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Bacterial decomposition of insects post-*Metarhizium* infection: Possible influence on plant growth

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ABSTRACT

Strains of entomopathogenic fungi may have substantial differences in their final stages of mycosis. Insect cadavers are usually overgrown with mycelium after colonization of the insect body, but in many cases, bacterial decomposition of the colonized hosts occurs. We used two *Metarhizium robertsii* strains in the work: Mak-1 (cadavers become overgrown with mycelium and conidia) and P-72 (cadavers decay after fungal colonization). We conducted a comparative analysis of gut and cadaver microbiota in Colorado potato beetle larvae using 16S rRNA gene sequencing after infection with these strains. In addition, we estimated the content of different forms of nitrogen in cadavers and the influence of cadavers on the growth of *Solanum lycopersicum* on sand substrates under laboratory conditions. It was shown that infections did not lead to a significant shift in the midgut bacterial communities of infected insects compared to those of untreated insects. Importantly, bacterial communities were similar in both types of cadaver, with predominantly enterobacteria. Decomposing cadavers (P-72) were characterized by increased nitrate and ammonium, and they had a stronger growth-promoting effect on plants compared to cadavers overgrown with mycelium and conidia (Mak-1). We also estimated the colonization and growth of plants after treatment with conidia of both strains cultivated on artificial medium. Both cultures successfully colonized plants, but strain P-72 showed stronger growth promotion than Mak-1. We propose that the use of deviant strains that are unable to sporulate on cadavers leads to a faster (though only passive) flow of nitrogen from killed insects to plants.

1. Introduction

The entomopathogenic fungi *Metarhizium* are widespread in various climatic zones and a large number of terrestrial ecosystems worldwide. Several species of this genus, such as *Metarhizium brunneum*, *Metarhizium robertsii*, and *Metarhizium anisopliae*, have a broad host range and are actively used in the biological control of economically important insects (Hu et al., 2014; Lacey et al., 2015). Moreover, the fungi species enable the colonization of the rhizosphere and aboveground parts of various plants that lead to the promotion of growth, increased resistance to phytopathogens, and other positive effects on plant physiology (reviewed by Moonjely et al., 2016; Bamisile et al., 2018; Vega, 2018). Interestingly, one of the mechanisms of plant growth promotion is the transfer of nitrogen from killed insects to plant roots via fungal mycelia (Behie et al., 2012; Behie and Bidochka, 2014). Application of *Metarhizium* fungi to soil can mitigate nutrient deficits in soil, thereby improving plant productivity (e.g., Krell et al., 2018).

It is important to note that the development of mycosis caused by *Metarhizium* can be substantially different in the final stages. The classic development of mycosis is finished by the formation of areal mycelium and conidia on/within cadavers. However, *Metarhizium* strains are frequently unable to sporulate on/in killed hosts, or only

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a low portion of cadavers become overgrown by fungus (e.g., St. Leger et al., 1996; Kershaw et al., 1999; Huang et al., 2015; Ríos-Moreno et al., 2017, 2018). These abnormalities often occur among natural fungal isolates and, especially among genetically engineered strains (see below). Cessation of fungal growth may occur at different stages of mycosis. It may occur at a stage of penetration and the beginning of host colonization when fungi cannot compete with the midgut or cuticular microbiota entering the hemocoel (Zacharuk, 1973; Vey and Fargues, 1977). Moreover, cessation of fungal growth may occur after complete colonization and the formation of sclerotia (a dense entanglement of hyphae within the cadaver) (Kryukov et al., 2011; Huang et al., 2015). The abnormalities may occur for many reasons: 1) infection of insects with a high dosage of conidia, leading to strong activation of the phenoloxidase cascade and the death of fungus together with the host (Soderhall and Ajaxon, 1982); 2) overexpression of cuticle-degrading proteases by the fungus, which also leads to the activation of the phenoloxidase cascade (St. Leger et al., 1996); 3) an influx of bacteria through cuticles injured by mycosis development (Zacharuk, 1973; Vey and Fargues, 1977); 4) high production of some fungal metabolites (e.g., destruxins) (Kershaw et al., 1999; Charnley, 2003); 5) gene deletions involved in the production of some fungal toxins (e.g., oosporein), which suppress the proliferation of bacteria in insect cadavers (Fan et al., 2017); or 6) inactivation of genes involved in the fungal pH response, chitinase activity (Huang et al., 2015), and heat tolerance (Xie et al., 2019). It is likely that mycelia and conidia formation on insect cadavers is a very delicate process that can be disrupted as a result of so many genetic deregulations. Because the abnormality is genetically determined, it can be a stable characteristic of certain fungal strains.

The phenomena of decomposition are usually classified as “mortality caused by other means” and are associated with the development of saprotrophic and opportunistic microorganisms in cadavers. Among the cultivatable bacteria, *Enterococcus*, *Stenotrophomonas*, *Pantoea*, *Serratia*, *Erwinia*, *Enterobacter*, and *Pseudomonas* predominate in insects killed by the entomopathogenic fungi *Metarhizium* and *Beauveria* (Zacharuk, 1973; Fan et al., 2017; Kryukov et al., 2011). The predominance of certain bacteria in decomposed cadavers depends on the host species (Kryukov et al., 2011). However, the species composition of the saprotrophic biota has been insufficiently studied, and deep investigations of microbiota structure in insects killed by fungi have not been conducted. From an ecological perspective, it is possible to compare microbiota in cadavers that were decomposed and overgrown by fungi. It is well known that bacterial decomposition is accompanied by the release of inorganic nitrogen from dead organisms. Therefore, nitrogen transmission could occur not only through fungal mycelia (as reported Behie et al., 2012) but also through the decomposition of cadavers. The decomposition could provide a faster supply of nitrogen to soil and plants. Therefore, it is important to compare the content of different forms of nitrogen in insects that have been decomposed and overgrown by mycelium and to estimate their influence on plant growth after entering the soil. In addition, it is unknown how effectively the “deviant” strains colonize plants and affect plant growth under treatment by conidia cultivated on artificial media.

We previously found that the highly virulent strain P-72 *M. robertsii* was unable to sporulate on a range of insects, with the exception of some lepidopterans (ESM, Fig. S1; Table S1). Fungus death was mainly observed after host colonization and sclerotia formation (Kryukov et al., 2011). In this work, we studied the microbiota of insects killed by P-72 and compared it with that of insects killed by a strain that forms aerial mycelium and conidiation on killed insects (Mak-1). Moreover, we also investigated gut microbiota because the gut is the main reservoir of bacteria in insects, and we hypothesized that bacterial decomposition of cadavers may be linked to changes in the gut communities of bacteria during mycosis. In particular, some bacteria may penetrate the trough gut into the hemocoel during fungal infections, as recently reported by Wei et al. (2017). In addition, we estimated the content of different forms of nitrogen in decomposing (P-72) and overgrown by mycelium (Mak-1) cadavers and the influence of these cadavers on plant growth. Differences in plant colonization and plant growth after treatment of soil with P-72 and Mak-1 were also evaluated. Larvae of Colorado potato beetle (CPB) *Leptinotarsa decemlineata* and tomato plants (*Solanum lycopersicum*) were used as model objects because *Metarhizium* species are prospective for CPB biocontrol (Yaroslavtseva et al., 2017; Kryukov et al., 2017) as well as for increased productivity of Solanaceae plants (García et al., 2011; Krell et al., 2018).

