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Biological *Diabrotica* Management and Monitoring of *Metarhizium* Diversity in Austrian Maize Fields Following Mass Application of the Entomopathogen *Metarhizium brunneum*

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Abstract: Inundative mass application of *Metarhizium brunneum* BIPESCO 5 (Hypocreales, Clavicipitaceae) is used for the biological control of *Diabrotica v. virgifera* (Coleoptera, Chrysomelidae). Long-term field trials were performed in three Austrian maize fields—with different cultivation techniques and infestation rates—in order to evaluate the efficacy of the treatment to control the pest larvae. In addition, the indigenous *Metarhizium* spp. population structure was assessed to compare the different field sites with BIPESCO 5 mass application. Annual application of the product Granmet-P^{TM} (*Metarhizium* colonized barley kernels) significantly increased the density of *Metarhizium* spp. in the treated soil above the upper natural background level of 1000 colony forming units per gram dry weight soil. Although a decrease in the pest population over time was not achieved in heavily infested areas, less damage occurred in treated field sites in comparison to control sites. The *Metarhizium* population structure was significantly different between the treated field sites. Results showed that inundative mass application should be repeated regularly to achieve good persistence of the biological control agent, and indicated that despite intensive applications, indigenous populations of *Metarhizium* spp. can coexist in these habitats. To date, crop rotation remains the method of choice for pest reduction in Europe, however continuous and preventive application of *M. brunneum* may also present an alternative for the successful biological control of *Diabrotica*.

Keywords: *Metarhizium* spp.; *Diabrotica v. virgifera*; inundative application; abundance; population genetics

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1. Introduction

The western corn root worm *Diabrotica virgifera virgifera* LeConte (Coleoptera, Chrysomelidae), an accidentally introduced, but now firmly established maize pest, causes major damage in maize growing areas in Austria, particularly in regions of Southeast Styria with continuous maize cultivation. The application of the biocontrol agent *Metarhizium brunneum* (Petch) against *D. v. virgifera* larvae has been investigated in a few studies [1–3], but very limited data is available on long-term field trials using only the entomopathogenic fungus (EPF) as insecticide against the maize pest *Diabrotica*. The inundative use of *Metarhizium* aims at controlling the pest within a short period of time and the application has to be repeated if the pest population increases again, because reproduction and/or a permanent establishment of the fungus is not expected. This strategy is mostly used for short-term crops where high population densities of the pest need to be controlled to prevent damage [4]. Large amounts of the biocontrol agent are necessary to achieve a control effect, as soil dwelling insects can only be infected by direct contact with spores.

However, annual field processing such as mechanical cultivating or ploughing bears the risk of substantially diminishing the applied microbial agent [5,6]. It was suggested by Rauch et al. [1], that the fungus should be applied preventively, before the pest has established a large population, and pest pressure is still low (i.e., number of beetles should not exceed the economic threshold value of approximately one beetle per ten plants for continuous maize cultivation).

Soil is an extremely complex milieu, an environment with a high number of diverse microorganisms [7,8]. The presence of a viable soil microbiota have an impact on the persistence and/or efficacy of entomopathogenic fungi and vice versa. Therefore, studies on diversity and distribution of soil inhabitants, especially *Metarhizium*, are requested and further knowledge is needed [9–11]. The *Metarhizium* community is influenced by changes in agricultural practice, e.g., abundance changes depending on the crop [10] as well as on type of land-use [12]. After application in high doses, biocontrol agents are exposed to resource restrictions and compete with the well-established indigenous microorganisms [5] including native *Metarhizium* strains. Mayerhofer et al. [9,13] investigated effects of *M. brunneum*-based control agents on microbial communities in pot and field experiments. They found small effects in some treatments, but these were attributed to the product formulation and not to the activity of the fungus itself. However, knowledge on microbial interactions is still limited. Further studies are necessary to assess on the one hand possible effects of mass application of specific strains on microbial communities [10,13], and on the other hand how microbial communities may affect the establishment and development of applied EPF strains.

In this study, we investigated whether long-term inundative mass application of the biocontrol agent *M. brunneum* BIPESCO 5 improves the efficacy of this EPF to control *D. v. virgifera.* In addition, the persistence of the application strain was evaluated with and without the presence of target pest and in co-occurrence with the indigenous *Metarhizium* species and genotypes. In Styria, the pest pressure was high at the beginning of our investigations in 2012, the fungus was applied annually in a six-year long efficacy study to investigate the long-term control effect of the fungus against the larvae of *Diabrotica*. In addition, a preventive application strategy was tested in Tyrol, by annually applying the product in a maize field in a noninfested area over a period of three years.

The necessary information was gained by isolation and cultivation of *Metarhizium* species from the soil, by molecular genetic analyses of isolates, by evaluation of fungal and pest densities, as well as by evaluation of effects on plant damage caused by larval feeding.

