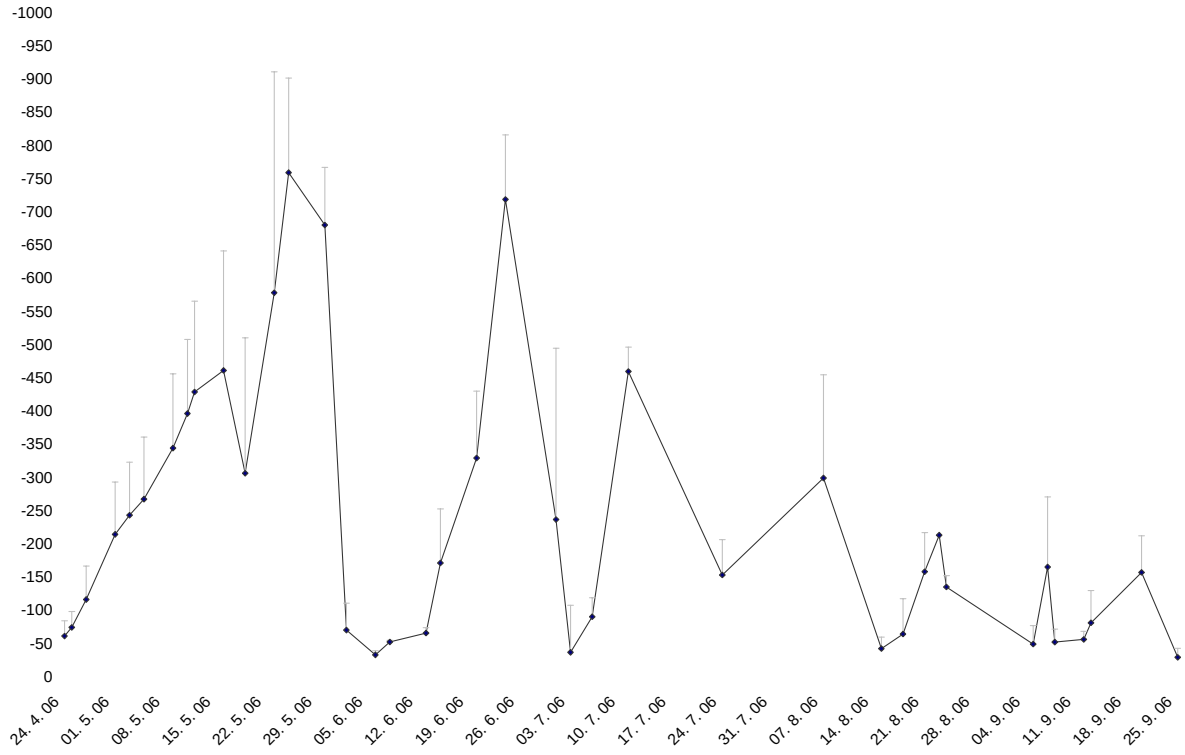

Figure 10: Mean values from five tensiometers (suction power; hPA) at a depth of 12-15cm on the location A in 2006.

In the year 2007 suction power values of -24 to -370 hPa were measured during the observation period. No critical time span (above -750 hPa) of water stress for plants could have been observed. The maximum value of -24 hPa was measured beginning of field season on

April 19th, while the minimum with -370 hPa was found beginning of June (6. to 8.6. 2007).

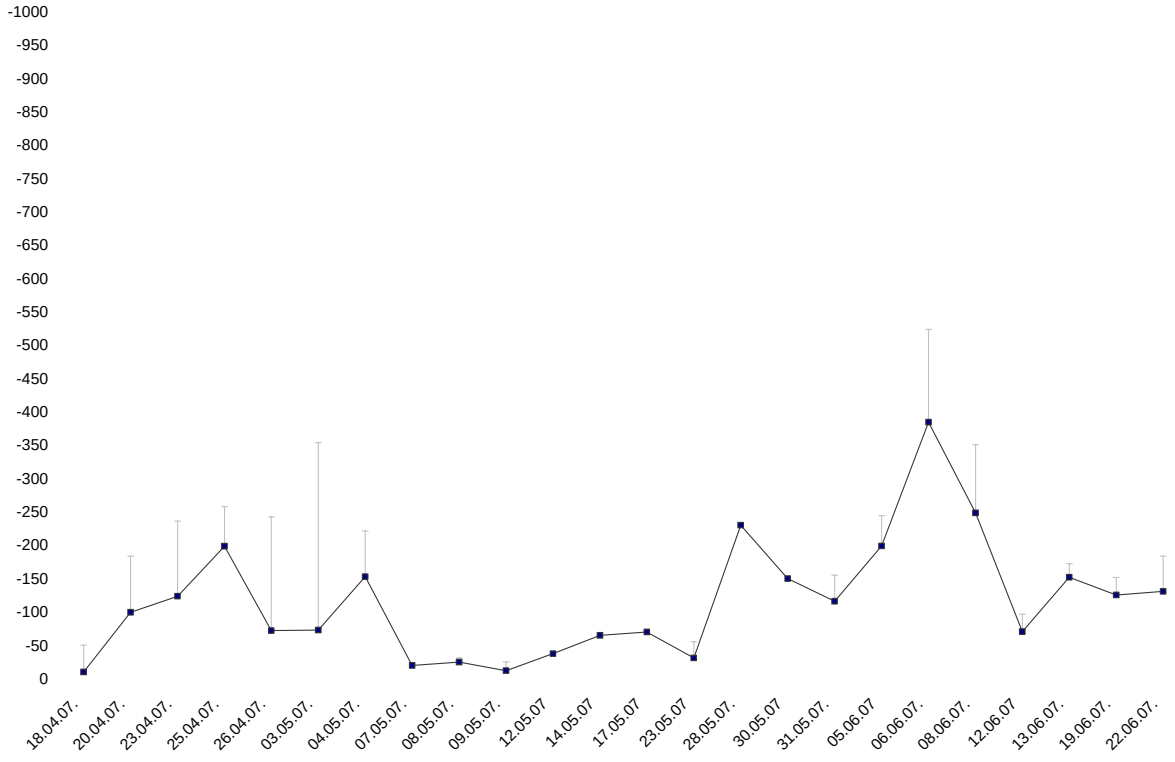

Figure 11: Mean value of five tensiometers (suction power, hPa) at a depth of 12-15cm on location A in 2007.

Discussion

Successful establishment and persistence of spores of a biological control agent are important criteria for the control of soil – dwelling pests. The effectiveness of entomopathogenic fungi for crop protection partly depends on the persistence of the applied inoculum in the field (Inyang et al., 2000).

Results of these field trials showed that after the application of fungus colonized barley kernels into the soil fungal densities increased in the soil and could be measured over the whole observation period in maize fields. The two applied strains (strain 2277 and strain Bipesco5) were the most effective ones in bioassays against *Diabortica v. virgifera* larvae and adults (Pilz et al. 2007). The present data show that they also fulfil the aspect of a biological control agent in respect to establishment, multiplication and persistence in the environment during the “critical time”, when the target insect perform its life cycle in the soil (Vidal and Kuhlmann, 2005). After application in April 2006 with a maize drill machine higher fungus concentrations in relation to the control were found up to fifteen months afterwards. Maize is considered to be an intensively cultivated, non native field crop, providing feed for only a few species of potential hosts (Journey and Ostlie, 2000). Also,

intensive tillage and cropping have a negative impact on the soil biota (Brust, 1991). Therefore, we suggest that *M. anisopliae,* which depends on host insect, could multiply and persist because of the natural occurrence of a *D. v. virgifera* population or the presence of alternative hosts at this location.

M. anisopliae occurs worldwide and has been isolated from several hundred insect species including pests and beneficials (Zimmermann, 1993). Therefore, also the question of "non-target effects" after application of *M. anisopliae* spores has to be answered (Vestergaard and Eilenberg, 2000; Steenberg et. al. 1995). In this study we concentrated our investigations on the influence of applied *M. anisopliae* strains on naturally occurring isolates of this fungus by using genetic markers. *M. anisopliae* is a cosmopolitan soil borne pathogen. It was proofed that the higher fungal density after application of fungal colonized barley kernels, which increased significantly to a 100 times higher concentration than before (average: 1.3×10^4 CFU/g soil) was caused by the application. Molecular markers, like simple sequence repeats (SSR) are able to distinguish between strains within a species (Enkerli et al., 2005). Molecular biological techniques, such as microsatellite markers are useful tools to study dissemination of fungal strains in the soil. Our results showed that after application of FCBK the fungal densities increased. Although only a limited number of isolates was analysed with genetic markers, the molecular data confirmed that this increase was mainly due to the applied fungal isolates. Further, data suggest that the application of a fungus product had no impact on indigenous isolates; i. e. indigenous isolates were not replaced and could coexist with applied strains at least for the time monitored.

A lot of studies about biotic and abiotic factors influencing the persistence of *M. anisopliae* have been carried out (Butt et al., 2001). *M. anisopliae* occurred naturally at a relative high density in the Hungarian maize fields ($0.2\text{-}7 \times 10^3$ CFU/g soil). In Switzerland Keller et al. (2003) and Rodrigues (2005) report lower densities of *M. anisopliae* in arable land (about $0.02\text{-}1.1 \times 10^3$ CFU/g soil) than in orchards and grassland, where they found densities up to 3.6×10^3 CFU/g soil. The density at our Swiss location D (3×10^2 CFU/g soil) is within this range. Our studies are in accordance with reports that *M. anisopliae* can persist for a long time in the surface layer of soils (Li and Holdom, 1993). Vänninen et al. (2000) showed that conidia of *M. anisopliae* survived for more than 3 years in Finnish soils. Optimal growth temperature for *M. anisopliae* is described near 27-28°C (Ferron, 1981). Keller et al. (2003) also suggest an influence of the soil type on the density and distribution of *M. anisopliae*, whereas Milner et al. (2003) report of negligible effects of soil type and rainfall, when testing persistence of different formulations of *M. anisopliae* in sugar cane fields in Australia.

A positive correlation between the number of infective spores and mortality by mycosis has been established (Ferron, 1978). Keller (2002) and Kessler (2004) reported that a good control of *Melolontha melolontha* with *Beauveria brongniartii* in grassland was achieved with fungus densities of 10^3- 10^4 CFU/g soil. Müller-Kögler and Stein (1970, 1976) in Burges (1981) recommend for a small, short living soil insect, like *Diabrotica v. virgifera*, more inoculum of *M. anisopliae* (10^{16} to 10^{17} conidia/ha) than for larger insects like *M. melolontha* larvae, which were controlled with about 5 x 10^{14} conidia/ha of *B. brongniarti* (Ferron, 1978).With fewer spores, the green muscardine disease develops slowly and affects only older larvae or adults, but disturbance in fecundity and diapause of surviving adults can occur ((Primak, 1967; Müller-Kögler und Stein, 1970; Bajan und Kmitowa, 1972; Sikura and Gritsaenko, 1973; Litvinenko, 1974; N`Doye, 1976: Faizy, 1978), data compiled in Burges, 1981)). We applied about 3.6-6.9 x 10^{13} spores/ha, as one g of sporulating fungus colonized barley grain produces on average 7x10^8 spores (Pilz, unpubl.). We also suggest that the body of a *D. v. virgifera* larvae, which decompose after infection by EPF very fast, is too small for a high propagation of the EPF in the soil and for increasing the infection pressure. The fungal spores have to be placed in the main habitat (maize roots) of the target pest, because larvae even mine in roots and have therefore a great chance to avoid contact with fungal spores.

Reasons for the faster peak of the fungal density at location A in the year 2007 compared to the former year and at location B are not known. Soil type, climatic conditions and especially humidity could have had an influence on the sporulation time. The average temperature in 15 cm soil depth at location A between the years was similar with an average of 19-22°C in May and June 2006 and 2007. Temperature optima for infection, growth and sporulation of entomopathogenic fungi are generally between 20 and 30°C (Goettel et al., 2000). A reason for the faster sporulation and afterwards significantly decrease of fungal densities between May and June 2007 could be the higher precipitation in May and afterwards the dehydration of the soil, which is very critical for the fungus. We measured soil humidity with tensiometers and found critical soil moisture values of more than 750hPA (suction pressure) in this time span (Lawrence and Hornberger, 2007). Water is critical for entomopathogenic fungi at all life stages, so that the relative humidity has a great influence on efficacy, fast decomposition of the infected individual and transmission of the entomopathogens within the host population (Milner and Lutton, 1986). Humidities >96% are essential for spore germination and vegetative growth (Ibrahim et al., 1999). Infections of insects by *M. anisopliae* are also influenced by fluctuation of humidity and temperatures (Butt, 2002). These climatic conditions also influenced the target pest. In 2007 a faster development and therefore earlier emergence of *D. v. virgifera* could be registered at location A (first adult

found in the field on the 12th June in 2007 and on the 20th June in 2006). At the time of egg hatching (middle of May) we reached densities of 2 x 10^3 CFU/g soil in 2006, which increased afterwards significantly to 1.4 x10^4 CFU/g soil in July, whereas in May 2007 already densities of 1.8 x 10^4 CFU/g soil were found.

It is conspicuous that in fields, where no natural population of *D. v. virgifera* existed and especially the natural density of *M. anisopliae* in the soil was higher (location B: average of 1.4 x 10^3 CFU/g soil) compared to the other ones the application of fungus colonized barley kernels failed, respectively didn't show increases of fungus densities during the observation period. Competition with and suppression by soil biota (Kessler et al., 2003) and absence of specific hosts for the selected strain could have been reasons for this.

In general the application of FCBK in one step with the sowing of maize grains increased fungal densities over the period when the pest insect hatched and developed through its larval stages in the soil. This method is also the easiest and most economic procedure for farmers.

Fungal densities on the soil surface also increased after the application of a spore suspension on maize leaves. A high fungal inoculum on the soil surface raises the probability to infect the pest when it emerges from soil. The susceptibility of adult *D. v. virgifera* for entomopathogenic fungi is higher than for larvae (Pilz et al., 2007).

One day after application on maize leaves it rained heavily, which could be another reason for the decrease of spores on leaves within 24 hours. Rainfall and dew in the morning can dislodge and disperse conidia from plants on the soil surface and even into deeper layers of soil (Butt and Goettel, 2000; Inyang et al., 2000; Vestergaard et al., 2003). Astonishing, we found *M. anisopliae* spores one hour after application of the spore suspension on control leaves too. Spores obviously drifted by wind during application from treated to control plots, the distance between the two plots was about 10 meters and obviously too small. One of the most important parameter affecting propagule persistence on maize leaves is inactivation by solar radiation (Zimmermann, 1982; Klingen et al., 2002; Fargues et al., 1996). Formulations to protect conidia from harmful radiation have to be tested too; several oils or oil-soluble sunscreens significantly increased survival (Inglis et al., 1998, Ibrahim et al., 1999; Ferron, 1981). Additional influencing factors for the short-term persistence of spores on maize leaves are mainly high temperature, low humidity, fungicidal substances, plant growth (dilution of inoculum), adhesion of conidia, morphology and chemistry of the plant as well as rainfall and crop density (Butt and Goettel, 2000; Inyang et al., 2000; Vestergaard et al., 2003; Zimmermann, 1982; Klingen et al., 2002; Fargues et al., 1996; Rangel et al., 2004). After 24 hours just a low density of viable *M. anisopliae* spores could be still detected on maize

leaves. Similar results were obtained when a concentration of 2 x 10^7 spores/ml were applied on rape leaves; the highest densities of about 1 x 10^4 CFU/g leave was found one hour after application (Pilz, 2005).

In conclusion we found in these two years of field trials an establishment and persistence of our applied *M. anisopliae* products in the soil of fields, where a host population was present for at least one vegetation period. The short term persistence of *M. anisopliae* spores on maize leaves needs more precise studies about formulations of spores as well as application techniques, application times and dose-related experiments to optimise the persistence and the control of adult *D. v. virgifera*. Molecular tools like simple sequence repeats (SSR) represent a useful and valuable tool to study fungal communities and their relations.

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*Field trials with the entomopathogenic fungi *Metarhizium anisopliae* for Western Corn Rootworm *Diabrotica virgifera virgifera* control

Summary

First results of an application of the entomopathogenic fungus *Metarhizium anisopliae* for Western Corn Rootworm *Diabrotica v. virgifera* control in Europe are shown. Field trials with spores of two *M. anisopliae* strains formulated either as fungus colonized barley kernels against larvae or as spore suspension against adults of *D. v. virgifera* were carried out in Hungarian maize fields. After application of fungus colonized barley kernels at a concentration of 50 kg/ha emergence of adults could be reduced by 43 % and differed significantly to emergence rates in the untreated control. Root injuries in different treatments were investigated as well as reduction of cob yield.