2. Materials and methods

2.1. Fungi, insects and plants

Fungi *M. robertsii* from collection of microorganisms of the Institute of Systematics and Ecology of Animals (Siberian Branch of the Russian Academy of Science) were used in the work. Strain P-72 was isolated from CPB in Latvian territory in 1972 (Serebrov et al., 2007), and strain Mak-1 was isolated from *Calliptamus italicus* in south of West Siberia in 2001. Both strains belong to the same EF1a haplotype (Kryukov et al., 2017). Strains were stored on agarized Saburoad and Czapek-Dox media with annual transfer until 2011. After 2011, strains were stored at −80 °C in water−glycerol solution (10 %). For bioassay, conidia were cultivated for 14 d on twice-autoclaved millet, collected by sifting using a 2 mm soil sieve, dried at 26 °C and 15 % RH for 10 d and stored at 4 °C. For inoculation, conidia were suspended in water-Tween-20 solution (0.03 %). Concentrations of conidia were determined by Neubauer hemocytometer. Larvae of CPB were collected in the Novosibirsk region on private potato fields (N 53.741, E 77.655) free from chemical and biological insecticides. Larvae were maintained in 300 mL ventilated plastic containers (10 larvae per container) with potato leaves (6–7 g) at 26 °C and a photoperiod of 12:12. Potato leaves were changed daily. Tomato seeds (breed – Belyi Naliv 241, Seeds of NK LLC, Russia) were used for estimation of effects on the plants.

2.2. Infection of insects and obtaining of cadavers

Larvae of IV instar (4–6 h post molt) were infected by dipping in a water-tween suspension of fungi (10^6 conidia/mL) for 10 s. Control larvae were treated with the water-tween solution. After inoculation, larvae were immediately placed on potato plants in plastic containers and maintained as described above. Mortality was registered over 12 d. Sixty insects in each treatment were used for virulence estimation, and the whole experiment was repeated more than 5 times. At 8–12 d post-treatment, cadavers were collected and dried at room temperature for 1 d. Then, the cadavers were surface sterilized by 70 % ethanol, cut to detect sclerotia (compact entanglements of hyphae) and placed in 90 mm Petri dishes with filter paper moistened by sterile water to observe mycelial growth or bacterial decomposition. At least 4000 insects killed by each strain were analyzed for the possibility of forming mycelia.

2.3. Midgut and cadaver bacterial communities

The bacterial communities of midguts were analyzed on larvae infected with Mak-1 and P-72 as well as on uninfected insects

(control). Ninety-six hours after fungal inoculation, larvae were surface-sterilized with 70 % ethanol and dissected. Midguts with their contents were extracted and frozen in liquid nitrogen (5 midguts = one sample). The bacterial communities of cadavers were analyzed on larvae killed by Mak-1 and P-72 after 48 h exposure in moisture chambers. The whole bodies of the cadavers (3 insects per sample) were frozen in liquid nitrogen. Three samples of each treatment were used for analysis.

DNA was isolated using a PowerSoil DNA Isolation Kit (Mo Bio) according to the manufacturer protocol. The V3–V4 region of the 16S rRNA gene was amplified with the primer pair 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) combined with Illumina adapter sequences (Fadrosh et al., 2014). PCR amplification was performed as described previously (Brouchkov et al., 2017). A total of 200 ng PCR product from each sample (mix of three technical replicates) was pooled together and purified using the MinElute Gel Extraction Kit (Qiagen). The 16S libraries were sequenced with 2 × 300 bp paired-ends reagents on MiSeq (Illumina) in the SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia).

Raw sequences were analyzed with the UPARSE pipeline (Edgar, 2013) using Usearch v10.0.240. The pipeline included merging of paired reads, read quality filtering, length trimming, merging of identical reads (dereplication), discarding singleton reads, removing chimeras and OTU clustering using the UPARSE-OTU algorithm (Edgar, 2016b). The OTU sequences were assigned a taxonomy using the SINTAX (Edgar, 2016a) and the 16S RDP training set v16 (Wang et al., 2007) as a reference. Alpha diversity metrics were calculated in Usearch. Rarefaction and extrapolated curves were generated using the "iNEXT" package (Hsieh et al., 2016). OTUs classified as chloroplasts were removed. The final dataset contained 43 OTUs and 92774 reads (6185 ± 509 reads per sample) (see Dataset). Rarefaction curves have a tendency to approach the saturation plateau, which indicated a reasonable volume of the sequenced reads (Fig. S3). Raw MiSeq reads were deposited in GenBank under the study accession PRJNA529958 and sequence read archive (SRA) accession number SRP190177.

2.4. Number of colony forming units (CFUs) in midguts and cadavers

Midguts and cadavers were obtained as described in the previous section. Midguts (one sample = 3 midguts) and cadavers (one sample = one cadaver) were homogenized in 1 mL of 0.1 M phosphate buffer. Then, the suspension was diluted to 10^{-4} and 10^{-5}. One-hundred-microliter aliquots of the midgut dilutions were inoculated onto the surface of media selective for Enterobacteriaceae (Endo agar, HiMedia, India) because this group of bacteria predominated in midgut and cadavers (see below). Petri dishes were incubated at 28 °C for 72 h. Then, enumeration of the colonies was performed. Fifteen samples of midguts and six samples of cadavers were used for analysis.

2.5. Measuring of nitrogen in cadavers

Decomposed (P-72) and conidia-formed (Mak-1) cadavers were obtained after incubation in moisture chambers for 5 d. Conidia were removed from the surface of Mak-1 cadavers by vortexing in water-Tween solution (0.05 %). Then, cadavers were lyophilized, homogenized by mortar and pestle and stored at −20 °C until analysis. The content of total carbon and total nitrogen were determined by the CHNS/O analyzer PerkinElmer 2400 Series II (PerkinElmer, USA). Acetanilide (PerkinElmer) ranging from 1.5 to 3 mg was used as a standard. The labile forms of macronutrients (N-NO_3, N-NH_4) were determined by conservative methods described by Maynard et al. (2007). Briefly, the quantity of nitrate was determined potentiometrically after extraction of 2 g of the dry cadaver material by 20 mL of 0.03 M K_2SO_4. Ammonium content was determined colorimetrically after extraction of 2.5 g of the cadaver material by 50 mL of the 2 N KCl. Each treatment was replicated three times.

2.6. Influence of cadavers on plant growth

Before bioassays, *Metarhizium* CFU counts in/on cadavers were determined. Decomposing (P-72) and conidia-forming (Mak-1) cadavers (48 h post incubation in moisture chambers) were homogenized by mortar and pestle, vortexed in sterile water and plated on modified Sabouraud agar (10 g peptone, 40 g D-glucose anhydrous, 20 g agar, 1 g yeast extract) supplemented by antibiotic cocktail (cetyl trimethyl ammonium bromide 0.35 g/L; cycloheximide 0.05 g/L; tetracycline 0.05 g/L; and streptomycin 0.6 g/L) for the inhibition of bacteria and saprotrophic fungi. The CFU count for Mak-1 was 1×10^7 per cadaver, and the CFU count for P-72 was zero per cadaver.