2. Materials and Methods

2.1. Field Sites and Cultivation

The field trials were performed in two fields in Styria (Bad Radkersburg) referred to as "Styria 1" (46°41′1.9608″ N, 16°1′6.7008″ E) and "Styria 2" (46°42′42.2028″ N, 15°55′51.798″ E) and one field in Tyrol (Oberndorf/St. Johann in Tirol; 47°30′23.1552″ N, 12°23′32.3844″ E) referred to as "Tyrol". Both fields in Styria were 2.5 ha in size and 6.6 km apart; the field in Tyrol was 13.5 ha in size. The soil type in all field sites was either a mixture of loamy sand and loamy silt (Styria1, Tyrol) or loamy silt (Styria 2). The region Bad Radkersburg was known for decade-long continuous maize cropping, before an official regulation on crop rotation went into effect in 2019. This region is therefore heavily infested with *D. v. virgifera* since 2009 [1]. Thus, a natural population of *Diabrotica* could be expected in all experimental fields and no artificial infestation with any stage of *Diabrotica* was carried out on either the trial or control fields. Up to the start of this study *D. v. virgifera* has not infested the region Tyrol. Consequently, preventive biological pest control in Tyrol was also carried out in the absence of *Diabrotica* infestations.

While in the field Styria 1 maize and *Cucurbita pepo* L. *var. styriaca* were grown in rotation (2012–2014, 2016, 2018 maize; 2015, 2017 pumpkin) in fields Styria 2 and Tyrol maize was grown annually. All the fields were prepared according to common agricultural practice before sowing (i.e., seeding rate was 70,000 seeds ha^{-1} in all years. manure and

the mineral fertilizer Nitramoncal™ (13.5% ammonium and 13.5% nitrate; Borealis L.A.T, Austria) were used as fertilizing elements in April and May each).

Control field sites in Styria and Tyrol (both approx. 3.5 ha) were in close proximity to the treated field sites (Styria 2 and Tyrol; <1500 m air distance) and cultivated with maize annually. The following maize seed varieties were sown: Pharaonix RZ 480, Pioneer Hi-Breed Services, in Styria until 2016, thereafter Mexini RZ 450, RAGT Saaten Österreich; in Tyrol only ATLETICO RZ 280; KWS Austria Saat GmbH. was used. The seeding rate in Tyrol was 80,000 seeds ha^{-1} in all years. Manure and DAP 18/46 (EuroChem Agro GmbH, Germany, Mannheim) was used for fertilizing in April each year. The herbicide Laudis® + Aspect® Pro (Bayer Agrar Austria, Austria, Vienna) was applied at a maize growth stage of 13–15 according to the BBCH scale. More detailed agronomical information on the field sites in Styria can also be found in the full paper of Rauch et al. [1]. Weather stations located in the neighborhood of the experimental fields in Styria and Tyrol recorded air temperature, precipitation, relative humidity, daily sunshine duration, and global radiation throughout the whole study.

2.2. Treatment with M. brunneum

The entomopathogenic fungus *M. brunneum* strain BIPESCO 5 cultivated on barley kernels and commercialized as GranMet-P™ (Agrifutur, Italy, Alfianello) was used for all the treatments. The product, registered according to Article 53 of Regulation No. 1107/2009 of the European parliament and of the council (emergency situations in plant protection) in Austria for *Amphimallon solsitiale* and *Phyllopertha horticola* control since 2006, was applied using a RAUCH fertiliser spreader AXIS LTC [final dosage of 50 kg ha^{-1}—corresponding to 2×10^{12} colony forming units (CFU) per ha] and ploughed in the soil using a HORSCH Terrano 5 FM cultivator to a final depth of 5–10 cm, before maize was sown. All field sites were treated annually in spring throughout the years 2016–2018. Styria 2 was treated annually since 2012, Styria 1 was treated once less with the product GranMet-P™ in the same period due to inadequacies in operational management in 2014. Control sites remained untreated. The quality of the applied active agent was confirmed each year by assessing spore density, colonization ability, pureness, and strain identity [14].

2.3. Assessment of Metarhizium spp. Abundance in the Field

The abundance of the applied fungus in the soil was assessed by analyzing the CFU from pooled soil samples taken with a soil corer three times a year (n = 9, sample size $\geq$ 40 cores ha^{-1}, drawn in a Z-shape across the field area). Sampling was done in spring (before GranMet-P™ application), in midsummer and before harvesting. More than 40 cores per ha were taken in a sandglass shape and pooled for each field before analysis. At least one pooled sample was taken each sampling (n_{total} = 90). Those samples were processed after Längle et al. [14]. In short, the samples were sieved, diluted in 0.1% (wt/vol) Tween® 80 and plated out in four parallels on selective Sabouraud−4%—Glucose agar medium. Colonies morphological identified as *Metarhizium* spp. were counted after incubation at 25 °C and 60% RH for two weeks and, based on the results, the CFU per gram soil dry weight (CFU g^{-1} dry weight) were calculated.