Also the treatment of adults with spores, sprayed on leaves resulted in a reduction of about 35%. The potential of a virulent strain of *M. anisopliae* against different life stages of the pest insect is discussed.

Keywords: *Metarhizium anisopliae*, *Diabrotica virgifera virgifera*, microbial control, fungus colonized barley kernels, efficacy, application, field trials, maize, root damage, IOWA scale

*All references are listed in chapter 6: References

Introduction

Diabrotica virgifera virgifera Le Conte (Western Corn Rootworm) (Coleoptera: Chrysomelidae) is a serious quarantine pest of maize recently introduced into Europe (Kiss, 2005). The damage of this chrysomelid beetle was first observed close to the airport of Beograd in 1992 (Baca, 1994) by finding plant lodging, caused by the larvae feeding on the maize roots. The larvae of *D. v. virgifera*, which are hatching after the winter diapause of the eggs, develop through three larval instars and a pupal stage into adults at the middle till the end of July. Adult beetles can occasionally damage maize fields by feeding on pollen, silks, immature kernels and leaves (Chiang, 1973). At present, *D. v. virgifera* occurs nearly all over Central Europe and beetles are still migrating and expanding into new European regions (Miller et al., 2005). In North America control measures using granular insecticides, seed treatments with insecticides and crop rotation, were partly successful, but failed to solve the problem at long terms, because of the development of adaptation to chemical insecticides and crop rotation (Spencer et al., 2005). An important alternative control measure providing environmentally friendly and sustainable plant protection is the use of biological control agents like pathogens, parasitoids or predators (Susurluk, 2005). Entomopathogenic fungi (EPF) like *Beauveria* spp. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metsch.) Sorokin (Hypocreales: Clavicipitaceae) (Humber, 2007), which are ubiquitous soil borne pathogens (Müller-Kögler, 1965) were found to attack this host (Pilz et al., 2008). *M. anisopliae* has a broad host spectrum; it was isolated from more than 200 insect species mostly of the order Coleoptera (Samson et al., 1988). Successful applications of EPF against different pest insects have been reported by Ferron (1981) and Butt (2002).

M. anisopliae was found in maize fields, causing infections of larvae and adults of *D. v. virgifera* (Pilz et al., 2008). In bioassays, strains of *M. anisopliae* originating from this host showed that adult *D. v. virgifera* were more sensitive to spore suspensions than larvae (Pilz et al., 2007). The aim of this study was to test the impact of *M. anisopliae* strains, formulated as fungus colonized barley kernels, against soil living stages and, formulated as spore suspension, against the adult stages of the western corn rootworm *D. v. virgifera* under field conditions in Hungary.

Material and Methods

Field sites

The impact of the biological control agent *M. anisopliae* on the pest insect *D. v. virgifera* (WCR) was tested in three experiments, referred to as experiments A, B and C. They were carried out in maize fields at two locations in Hungary in the years 2006 and 2007 (Table 1). Experiment A and B targeted the soil living stages, whereas experiment C targeted the adult stage of *D. v. virgifera*. Experiment A and C were carried out at Hodmezòvasarhely in 2006 and 2007, whereas experiment B was conducted in a maize field at Orosazha in the year 2007 (Table 1). Untreated maize seeds of the hybrid variety Magister (UFA Semences, Bussigny, Switzerland) were sown between middle and late April of each year. Seeds of experiment A have been treated against feeding damages by pheasants, *Phasianus colchicus* L. with Mesurol (1 l/100 kg seeds, containing 500 g/l Methiocarb, FS). Individual maize grains were sown every 150 mm in rows, the distance between rows was 750 – 800 mm. Fields were treated once with 0.16 l of the herbicide Merlin SC (75% Isoxaflutol, Bayer CropScience, Germany) per hectare at maize stage 3 to 5 (BBCH: 14-16; Weber & Bleiholder, 1990). Experiment A was carried out in a field where only maize was cultivated for at least 5 years and an indigenous population of the pest insect had already established. For the experiments A and B plants were artificially infested with *D. v. virgifera* eggs.

Table 1: Details of the three experiments (A, B, C) carried out in maize fields at two locations in Hungary in the years 2006 and 2007.

Parameter	Experiment		
	A	**B**	**C**
location	*Hodmezòvasarhely*	*Orosazha*	*Hodmezò-vasarhely*
number of replicates	3	2	2
number of treatments	5	2	3
coordinates	46°25.99`N 20°20.34È	46° 30 N 20° 44 E	46°25.99` N 20°20.34 È
altitude	83	92	83
type of experiment	Application of fungus colonized barley kernels	Application of Fungus colonized barley kernels	Application of spore suspension
target stage of *D. v. virgifera*	soil living stages	soil living stages	adult stage
applied treatments	Two fungal strains (2277; Bipesco5), soil insecticide, control (sterile barley); untreated (no treatment)	One fungal strain (2277), control (sterile barley)	Two fungal strains (2277, Bipesco 5), control (0.05% Tween 80)
Concentration of fungal strains	50 kg FCBK, i. e. 4 – 7 x 10^{13} sp/ha	50 kg FCBK, i. e. 4 – 7 x 10^{13} sp/ha	2 x 10^{7} sp/ml
application time	April 2006 and 2007	April 2007	June 2007, July 2007
Date of infestation with eggs (# eggs per plant)	14. – 16. May 2006 and /2007 (2 x 80)	16. May 2007 (2 x 225)	-
soil fractions (% clay /silt/ sand)	clay loam soil (31.5/35.3/29.2)	sandy loam (15/18.6/64.7)	-

Infestation of Diabrotica v. virgifera eggs

In the experiments A and B maize plants were artificially infested with eggs of *D. v. virgifera*. The eggs were obtained from beetles collected the year before in south Hungary and reared in cages (25°C day, 15-20°C night, L: D: 14:10, 40% r.h.). Eggs which hibernated at 6°C (± 2°) in moist sand were incubated at 25°C (± 2°C) three weeks before inoculation in the field. The eggs were separated from the sand by sieving through a 250 micrometer mesh. Afterwards, they were suspended in 0.15 % water agar. Maize plants were infested in the mid

of May (1-3 leaf stage; BBCH- Code: 12-13; Weber and Bleiholder, 1990) with about 160 - 450 viable eggs/plant using a standard pipette (Eppendorf Company, Germany) and about 2 ml water-agar. The eggs were applied in two opposite holes with a depth of about 10-15 cm and a distance of 8-10 cm from the maize plant. In the laboratory (25°C) the percentage of hatching eggs was recorded with samples of the same egg batches as applied in the field experiments. Larvae hatched from about 80 % (± 10%) of the eggs.

Tested fungal strains and formulations

Strain 2277 was isolated from a *Diabrotica v. virgifera* larva during field surveys in Hungary in 2005 (Pilz et al., 2008), while the *M. anisopliae* strain Bipesco 5 originated from *Cydia pomonella* collected in Austria (Eilenberg et al., 2007). These two strains showed good virulence against larvae and adults of *D. v. virgifera* as well as good growth characteristics on artificial medium (Pilz et al., 2007). Depending on the target life stage the fungus material was formulated either as fungus colonized barley kernels (FCBK) or as spore suspension.

Production of fungus colonized barley kernels (FCBK) in the lab

To apply the fungus into a soil depth of 10-12 cm both fungus strains were formulated as so called "fungus colonized barley kernels" (FCBK). FCBK were produced in the laboratory of the Swiss Federal Research Station Agroscope Reckenholz-Tänikon ART one month before field application. After a host passage through *Diabrotica v. virgifera* larvae, the strains were cultivated in Petri dishes on selective medium (Strasser et al., 1996), incubated at 22°C (± 2°C), 70% r.h. and in darkness. Spores of fully overgrown medium plates were washed off with 6 ml 0.05 % Tween 80 (Merck, Germany) into 250 ml Erlenmeyer flasks containing 100 ml of a modified liquid medium originally described by Blachère et al. (1973). Solution 1 contained 1000 ml tap water, 40 g corn steep, 4.52 g KH_2PO_4, 7.6 g Na_2HPO_4. Solution 2 contained 1000 tap water, 60 g sucrose (commercial sugar). The two solutions were mixed (ratio 1:1) after autoclaving when the liquids were cooled down to about 60°C.

The Erlenmeyer flasks were shaken for 7 days on a longitudinal shaker (130 rpm, 22°C ± 2°C) to produce submerse spores and mycelium, which were used to inoculate peeled barley kernels in autoclaved plastic bags. The production of fungus colonized barley kernels is described in detail by Keller (2004). They were transported to the field sites in a cool box.

For quality control 100 kernels per bag were transferred to sand containing 10% water. After 10 days incubation at 22°C and 70% r.h. the percentages of sporulating, none sporulating and contaminated kernels were determined. Only bags with more than 90% sporulating fungus kernels were used in the field.

Production of M. anisopliae spore suspension in the lab

The fungus was cultivated in Petri dishes. Pure cultures completely covering the selective medium were washed off with 0.05% Tween 80 (Merck, Germany). The spore suspension was stirred and filtered through gauze (mesh: 500 µm) to remove mycelium particles. After counting the concentration in a haemocytometer (Thoma chamber) the suspension was adjusted with 0.05% Tween 80 to a final concentration of 2 x 10^7 sp/ml. The spore suspension was stirred again before the application.

Spore traps constructed of microscopic slides with a layer of 2% water agar were fixed on randomly selected maize plants in the axil of the third or fourth leave. After application they were incubated in a chamber with 90% r.h. at 25°C (± 2°C) for 24 and 48 hours to measure viability of spores (germination rate). A spore was called germinated, when the germ tube was as long as the diameter of the spore. Determination was done with a normal light microscope at a magnification of 200x.

Experimental design and application

Experiment A: The impact of two fungal strains of *M. anisopliae* (strain 2277 and strain Bipesco 5), applied as fungus colonized barley kernels (FCBK) by hand, compared with a control and a soil insecticide, was tested against soil stages of *D. v. virgifera*. Additionally, plots without "any treatment" were added to determine indigenous population of the pest insect at this site, called as "untreated" (Table 1). The whole experiment needed an area of about 100 m^2 (treatments and buffer zones) and the whole set up was repeated three times (once in the year 2006 and twice in the year 2007). All plants, excluded the treatments with indigenous populations, were infested with *D. v. virgifera* eggs on the 16th and 14th May in 2006 and 2007 respectively. The impact of the five different treatments was evaluated by catching emerging adults in gauze cages placed over each plot measuring about 2m^2.

Experiment B: The impact of the fungal strain 2277, formulated as FCBK, on soil living stages of *D. v. virgifera* was evaluated at a larger scale by machine application. The whole experiment needed an area of 1920 m^2 and was repeated two times. The fungus material was applied at a concentration of 50 kg FCBK/ha on a surface of 960 m^2. An area with the same size was left untreated. At leaf stage 3-5 (BBCH Code: 14-16; Weber & Bleiholder, 1990) 160 randomly chosen maize plants in the untreated and the treated area were inoculated with *D. v. virgifera* eggs. Infested maize plants were marked. In August 2007, when most of *D. v. virgifera* adults had already emerged, maize plants were dug out, adhering soil was washed off and the damage on roots caused by larval feeding was evaluated using the IOWA-scale.

Additionally, all maize cobs from the same plants were weighted and the yield between the treated and the untreated area was compared.

Experiment C: The susceptibility of adult *D. v. virgifera* to spores of the strains 2277 and Bipesco 5 was tested in pot trials. One single untreated maize seed (Magister, UFA Bussigny, Switzerland) was planted in each plastic pot (d: 40 cm), watered and kept under field conditions till 5 to 6 leaves were developed. At that stage the plants were covered with gauze cages (sleeve cages, about 1m height) with a zip in front. The cages were attached with their top on a wire and their bases were fixed with adhesive tape on the pots. Adult beetles were collected at the beginning of July in a field in Hungary (Kondoros, Csongrad County). These beetles were kept for about 2 weeks in cages with artificial diet (Singh and Moore, 1985), water agar and maize cobs. After two weeks of quarantine, beetles were removed from the cage with an exhauster and transferred to the maize plants, covered by gauze nets. Twenty-five beetles per plant were used and each fungal treatment was repeated ten times. The trial was repeated twice. In the control the beetles were treated with 0.05% Tween 80.

Application of products

Experiment A: Field experiments started on the 20th April 2006 and on the 18th April 2007 together with the sowing of maize grains. The rows were opened with a rotator to a depth of 15 cm. Five different treatments were carried out: At first maize seeds were sown and then the two *M. anisopliae* strains, formulated as FCBK were applied in the same still open furrow at a concentration of 50 kg/ha. In the treatment “control” the same procedure was done with sterilized barley kernels and in treatment “insecticide” the granular soil insecticide 1.5 G Force (Tefluthrin, Syngenta, Switzerland) was applied at a concentration of 1.05g m^{-1}. The furrow was closed by hand and pressed by walking a couple of times on the soil surface. The treatment “untreated” (without any treatment) served as a control for the presence of a indigenous *D. v. virgifera* population in the experiment. A treatment consisted of six to eight plants in a row (about 1.4 row meters) and each treatment was repeated five times.

Experiment B: For the application of FCBK (*M. anisopliae* strain 2277) a sowing machine (John Deere, MaxEmerge) with six application tanks for maize (distance between rows 80 cm) and six additional smaller tanks for granulated fertilizers was used. Maize seeds and FCBK (placed in the fertilizer tanks) were applied at the same time and at the same depth of about 10 cm – 12 cm. The fungal strain 2277 was applied on 25th April 2007 at a concentration of 50 kg FCBK/ ha on 960 m^2.

Experiment C: The applications were performed always in the evening between 7 pm and 9 pm. Each plant was inoculated with 10 ml of spore suspension with a concentration of 2 x 10^7 sp/ ml with a sprayer (type: DPZ Profi 1.5l, KC GmbH, Germany). Sleeve cages, which covered the maize plants, were opened and 10 ml spore suspension was applied from the top with a distance of 20 cm from the plants. After treatment the zip was closed again. The cages were placed between maize plants in a maize field and left there under field conditions for two weeks.

Evaluation of the impact of applied M. anisopliae strains against D. v. virgifera

Experiment A: The six to eight maize plants per treatment were cut at a height of 100 cm and covered with gauze cages (130-145 cm x 75 cm x 150 cm). Cages were installed on the 20th June 2006 and 18th June 2007, just before adult beetles started to emerge from the soil (Toepfer and Kuhlmann, 2006). Emerging rates of adult *D. v. virgifera* were recorded weekly by collecting and removing all beetles from the cages. The observation period lasted eight weeks from end of June till early August in both years. The total emerge of adults was correlated to 100 inoculated eggs per plant. The efficacy of the treatments was calculated as the reduction of *D. v. virgifera* emergence compared to the control and the plants without any treatment (indigenous population) (Abbott, 1925). To evaluate the root damage caused by the pest insect all experimental plants were dug out at the end of August. The soil was removed using a high pressure water sprayer and the root damage was rated according to the traditional 1-6 IOWA scale (Hills and Peters, 1971), 1 stands for the least, 6 for the greatest damage. The roots were evaluated by at least two independent persons without information of the origin. The average root damage was calculated for each treatment and compared with the other ones. The efficacy of each treatment was calculated as the reduction of root damage compared to the untreated control (Abbott, 1925).

Experiment B: In this experiment only the root damage rates and the weight of maize cobs were evaluated. Experimental maize plants from the treated and untreated area were dug out in the middle of August. The maize cobs were cut off the stem and put into plastic bags, separately for each plant and transferred to the laboratory. Roots of the experimental plants were separated from the stem and also transferred to the laboratory. The soil was removed from the roots using a high pressure water sprayer. Damage was recorded by the traditional 1-6 IOWA scale as described in experiment A. Additionally; all maize cobs per plant were weighted. Root damage rates and weight of cobs per plant were compared between treated and untreated area.

Experiment C: Sleeve cages with inoculated adults were checked every second day for two weeks. All dead beetles were counted and removed from the cages. They were transferred to moist filter paper and incubated at 22°C (±2°C), 55% r.h. to induce fungal growth. Two weeks after application the sleeve cages were removed and the numbers of all beetles (dead and still alive beetles) were recorded. All of them were transferred to moist filter paper and fungal infections were observed for three weeks after the experiment has been finished, assuming that fungal symptoms are seen within 10 days after death on insects (Pilz et al., 2007).