In a preliminary experiment, we assayed cadaver water suspensions on tomato seedlings to determine the range of stimulation/inhibition of plant growth. Briefly, cadavers (incubated in moisture chambers for 48 h) were homogenized in water, and suspensions were applied directly to the substrate, close to the base of the shoot of the tomato seedlings. Three doses were used for inoculation: 10, 2 and 0.4 cadavers per 10 mL per 10 plants growing in 84 g of sterile sand. At 14 d posttreatment, the dry weight of roots and shoots was determined. Full details are provided in the ESM, Text S1.

In a basic experiment, we estimated the influence of unbroken cadavers on tomato growth. Tomato seeds were surface-sterilized with hypochlorite and ethanol as described by Posada et al. (2007). Seeds were planted in washed and sterile sand in plastic containers with a volume of 1000 mL with 180 g of sand moistened with 25 mL of distilled water. In each container, eight seeds were planted in a circle (diameter 4.5 cm). Cadavers were incubated for 24 h in moisture chambers to detect the start of mycelial formation or decay. Three days post planting, we embedded the cadavers in a groove (depth − 1.2 cm) located in the center of the circle. Grooves with cadavers were sprinkled with sand. Three treatments for each type of cadaver (1, 4 and 16 cadavers per groove) were used. No cadavers were placed in grooves for the control treatment. Containers with plants were incubated at 20–22 °C, 40 % rH and a 16:8 (light/dark) photoperiod. Six milliliters of distilled water was added to each container every day. At 14 d posttreatment, plants were washed with the substrate and divided into roots and shoots. Then, the organs were lyophilized, and the dry weight was determined to an accuracy of 0.01 mg. Seedlings that were delayed for two or more days were excluded from the analysis. The final dataset contained at least 25 plants of each treatment.

2.7. Treatments of plants with conidia, colonization and growth promotion assays

Tomato seedlings were planted in 500 mL containers with 84 g of washed sterile sand moistened with 14 mL of distilled water. Water-tween suspensions (0.03 %) of Mak-1 and P-72 (5×10^7 conidia/mL) were applied to the substrate, close to the base of the shoot of 3-d-old seedlings (1 mL/1 plant). Control plants were treated with water-tween solution. Containers with plants were incubated as described in the previous section. After 14 d of incubation, the plants were washed of the substrate and divided into roots, shoots and leaves. The organs were sterilized by hypochlorite and ethanol (Posada et al., 2007), and then the organs were imprinted (McKinnon et al., 2017) and plated on Saburoad agar

with antibiotics (see previous section) in 90 mm Petri dishes (one plant per dish). The dishes were incubated at 24 °C for 7 d, and the number of plants with *Metarhizium* was calculated. Samples in which fungal growth was registered on imprints were excluded from analysis. At least 30 plants in each treatment were finally used in the dataset. Simultaneously, using the same method of treatment and incubation, we grew tomato plants to estimate the growth-promoting effects of P-72 and Mak-1 conidia. Forty plants were used in each treatment.

2.8. Statistics

Data were analyzed using the program Statistica 8.0 (StatSoft Inc., USA), SigmaStat 3.1 (Systat Software Inc., USA) and PAST 3. Differences in mortality dynamics were estimated by Kaplan–Meier survival analysis followed by a log-rank test with Holm-Sidak adjustment. Data on bacterial taxa abundance, CFU counts in the midgut and plant growth were analyzed by one-way ANOVA followed by Fisher's LSD posttest. Under abnormal distribution (Shapiro–Wilk W test $P < 0.05$), data were transformed by square root (percentage) or Lg10 (other data). Data on bacterial taxa abundance, CFU count and nitrogen content in cadavers were analyzed by *t*-test (under normal distribution) and Mann–Whitney *U* test (if distribution was abnormal). The chi-square test was applied to compare proportions of plants colonized by fungi.

3. Results

3.1. Development of mycoses

Infection with both cultures of *M. robertsii* led to a decrease in larval survival relative to the control (log rank test, $\chi^2 > 55.3$, $P < 0.0001$, Fig. 1). Strain P-72 was more virulent than Mak-1. The median survival time (ST_{50}) was 8 ± 0.3 d for larvae infected with P-72 and 11 ± 0.5 d for larvae infected with Mak-1 ($\chi^2 = 11.5$, $P < 0.001$). Both infections finished with the formation of sclerotia within the dead insects (Fig. 2A). However, the subsequent development of the sclerotia essentially differed between the two strains. In moist chambers, 91 % of the sclerotia of Mak-1 (n = 4000) were overgrown by aerial mycelium with conidia formation afterwards. In contrast, all the sclerotia of P-72 (n > 10 000) decomposed with the appearance of a specific ammoniac smell.

3.2. Bacterial communities in midguts and cadavers

Classification based on 16S rRNA gene sequencing revealed that the predominant groups were unclassified Enterobacteriaceae (Proteobacteria) and *Spiroplasma* (Tenericutes, Spiroplasmataceae) (Fig. 2B, see also Dataset). BLAST analysis showed 100 % identity of *Spiroplasma* (OTU 3) with *Spiroplasma leptinotarsa* strain LD-1 isolated from the *L. decemlineata* gut (Hackett et al., 1996). Unclassified Enterobacteriaceae were present in two OTUs (OTU 1 and OTU 16). OTU 1 had 100 % identity with different species of *Enterobacter* and *Klebsiella*. OTU 16 showed 100 % identity with *Citrobacter braakii* and *Citrobacter freundii*.

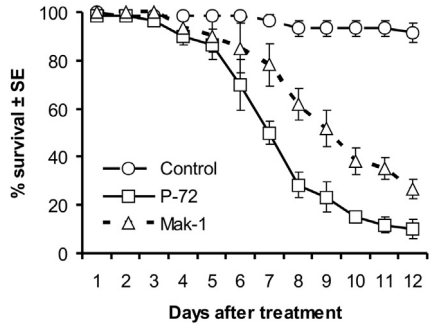

Fig. 1. Survival dynamics of Colorado potato beetle larvae after topical infection with *Metarhizium robertsii* strains Mak-1 and P-72. Differences between all treatments were significant (log-rank test: $\chi^2 > 11.5$, $P < 0.001$).

Neither infection (P-72 or Mak-1) significantly changed the midgut microbiota structure relative to that of the untreated larvae at 4 d after treatment (DAT) (Fisher LSD, $P > 0.12$, Fig. 2B). However, some significant differences were revealed between the infections caused by Mak-1 and P-72. In particular, strain Mak-1 led to an elevation of the enterobacteria portion (differences between strains: $P < 0.038$), whereas strain P-72 led to an increase in the relative abundance of *Spiroplasma*, but the differences only reflected trends ($P = 0.07$). The ratio of Enterobacteriaceae/*Spiroplasma* was significantly different ($P = 0.05$) between mycoses caused by Mak-1 and P-72.

In cadavers, Enterobacteriaceae was the predominant group (Fig. 2B). In addition, *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas* and *Serratia* were present in a significant portion (>10 %). *Spiroplasma* had low relative abundance (<0.9 %). Surprisingly, the bacterial communities of decayed (P-72) cadavers and cadavers overgrown by hyphae (Mak-1) were similar. In both cases, the ratio between both Enterobacteriaceae OTUs was close. Significant differences between Mak-1 and P-72 cadavers were linked with *Pseudomonas*, which was most abundant in Mak-1 ($t = 4.3$, df = 4, $P = 0.01$). Interestingly, *Spiroplasma* abundance was 8-fold higher in cadavers overgrown by fungus (Mak-1) compared to decaying cadavers (P-72); however, the differences were marginally significant ($t = 2.6$, df = 4, $P = 0.06$).