2.4. Evaluation of the Control Efficacy of the Entomopathogen M. brunneum

Direct efficacy assessment is hardly possible due to the small size and fragile texture of mycosed larvae. Instead, indirect methods were used: assessment of adult *Diabrotica* emerging from soil and plant damage due to larval root feeding. The number of adult beetles emerging from soil was assessed in all fields in 2016 and 2017 using the trap system published by Rauch et al. [15] which covered an area of 1.1 m^2, equal to 5 cut down maize plants. Traps were installed in late June with a distance of 15 (Styria) and 35 (Tyrol) rows, respectively. Per row up to eight trap systems were established, corresponding to a final trap number of 50 traps per ha in Styria and 24 traps per ha in Tyrol. Emerging beetles were counted at least once a week until week 34 (Styria) and 37 (Tyrol). Additionally, PAL

Pheromon-sticky trap systems (CSALOMON™, 1525 Budapest, Hungary) were installed in Styria and Tyrol to monitor the number of beetles on the field sites in the region.

Plant damage was assessed one week before harvesting (in Styria calendar week 35, in Tyrol cw 37), according to BBCH coding 87–91 [16], after Rauch et al. [1], ranking from completely upright (Class 1) to not harvestable due to lodging (Class 4). More than 3000 plants per site and year were assessed.

2.5. Genetic Identification of Metarhizium spp. Isolates

Two *Metarhizium* isolates were randomly selected for multilocus genotyping (MLG) from plates used for CFU counting (n = 653). DNA extraction was performed after Kepler et al. [17] using an ACME extraction buffer containing 0.05 g diatomaceous earth per 50 mL extraction buffer. DNA extracts were stored at −20 °C until further use. Simple sequence repeat (SSR) PCR was performed according to Mayerhofer et al. [18], using set I and V of the published SSR marker sets (Ma2049, Ma2054, Ma2063, Ma195, Ma327, and Ma2287). PCR products were examined using an ABI 3130xL (Applied Biosystems, Waltham, MA, USA) and the amplicon sizes determined using the software GeneMarker (SoftGenetics; State College, PA, USA).

For each MLG one isolate was selected to determine species allocation by sequencing the 5′ end of nuclear translation elongation factor-1α (5′-TEF-1α) and subsequent sequence alignment with sequences of reference strains as described by Fernandez et al. [12]. The 5′-TEF-1α was PCR amplified using alignment with reference sequences primers EF2F (5′-GGAGGACAAGACTCACAT-CAACG-3′) and EFjmetaR (5′-TGCTCACGRGTCTGGC-CATCCTT-3′). The PCR was performed in volumes of 20 µL containing 3 µL DNA extract, 1× Phusion HF Buffer with 7.5 mM $MgCl_2$, 3% DMSO, 0.2 mM dNTPs, 0.2 µM of each primer, and 0.4 U Phusion Polymerase HotStart II. PCR amplification included an initial denaturation at 98 °C for 30 s, followed 38 cycles of denaturation at 98 °C for 5 s, annealing of the primer at 58 °C for 20 s and elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. Quality of the PCR products was verified by agarose gel electrophoresis. PCR products were purified using a Millipore MultiScreen® 96-well filtration plate (Millipore, Darmstadt, Germany) according to the manufacturer´s protocol. Sequencing was performed with the primers mentioned above using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA). Sequencing products were purified with the XTerminator Purification Kit (Applied Biosystems, Waltham, MA, USA) and analyzed using an ABI 3130xL genetic analyzer. Complimentary sequences were assembled using DNA Baser Assembler v4.36.0 (Heracle BioSoft, Mioveni, Romania). Sequences were aligned with reference sequences obtained from the GenBank database representing the different species of the *M. anisopliae* species complex [19,20] using BioEdit [21], a phylogenetic tree was calculated based on the alignment using MEGA X [22].

2.6. Data Analysis

Statistical analyses were performed using IBM SPSS Statistics version 23 (IBM Corporation, Armonk, NY, USA), OriginPro 2015G (OriginLab Corporation, Northampton, MA, USA), and R version 1.4.1717 (Free Software Foundation, Inc., Boston, MA, USA). The influence of treatment and time on CFU was analyzed with ANOVA. The correlation between CFU and percentage of BIPESCO 5, CFU and time and temperature and radial growth was assessed with Pearson correlation calculation. Minimum spanning network was created using the "poppr" package of R. For further analysis (e.g., NMDS, PERMANOVA) the package "vegan" was used. The differences of the *Metarhizium* population structure between locations were assessed with the "adonis" function within the "vegan" package based on abundance of SSR derived multilocus genotypes and Bray–Curtis dissimilarity matrices.

3. Results

3.1. Evaluation of Metarhizium spp. Abundance

The *Metarhizium* spp. abundance in all treated field sites increased after application of the production strain. Although achieved CFU values in field site Styria 2 fluctuated during the seasons between 1480 and 53,850 CFU g^{-1} dry weight soil (Figure 1A), this site, continuously planted with maize and treated annually with GranMet-P^{TM} since 2012, has consistently shown significantly higher *Metarhizium* CFU values than the untreated control site in all samples taken since spring 2015. A weak to moderate positive correlation between CFU and time (r = 0.4, $p < 0.001$) was determined for this field site (from first to last sampling). Styria 1 showed the highest variation in *Metarhizium* CFU (Figure 1B; mean values of 720 up to 85,580 CFU g^{-1} dry weight soil after first application). Although high CFU levels were not able to persist (no correlation between CFU and time; r = 0.06, p = 0.61), development of CFU was significantly different from control site ($p < 0.001$). In all Styrian field sites, including the control, *Metarhizium* CFU increased significantly since first sampling in 2012 ($p < 0.001$)—where less than 100 CFU were found—up to at least 2800 CFU (evaluated in the soil of the control field in 2018).