Statistical analysis

To compare the five different treatments in experiment A the number of emerged adults per plant was set in relation to 100 inoculated eggs. The three replicates were pooled and log-transformed before analysis. The statistical program SPSS, version 11.5, was used to compare treatments with the within-subjects ANOVA and the LSD post hoc test (Kinnear and Gray, 2000).
Root injuries between treatments were compared with a one way ANOVA and the LSD post hoc test. To compare two treatments (one fungal treatment and the control) the independent t-test was used (experiment B). Data of the spore applications (two fungal strains and control) were analysed with the one way ANOVA and the LSD post hoc tests.
The efficacy of the treatments was calculated using Abbott's formula (1925).

Results

Impact of M. anisopliae on soil inhabiting stages of D. v. virgifera

Reduction of Diabrotica v. virgifera emergence

The term "beetles/plant" always means "number of emerged beetles per 100 inoculated eggs per plant".
Experiment A: Firstly, a general overview is given, where all three replicates of this experiment (one in 2006 and two in 2007) are pooled and described. After that each experiment is described separately in detail, as we observed significantly different emergence rates between the two years.

The applied fungal strains 2277 and Bipesco 5 as well as the soil insecticide reduced *D. v. virgifera* emergence compared to the control (Figure 1). Best reduction rates resulted from the fungal treatment Bipesco 5 and the application of the soil insecticide, where significantly less beetles emerged ($p = 0.00$). Fungal strain 2277 significantly differed from the control ($p = 0.012$) and from the soil insecticide 1.5G Force ($p= 0.005$), while fungal strain Bipesco5 had a similar efficacy as the soil insecticide ($p = 0.2$) and did also not differ from strain 2277 ($p = 0.67$). In the treatment control the highest emergence rate with on average of 2.3 beetles/plant (median of 1.46 beetles/plant; sum: 285 beetles) was recorded. Strain 2277 showed an efficacy of about 31% (median: 1.55 beetles/plant; average: 1.55 beetles/plant; SD ± 0.9 (sum: 163)) and strain Bipesco 5 significantly reduced emergence by 43% (median: 0.92 beetles/plant; average: 1.29 beetles/plant; SD ± 0.98 (sum: 136)). The soil insecticide reduced the emergence of adults by 68%, where on average 0.72 beetles/plant (median: 0.67 beetles/plant) emerged (SD ± 0.59; sum: 93). An indigenous population of the pest insect existed at this site; in the untreated plots 16 beetles corresponding to 0.2 beetles /plant emerged.

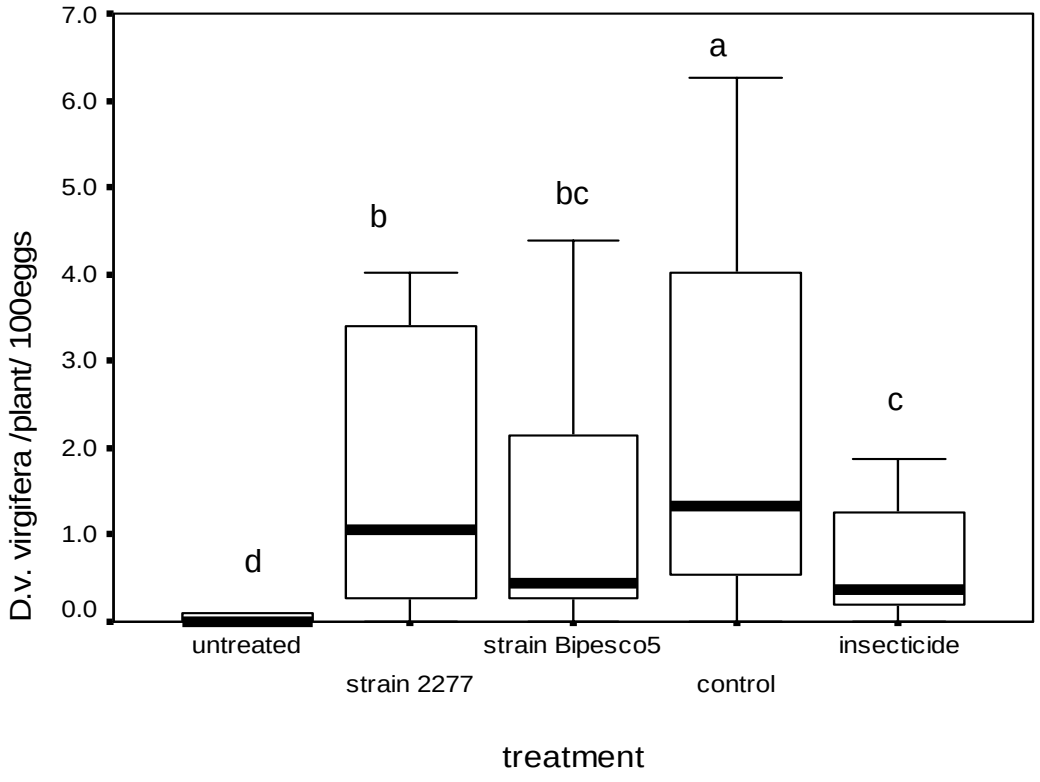

Figure 1: Emergence of *D. v. virgifera* per plant and per 100 inoculated eggs after application of the two entomopathogenic fungal strains 2277 and Bipesco5 and the soil insecticide Force 1.5G (data pooled for 2006 and 2007), n = 15 replicates/ treatment; control = application of sterile barley; untreated = without any treatment. The bars represent the median (50th percentile), boxes are 25th (lower) and 75th percentile (upper), whiskers are smallest and largest value, which are not outliers.

Replicate 1 (2006): Significantly higher numbers of beetles emerged in the control than in the fungal treatments (p = 0.007) and in the treatment soil insecticide (p = 0.00). The two fungal treatments (strains 2277 and Bipesco 5) showed an efficacy of about 49% and 44% respectively and didn't differ from each other (p = 0.80). In treatment 2277 the median emergence rate (0.09 beetles/plant, average: 0.23 beetles/plant; SD ± 0.31) was slightly lower than in treatment Bipesco 5 (0.18 beetles/plant; average 0.25 beetles/plant, SD ± 0.25).
Highest efficacy with 89% was obtained in the plots treated with the soil insecticide (median: 0.05 beetles/plant; average: 0.04 beetles/plant, SD ± 0.05) and just one beetle was found during the whole observation period in untreated plots, indicating an indigenous population of *D. v. virgifera* at this site. On average significantly lower numbers of beetles emerged in all treatments as compared to the replicates in the year 2007.

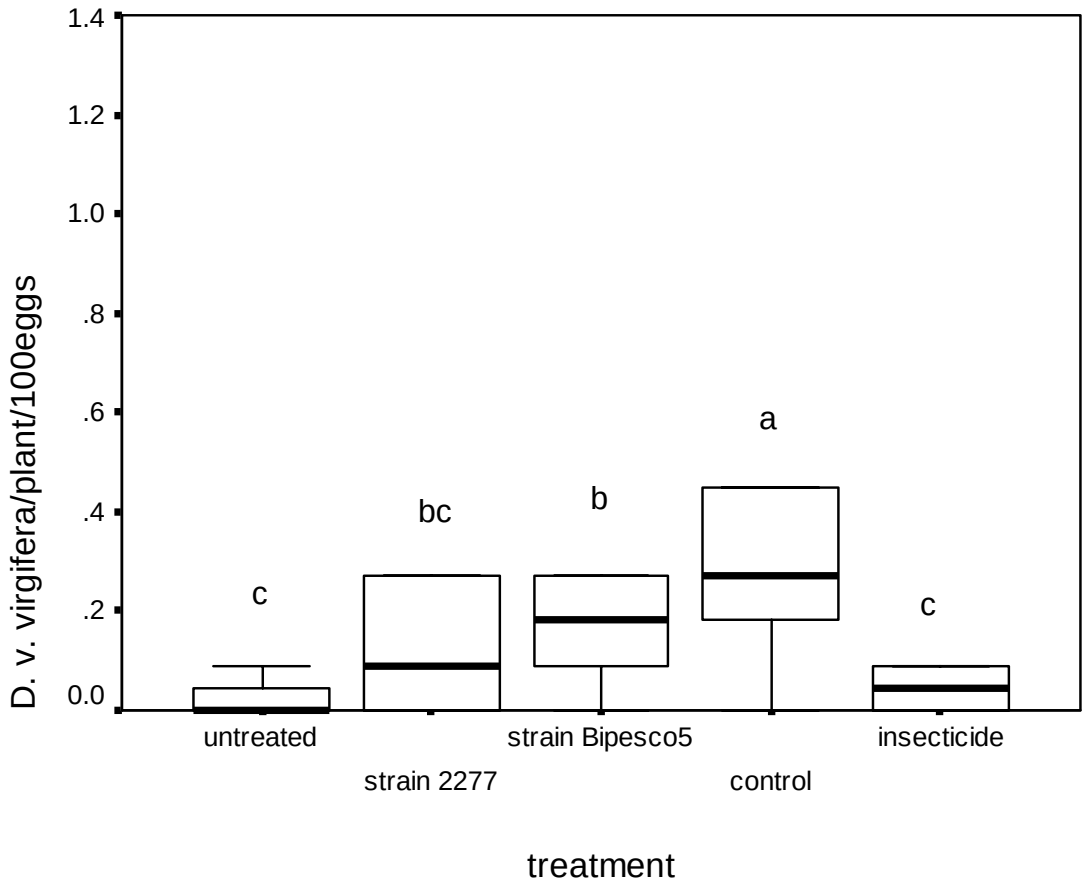

Figure 2: Emergence of *D. v. virgifera* per plant and per 100 inoculated eggs after application of the entomopathogenic fungal strains 2277 and Bipesco5 and the soil insecticide Force 1.5G, n = 5 replicates/treatment in replicate 1 in 2007. Control = application of sterile barley; untreated = without any treatment. The bars represent the median (50^{th} percentile), boxes are 25^{th} (lower) and 75^{th} percentile (upper), whiskers are smallest and largest values which are not outliers.

Replicate 2 (2007): Similar numbers of emerged beetles could be recorded, no matter if treated by fungal strains or by the insecticide. Just in the treatment, where the indigenous population of *D. v. virgifera* was observed (untreated), significantly less beetles (p = 0.001) with a median of 0.58 beetles/plant (average of 0.58 beetles/plant, SD ± 0.54) were found. Also treatment Bipesco 5 reduced the amount of emerging beetles by about 30%, but did not differ significantly from the other treatments. Emergence rates with a median of 0.45 beetles/ plant (average: 1.16 beetles/ plant, SD ± 1.37) were found there. The application of strain 2277 as well as the soil insecticide failed at this site. Emergence rates with a median of 1.16 beetles/plant (average: 1.68 beetles/plant, SD ± 1.27) were found in treatment strain 2277 and 1.25 beetles/plant (average: 1.59 beetles/pant, SD ± 1.25) were observed in treatment soil insecticide, which were similar to the control (median: 1.16 beetles/plant; average: 1.66 beetles/plant, SD ± 1.66) (Figure 3).

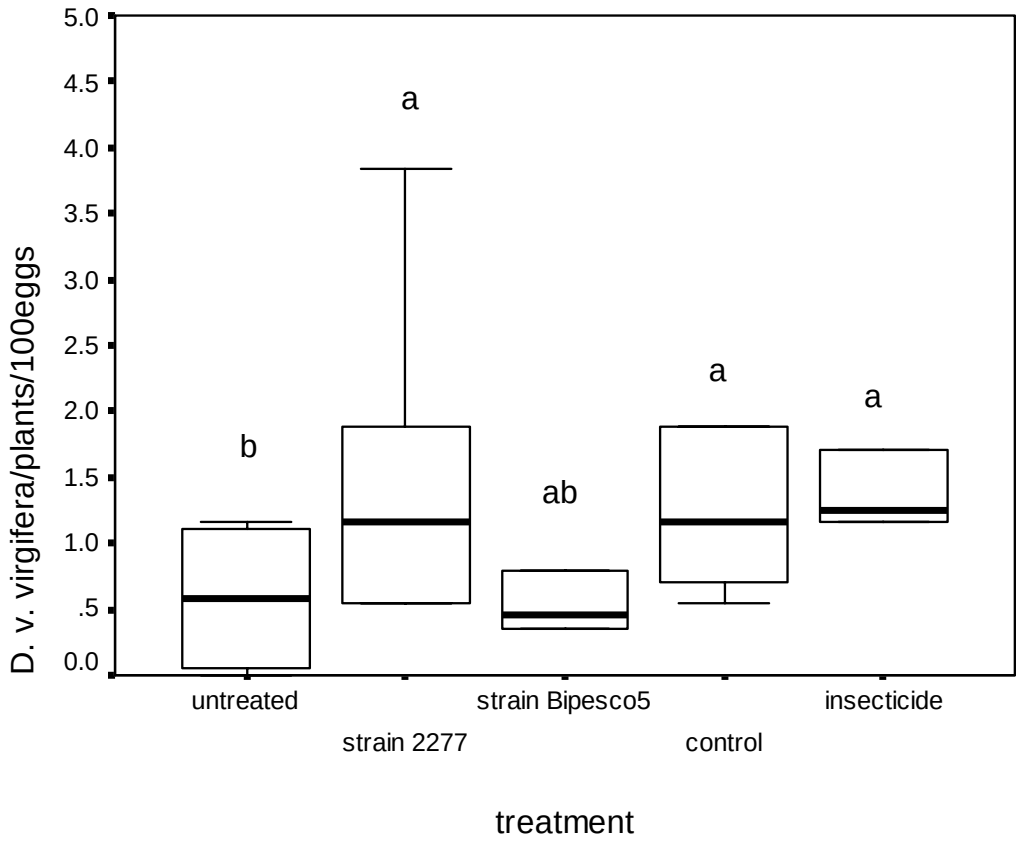

Figure 3: Emergence of *D. v. virgifera* per plant and per 100 inoculated eggs after application of the entomopathogenic fungal strains 2277 and Bipesco5 and the soil insecticide Force 1.5G, n = 5 replicates/treatment in replicate 2 in 2007. Control = application of sterile barley; untreated = without any treatment. The bars represent the median (50^{th} percentile), boxes are 25^{th} (lower) and 75^{th} percentile (upper), whiskers are smallest and largest values, which are not outliers.

Replicate 3 (2007): No indigenous population of *D. v. virgifera* was observed at this site. Significantly higher emergence rates (median: 2.95 beetles/plant; average 3.5 beetles /plant) were found in the treatment control than in the fungal treatment Bipesco 5 ($p = 0.04$) and in the treatment soil insecticide ($p = 0.00$). In fungal treatment Bipesco 5 emergence rates with a median of 2.14 beetles/plant (average: 2.4 beetles/ plant, SD ± 1.45) could be found, which led to a reduction of emerged beetles by about 32% compared to the control. Also fungal strain 2277 reduced on average the emergence of beetles by about 20% (median: 3.39 beetles/plant; average 2.8 beetle/plant, SD ± 1.1), but did not differ significantly from the control. The soil insecticide had the best efficacy with 83%, the emergence rate was 0.6 beetles/plant (median: 0.02, SD ± 0.6) (Figure 4).