The principal component analysis of the microbial communities showed clear clustering between the midguts and cadavers (Fig. 3). The first component explained 82 % of the variation in the data, which was due to changes between the Enterobacteriaceae and *Spiroplasma* portions. The second principal component explained only 12 % of the variation, which was caused by increasing *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas* and *Serratia* abundance in cadavers.

The diversity of the bacterial community was slightly decreased in midguts in response to infection caused by P-72 (Table 1); however, the differences were not significant (Fisher LSD, OTU counts – $P = 0.16$, Chao index – $P = 0.12$, Shannon index – $P = 0.27$ compared to control). Infection with Mak-1 did not lead to changes in midgut bacterial diversity ($P > 0.46$ compared to control for the above-mentioned parameters). Greater values for the diversity indexes were registered in Mak-1 cadavers compared to those in P-72 cadavers (Table 1); however, the differences were not significant (*t*-test, $P > 0.09$).

3.3. Enterobacteria CFU in midguts and cadavers

There were no significant differences in enterobacterial CFU in the midguts of control and infected larvae ($F_{2,41} = 0.9$, $P = 0.40$, Fig. 4A). A slight tendency toward decreasing CFUs was observed in the midguts of larvae infected by P-72 compared to those infected by Mak-1 and control larvae (Fisher LSD, $P > 0.18$). Enterobacteria were present in considerable amounts in both types of cadavers (Fig. 4B). In particular, the concentration of enterobacteria in one cadaver overgrown by hyphae (Mak-1) was $1.1 \pm 0.2 \times 10^8$ CFUs, and in a decomposed cadaver, it was $2.7 \pm 0.6 \times 10^9$ CFUs ($t = 4.0$, df = 10, $P = 0.003$).

3.4. Nitrogen content in cadavers

The content of total nitrogen was elevated 1.3-fold in cadavers overgrown by fungus (Mak-1) compared to the nitrogen content in decomposed cadavers P-72 ($t = 46.6$, df = 4, $P < 0.0001$, Fig. 5). The

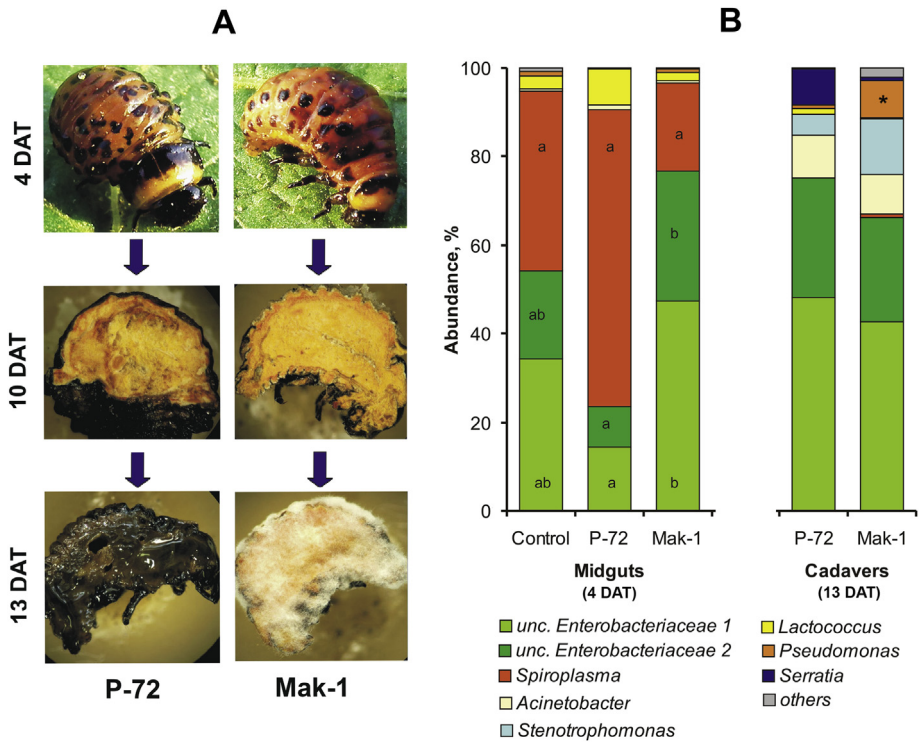

Fig. 2. Bodies of Colorado potato beetle larvae and cut cadavers after infection with *Metarhizium robertsii* strains Mak-1 and P-72 (A), and microbiota structure of midguts (4 d after treatment (DAT)) and whole cadavers (2 d after exposure in moist chamber). Data shown as arithmetic mean calculated for 3 biological replicates. The same letters indicate insignificant differences in the abundance of predominant OTUs of gut bacteria – *Spiroplasma* and unclassified Enterobacteriaceae (Fisher LSD, $P > 0.05$). *Significant differences in the abundance of OTUs between cadavers (*t*-test, $P < 0.01$).

C/N ratio was 1.4-fold higher in decomposed cadavers (P-72) relative to Mak-1 cadavers ($t = 62.3$, df = 4, $P < 0.0001$). The contents of nitrate and ammonium nitrogen were significantly higher in decomposing cadavers (P-72) than in cadavers overgrown by fungus (Mak-1) (2.8-fold for nitrate and 1.8-fold for ammonium, $Z > 1.96$, $P < 0.05$, Fig. 5).

3.5. Influence of cadavers on plant growth

A preliminary assay in which potato plants were treated with water suspensions of cadavers showed both stimulation and inhibition of plant growth within the range of 0.4–10 cadavers per 10 plants per 84 g of sand (Fig. S3). At low concentrations (0.4–2 cadavers), we registered stimulation of plant growth, but at high concentrations (10 cadavers), inhibition of growth was noted. It should be noted that increased root weight was observed after treatment with the lowest concentration of decomposing (P-72) cadavers but not conidia-forming (Mak-1) cadavers (Fisher LSD, $P < 0.001$). The total plant weight at this concentration was also higher after treatment with P-72 cadavers than that after treatment with Mak-1 cadavers ($P = 0.01$). Intermediate concentrations of Mak-1 and P-72 cadavers increased shoot weight ($P < 0.003$, compared to control), whereas root weight was not changed ($P > 0.20$). The highest concentration of Mak-1 cadavers was more toxic to plants than the same concentration of P-72 cadavers (Fig. S3).