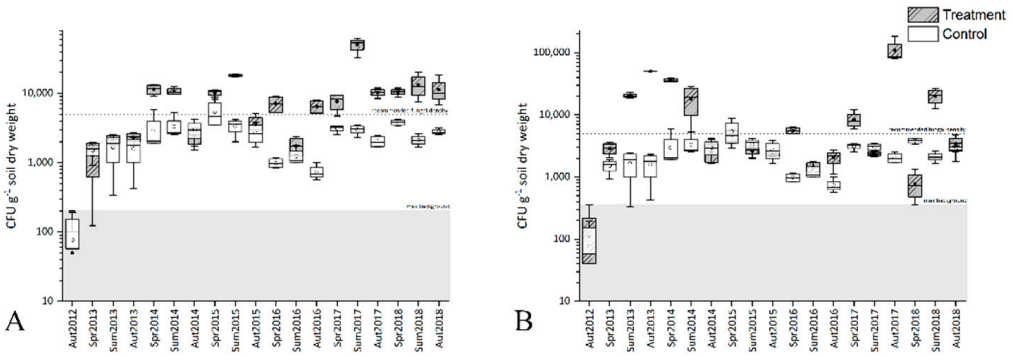

Figure 1. *Metarhizium* spp. abundance in GranMet-P^{TM} applied Styrian field sites. (**A**) shows soil samples taken from field site Styria 2, (**B**) Styria 1, both compared to untreated control site. Samples were taken in spring (Spr), summer (Sum), and autumn (Aut) from autumn 2012 to autumn 2018. The grey box indicates maximal background CFU levels before treatment, the dotted lines show recommended fungal density for sustainable control in the soil [1]. Lines and dots in box plots show median and mean CFU g^{-1} soil dry weight, respectively; boxes show the 25th and 75th percentiles, whiskers the 10th and 90th percentiles.

After the first application in Tyrol (March 2016), the abundance of *Metarhizium* spp. increased from a maximum background value of 2500 CFU g^{-1} dry weight soil to densities above the recommended abundance of 5000 CFU g^{-1} dry weight soil (Figure 2). Significantly higher values were achieved after the second treatment and could be established throughout the last year of the field trial with a final fungal density of 11,386 CFU g^{-1} dry weight soil. In comparison to the increase of the *Metarhizium* spp. density in the treated field site (r = 0.41; $p < 0.001$), the CFU in the control fields in Tyrol showed a negative correlation of CFU and time ($r = -0.48$; p = 0.003). A decrease in CFU by a factor of five could be observed in samples from autumn 2018 (366 $\pm$ 223 CFU) compared to the first sampling in 2016 (1912 $\pm$ 536 CFU) on this experimental site.

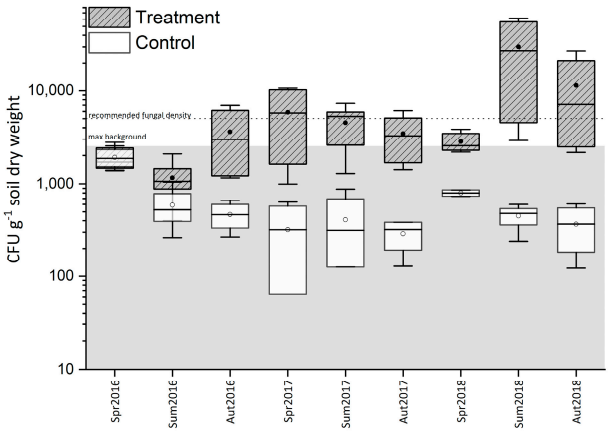

Figure 2. *Metarhizium* spp. abundance in Tyrolean field sites. Samples were taken in spring (Spr), summer (Sum), and autumn (Aut) from 2016 to 2018. Grey box indicates maximal background CFU levels before treatment, dotted line shows recommended fungal density. Lines and dots in box plots show median and mean CFU g^{-1} soil dry weight, respectively; boxes show the 25th and 75th percentiles, whiskers the 10th and 90th percentiles.

A significant positive correlation between percentage of isolates identified as BIPESCO 5 and amount of CFU was assessed for Styria 1 ($r = 0.996$; $p < 0.001$) and Tyrol ($r = 0.742$; $p = 0.03$). A moderate, but non-significant correlation occurred at the field site Styria 2 ($r = 0.527$; $p = 0.18$). The sustainable establishment of BIPESCO 5 varied between the field sites: Styria 1, which showed the highest variability of *Metarhizium* spp. abundance, also showed poor persistence of BIPESCO 5 over time, while in all the other treated fields BIPESCO 5 was able to persist throughout the season.