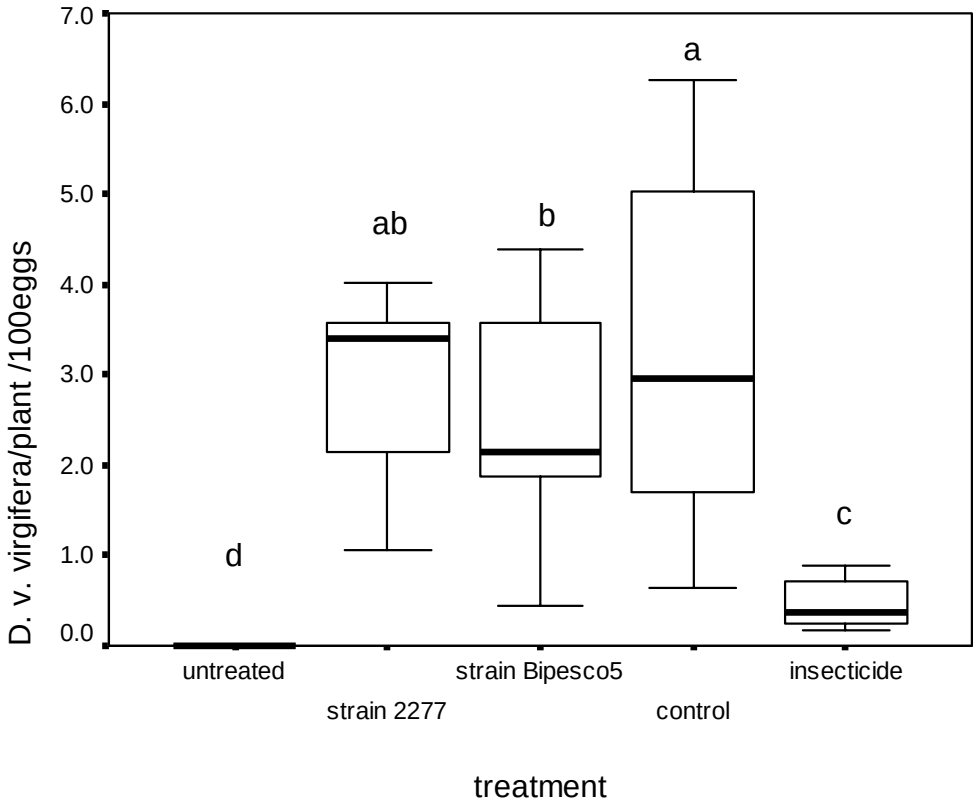

Figure 4: Emergence of *D. v. virgifera* per plant and per 100 inoculated eggs after application of two entomopathogenic fungal strains and the soil insecticide Force 1.5G, n = 5 replicates /treatment in replicate 3 in 2007. Control = application of sterile barley; untreated = without any treatment. The bars represent the median (50^{th} percentile), boxes are 25^{th} (lower) and 75^{th} percentile (upper), whiskers are smallest and largest values, which are not outliers.

Reduction of root damage, caused by larval feeding

Experiment A: Data of all three replicates of the experiment are pooled and described for a general overview. Afterwards each experiment is described separately in detail. Root damage was evaluated on the same plants, which were used to study the efficacy of different treatments on adult emergence.

No differences in root injuries between fungus treated plants and control plants could be found, while plants treated with the soil insecticide 1.5G Force showed significantly less feeding damage than all other treatments ($p = 0.017$) (Figure 5). As there was a low indigenous population of *D. v. virgifera* established, low damage was recorded on untreated plants too.

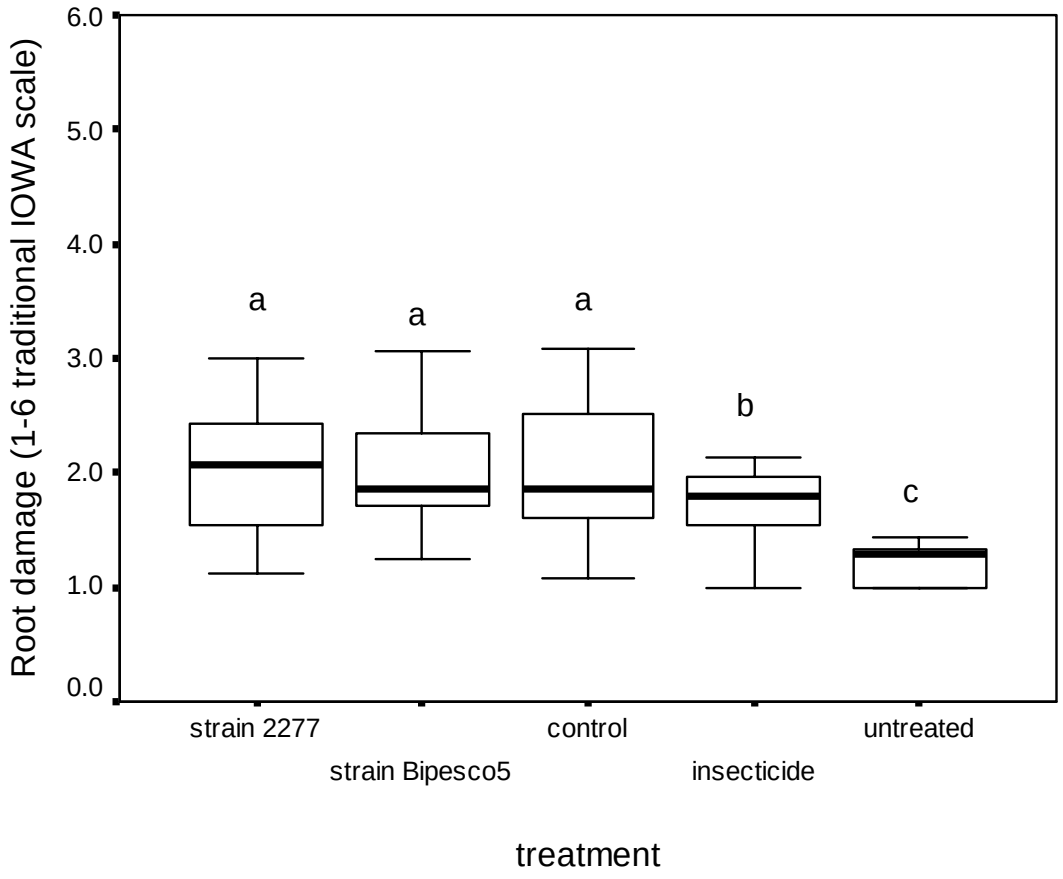

Figure 5: Root injury by *D. v. virgifera* larval feeding measured with the traditional 1-6 IOWA scale on plants in the five treatments in experiment A (pooled data of three replicates). Two fungal strains (strain 2277 and Bipesco5; control: sterile barley and the soil insecticide 1.5G Force as well as in the untreated control (indigenous population). The bars represent the median (50^{th} percentile), boxes are 25^{th} (lower) and 75^{th} percentile (upper), whiskers are smallest and largest values, which are not outliers.

Replicate 1 (2006): No significant differences between treatments and control were observed. The highest root damage was even recorded in the fungal treatment Bipesco 5 with a median of 1.64 (average: 1.61, SD ± 0.25), which differed slightly from the control (median: 1.35; average: 1.42; SD ± 0.34; p = 0.280) and from the treatment with strain 2277 (median: 1.50; average: 1.41; SD ± 0.18; p = 0.260). The lowest root damages were recorded in the plots treated with the „soil insecticide"(median: 1.12; average: 1.13; SD ± 0.18), which was significantly lower than in fungal treatment Bipesco 5 (p = 0.043). In the untreated plots (indigenous population) root damage rates with an average of 1.37 (SD ± 0.05) were recorded. This replicate showed significantly lower root damages than the successive ones in the year 2007, which corresponds with the lower emergence rates of adults (Figure 2 and Figure 6).

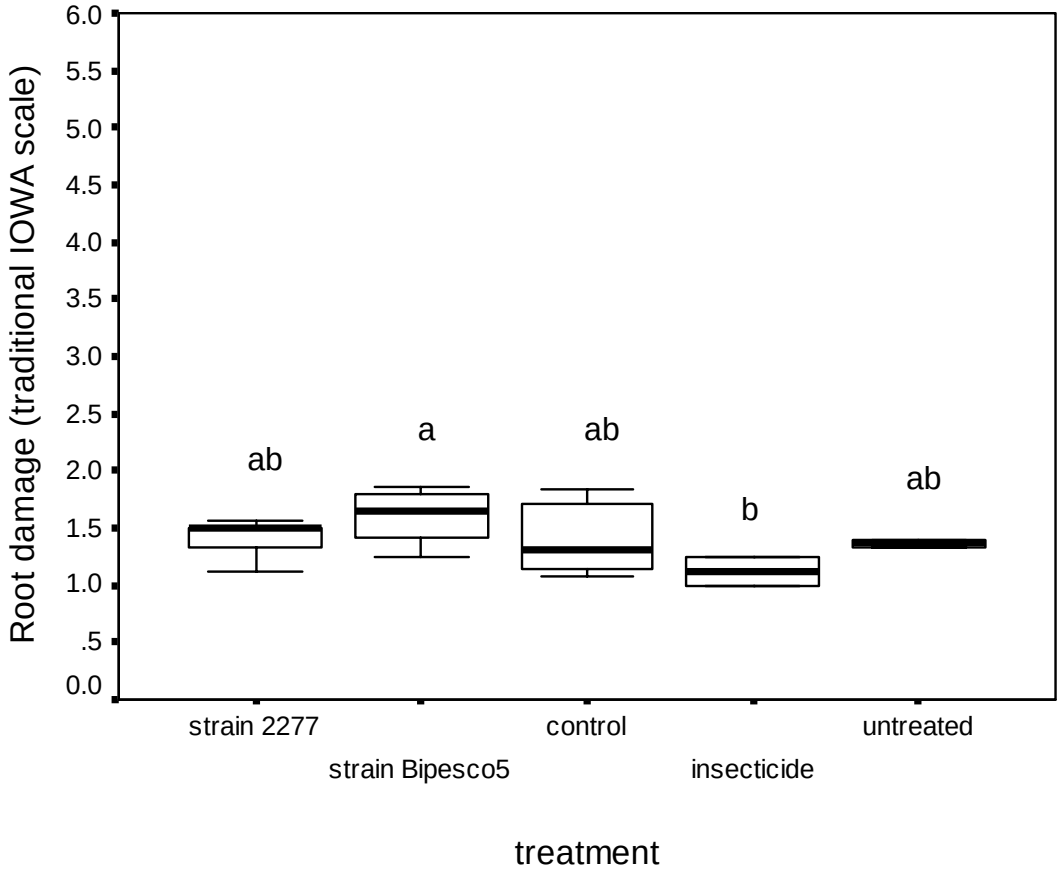

Figure 6: Replicate 1 in 2006. Root injury by *D. v. virgifera* larval feeding measured with the traditional 1-6 IOWA scale on plants in the five treatments [(strains 2277 and Bipesco5, the control (sterile barley) and the soil insecticide 1.5G Force as well as in the untreated control (indigenous population)] in the year 2006. The bars represent the median (50th percentile), boxes are 25th (lower) and 75th percentile (upper), whiskers are smallest and largest values, which are not outliers.

Replicate 2: Also this replicate showed similar root injury, no matter if treated by FCBK or soil insecticide (0.128 <p> 0.911). Only the roots in the untreated plots with an indigenous population were significantly less damaged (0.001 <p> 0.016). The median root damage in the control had a value of 1.95 (average: 2.1, SD ± 0.6) on the IOWA scale, while the highest root ratings with a score of 2.48 (average: 2.3, SD ± 0.3) were found on roots in treatment strain 2277. Slightly lower damages were observed in the treatments Bipesco 5 (median: 1.8; average: 1.9, SD ± 0.4) and the soil insecticide (median: 1.8; average: 1.9, SD ±0.2) (Figure 7). These results also correlate with the emergence rates at this site (Figure 3).

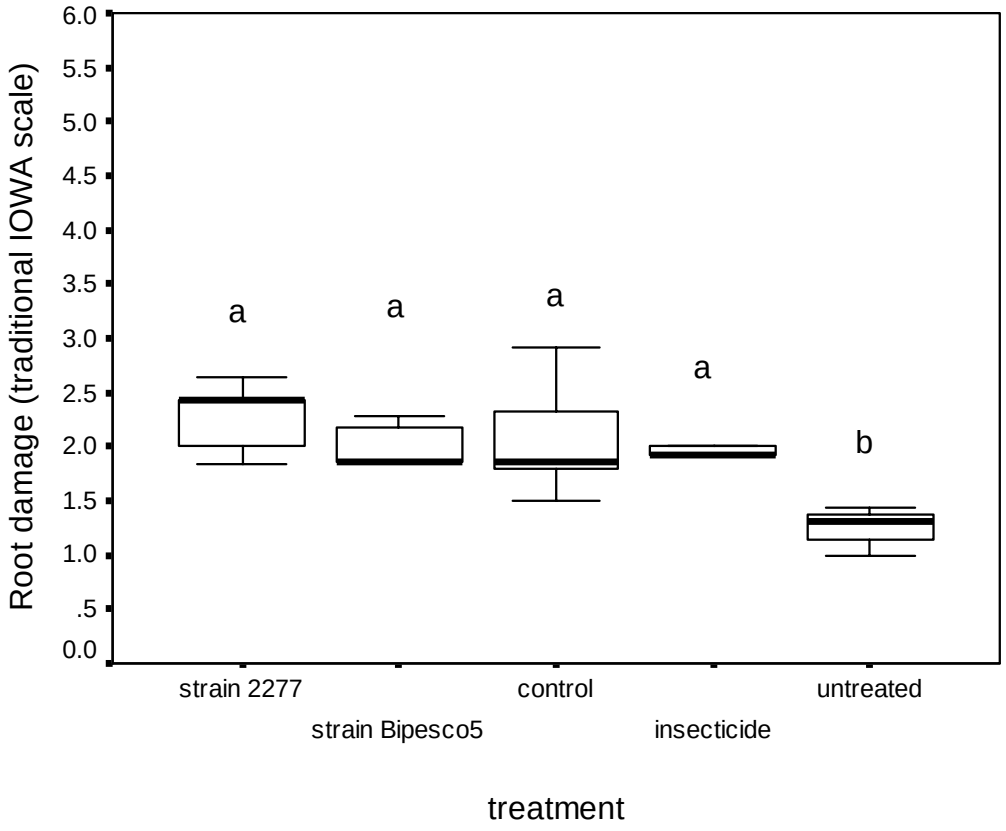

Figure 7: Replicate 2 in 2007. Root injury by *D. v. virgifera* larval feeding measured with the traditional 1-6 IOWA scale on plants in the five treatments ((strains 2277 and Bipesco5, the control (sterile barley) and the soil insecticide 1.5G Force as well as the untreated control (indigenous population)) in the year 2007. The bars represent the median (50th percentile), boxes are 25th (lower) and 75th percentile (upper), whiskers are smallest and largest values which are not outliers.

Replicate 3: Similar results as in the former replicates were observed; no differences between the application of the two fungal strains and the control could be recorded. Root injuries recorded in the treatment control (median: 2.62; average: 2.57, SD ± 0.41) and the two fungal treatments strain 2277 (median: 2.46; average 2.46, SD ±0.34) and Bipesco5 (median: 2.50; average 2.59, SD ± 0.32) were significantly higher than in the treatmentsoil insecticide (p = 0.002), where a median value of 1.62 (average: 1.75, SD ± 0.24) on the IOWA scale was

recorded. On untreated roots (indigenous population) a median value of 1.38 (average: 1.00, SD ± 0.20) was found (Figure 8).

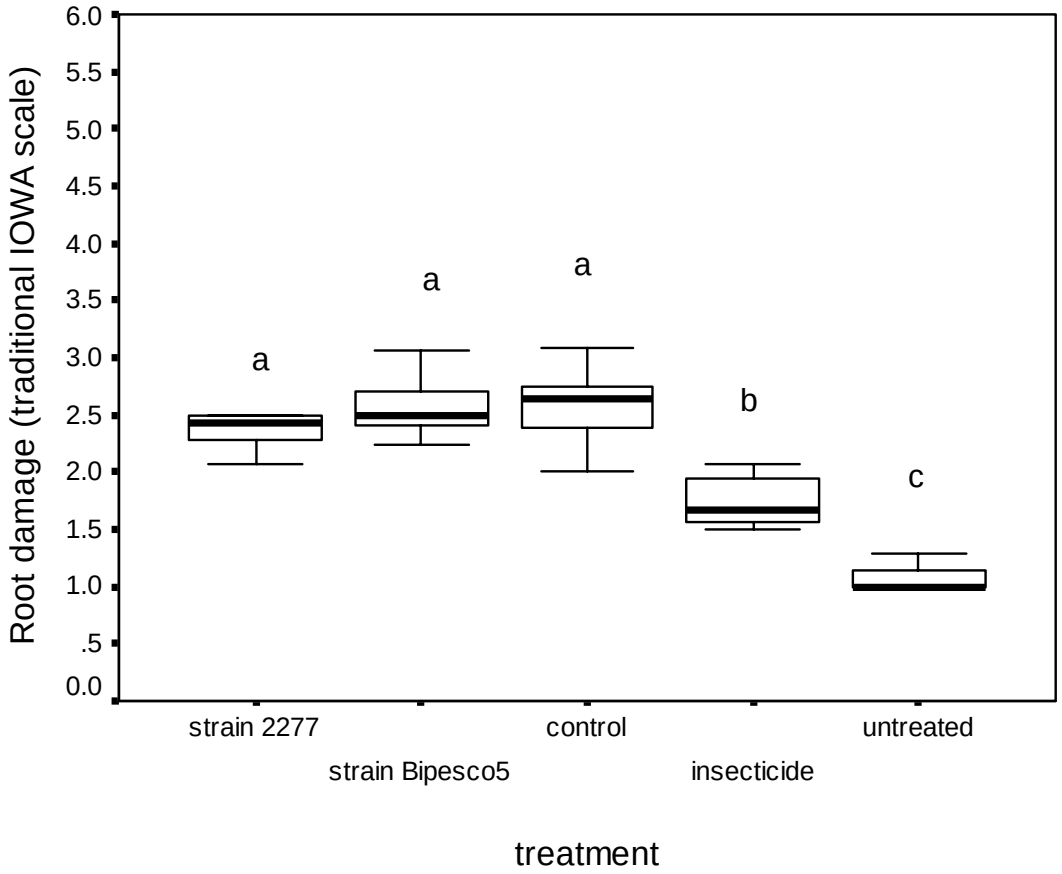

Figure 8: Replicate 3 in 2007. Root injury by *D. v. virgifera* larval feeding measured with the traditional 1-6 IOWA scale on plants in the five treatments ((strains 2277 and Bipesco5, the control (sterile barley) and the soil insecticide 1.5G Force as well as the untreated control (indigenous population)) in the year 2007. The bars represent the median (50th percentile), boxes are 25th (lower) and 75th percentile (upper), whiskers are smallest and largest values which are not outliers.