Assays with unbroken cadavers showed that the results were consistent except at the highest concentration. At low concentrations (1 cadaver per 8 plants per 180 g of sand), decomposing cadavers (P-72) led to stronger tomato growth promotion compared

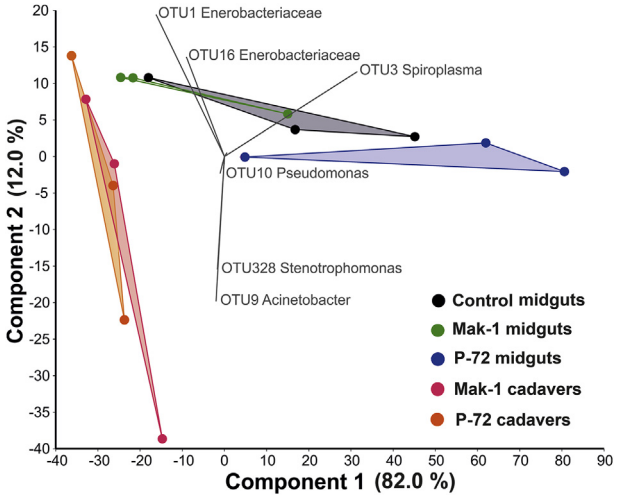

Fig. 3. Principal component analysis of Colorado potato beetle microbial communities after infection with *Metarhizium robertsii* strains Mak-1 and P-72. The plot was generated for the OTU level.

Table 1
Diversity characteristics of the microbiota in Colorado potato beetle larvae midguts at 96 h post-infection with *Metarhizium robertsii* strains and in cadavers exposed for 48 h in moist chambers.

	OTU count	Chao Index[a]	Shannon Index[a]
Midguts			
Control	14 ± 1.2	16.2 ± 0.9	1.2 ± 0.11
P-72	10 ± 1.0	10.8 ± 1.0	0.8 ± 0.36
Mak-1	12 ± 1.2	15.7 ± 2.3	1.2 ± 0.05
Cadavers			
P-72	17 ± 2.3	17.8 ± 2.3	1.2 ± 0.25
Mak-1	22 ± 2.7	27.0 ± 3.2	1.6 ± 0.25

The standard error (±SE) was calculated for three replicates.
[a] The indexes were calculated for the OTU level.

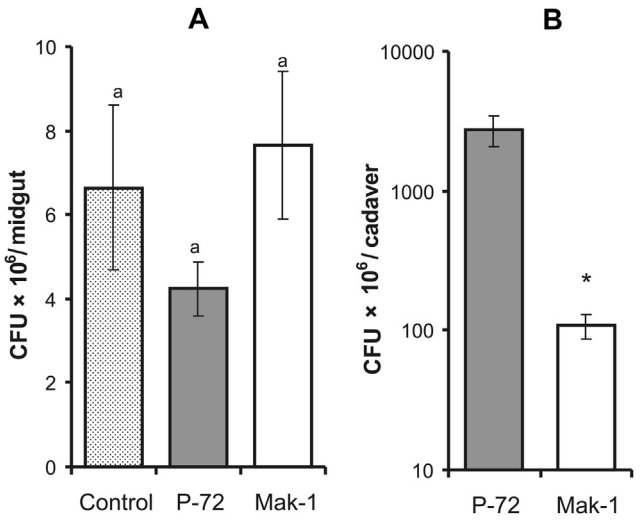

Fig. 4. Number of colony forming units (CFUs) of Enterobacteriaceae in Colorado potato beetle midguts at 4 d after treatment with *Metarhizium robertsii* strains Mak-1 and P-72 (A) and in cadavers at 2 d after exposure in moist chamber (B). The same letters indicate insignificant differences (Fisher LSD, $P > 0.05$), *significant differences in CFUs ($t = 4.0$, $P = 0.003$).

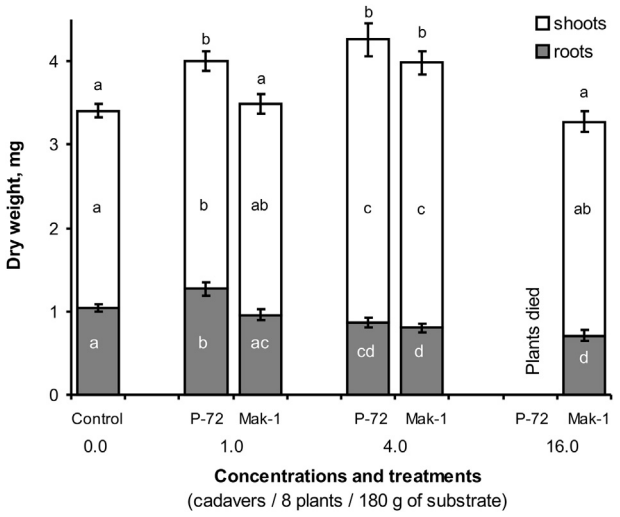

Fig. 6. Effect of cadavers overgrown with fungus (Mak-1) and decomposed with bacteria (P-72) on tomato growth in sand substrate. Treatment was conducted at 3 d post planting. Parameters were measured at 14 d after treatment. The same letters indicate insignificant differences between all treatments for shoots, roots and total weight (Fisher LSD, $P > 0.05$).

to conidia-forming (Mak-1) cadavers (Fig. 6). In particular, P-72 cadavers caused a 1.16-fold increase in dry shoot weight and a 1.22-fold increase in dry root weight compared to those of the control (Fisher LSD, $P < 0.05$). The same concentration of Mak-1 cadavers did not lead to any significant changes in shoot or root weight relative to the control ($P > 0.20$). The total weight of plants (root + shoot) was higher after treatment with P-72 cadavers compared to that after treatment with Mak-1 cadavers ($P = 0.03$).

At the intermediate concentration (4 cadavers per 8 plants), we observed a significant increase in shoot weight after treatment with both types of cadavers (1.35–1.44-fold compared to the control, $P < 0.0001$). However, both types of cadavers led to a 1.2–1.3-fold decrease in root weight ($P < 0.02$ compared to the control). The total weight of plants treated with both types of cadavers was higher than that of the control plants: P-72 – 1.25-fold ($P = 0.002$), Mak-1 – 1.16-fold ($P = 0.03$). However, differences between treatments with P-72 and Mak-1 cadavers were not significant ($P = 0.33$).

The highest concentration (16 cadavers per 8 plants) led to the inhibition of plant growth by Mak-1 cadavers. The weight of shoots decreased to the control level, and the weight of roots dropped to 1.5-fold lower than that of the control ($P < 0.0001$). Plants incubated with the same number of P-72 cadavers died at 5–6 DAT.

3.6. Colonization of plants and growth promotion after treatments with conidia

Systemic colonization of tomato plants was registered after treatment of growth substrate with conidia by both strains, P-72 and Mak-1 (Fig. 7A). Fungi were detected in the roots, shoots and leaves of 79–91 % of plants. There were no significant differences in colonization level between P-72 and Mak-1 ($\chi^2 < 0.29$, $P > 0.59$). No *Metarhizium* growth was registered in control plants. A slight and insignificant increase in plant dry weight was observed after treatment with Mak-1 conidia (1.1-fold, $P = 0.30$, compared to control, Fig. 7B). However, treatment with P-72 led to significant elevation of tomato dry weight (1.3-fold, $P < 0.0001$ compared to control, and 1.2-fold, $P = 0.002$ compared to treatment with Mak-1 conidia). Thus, despite equal colonization of tomato by the strains, P-72 conidia promoted growth more strongly than Mak-1.