3.2. Pest Abundance and Plant Injury

In the heavily infested areas in Styria the *D. v. virgifera* population density continued to increase over the years. On average, the number of caught beetles doubled every year, up to 130 beetles per m^2 caught with the emergence trap system in 2018 on the untreated maize field. Only the crop rotation in combination with the biocontrol agent in Styria 1 ensured a significant reduction of the pest, with only five emerging beetles per m^2. The number of adult *Diabrotica* evaluated in Styria was significantly different between all field sites ($p < 0.001$, data not shown). Although *Diabrotica* population pressure in Styrian maize fields was very high (Table 1), both treated field sites showed no or only low damage of maize plants. As for the untreated control area, the extent of the damage was affected by the prevailing weather conditions. In the field season 2016 more than 30% of the maize plants showed plant lodging. In 2017, no lodging was observed, but due to the lack of water, plants dried up, were low growing, and fewer-to-no corn cobs had developed. In 2018, less than 1.25% of plants showed lodging due to the sufficient precipitation during this season (Figure 3). Overall, no root injuries were observed in 2018.

Table 1. Number of beetles in the trial region evaluated with PAL sticky traps (CSALOMON^{TM}, Hungary) in the regions Bad Radkersburg and Oberndorf/St. Johann in Tyrol from 2016 to 2020. Shown is the mean number of beetles over the season per trap with minimum (min) and maximum (max) number of beetles per week.

	Bad Radkersburg (Styria)						Oberndorf/ St. Johann I. T. (Tyrol)					
Year	Mean N° beetles total	Min beetles per week	Max beetles per week	Increase to previous year	cw of monitoring	cw of max catching	Mean N° beetles total	Minbeetles per week	Max beetles per week	Increase to previous year	cw of monitoring	cw of max catching
2016	x	<250	>250	-	27/41	-	260	0	226	-	27/38	36
2017	4429	0	980	-	27/38	36	749	0	549	2.88	27/37	31
2018	7336	0	1488	1.66	27/38	36	1008	0	475	1.35	27/38	32
2019	4588	10	1041	0.63	27/38	36	753	0	250	0.75	28/40	37
2020	7023	72	1193	1.53	27/38	37	y	-	-	-	-	-

cw calendar week; x the exact number of beetles was not evaluated, only classified as <250 or >250 beetles per trap; y not evaluated;—not calculated due to missing data.

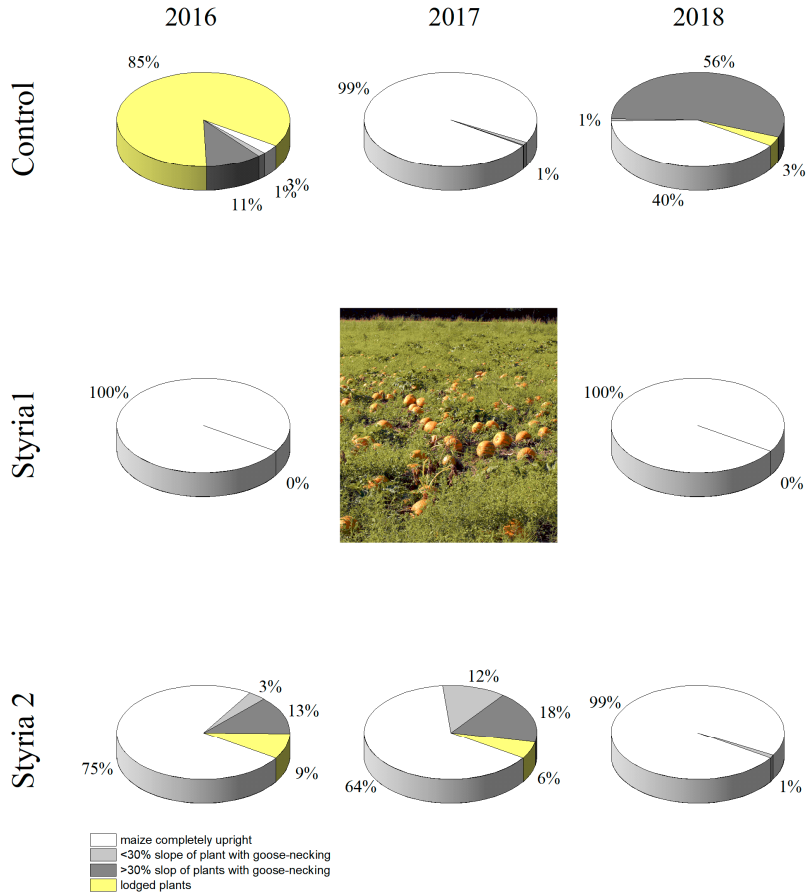

Figure 3. Percentage of plants for each damage level assessed in the years 2016 to 2018 in Styria. Healthy plants are indicated as upright, completely fallen plants as lodging. The damage for Styria 1 in 2017 could not be surveyed due to the cultivation of pumpkin (*Cucurbita pepo* L. *var. styriaca*) as part of the crop rotation.

In Tyrol, a total of only two beetles were caught in the emerging trap, confirming that the pest has reached Tyrol, however, in such small numbers that damage to the crop was not to be expected: plant health was not yet affected by larval root feeding, all plants were scored class 1 according to Rauch et al. [1]. Nevertheless, data obtained from the pheromone traps revealed a two-to-three-fold increase in *Diabrotica* density per year (Table 1).