Experiment B: In general root injuries between fungus treated and untreated plots did not differ significantly. However, a slightly higher root injury was observed on control plants than on fungus treated ones (strain 2277) ($p = 0.056$). Control plants showed a median root injury score of about 1.28 (average 1.45; SD ± 0.53) on the traditional 1-6 IOWA scale, while the treated ones had a median score of 0.98 (average 1.31; SD ± 0.42) (Figure 9).

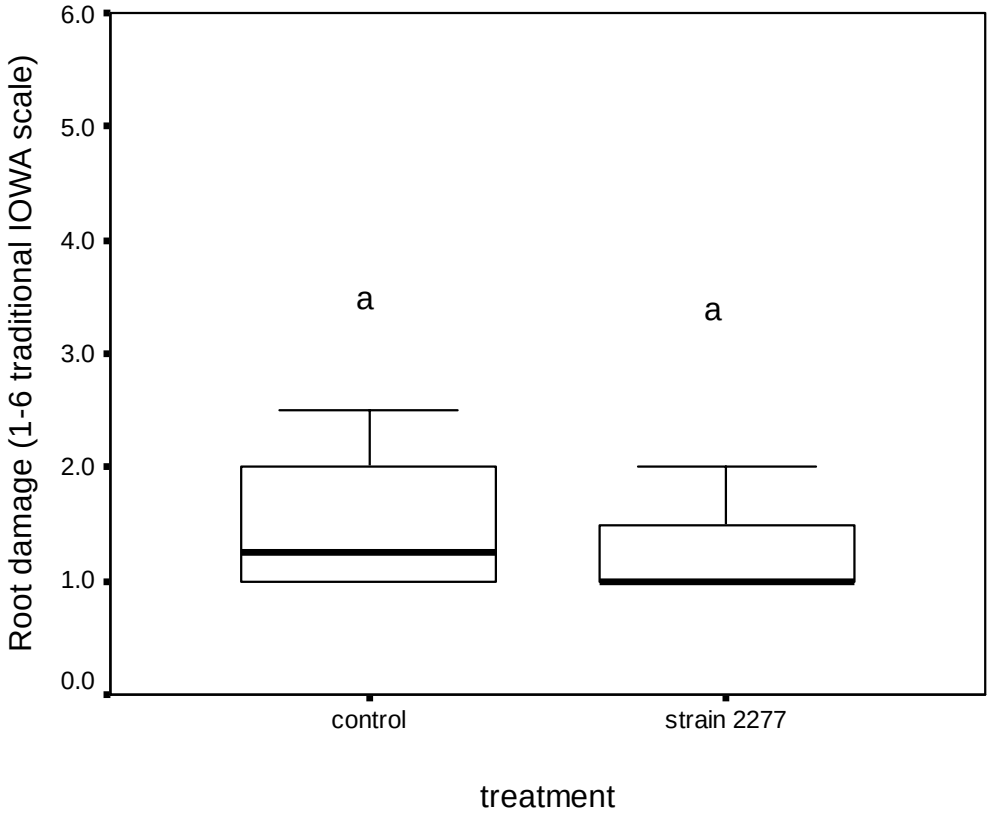

Figure 9: Root injury score from roots from fungal treated (strain 2277) and untreated (control) plants in experiment B (Orosazha). Data of two replicates (n = 320 plants/ treatment). The bars represent the median (50^{th} percentile), boxes are 25^{th} (lower) and 75^{th} percentile (upper), whiskers are smallest and largest values, which are not outliers.

The same situation was observed; when maize cobs from fungus treated and control plots were weighted. Fresh maize cobs from plants from fungal treated plots (strain 2277) did not differ significantly from those from control plots ($p = 0.44$). On average a difference of 7 g/plant between the treatments could be observed. The cobs from plants from the fungus treatment had a mean weight of 133 g/plant (median: 1.45g, SD± 62.5), while those from untreated plots yielded on average 126 g/plant (median: 1.30g, SD ± 64.1) (Figure 10).

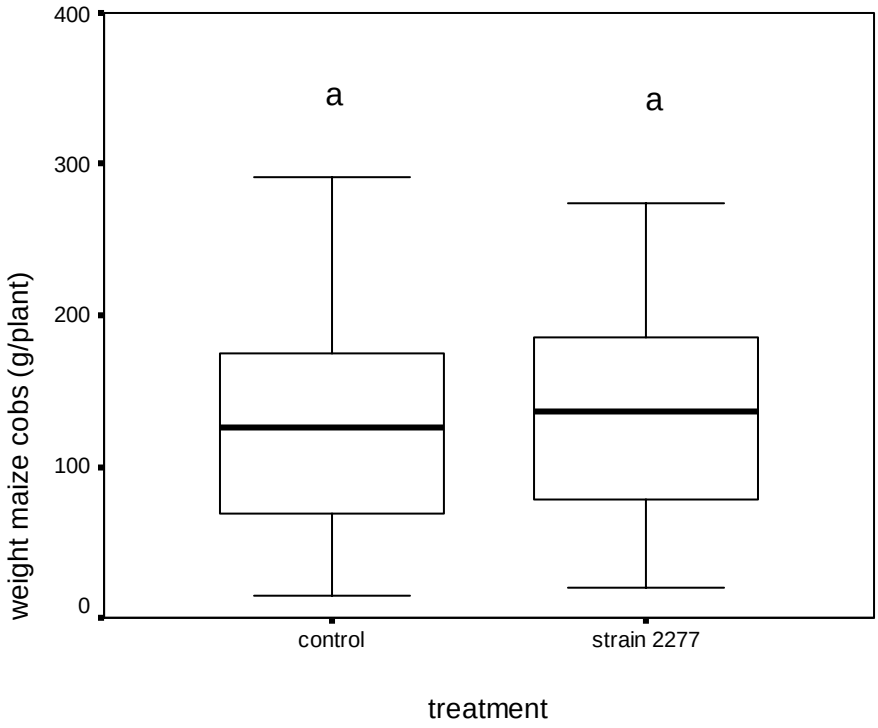

Figure 10: Fresh weight of maize cobs (gram) per plant from untreated and treated (strain 2277) plants in experiment B at *Orosazha* in Hungary 2007. Data of two replicates (n= 320 plants/treatment). The bars represent the median (50th percentile), boxes are 25th (lower) and 75th percentile (upper), whiskers are smallest and largest values which are not outliers.

Impact of M. anisopliae spore application on adult D. v. virgifera

Germination rates on 2% water agar at the time of application were for both strains (2277 and Bipesco5) between 85% and 99%. The application of *M. anisopliae* spores resulted in a significant reduction of beetles in both fungal treatments. *M. anisopliae* spore application on maize leaves colonised with adults resulted in a mean efficacy of 35% for strain 2277 and 24% for strain Bipesco 5. A significantly higher number of beetles survived in the control ($0.00 < p > 0.018$). A median of 11 living beetles/plant (average 10.6, SD ± 4.6) were recovered in the control, while in treatment strain 2277 a median of 7.2 living beetles/plant (average; 6.6, SD ± 2.6) and in treatment Bipesco 5 8.2 living beetles/plant (average: 7.9, SD ± 2.55) were found two weeks after application. The mortality between the two fungus strains did not differ significantly ($p = 0.372$), although strain 2277 was slightly more efficient (Figure 11).

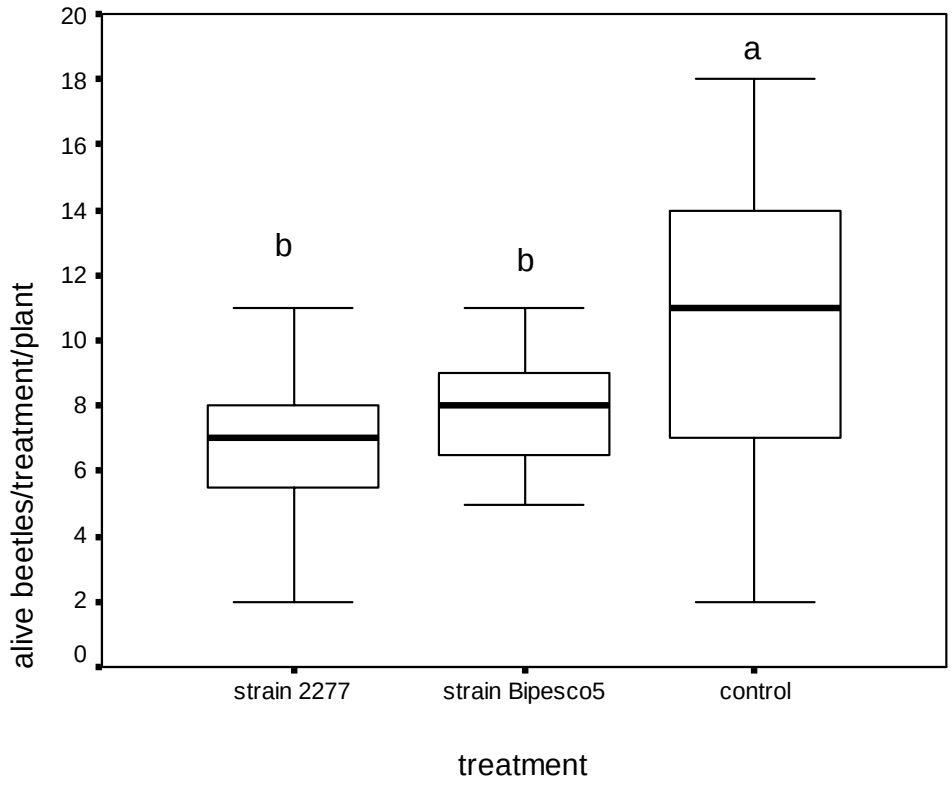

Figure 11: Number of living beetles per treatment after application of spore suspensions of *M. anisopliae* strain 2277 and strain Bipesco5 with a concentration of 2 x 107 sp/ml. Each treatment was replicated 10 times and each plant was infested with 25 beetles. The whole set up was repeated two times (n = 500 beetles/treatment). The bars represent the median (50th percentile), boxes are 25th (lower) and 75th percentile (upper), whiskers are smallest and largest values which are not outliers.

Discussion

The data presented here are the first results of field trials with entomopathogenic fungi (EPF) to control the Western Corn Rootworm (WCR) *Diabrotica v. virgifera* in Europe. We followed a step by step approach. Firstly, naturally occurring isolates of *M. anisopliae* were evaluated in the lab and the most effective ones were selected for field trials (Pilz et al., 2007, 2008). Different formulations of the fungus (fungus colonized barley kernels and spore suspension) to target different life stages of the pest insect were tested. The applied concentrations were related to economic aspects and to long term experiences in the control of *Melolontha melolontha* with the entomopathogenic fungus *Beauveria brongniartii* (Keller et al., 2002). The application of EPF showed the following effects against the larval and adult stages of WCR: Significantly higher numbers of the target pest were killed by the fungus treatment. Also the establishment and the persistence of *M. anisopliae* for an effective, cost saving and sustainable control was proved. The fungus spores were able to establish and

persist in the soil for at least fifteen months and highest spore concentrations were measured at the time, when *D. v. virgifera* was in its soil dwelling stage (Pilz et al., in prep.). The fungus application as FCBK had a mean efficacy of 43% on soil living stages when a concentration of 50 kg/ha was used. The results were obtained in field trials using simple standard equipment and in a "one-step-application" together with the sowing of maize. This demonstrates that a user friendly application technique is available and can lead to an effective control. Besides the virulence of the strain and its impact on the target pest the application technique and the training of farmers is essential for the control success.

Different results between the years were observed and could have been the result of different climatic conditions. For example, less rain falls during the field season 2007 could have influenced the germination of *M. anisopliae* spores and subsequently the infection of the insect. It is known that the first 24 hours are critical for germination on insect cuticles to cause infection and mortality (Milner and Lutton, 1986; Helyer et al., 1992; Fargues and Luz, 2000; Butt, 2002). The chemical control with the soil insecticide 1.5 G Force was found to be the most effective strategy to reduce the population density and the root injuries. However, this soil insecticide is banned in organic farming. The treatment with a fungus product offers a valuable alternative.

Infections of larvae are depended on a range of factors, like initial spore concentration in the soil, humidity, virulence of the strain as well as size of the insect, insect behaviour (movement) and the morphology of the cuticle. For the efficient control of the cockchafer *M. melolontha* spore densities of about 10^4 CFU/g soil of the entomogenous fungus *B. brongniartii* were successful (Kessler, 2004). However, *D. v. virgifera* is a relatively small insect and the life stages in the soil last for a short time of only 1 to 1.5 months. According to Ferron (1978) such small insects require an initial concentration of 10^{16} to 10^{17} spores of *M. anisopliae*/ha. Further research with increased amounts of FCBK and therefore also increased infection pressure has to be done. Increased spore concentrations in the soil increase the probability that this small insect gets in contact with the fungus. The chances are given during the first two larval instars (the last larval instars penetrate into maize roots), when the fully-grown larvae move away for pupation and when the adults emerge and move to the soil surface.

In replicate 2 of experiment A the application of the soil insecticide 1.5G Force as well as that of the fungus strain 2277 failed. Reasons are not known as this replicate was applied and handled in the same way as the other replicate in 2007. Rozen and Ester (2007) conclude that an insecticide has no effect on *D. v. virgifera* if the period between application and emergence of *D. v. virgifera* exceeds six weeks. Biological factors such as biodegradation,

behaviour and susceptibility can impact the efficacy of soil insecticides too (Krysan and Miller, 1986). No significant effects of the treatments were observed on the root injuries and weights of maize cobs. However, a positive correlation between the number of emerged adults and the damage on maize roots was found.

Techniques developed to measure the efficacy of corn rootworm larval control strategies include larval counts (Weiss and Mayo, 1983), collecting adults as they emerge from the soil (Hein et. al., 1985), root damage ratings (Hills and Peters, 1971), root size and regrowth ratings (Rogers et al., 1975), recording the percentage of lodged plants (Hill et al., 1948) and measuring grain yield (Oleson et al., 2005). Root damage ratings are generally adopted, as this method is relatively efficient, whereas the other evaluation techniques can be highly variable due to interactions with environmental conditions, soil type, moisture and maize varieties (Mayo, 1986). Root injuries of above 2.5 - 3 on the Iowa scale result in economic reductions in grain yield (Turpin et al., 1972; Stamm et al., 1985; Foster et al., 1986; Mayo, 1986). In our trials we measured in general low feeding injuries, mostly beyond the economic threshold. But, slightly higher weights of fresh maize cobs were observed in plots treated with FCBK.