4. Discussion

The work showed that bacterial decomposition of CPB larvae infected by *M. robertsii* was mainly caused by the proliferation of enterobacteria. Enterobacteriaceae are present in considerable

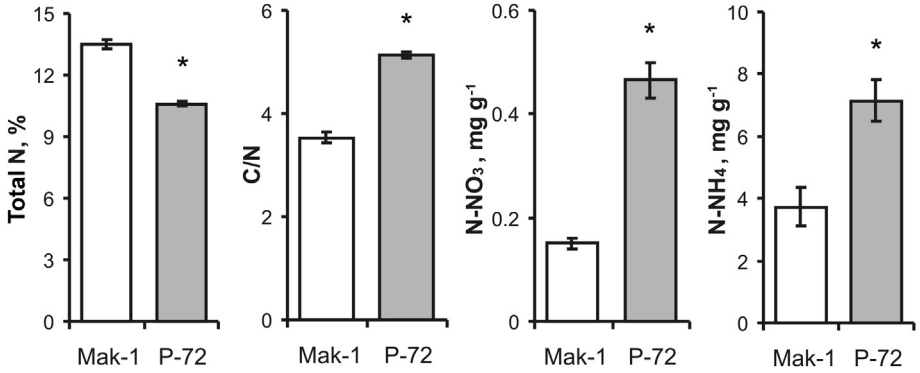

Fig. 5. Content of different types of nitrogen in Colorado potato beetle cadavers killed by *Metarhizium robertsii* strains Mak-1 and P-72 and exposed in moist chambers until conidia formation (Mak-1) or bacterial decomposition (P-72). *Significant differences in nitrogen content ($t > 46.6$, $P < 0.0001$ for total N and C:N ratio; $Z > 1.96$, $P < 0.05$ for $N\text{-}NO_3$ and $N\text{-}NH_4$).

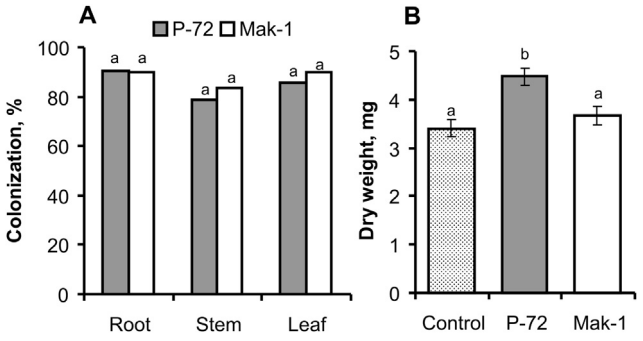

Fig. 7. Effect of treatment of sand substrate with conidia of *M. robertsii* (strains Mak-1 and P-72) on tomato colonization (A) and growth (B). Parameters were measured at 14 d after treatment of 3-d-old seedlings. The same letters indicate insignificant differences between treatments ($\chi^2 < 0.29$, $P > 0.59$ for colonization; Fisher LSD, $P > 0.05$ for dry weight).

amounts in midguts and cadavers overgrown by hyphae (10^8 CFUs per cadaver). However, their concentration strongly increases if fungi are unable to sporulate on cadavers. The bacterial decomposition of cadavers leads to the release of inorganic (nitrate and ammonium) nitrogen; therefore, decomposed cadavers have a stronger effect on plant growth promotion than conidia-formed cadavers in laboratory assays. In addition, treatment of plants with conidia of the deviant strain (P-72) led to successful colonization of plants and promotion of plant growth.

Previously, we showed that strain P-72 led to the decomposition of insects from various taxonomical groups (Orthoptera, Coleoptera, Diptera) after sclerotia formation; however, the strain was able to grow and form conidia on/in lepidopteran species such as *Galleria mellonella* and *Yponomeuta malinellus* (Kryukov et al., 2011; see also Table S1). Interestingly, passage of the strain through *G. mellonella* and the subsequent infection of CPB larvae did not also lead to sporulation on CPB (V. Y. Kryukov, unpublished). It is intriguing that strain specialization is linked to the necrotrophic stage of fungal development, but the phenomena require further investigation. Stopping the development of the strain after sclerotia formation may be caused by many reasons linked with different mutations (e.g., Huang et al., 2015; Fan et al., 2017). We propose that the phenomena may also be caused by long-term maintenance on artificial media, since the strain P-72 was maintained on artificial media for more than 40 y. Similarly, loss of capability to sporulate on insects under adaptation to artificial media was shown for entomophthoralen fungi (Voronina, 1997).

Fungi can interact with different members of the gut bacterial community during the development of mycoses in insects (Boucias et al., 2018). The composition of gut bacterial residents in the investigated CPBs was similar to that reported by other authors (Blackburn et al., 2008; Muratoglu et al., 2011; Chung et al., 2013, 2017; Sorokan et al., 2018). In particular, Chung et al. (2017) showed that OTU counts in the CPB gut vary from 16 to 69 depending on the host plant, and Enterobacteria OTUs were most abundant when feeding on potato, which is in agreement with our study. Unlike this work, we found a significant abundance of *S. leptinotarsa* in larval midguts, which could be caused by population differences (Hackett et al., 1992). Interrelations between insect pathogenic fungi and gut bacteria are mediated by changes in gut immunity under topical fungal infection as well as by the production of fungal toxins (Wei et al., 2017; Fan et al., 2017; Ramirez et al., 2018). Significant changes in bacterial composition in the guts of *Anopheles* mosquito and *Galleria* moth, expressed by an increase in opportunistic bacterial abundance (*Serratia*), were revealed after topical infection with *Beauveria bassiana*, but these alterations were observed under acute mycoses (Wei et al., 2017; Polenogova et al., 2019). In the present study, the structure of the CPB gut microbiota was not significantly altered in larvae infected by fungi relative to the control. We propose that this could be explained by the prolonged development of the mycoses (Fig. 1). However, we registered a shift in the *Spiroplasma* – Enterobacteriaceae proportion in insects infected by Mak-1 and P-72 that may indicate different effects of the strains on gut microbiota. Slight and nonsignificant changes in gut microbial composition of *Aedes aegypti* adults were also documented by Ramirez et al. (2018) after topical infection of the mosquito with fungi *Beauveria, Isaria* and *Trichothecium*.

We found that the cadaver bacterial community differed from the midgut community. In particular, if *S. leptinotarsa* was prevalent in midguts, *S. leptinotarsa* had very low abundance in cadavers. Bacteria *S. leptinotarsa* are specialized symbionts with CPB and usually require live cells for development (Hackett et al., 1992, 1996). Therefore, these bacteria did not participate in the bacterial decomposition of cadavers (P-72), and they were present in a low proportion in cadavers overgrown with mycelium (Mak-1). Interestingly, the microbiota composition was close between decaying (P-72) and overgrown by mycelium (Mak-1) cadavers. In both cases, enterobacteria were predominant, but a trend toward increased bacterial diversity was observed in Mak-1 cadavers. There are high quantities of cultivable enterobacteria (10^8) in overgrown cadavers, but the quantity was increased 25-fold in decaying cadavers. Obviously, many groups of symbiotic bacteria may be preserved by mummification following mycelia formation, whereas stopping fungus development and decaying of cadavers led to competition between bacteria and the prevalence of groups that are most adapted to necrotrophic consumption. Therefore, we registered high diversity in cadavers characterized by classical development of mycosis. Notably, the cadavers' microbiota may emerge from both insect bacteria and bacteria harbored within fungal cells. A recent study by Chen et al. (2016) showed the presence of bacteria (including *Serratia*, *Stenotrophomonas*, and *Acinetobacter*) within the hyphae and conidia of entomophthoralen fungi *Pandora neoaphidis* and *Pandora nouryi*. Bacterial communities of *Metarhizium* hyphae and conidia were not studied. We can speculate that bacterial symbionts of the fungi can participate in the bacterial decomposition of cadavers. It should be noted that bacterial communities of CPB larvae colonized by fungus and larvae that died before sclerotia formation were very different (Fig. S5). In the latter larvae, unclassified Enterobacteriaceae had low abundance, and the predominant groups were *Acinetobacter*, *Pseudomonas* and *Serratia*. Most likely, the bacterial decomposition of cadavers before and after sclerotia formation is accomplished by different taxa of bacteria. It should also be noted that in the present study, we did not analyze cuticular bacteria of CPB larvae, although they can also affect mycoses development and lead to bacterial decomposition in insects (Zacharuk, 1973; Vey and Fargues, 1977). Changes in integumental bacterial communities during mycoses could be the subject of future studies.