3.3. Metarhizium Genotyping

SSR-based genotyping and subsequent 5′-TEF-1α sequencing of 653 *Metarhizium* isolates revealed the presence of 31 multilocus genotypes (MLGs) in addition to the applied production strain (Figure 4). The MLGs represented three species, i.e., *M. brunneum*, *M. robertsii*, and *M. lepidiotae* (Table 2; Figure S1).

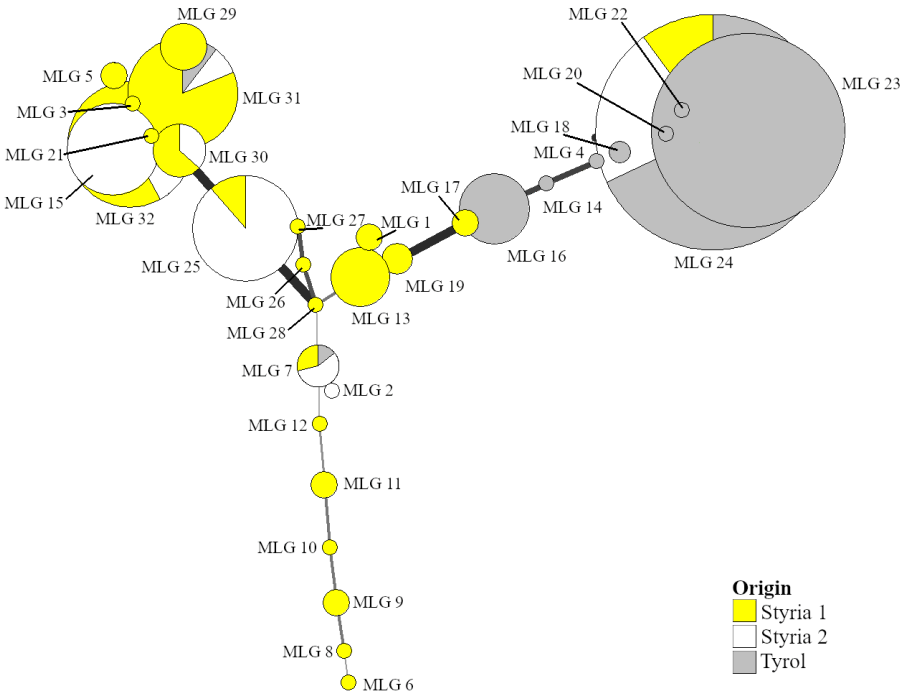

Figure 4. Minimum spanning network (MSN) showing the relationship between the SSR genotypes isolated from the treated field sites (Styria 1, Styria 2, and Tyrol). Circle sizes are proportional to the number of isolates belonging to one MLG, the thickness of the line is proportional to genetic SSR based similarity of genotypes. MLG 24 represents the genotype of the applied strain BIPESCO 5.

Table 2. Numbers of isolates and MLG identified as *M. brunneum*, *M. robertsii*, or *M. lepidiotae* from the treated field sites. The applied strain BIPESCO 5 is shown separated from *M. brunneum*.

		BIPESCO 5	***M. Brunneum***			***M. Robertsii***			***M. Lepidiotae***		
Origin	**Year**	**N**	**N**	**MLG**	**SG**	**N**	**MLG**	**SG**	**N**	**MLG**	**SG**
Styria 1	2016	0	25	1	0	47	7	2	2	2	2
Styria 1	2017	15	10	1	0	34	8	3	13	8	5
Styria 1	2018	0	8	2	0	16	3	2	1	1	1
Styria 2	2016	11	28	2	0	33	4	0	0	0	0
Styria 2	2017	24	29	2	0	19	4	3	0	0	0
Styria 2	2018	12	3	1	0	11	1	1	0	0	0
Tyrol	2016	20	130	5	3	5	1	0	0	0	0
Tyrol	2017	101	42	5	3	1	1	1	0	0	0
Tyrol	2018	30	2	2	2	0	0	0	0	0	0

N total number of isolates; MLG number of unique multilocus genotypes; SG MLG with a single isolate.

The MLG of the applied strain *M. brunneum* BIPESCO 5 was detected in 213 isolates (32.6%) and at least once in every treated field site after application of the product. The MLG composition without the applied strain was significantly different (PERMANOVA, $p < 0.001$) among the three locations (Figure 5). *M. brunneum* and *M. robertsii* were isolated from all field sites. *M. robertsii* was the dominant (54.3% in Styria 1, 67% in Styria 2) and, for Styria 2, genetically most diverse species in both Styrian trial sites, excluding the applied strain. Field site Styria 1 contained the highest diversity of MLGs from the three analyzed locations. Fifty-three percent of all MLGs were only found there. It was also the only field site where the species *M. lepidiotae* occurred (37.5% of the genotypes; but only corresponding to 9.9% of all isolates—the individual MLGs were usually found once). In Tyrol, *M. brunneum* was the dominating species isolated from the soil (96.7% of samples without BIPESCO 5), exhibiting one major genotype (82.1% of all isolates). Two genotypes (MLG 7, MLG 31) were found in all three sampling sites. The majority (22) of the 31 MLGs was only isolated in Styria. Most of the MLGs (84%) were only found in one of the field sites. None of the genotypes was present at every sampling point of the different sites, but in both Styrian fields two genotypes were isolated at least 75% of the sampling times (Styria 1: MLG 31, MLG 32; Styria 2: MLG 15, MLG 32). In Tyrol, only one MLG was found in 75% of the samplings (MLG 23), but another genotype was found at least at 62.5% of the sampling times (MLG 16). Out of ten different *M. brunneum* genotypes, only four clustered closely to the applied strain BIPESCO 5 (Figure 4; MLG 18, 20, 22, and 23). A maximum of two SSR loci differed in these genotypes by a maximum of four base pairs. The other, non-clustering *M. brunneum* genotypes contained at least five variable loci (out of six).