The application of *M. anisopliae* formulated as spore suspension against adults resulted in a mean efficacy of 35%. However, effects on adults do not reduce the economic losses and damages of the present year, but they can lead to a reduced fertility of females and consequently to reduced population sizes on long terms. Mulock and Chandler (2001) tested the effect of *Beauveria bassiana* on the fecundity of *D. v. virgifera*. They found that the egg production was reduced by 30% when females were treated with a spore suspension of 5 x 10^7 sp /ml and that the overall egg production capacity (mean numbers eggs/female) was significantly lower compared to the untreated females. According to Mulock and Chandler (2001) a properly timed application of the fungus causing 75% beetle mortality could potentially reduce the oviposition by approximately 70%. The pest insects should be target directly because of the short term persistence of *M. anisopliae* spores in the environment (Butt, 2002; Klingen et. al., 2002; Inyang et. al., 2000, Fargues et al., 1996; Goettel et al., 2000; Rangel et al., 2004). Mulock and Chandler (2000) got a mortality rate of about 50% when spores were applied directly on beetles, but only 24% of the beetles got infected when they were in contact with contaminated substrates. In our experiments spores were formulated in 0.05% Tween 80. Bateman et al., (1993), Burges (1998), Prior et al., (1988) and Ibrahim et al., (1999) found, that oil based formulations appear to be superior than water-based formulations. Oils reduce the dependency on saturated conditions, increase the persistence of fungal spores, and may have many other useful properties. Unformulated

spores applied on rape and maize leaves are rapidly inactivated (Pilz, 2005; Pilz et al., in prep.). However, formulations have to be tested which protect spores from UV light and dehydration and which attach them on leaves. Further, the effect of repeated applications on adults needs to be investigated.

As adults are more susceptible to *M. anisopliae* spores (Pilz et al., 2007) strategies have to be developed to reach the highest potential of the biological control agent. The timing of the application must consider the climatic conditions, as high temperatures and low air humidity are unfavourable for the germination of spores and for successful infections of the insect in the environment. A combination of formulation, application and selection of highly virulent isolates are considered as the "key of success" (www.lubilosa.org.).

Infections of adult *Diabrotica* spp. by entomopathogenic fungi have been reported by Consolo et al. (2003), who infected *D. speciosa* with *Beauveria* sp. and *M. anisopliae* strains at a concentration of 1x 10^8 sp /ml and got infection rates of 70% and 45% respectively. Applications against *D. v. virgifera* were mainly conducted with *B. bassiana* (Campbell et al., 1985; Krueger and Roberts, 1997; Mulock and Chandler, 2000, 2001; Bruck and Lewis, 2001, 2002; Consolo et al., 2003), except Silva-Werneck et al. (1995), who obtained higher infection rates when *D. speciosa* larvae in Brazil were treated with *M. anisopliae* than with *B. bassiana*. They got the highest infection rate of 30% with *M. anisopliae* spores at a concentration of 1 x 10^8 sp /ml. Most of these experiments were carried out under controlled conditions in the laboratory to select mainly species and strains for their virulence. Zimmermann and Baltruschat (1991) showed a mortality of 90% of the southern corn rootworm *D. undecimpunctata* by *M. anisopliae* spores, when the soil was mixed with dry fungal spores (1 x 10^{10} conidia per 1 l soil). Such a high concentration will not be reached by the application of FCBK in the field. Highest densities of about 1.7 x 10^5 colony forming units (cfu)/g soil of *M. anisopliae* were reported by Pernfuss et al. (2005) by a successive application of two times 50 kg/ha FCBK within two years to enrich the soil with enough infectious propagules for the control of the garden chafer *Phyllopertha horticola* in Austria. Krueger and Roberts (1997) successfully reduced the root damage (percent „goosenecked"plants) in field trials, when dry mycelium of *M. anisopliae* and *Beauveria bassiana* (9.3 g/m) were incorporated into a 15 cm deep soil layer against the southern corn rootworm *D. undecimpunctata*.

Our results gave the first evidence of the potential of the biological control agent *M. anisopliae* for the control of the Western Corn Rootworm *D. v. virgifera* in Europe. Fungal applications, either as fungal colonized barley kernels or as spore suspension showed an effect to different life stages of the pest insect. Further research on the "keys of success" like

optimal application technique and time as well as of a combination of application of FCBK into the soil and spore suspension on adults, and formulations of spores will be the next steps.

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Abstract: All three larval instars of Diabrotica virgifera virgifera LeConte (western corn rootworm, Coleoptera: Chrysomelidae) feed on roots of maize, Zea mays (L.). In order to assess the efficacies of the entomopathogenic fungus Metarhizium anisopliae (Metsch.) Sorokin (Hypocreales: Clavicipitaceae), the nematode Heterorhabditis bacteriophora Poinar (Nematoda: Rhabditida), a Tefluthrin-based soil insecticide, and Clothianidin - coated seeds in controlling these larvae, we applied these agents in a field plot experiment in southern Hungary in 2006 and 2007. Efficacy was assessed by comparing the number of emerging D. v. virgifera adults and root damage among treatments and untreated controls. The nematode and the two insecticides reduced D. v. virgifera adult emergence by 65 % ± 34 SD and the fungus by 31 % ± 7 SD on average across fields and years. According to damage ratings with the node injury scale, 23 to 95 % of root damage was

prevented by the applied agents. The results of this study indicate that the future commercialisation of biological agents could be a practical and cost-effective control strategy for D. v. virgifera.

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Comparative efficacy assessment of fungi, nematodes and insecticides to control western corn rootworm larvae in maize

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Introduction

The western corn rootworm (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) causes yield losses to maize (*Zea mays* (L.)) in large areas of the USA and Canada (Kim and Sappington 2005) as well as in Europe (Kiss et al. 2005). *Diabrotica v. virgifera* is hypothesized to have originated in Mexico, where several pestiferous *Diabrotica* species occur (Branson and Krysan 1981; Krysan and Smith 1987). With the expansion of maize-growing areas in the 20th century, *D. v. virgifera* became a major pest of maize in the USA (Krysan and Miller 1986; Levine and Oloumi-Sadeghi 1991), and was accidentally introduced from North America into Europe on several occasions between the 1980s and the early 2000s (Miller et al. 2005) It is now a threat to maize production in most European countries, particularly Austria, Hungary, Serbia, Romania and Italy (Kiss et al. 2005).

Diabrotica v. virgifera is a univoltine species with eggs that overwinter in the soil (Krysan and Miller 1986). After maize has germinated, the eggs hatch, and its three larval instars feed almost exclusively on maize roots (Moeser and Vidal 2005), often causing plant lodging and economically significant yield losses. Adults can occasionally reduce yields through intensive silk feeding, which interferes with maize pollination (Chiang 1973).

In an effort to prevent yield losses, European farmers began to apply synthetic insecticides against the root feeding larvae, such as granular soil insecticides or insecticide - coated maize seeds (Ward et al. 2004). In order to avoid increasing insecticide usage against this invader, two biological control products are currently under development, one based on entomopathogenic fungi (Pilz et al. 2008) and another one based on entomopathogenic nematodes (Babendreier et al. 2006; Toepfer et al. 2008).

Entomopathogenic fungi, like *Beauveria* spp. (Hypocreales: Cordycipitacea) and *Metarhizium* spp. (Hypocreales: Clavicipitaceae) are ubiquitous soil borne pathogens (Müller-Kögler 1965), and have been found to naturally attack *D. v. virgifera* in maize fields in Europe (Pilz et al. 2008) and North America (Toepfer et al. 2009). Moreover, field studies conducted in the USA

on the efficacy of *Beauveria* and *Metarhizium* spp. against larvae of *Diabrotica* pest species (Campbell et al. 1985; Silva-Werneck et al. 1995; Krueger and Roberts 1997; Mulock and Chandler 2000, 2001; Bruck and Lewis 2001, 2002; Consolo et al. 2003) have revealed their potential as biological control agents. Also, several strains of *M. anisopliae* (Metsch.) Sorokin and *Beauveria* spp. were found to be virulent to *D. v. virgifera* larvae and adults in Europe (Pilz et al. 2007). Currently, virulent and fast growing strains of *M. anisopliae* with high spore production are prioritized for the development of a biological control product.

Entomopathogenic nematodes, like Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) are commercially available and well-studied (Kuhlmann and van der Burgt 1998; Cabanillas et al. 2005), but natural host records in *Diabrotica* species are limited. *Heterorhabditis* species were recorded in larvae of *D. balteata* LeConte in South Carolina, USA (Creighton and Fassuliotis 1985), *D. u. howardi* Barber in North Carolina, USA (Brust 1991) and *D. v. virgifera* in Hungary (Pilz et al. 2008). *Steinernema* species were recorded in larvae of *D. u. howardi* in Virginia, USA (Fronck 1950) and *D. v. virgifera* in Hungary (Pilz et al. 2008). As with fungi, North American field studies on the efficacy of nematodes against larvae of *Diabrotica* (Poinar et al. 1983; Creighton and Fassuliotis 1985; Kaya et al. 1989; Thurston and Yule 1990; Ellsbury et al. 1996; Jackson 1997) have revealed their potential as biological control agents. Also, in Europe, several commercially available nematode species or strains were found to be highly virulent to *D. v. virgifera* larvae (Toepfer et al. 2005). Several of these species / strains are commercially produced in liquid culture (Ehlers 2003), potentially allowing their use at the field scale. Currently, *Heterorhabditis bacteriophora* Poinar is prioritized for the development of a biological control product, due to its efficacy against *D. v. virgifera* larvae in plant scale field experiments (Toepfer et al. 2008), and because it belongs to the cruising type of nematodes (Gaugler 2002), which can actively search for *D. v. virgifera* larvae.

Entomopathogenic fungi or nematodes will only have commercialization potential if they can reach efficacies comparable to commonly used chemical control strategies (Levine and Oloumi – Sadeghi 1991; Rozen and Ester 2007). Several soil insecticides are registered for the control of

Diabrotica spp. larvae in the USA (Boetel et al. 2003; Sutter et al. 1989; Witmer et al. 2003), some are already registered in Europe, such as the pyrethroid Tefluthrin and the nicotinoid Clothianidin (Furlan et al. 2006; Rozen and Ester 2007), and further chemical control strategies are under development (Andersch and Schwarz 2003). Borani et al. (2006) reported that most soil insecticides work better than insecticide-coated seeds, although soil insecticides do not necessarily control larval populations, but instead protect root systems from damage (Bergmann 1981). However, because insecticides only protect maize roots in a limited zone around the site of application, larvae may survive and complete development outside the treated band, or on alternative hosts (Felsot et al. 1982). Seed coatings with insecticides provide adequate control under low to moderate pest pressure, but show variable and insufficient results at high pest levels (Furlan et al. 2006; Rozen and Ester 2007).

Unfortunately, all control strategies, whether chemically or biologically based, often produce inconsistent results, with some studies showing high efficacies, and others low efficacies (e.g. for fungi: Kinney et al. 1989; Krueger and Roberts 1997; for nematodes: Georgis and Gaugler 1991; Wright et al. 1993; Jackson and Brooks 1995; Riga et al. 2001, for soil insecticides and seed coatings: Borani et al. 2006; Furlan et al. 2006; Rozen and Ester 2007). Occasional failures of fungi or nematodes are usually explained by the use of species or strains that are not adapted to the host or to local conditions (Jackson 1995; Goude and Shapiro-Ilan 2003), by soil aridity and soil texture (Ellsbury et al. 1996; Kessler et al. 2003) as well as by the lack of alternative hosts (Brust 1991; Susurluk 2005). Additionally, fungi and nematodes often require high soil moisture (Milner and Lutton 1986; Helyer et al. 1992; Fargues and Luz 2000; Butt 2002) and are sensitive to UV radiation (Ferron 1978; Burges 1981; Butt 2002). Occasional failures of soil insecticides are usually explained by the adsorption of insecticides to organic soil particles (Felsot and Lew 1989), chemical volatilization and degradation at high soil temperatures (Getzin and Shanks 1970), surface runoff or leaching during heavy rainfalls (Gorder et al. 1982) or biodegradation (Felsot et al. 1982;

Harris et al. 1988; Felsot and Lew 1989). All these factors are influenced by the formulations of the chemical agent, the level of insecticide solubility and the insecticide placement.

Because of this inconsistency, results from previous studies can not be used to compare the efficacy of fungi, nematodes, soil insecticides and insecticide seed coatings at managing *D. v. virgifera*. To allow this comparison we used similar experimental setups for each agent, applying them with sowing of maize in the same experimental area in Csongrad County in southern Hungary. Their efficacies were assessed by measuring the reduction of *D. v. virgifera* adult emergence in the maize fields as well as the prevention of root damage. From this study, the efficacy of the potential biological control products can be estimated in comparison to the efficacy of already used chemical control options. The direction of future research for the development and commercialisation of environmentally safe and sustainable control strategies against *D. v. virgifera* larvae can be concluded.

Material and Methods

Field sites

This study was carried out in three conventionally managed maize fields in southern Hungary in 2006 and 2007 (Tab. 1). None of the fields had a geographic relief drift and thus soil texture was assumed to be relatively homogenous. All fields were ploughed and harrowed before sowing. Maize grains of the hybrid Magister (middle late silage / grain maize, UFA Semences, Bussigny, Switzerland) were sown between late April and early May (Tab. 2). The roots of the hybrid Magister emit the nematode – attracting volatile ß - caryophyllene upon feeding of *D. v. virgifera* larvae (Rasmann et al. 2005). All grains were fungicide treated except in plots where *M. anisopliae* was applied (see below). Individual maize grains were sown every 150 mm in rows separated by 750 mm. Fields were treated once with 0.16 litres herbicide per hectare at the 2 to 5 maize leaf stage (Merlin SC, 75% izoxaflutol,

Bayer Crop Science). Mechanical weeding was carried out once in May. Temperature and rainfall were recorded hourly from April to September of each study year (Davis Instruments Corp., Hayward, CA, USA) (Tab. 1).

Table.1. >>

Source and handling of Diabrotica v. virgifera

Diabrotica v. virgifera eggs were obtained from a laboratory culture of field-collected beetles in southern Hungary in 2005 and 2006 (for procedures see Singh and Moore (1985)). *Diabrotica v. virgifera* eggs were hibernated at 6 to 8°C in moist sand, and diapause was broken early April of the following experimental year by transferring them to 25°C. The sand was sieved through a 250 micrometer mesh, and recovered eggs were mixed into a solution of water and 0.15% agar. Then, maize plants of each field were infested with 189 ± 34 SD viable and ready-to-hatch eggs (in 2 ml water - agar) in early May (1 - 3 leaf stage) (Tab. 2). The eggs were applied with a standard pipette (Eppendorf company, Hamburg, Germany) in half portions into two 100 to 140 mm-deep holes at a distance of 50 to 80 mm from both sides of the maize plant. A portion of eggs was kept at 25 °C in the laboratory to assess emergence rates (~ 80 % hatched). In the field, larvae were expected to emerge between the middle and end of May and second instar larvae were expected in June (Toepfer and Kuhlmann 2006).

Experimental design

The efficacy of the biological agents *M. anisopliae* and *H. bacteriophora* as well as of the chemical insecticides Tefluthrin and Clothianidin was studied on *D. v. virgifera* larvae in five experiments

(field 1 and 2 used twice, field 3 used once; Tab. 1, 2). This study was conducted according to the efficacy evaluation standards PP 1/212 and PP 1/152 of EPPO (Anonymous 1999, 2007). In each experiment, four to five randomized plots of 3 rows of 2.5 metres each were usually used per treatment and per untreated control. In each plot, six to seven successive maize plants (≈ 1.3 meters) were artificially infested with ready-to-hatch *D. v. virgifera* eggs as described above. All agents were applied with the sowing of maize at about 100 to 150 mm depth in late April or early May (see for each agent below). The experiments in 2007 were located in different parts of the fields 1 and 2 than in 2006 in order to avoid long term influences of treatments.

Treatment with M. anisopliae

Spores of the *M. anisopliae* strain Bipesco 5, originally isolated from *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in Austria (Eilenberg et al. 2007) were prepared for field applications as follows. The strain was passed through *D. v. virgifera* larvae in laboratory, and then isolated on selective medium (Strasser et al. 1996). Medium plates were incubated in darkness for 14 days at 22°C ± 2 SD and 70% r.h. Spores were then washed off the overgrown media plates with 6 ml 0.05 % Tween 80 (polyoxyethylene-20-sorbitan monooleate, Croda International Plc) into 100 ml of a liquid medium. The medium was a mixture of (A) 1,000 ml tap water with 40 g maize starch, 4.5 g KH_2PO_4, 7.6 g Na_2HPO_4, and (B) 1,000 ml tap water with 60 g sucrose, autoclaved and mixed at 60°C. After cooling the liquid medium was inoculated with spores and shaken for 7 days on a longitudinal shaker (130 rpm, 22°C ± 2°C) to produce submersed spores and mycelia. About 100 ml of spore-mycelia suspension were then used to inoculate 1 kg of peeled sterilized barley kernels. The fungus-colonized-barley-kernels (FCBK) were produced according to the protocol of Keller (2004) at the Swiss Federal Research Station Agroscope Reckenholz-Tänikon ART, Switzerland, one month before field application, and shipped in a cool box to Hungary.