The results of enterobacteria predomination in decomposed CPB larvae after sclerotia formation are in agreement with previous data on cultivatable bacteria. In particular, infection of the Middle-Asian CPB population with strain P-72 led to cadaver decomposition by *Erwinia* sp., *Serratia grimesii* and *Serratia* sp. (Enterobacteriaceae) (Kryukov et al., 2011). Additionally, bacteria *Yersinia kristensenii* and *Providencia rettgeri* (Enterobacteriaceae) were found in decomposed larvae from the West Siberia population of CPB after infection with P-72 (V.V. Morozova, personal communication). It is likely that enterobacteria are essential destructors of the larvae killed by the investigated fungus; however, their genus and species composition may vary depending on certain environmental conditions.

Decaying cadavers are characterized by a significantly decreased content of total nitrogen but increased concentration of nitrate and ammonium nitrogen compared to cadavers overgrown with fungus. This was expected because microbial decomposition is accompanied by nitrate and ammonium release. Gammaproteobacteria can participate in ammonification and heterotrophic nitrification (Stein, 2011). *Enterobacter* species in particular exhibit simultaneous nitrification and aerobic denitrification (Padhi et al., 2017; Wan et al., 2017). We showed that the application of both types of cadavers to soil led to an increase in tomato shoot weight. However, treatment by decomposing cadavers (P-72) also increased root weight. It is known that inorganic nitrogen (NO^{3-}) can have both positive and negative effects on the growth of lateral roots (Zhang et al., 1999; Sun et al., 2017). Severe nitrogen deficiency and high nitrate supply display inhibitory effects on lateral root development; however, mild nitrogen deficiency led to stimulation of their growth (Sun et al., 2017). This is consistent with our experiment performed on inert substrate-washed sand, which is characterized by severe nitrogen deficiency (Budianta et al., 2013). An increase in root weight (compared to that in the control) was observed at low concentrations of P-72 cadavers; however, higher concentrations of P-72 cadavers led to inhibition of root growth. Increased shoot weight post-treatment with Mak-1 cadavers is expected because treatment with *Metarhizium* conidia as a rule increased the growth of plant aboveground organs (Kabaluk and Ericsson, 2007; García et al., 2011; Liao et al., 2014; Jaber and Enkerli, 2016, 2017). The highest dosages of P-72 and Mak-1 cadavers led to the inhibition of tomato growth or the death of plants, which was evidently caused by excess nitrogen.

Previously, active nitrogen transfer from cadavers to roots via *Metarhizium* mycelia was reported (Behie et al., 2012; Behie and Bidochka, 2014). Microbial decomposition of fungal and insect material may be an alternative method of inorganic nitrogen formation under mycosis development. We showed that in laboratory conditions using a nitrogen-deficient substrate, this method may be more effective for plant growth promotion than sporulating cadavers. However, in field conditions, nitrates can be quickly removed due to washing-off by precipitation and denitrification by soil microorganisms (Lal, 2010; Yakutina et al., 2015). Therefore, field assays are required for further comparative analyses. The present work was performed on CPB larvae; however, we can expect similar results on other species (including soil insects, feeding by roots) because bacterial decomposition was observed in different insect taxa after infection with strain P-72. It should be noted that inundative biocontrol focuses on the high-speed of killing of target insects. Early insect mortality after fungal infection is not favorable for fungal survival and persistence due to decreasing sporulation on killed insects or their bacterial colonization (Boucias et al., 2018). In particular, genetically engineered strains with increased virulence are characterized by reduced or lost mycelium formation and conidiation on killed insects (e.g., St. Leger et al., 1996; Pava-Ripoll et al., 2008). Using such fungi against herbivorous insects should lead to the predomination of a passive flow of nitrogen to plants.

In conclusion, we revealed the similarity between bacterial communities in insects killed and colonized by *M. robertsii* strains, which have different final stages of mycoses development, the decaying of cadavers (P-72) or the formation of mycelium on the cadavers (Mak-1). Enterobacteria were the predominant group in both types of cadavers. Bacterial decomposition of cadavers led to more rapid mineralization of nitrogen, which led to plant growth promotion in laboratory conditions. It is important that the treatment of plants with conidia of the deviant strain (P-72) led to successful colonization of plants and showed an even stronger growth-promoting effect than treatment with the strain with a typical developmental cycle on insects (Mak-1). Intriguingly, some strains of *M. robertsii* may be more adapted to insects, whereas other strains are more adapted to plants. Further investigation could be focused on the mechanisms of physiological interactions between plants and fungi with different pathogenic strategies toward insects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.09.012.

References

Bamisile, B.S., Dash, C.K., Akutse, K.S., Keppanan, R., Afolabi, O.G., Hussain, M., Qasim, M., Wang, L., 2018. Prospects of endophytic fungal entomopathogens as biocontrol and plant growth promoting agents: an insight on how artificial inoculation methods affect endophytic colonization of host plants. Microbiol. Res. 217, 34–50. https://doi.org/10.1016/j.micres.2018.08.016.

Behie, S.W., Bidochka, M.J., 2014. Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. Appl. Environ. Microbiol. 80, 1553–1560. https://doi.org/10.1128/AEM.03338-13.

Behie, S.W., Zelisko, P.M., Bidochka, M.J., 2012. Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. Science 336, 1576–1577. https://doi.org/10.1126/science.1222289.

Blackburn, M.B., Gundersen-Rindal, D.E., Weber, D.C., Martin, P.A., Farrar Jr., R.R., 2008. Enteric bacteria of field-collected Colorado potato beetle larvae inhibit growth of the entomopathogens *Photorhabdus temperata* and *Beauveria bassiana*. Biol. Cont. 46, 434–441. https://doi.org/10.1016/j.biocontrol.2008.05.005.

Boucias, D.G., Zhou, Y., Huang, S., Keyhani, N.O., 2018. Microbiota in insect fungal pathology. Appl. Microbiol. Biotechnol. 102, 5873–5888. https://doi.org/10.1007/s00253-018-9089-z.

Brouchkov, A., Kabilov, M., Filippova, S., Baturina, O., Rogov, V., Galchenko, V., Mulyukin, A., Fursova, O., Pogorelko, G., 2017. Bacterial community in ancient permafrost alluvium at the Mammoth Mountain (Eastern Siberia). Gene 636, 48–53.

Budianta, D., Gofar, N., Andika, G.A., 2013. Improvement of sand tailing fertility derived from post tin mining using leguminous crop applied by compost and mineral soil. J. Trop. Soils 18. https://doi.org/10.5400/jts.2013.18.3.217.