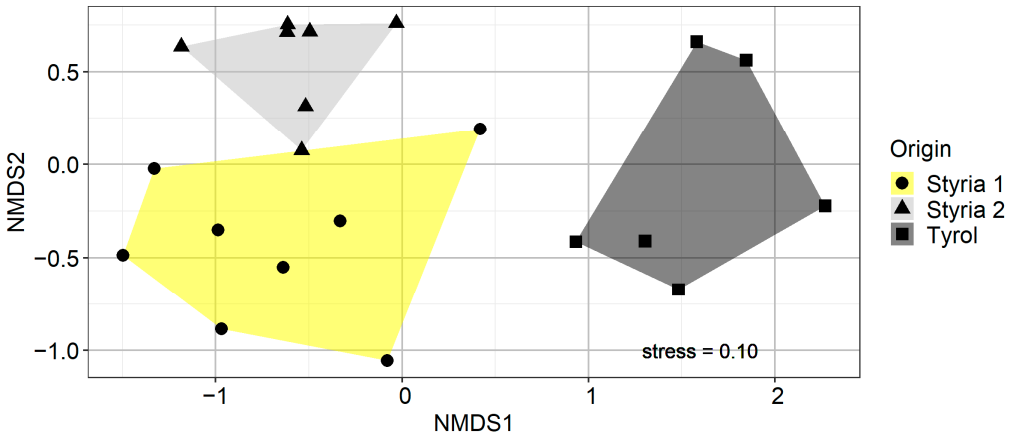

Figure 5. Non-metric multidimensional scaling (NMDS) using abundance of MLGs based on Bray–Curtis dissimilarities showing a different *Metarhizium* population structure at the treated field sites Styria 1 (circles), Styria 2 (triangle), and Tyrol (square). Each symbol represents the population composition at a sampling time. Data shown is without the applied strain and from samples after the first application of the product. Hulls illustrate the different field sites Styria 1 (yellow), Styria 2 (light grey), and Tyrol (dark grey).

4. Discussion

Diabrotica virgifera virgifera has become one of the most important maize pests in Europe and many different studies have been carried out to obtain information on the biology and behavior of the pest, as well as control options against it [1,23–25]. In this study, efficacy of the entomopathogenic fungus *M. brunneum* BIPESCO 5 against *Diabrotica* was compared under the following conditions: the product GranMet-P^{TM} (registered for *Amphimallon solstitiale* and *Phyllopertha horticola* control) was applied at the time of the general tillage in March/April with traditional agricultural equipment, reasonable workload for the farmers, heavily *Diabrotica* infested fields with continuous maize cultivation or regular crop rotation. In addition, continuously cultivated maize fields not yet heavily infested were treated to ensure the establishment of the entomopathogenic fungus without the presence of the target pests as a preventive measure by increasing the pest antagonist.

We found significant differences in emerging adults in all heavily infested areas. The lowest number of adult beetles was observed in the field site with crop rotation ($\geq$5 adults m^{-2}). This low number of adults per m^2 compared to an at least six times higher number found in continuous maize fields (treatment and control) is in accordance with results reported by Szalai et al. [26]. Although oviposition into non-maize fields near heavily infested maize fields occurs and therefore adult emergence in first-year maize can be observed, crop rotation still is the most effective method to quickly decrease *Diabrotica* population in maize fields in Europe [26–28]. As already reported by Rauch et al. [1], *M. brunneum* alone was not able to reduce the *Diabrotica* population below an acceptable/zero-damage threshold level in our study due to the high pest population density (i.e., economic threshold value: >1 beetle per plant during any weekly counts in July and August, [29]). Nevertheless, plant health was better, and less lodging occurred in *Metarhizium* treated field sites. The beneficial effect of *Metarhizium* on different plants was also recognized in studies on rhizosphere colonization of the entomopathogenic fungus; results showed extensive root development, increased root length, improved plant growth, decreased stress in plants and improved availability of nutrients [30–32]. For maize crops in particular, it was shown, that, for instance, plant-growth-promoters were activated by the production of auxins at the roots by *Metarhizium* spp. [31]. Furthermore, entomopathogenic fungi could colonize niches which otherwise are occupied by plant pathogens [31,33].

Persistence of *M. brunneum* at elevated abundance of approximately 5000 CFU g^{-1} dry weight in soil is important for the successful control of soil-borne pests such as *Diabrotica* [1]. In Tyrol and Styria 2, BIPESCO 5 could be established (Table 2) and persisted in this density. The strain also persisted in the field site Styria 1, but not as sustainably as in the other field sites—here, annual reapplication was necessary to ensure the persistence of the strain throughout the planting season.