Before application, subsamples of 100 FCBKs were taken per bag to determine the quality of the fungi material. After 10 days incubation at 22°C in sand with 10% water, the percentages of

sporulating, non-sporulating or contaminated FCBKs were determined. Only bags with more than 90% sporulating and non-contaminated FCBKs were used for the experiments.

FCBKs were applied by hand at about 150 mm depth into the soil just before the grains of maize were placed at 100 mm depth. About 4 g or 133 FCBKs were applied, which corresponds to 3.1 – 5.4 x 10^9 spores/ml, or about 4 - 7 x 10^{13} spores or 50 kg of FCBKs per hectare (Tab. 2).

Treatment with H. bacteriophora

Infective juveniles of a hybrid of European and US strains of *H. bacteriophora* were provided from a liquid culture by e-nema company, Raisdorf, Germany, and were shipped in clay in a cool box from the producer to Hungary. It was stored in its shipping material at 7 to 9 °C in darkness until use. Approximately 2 to 3 hours before application, the infective juveniles were diluted together with the carrier material in tap water to the required dose (Tab. 2).

Before application, sub samples of nematodes were taken to determine the quality of the shipment batches as follows. From each shipment batch, three plastic cups (d: 40 mm, h: 60 mm) were filled with 200 g moist, sterilised sand, five larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) and 100 infective juveniles. Mortality of *Galleria mellonella* larvae was assessed after one week in darkness and at 22°C. Mortality of 70 to 100% of *G. mellonella* was found for all nematode batches, which was considered sufficient for us to use data from all nematode applications of this study for analyses.

Infective juveniles were manually applied as a row spray in a solid stream at about 100 mm depth into the soil together with the sowing of maize (see Tab. 2 for replicates). About 0.29 x 10^6 ± 0.06 SD infective juveniles were applied in a volume of 0.2 litres of water per row metre. This was the equivalent of 3.8 x 10^9 ± 0.76 SD juveniles in about 2600 litres of water per hectare. All applications were carried out in the evenings or during cloudy afternoons to avoid destruction of nematodes by UV radiation.

Treatment with Tefluthrin

Granules of the soil insecticide Tefluthrin, i.e. the pyrethroid 2,3,5,6-Tetrafluoro-4-methylbenzyl(Z)-(1RS,3RS)-3-(2-chloro- 3,3,3-trifluoro-1-propenyl-2,2-dimethylcyclopropanecarboxylate (Force 1.5 G, Syngenta, Switzerland), were applied by hand along rows at about 100 mm depth into the soil shortly after the grains of maize had been placed (Tab. 2). About 1.05 g of Force granules were applied per row meter, the equivalent of 15 kg granules per hectare.

Treatment with Clothianidin

Maize seeds coated with Clothianidin, i.e. the nicotinoid (E)-1-(2-Chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidin (Poncho FS 600, Bayer Crop Science Hungaria KFT, Budapest, Hungary) were sown by hand as described above (Tab. 2). Each seed was coated with about 2.08 microlitres of Poncho FS 600 (dose 600 g/l Clothianidin). This was the equivalent of about ~ 0.19 litre Poncho (107 g Clothianidin) per 86,000 grains per hectare.

Table.2. >>

Assessment of the efficacy of M. anisopliae, H. bacteriophora, Tefluthrin and Clothianidin

Diabrotica v. virgifera infested maize plants in each experimental plot were cut to a height of 1,000 mm and covered with gauze cages (l.: 1,300 mm, w.: 750 mm, h.:1,500 mm) just before the predicted start of adult emergence (Toepfer and Kuhlmann 2006). Adult emergence within the cages was recorded weekly from 27 June to 16 August 2006 and from 20 June to 9 August 2007

(Anonymous 1999, 2003, 2007). Total adult emergence was normalised to 100 eggs per plant. Then, the adult emergence was compared among treatments and controls in each experiment (Fig. 1) using the non parametric M. Whitney U test as the data did not conform to normality even after transformations. Then, the mean efficacy of each treatment was calculated as the reduction of *D. v. virgifera* compared to the untreated control (corrected efficacy % = (1- beetles in treated plots / beetles in the control) x 100) (Abott 1925). The efficacy among treatments was compared using the independent sample T test for non equal variances according to Levene's test for equality of variances (Kinnear and Gray 2000) (Fig. 2).

In August, after removing the emergence cages, the root systems of all infested maize plants (ca. 250 x 250 and 200 mm depth) were dug out in order to rate the root damage. Adhesive soil was removed from the roots by gentle shaking and beating, being careful not to break off any of the crown roots, and any remaining soil was then removed using a high-pressure water sprayer. Damage was rated using two scales recommended by EPPO (Anonymous 1999): (1) the non-linear 1 to 6 Iowa scale (Hills and Peters 1971) which is the most commonly used scale, but overestimates small amounts of damage such as feeding scars; and (2) the linear decimal 0.00 to 3.00 node injury scale (Oleson et al. 2005) which measures only destroyed roots or nodes (Fig. 3). The economic threshold level in conventional grain maize is reached at a rating of 3 according to the Iowa scale and 0.25 according to the node injury scale (Oleson et al. 2005). To avoid subjective bias on these ratings, root damage was estimated independently by two experts, neither which knew whether the roots came from treated or untreated plots. Average root damage was calculated for each experimental plot. Then, the mean efficacy of each treatment was calculated as the reduction in root damage compared to the control (corrected efficacy % = (1- root damage in treated plots / root damage in the control) x 100). The efficacy among treatment was compared using the independent sample T test for non equal variances according to Levene's test for equality of variances (Kinnear and Gray 2000).

Results

All tested agents, whether biological or chemical, were able to significantly reduce adult emergence of *D. v. virgifera* (Fig. 2). The choice of agent, however, influenced the degree to which *D. v. virgifera* emergence was reduced (ANOVA: $F_{(4,116)} = 4.6$, $p = 0.001$). Beetle emergence data varied strongly across fields and years. The granular soil insecticide Tefluthrin, the entomopathogenic nematode *H. bacteriophora* and the entomopathogenic fungus *M. anisopliae* failed at least once at controlling *D. v. virgifera* (Fig. 1).

On average across fields and years, Tefluthrin, *H. bacteriophora* and Clothianidin reduced adult emergence of *D. v. virgifera* by 65 % ± 34 SD (Fig. 2), with 0.6 ± 0.5 SD beetles emerging per 100 inoculated eggs in the treated plots, compared to 1.6 ± 1.3 SD beetles per 100 eggs in the untreated control plots. *Metarhizium anisopliae* reduced *D. v. virgifera* emergence by a significantly lower extent than the other agents, i.e. by 31 % ± 6.6 SD (Fig. 2), with 1.3 ± 1.1 SD beetles emerging per 100 eggs, compared to 1.6 ± 1.3 SD beetles per 100 eggs in the untreated control plots.

The reduction of the *D. v. virgifera* populations also resulted in a reduction of root damage (Fig. 3). However, root damage was not totally prevented. When small feeding scars were included in the root damage rating (1 – 6 Iowa scale), 5 to 45 % of the average root damage was prevented by the tested agents (Fig. 3). Clothianidin proved to be the most effective with 45.4 % ± 41 SD damage reductions, followed by Tefluthrin with 25.4 % ± 14.2, *H. bacteriophora* with 15.1 % ± 27.6, and *M. anisopliae* with 5.1 % ± 4.1. The average root damage in the untreated control plots was rated 2.1 ± 0.06, below the recommended economic threshold value of 3 (Oleson et al. 2005).

When only fully destroyed roots or nodes were included in the rating method (0.00 – 3.00 node injury scale), 23 to 95 % of root damage was prevented (Fig. 3). Again Clothianidin was the most effective, reducing damage by 95% ± 4.5, followed by Tefluthrin and *H. bacteriophora*, with reductions of 82 % ± 22 and 76 % ± 26, respectively, and *M. anisopliae* with 23 % ± 11. The average root damage in the untreated control plots was rated 0.13 ± 0.09, again below the recommended economic threshold value of 0.25 (Oleson et al. 2005).

Fig. 1 >>

Fig. 2 >>

Fig. 3 >>

Discussion

The experiments presented here show for the first time comparative results for the control of *D. v. virgifera* larvae by using a nematode, a fungus, and two insecticides. All these tested agents, whether biologically or chemically based, resulted in a significant reduction of *D. v. virgifera* adult emergence, and kept or suppressed root damage far below the economic thresholds.

The Tefluthrin-based insecticide, which is currently the most common control strategy against *D. v. virgifera* in Europe (Rozen and Ester 2007), achieved over 60 % efficacy at reducing adult emergence of *D. v. virgifera* in this study. Comparable results were reported from trials in the USA (Jackson 1996). However, the application and efficacy of such soil insecticides depend on several environmental and application-related factors, preventing such a high efficacy from always being reached. For example, in field 1 of this study in 2007, there was no difference observed in adult emergence between the control and the Tefluthrin –treated plots (Fig. 1). Reasons for such a failure might have been a combination of: (a) the heavy precipitation in 2007 (Tab. 1) potentially leading to a surface runoff or leaching of the insecticides (Gorder et al. 1982), and (b) a relatively high proportion of organic matter in the soil of field 1 (Tab. 1) potentially increasing an adsorption of insecticides to soil particles (Getzin and Shanks 1970).

The seeds coated with Clothianidin achieved over 70 % efficacy at reducing *D. v. virgifera* in this study. Comparable results were reported from trials in the USA by Andersch and Schwarz (2003). Although several authors reported that the Clothianidin seed coating can occasionally fail at reducing *D. v. virgifera* (Furlan et al. 2006), this was not obvious in our study.

As with the insecticides, the efficacy of the two tested biological agents; i.e. the nematode and fungus, may be influenced by soil-related (Jackson 1995) or application-related factors (Brust 1991; Ellsbury et al. 1996; Susurluk 2005). This may be the reason that several authors reported inconsistent results when using such biological agents (Georgis and Gaugler 1991; Wright et al. 1993; Jackson and Brooks 1995; Riga et al. 2001). These factors may also be the reason that both the fungus and the nematode failed once in field 1 of this study. Such failure may be attributed to the comparatively small amount of rainfall in 2006 as well as the relatively high amount of organic matter in field 1 which may have negatively influenced the fungus (Milner and Lutton 1986; Helyer et al. 1992) and the nematode (Ellsbury et al. 1996; Portillo Aguilar et al. 1999; Koppenhoefer and Fuzy 2006). However, on average across fields and years, the nematode achieved about 60 % efficacy at reducing *D. v. virgifera* emergence, which is comparable to studies with this nematode in the USA (Georgis et al. 1991) and to the efficacy of insecticides (Jackson 1996; Andersch and Schwarz 2003). Only the fungal application achieved lower mean efficacies in this study than the nematode or insecticides, i.e. a 31% reduction of *D. v. virgifera* emergence. This is probably not due to the wrong choice of fungal species or strain, because it has been found to be highly virulent to *D. v. virgifera* in laboratory screenings (Pilz et al. 2007). Moreover, other field studies showed that *M. anisopliae* can effectively control *Diabrotica* pest larvae. Zimmermann and Baltruschat (1991), for example, achieved a mortality of up to 90% of *D. undecimpunctata* using *M. anisopliae* spores, and Krueger and Roberts (1997) achieved successful reductions of plant lodging when dry mycelia of *M. anisopliae* and *Beauveria sp.* were incorporated into the soil against *D. undecimpunctata*. The relatively low efficacy of *M. anisopliae* in our study can likely be attributed to a low dose of applied fungi-colonised barley kernels. We used 50 kg, or about 4 to 7 x 10^{13} fungal spores per hectare, while Ferron (1978) stated that an initial dose of 10^{16} to 10^{17} *M. anisopliae* spores per hectare are needed for effective control of relatively small and short- lived insects such as *D. v. virgife*ra larvae. We therefore suggest the performance of further field experiments with a higher dose of this strain of *M. anisopliae*, by using either a higher initial dose or repeated applications. The latter was suggested by Pernfuss et al. (2005), who controlled the

garden chafer *Phyllopertha horticola* (L.) (Coleoptera: Scarabeidae) in Austria with two successive applications of 50 kg *M. anisopliae*- colonised barley kernels per hectare over two years.

In conclusion, the results presented here reveal that: (a) biological control agents, such as the nematode, can reach control efficacies comparable to commonly used insecticides; and (b) other agents, such as the fungus, have potential to reach such efficacies with improved application techniques. These results encourage further investigation into practical and cost-effective application techniques that could be used in the commercialisation of biological agents to control *D. v. virgifera* larvae.

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table

Tab.1. Characteristics of the three study fields in Csongrad County, southern Hungary in 2006 and 2007 (Soil samples were taken randomly around application and analysed either by the Soil Conservation Service, Szolnok, Hungary or University of Neuchatel, Switzerland or Agroscope ART, Reckenholz, Switzerland; Mean soil moisture ± SEM from May to July).

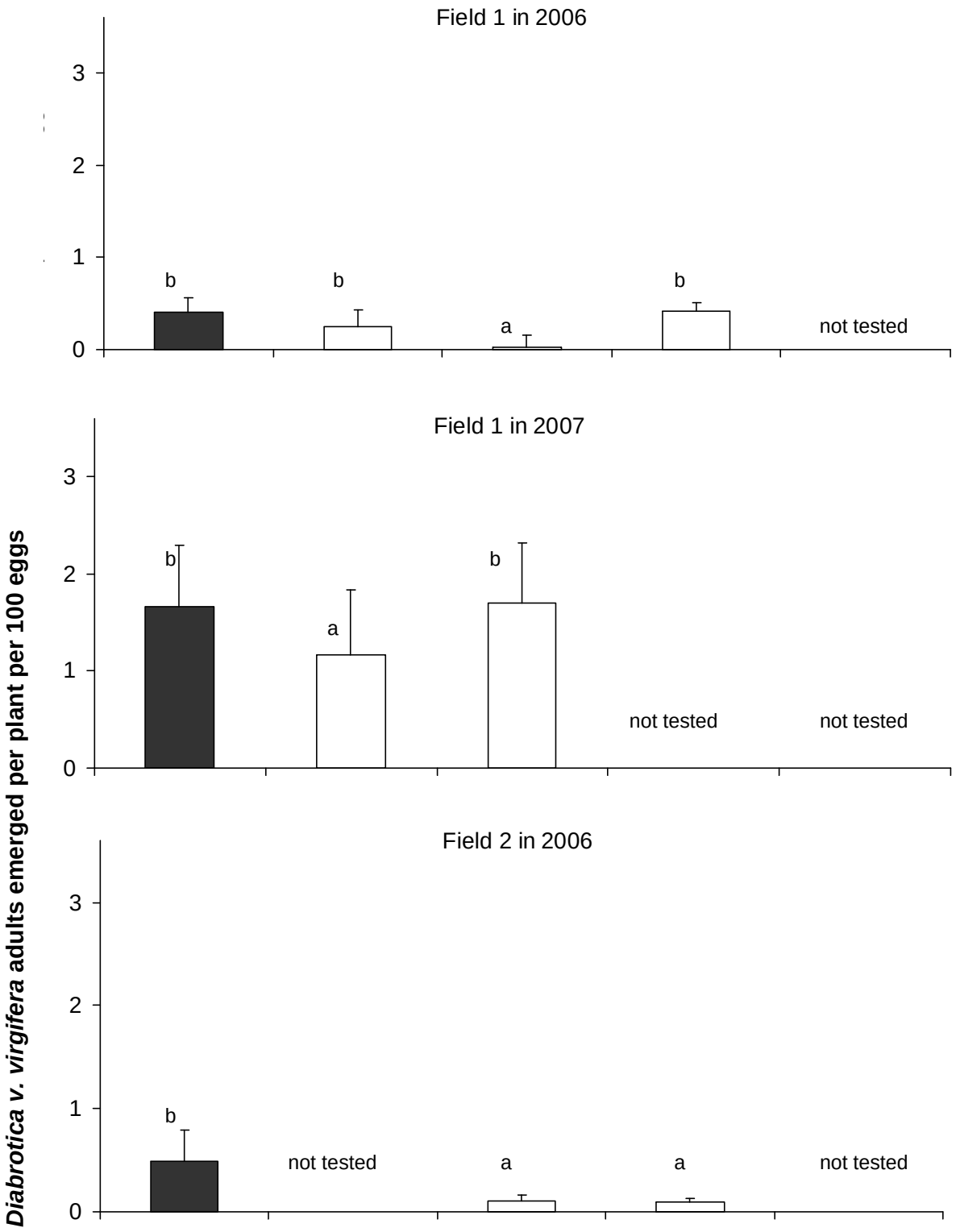
Field	1	2	3
Location	Hodmezovasarhely	North of Szatymaz	Hodmezovasarhely
Study years	2006 and 2007	2006 and 2007	2007
Coordinates	N 46° 25' 59.54''	N 46° 20' 56.50'	N 46° 25' 59.20''
	E 20° 20' 22.12''	E 20° 00' 34.00"	E 20° 20' 16.62''
Elevation (m)	83	87	83
Field size (ha)	0.2	0.2	0.1
Sand content (%)	29	86	21
Loam content (%)	35	4	38
Clay content (%)	32	9	39
PH (H_2O)	8.1	8.3	8.1
CaCO3 (%)	6.7	6	4.1
C organic (%)	2.3	0.7	1.6
Humus (%)	4	1.2	2.7
Soil bulk density (g/cm^3)	1.4 ± 0.15	1.6 ± 0.07	1.1 ± 0.18
Porosity (Pore Vol %)	45	39	48
Soil moisture (w% = grav %)	14.4 ± 5.2 ^{2006}	7.1 ± 2.5 ^{2006}	-
	no data for 2007	10.9 ± 6.3 ^{2007}	20.2 ± 2.8
Mean daily air temperature	17 ^{2006}	17 ^{2006}	-
May (C)	18 ^{2007}	18 ^{2007}	18
Mean daily air temperature	20 ^{2006}	20 ^{2006}	-
June (C)	22 ^{2007}	22 ^{2007}	22
Sum rainfall May (mm)	35 ^{2006}	35 ^{2006}	-
	104 ^{2007}	104 ^{2007}	104
Sum rainfall June (mm)	97 ^{2006}	97 ^{2006}	-
	85 ^{2007}	85 ^{2007}	85

Tab. 2 Application techniques of Metarhizium anisopliae, Heterorhabditis bacteriophora, Tefluthrin and Clothianidin against Diabrotica v. virgifera larvae in maize fields in southern Hungary (FCBK = fungus colonised barley kernels)

Agent	Field	Plots*	Eggs of *D. v. virgifera* / plant	Date of maize sowing and treatment	Application technique
M. anisopliae	1	5	160	20-Apr-06	3.1 – 5.4 x 10^9 spores, i.e. 133
	1	5	160	18-Apr-07	FCBKs (4g) / meter (= 4 - 7 x 10^{13}
	3	5	160	18-Apr-07	spores, i.e. 50 kg of FCBKs / ha)
H. bacteriophora	1	4	160	19-Apr-06	0.29 ± 0.06 million nematodes
	2	4	155	8-May-06	with 0.2 l water / row meter
	2	4	224	2-May-07	(= 3770 ± 765 Mill with 2600 l
	3	4	224	18-Apr-07	water / ha)
Tefluthrin	1	5	160	19-Apr-06	1.05 g Force 1.5G granules/ row
	1	5	160	18-Apr-07	metre (=15 kg /ha)
	2	5	155	8-May-06	
	2	4	224	2-May-07	
	3	8	224	18-Apr-07	
Clothianidin	2	4	224	2-May-07	2.08 micro litre Poncho FS600,
	3	4	224	18-Apr-07	i.e. 1.25 mg Clothianidin)/ seed,
					(= ~ 0.19 litre Poncho (107 g
					Clothianidin)/ 86,000 grains/ ha)

* Similar number of plots also for the corresponding controls

15


Field 1 in 2006
3
2
1
0
b
b
a
b
not tested
Field 1 in 2007
3
2
1
0
b
a
b
not tested
not tested
Field 2 in 2006
3
2
1
0
b
not tested
a
a
not tested
Diabrotica v. virgifera adults emerged per plant per 100 eggs

16

17

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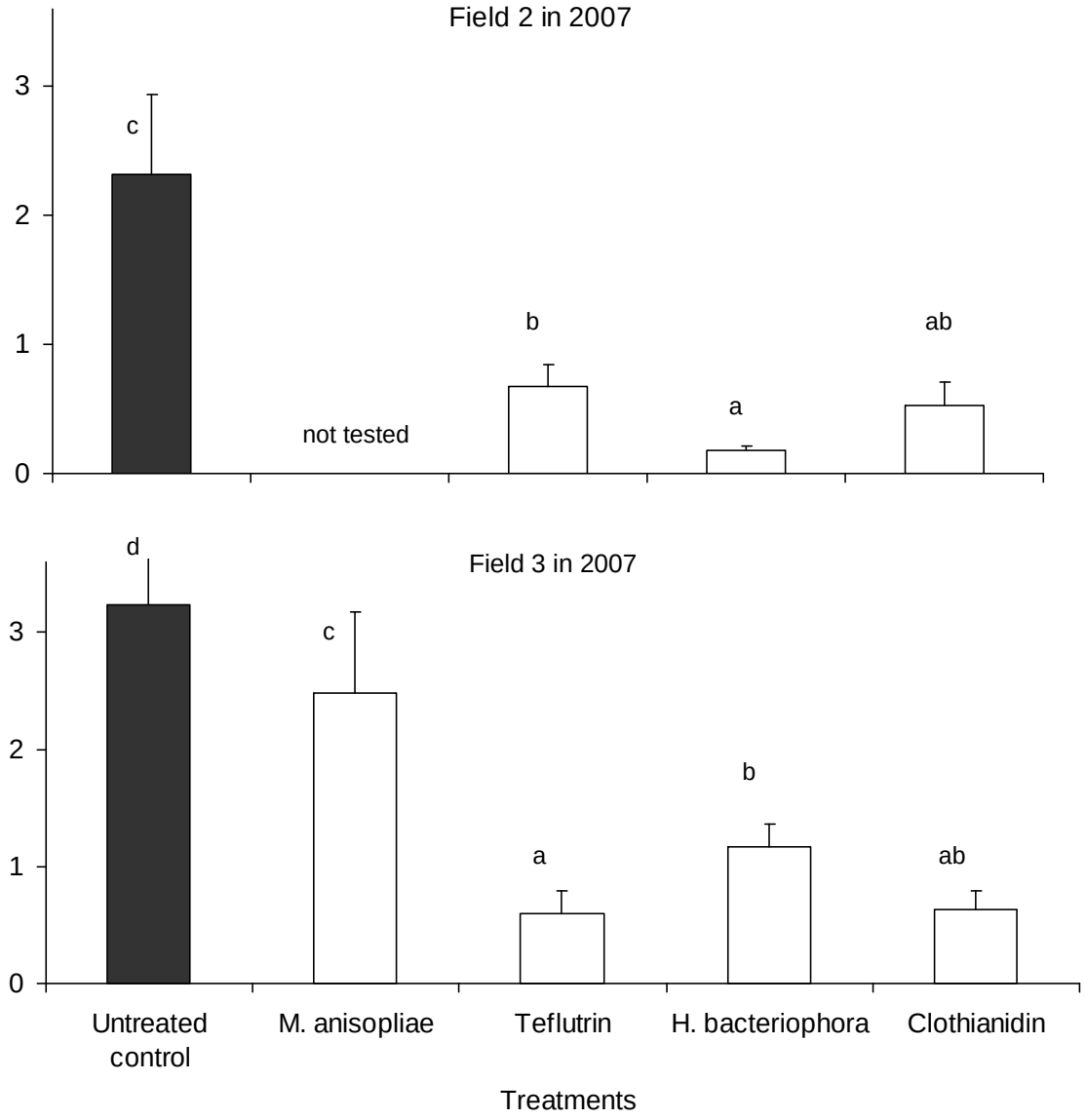

Fig. 1 Adult emergence of Diabrotica v. virgifera in maize plots treated with Metarhizium anisopliae, Heterorhabditis bacteriophora, Tefluthrin or Clothianidin, and in the untreated control plots. Adult emergence normalised to 100 eggs per plant. Fields in southern Hungary in 2006 and 2007; error bars = SEM; number of plots shown in Tab.2; letters above bars indicate significant differences according to M Whitney U test at $p < 0.05$.

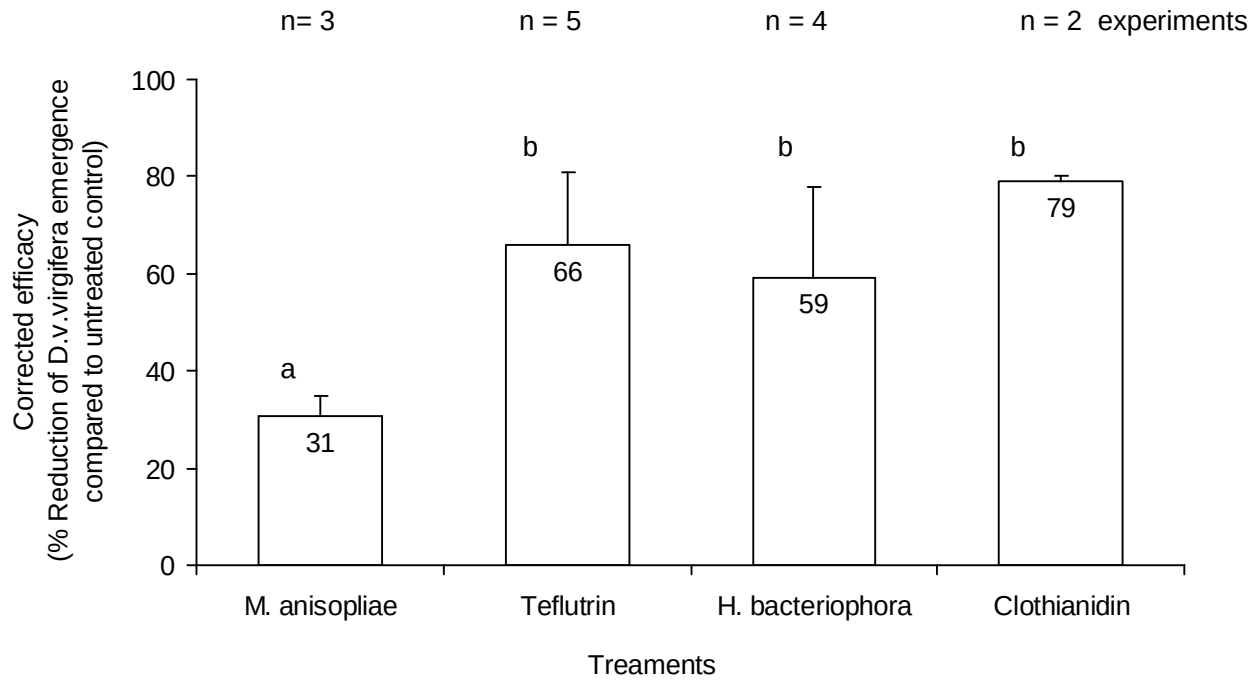

Fig. 2 Mean percent reduction of Diabrotica v. virgifera emergence due to applications of Metarhizium anisopliae, Heterorhabditis bacteriophora, Tefluthrin and Clothianidin in maize fields. Reduction of adult emergence shown in comparison to the controls (= Corrected efficacy). n = number of experiments in southern Hungary in 2006 and 2007 each with four to eight maize plots of six or seven successive plants per treatment and control; letters on bars indicate differences between application techniques according to the independent sample t test of unequal variances at $p < 0.05$; error bars = SEM.

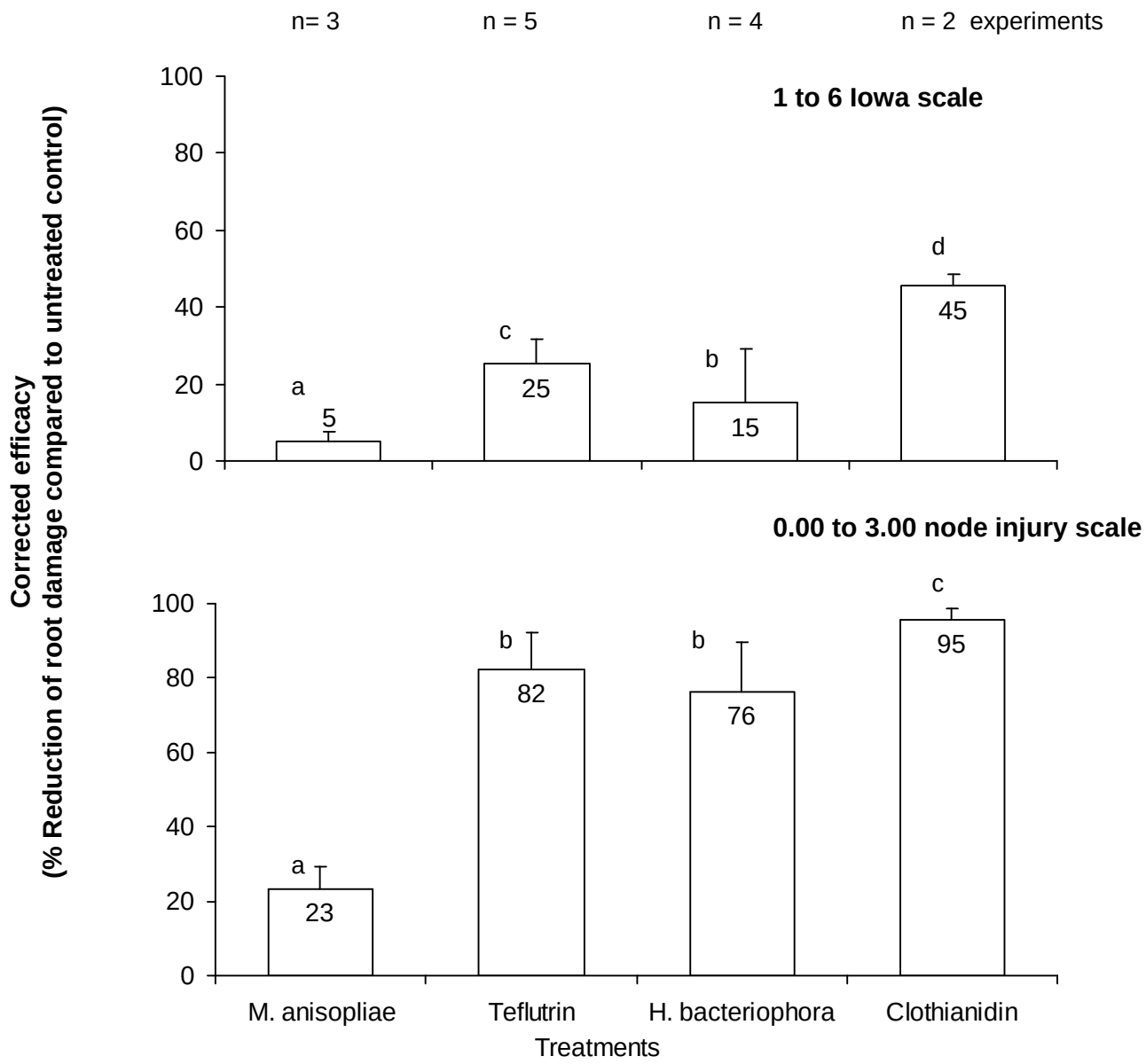

Fig. 3 Mean percent reduction of root damages due to applications of Metarhizium anisopliae, Heterorhabditis bacteriophora, Tefluthrin and Clothianidin in Diabrotica v. virgifera infested maize plots. Reduction of root damage measured by 1 to 6 Iowa scale and by the Oleson 0.00 to 3.00 node injury scale shown in comparison to the untreated controls (=corrected efficacy). n = number of experiment in southern Hungary in 2006 and 2007 each with four to eight maize plots of six or seven successive plants per treatment and control; letters on bars indicate differences between application techniques according to the independent sample t test of unequal variances at $p < 0.05$; error bars = SEM).

Curriculum vitae

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Publications

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