Investigations on the diversity of *Metarhizium* in soil has revealed a variable distribution of the different species worldwide, but with genetically closely related isolates across large distances [34]. Klingen et al. [35] also found higher diversity of entomopathogenic fungi in organically farmed soil compared to conventionally treated field sites. Liao et al. [31] reported that there is evidence that plant host associations play an important role in evolutionary divergence within the genus *Metarhizium*. In the USA, *M. brunneum* is associated with the rhizosphere of shrubs and trees, whereas *M. robertsii* is found in open fields and grassland [10,36]. In addition, *M. brunneum* was only found in agricultural and open field sites when *M. robertsii* was also present [36]. In contrast, *M. brunneum* has been reported to be the dominant species in agricultural and grassland fields in Europe [12,37]. This could also be observed in our field sites in Tyrol, with more than 96.7% of all isolates being *M. brunneum* (without the applied strain). Although a comparison of the distribution of *Metarhizium* species already published is difficult due to different sampling techniques, variable distribution patterns of dominant species were found in recent studies. The species *M. anisopliae*, *M. brunneum*, *M. robertsii,* and *M. pingshaense* are most frequently isolated [38], and even these species are found with local preferences: *M. pingshaense* being the most common species found in soil taken from various locations in Japan [39], *M. anisopliae* in agricultural soil in Brazil [40,41], and *M. robertsii* dominating in the USA [10], for example. Other species only occur in restricted areas. *M. flavoviride*, for example, was the most common species found in agricultural fields in Denmark [42]. These global distribution differences of *Metarhizium* species can also be found on a smaller scale in our test sites—which all had a significantly different *Metarhizium* population structure. The two chosen regions differ in their climate, landscape and vegetation—the wide-open space in Southeast Styria, 209 m above (Adriatic) sea level (Pannonic climate), and the mountainous region of North-Tyrol (659 m above sea level; Alpine climate). Overall, *M. brunneum* as well as *M. robertsii* and *M. lepidiotae* were isolated as indigenous species. At the sampling sites of Tyrol, most of the isolated colonies were classified as *M. brunneum*, while in Styria, *M. robertsii* dominated the *Metarhizium* community. Bidochka et al. [43] suggested that due to the saprophytic phase of the species, their population genetics shifted in accordance with

the ability to grow at low or high temperatures. This would correspond to our preliminary studies on the radial growth of the three species (Table S1) in which growth between 25 °C and 30 °C for *M. brunneum* (negative correlation of temperatures with $r = -0.559$, $p < 0.05$) and *M. robertsii* ($r = -0.018$, $p > 0.05$) stagnates or declines and *M. lepidiotae*, which only occurs in the warmer region of Styria, showing a larger colony diameter at 30 °C compared to incubation at 25 °C ($r = 0.641$, $p < 0.05$). These findings are supported by Kryukov et al. [44], who have reported, that *M. robertsii* and *M. brunneum* have different optimal growth temperatures, with *M. robertsii* preferring higher temperatures than *M. brunneum*. In contrast Steinwender et al. [37] have found that certain *Metarhizium* species are not necessarily dominant in sun exposed habitats but react differently to multiple abiotic factors. Regarding *Metarhizium* spp. abundance it is well documented that there is a high correlation between temperature, humidity and survival of entomopathogenic fungi [45]. These natural abiotic factors may have an influence on the survival and development of the *M. brunneum* production strain in both Austrian regions. Our data suggest that *M. brunneum* BIPESCO 5 is more persistent when applied in Tyrol. Meyling and Eilenberg [46] summarized that *Metarhizium* is more common in exposed and regularly disturbed soil but cannot extensively proliferate. In addition, different tillage systems lead to very variable soil densities. This may also be the cause for the fluctuation of CFU and genotypes isolated from soil of the sampling site Styria 1: due to the crop rotation applied, farming practices were different compared to the field sites in Tyrol and Styria 2, where, for instance, the tillage practice remained the same every year.

Crop rotation remains the option of choice for rapid pest population decline at high pest densities. However, both the preventive and continuous use of GranMet-P^{TM} can successfully increase the density of the entomopathogenic fungus in the soil, and therefore may decline the pest population in the regions. In addition, healthy and vigorous plant growth is promoted. The production strain of the GranMet-P^{TM} product—BIPESCO 5—has been successfully tested for western corn rootworm control in Austria, Germany, Hungary, Italy and Switzerland [1,3,25,47,48]. However, studies on the biological control of adult beetles and marketable products are still lacking, although preliminary studies [49] have shown promising results. Further findings in this area will contribute to an even greater success of biological control of *Diabrotica* populations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11209445/s1, Figure S1: Maximum likelihood phylogenetic tree based on the alignment of 5′-TEF-1α sequences representing 31 different multilocus genotypes (MLG) isolated from the soil (MLG 24 representing the applied strain BIPESCO 5) and reference strains with a total of 672 positions in the final dataset, Table S1: Radial growth (mm) of production strain BIPESCO 5 and isolates from Styria after 14d incubation.

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