Charnley, A.K., 2003. Fungal pathogens of insects: cuticle degrading enzymes and toxins. Adv. Bot. Res. 40, 241–321. https://doi.org/10.1016/S0065-2296(05)40006-3.

Chen, C., Chen, X., Xie, T., Louis Hatting, J., Yu, X., Ye, S., Wang, Z., Shentu, X., 2016. Diverse bacterial symbionts of insect-pathogentic fungi and possible impact on the maintenance of virulence during infection. Symbiosis 69, 47–58. https://doi.org/10.1007/s13199-015-0371-x.

Chung, B.H., Rosa, C., Scully, E.D., Peiffer, M., Tooker, J.F., Hoover, K., Luthe, D.S., Felton, G.W., 2013. Herbivore exploits orally secreted bacteria to suppress plant defenses. Proc. Natl. Acad. Sci. U. S. A. 110, 15728. https://doi.org/10.1073/pnas.1308867110.

Chung, S.H., Scully, E.D., Peiffer, M., Geib, S.M., Rosa, C., Hoover, K., Felton, G.W., 2017. Host plant species determines symbiotic bacterial community mediating suppression of plant defenses. Sci. Rep. 7, 39690. https://doi.org/10.1038/srep39690.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996. https://doi.org/10.1038/nmeth.2604.

Edgar, R.C., 2016b. UNOISE2: improved error-correction for illumina 16S and ITS amplicon sequencing. bioRxiv. https://doi.org/10.1101/081257, 081257.
Edgar, R.C., 2016a. SINTAX: a simple non-Bayesian taxonomy classifier for 16S and its sequences. bioRxiv. https://doi.org/10.1101/074161, 074161.
Fadrosh, D.W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R.M., Ravel, J., 2014. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome 2, 6.
Fan, Y., Liu, X., Keyhani, N.O., Tang, G., Pei, Y., Zhang, W., Tong, S., 2017. Regulatory cascade and biological activity of *Beauveria bassiana* oosporein that limits bacterial growth after host death. Proc. Natl. Acad. Sci. U. S. A. 114, E1578. https://doi.org/10.1073/pnas.1616543114.
García, J.E., Posadas, J.B., Perticari, A., Lecuona, R.E., 2011. *Metarhizium anisopliae* (Metschnikoff) sorokin promotes growth and has endophytic activity in tomato plants. Adv. Biol. Res. 5, 22–27.
Hackett, K.J., Henegar, R.B., Whitcomb, R.F., Lynn, D.E., Konai, M., Schroder, R.F., Gasparich, G.E., Vaughn, J.L., Cantelo, W.W., 1992. Distribution and biological control significance of Colorado potato beetle spiroplasmas in North America. Biol. Cont. 2, 218–225. https://doi.org/10.1016/1049-9644(92)90062-I.
Hackett, K.J., Whitcomb, R.F., French, F.E., Tully, J.G., Gasparich, G.E., Rose, D.L., Carle, P., Bove, J.M., Henegar, R.B., Clark, T.B., Konai, M., Clark, E.A., Williamson, D.L., 1996. *Spiroplasma corruscae* sp. nov., from a Firefly beetle (Coleoptera: Lampyridae) and Tabanid flies (Diptera: Tabanidae). Int. J. Syst. Bacteriol. 46, 947–950. https://doi.org/10.1099/00207713-46-4-947.
Hsieh, T.C., Ma, K.H., Chao, A., 2016. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods Ecol. Evol. 7, 1451–1456. https://doi.org/10.1111/2041-210X.12613.
Hu, X., Xiao, G.H., Zheng, P., Shang, Y.F., Su, Y., Zhang, X.Y., Liu, X.Z., Zhan, S., St Leger, R.J., Wang, C.S., 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc. Natl. Acad. Sci. U. S. A. 111, 16796–16801. https://doi.org/10.1073/pnas.1412662111.
Huang, W., Shang, Y., Chen, P., Gao, Q., Wang, C., 2015. MrpacC regulates sporulation, insect cuticle penetration and immune evasion in *Metarhizium robertsii*. Environ. Microbiol. 17, 994–1008. https://doi.org/10.1111/1462-2920.12451.
Jaber, L.R., Enkerli, J., 2016. Effect of seed treatment duration on growth and colonization of *Vicia faba* by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. Biol. Cont. 103, 187–195.
Jaber, L.R., Enkerli, J., 2017. Fungal entomopathogens as endophytes: can they promote plant growth? Biocont. Sci. Technol. 27, 28–41. https://doi.org/10.1080/09583157.2016.1243227.
Kabaluk, J.T., Ericsson, J.D., 2007. *Metarhizium anisopliae* seed treatment increases yield of field corn when applied for wireworm control. Agronomy J. 99, 1377–1381. https://doi.org/10.2134/agronj2007.0017N.
Kershaw, M.J., Moorhouse, E.R., Bateman, R., Reynolds, S.E., Charnley, A.K., 1999. The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J. Invertebr. Pathol. 74, 213–223. https://doi.org/10.1006/jipa.1999.4884.
Krell, V., Unger, S., Vidal, S., Patel, A.V., 2018. Endophytic *Metarhizium brunneum* mitigates nutrient deficits in potato and improves plant productivity and vitality. Fungal Ecol. 34, 43–49. https://doi.org/10.1016/j.funeco.2018.04.002.
Kryukov, V.Y., Dubovskiy, I.M., Yaroslavtseva, O.N., Levchenko, M.V., Slyamova, N.D., Belgibaeva, A.B., Khodyrev, V.P., Lednev, G.R., Glupov, V.V., 2011. Comparative analysis of two strains of entomopathogenic fungus *Metarhizium anisopliae* with different pathogenesis strategies. Mikol. Fitopatol. 45, 164–176 (in Russian).
Kryukov, V.Y., Yaroslavtseva, O.N., Tyurin, M.V., Akhanaev, Y.B., Elisaphenko, E., Wen, T.C., Tomilova, O.G., Tokarev, Y.S., Glupov, V.V., 2017. Ecological preferences of *Metarhizium* spp. from Russia and neighboring territories and their activity against Colorado potato beetle larvae. J. Invertebr. Pathol. 149, 1–7. https://doi.org/10.1016/j.jip.2017.07.001.
Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: back to the future. J. Invertebr. Pathol. 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009.
Lal, R., 2010. Enhancing eco-efficiency in agro-ecosystems through soil carbon sequestration. Crop Sci. 50, 120–131. https://doi.org/10.2135/cropsci2010.01.0012.
Liao, X., O'Brien, T.R., Fang, W., St Leger, R.J., 2014. The plant beneficial effects of *Metarhizium* species correlate with their association with roots. Appl. Microbiol. Biot. 98, 7089–7096. https://doi.org/10.1007/s00253-014-5788-2.
Maynard, D.G., Kalra, Y.P., Crumbaugh, J.A., 2007. Nitrate and exchangeable ammonium nitrogen. In: Carter, M.R., Gregorich, E.G. (Eds.), Soil Sampling and Methods of Analysis, second ed. CRC Press, USA, pp. 71–80.
McKinnon, A.C., Saari, S., Moran-Diez, M.E., Meyling, N.V., Raad, M., Glare, T.R., 2017. *Beauveria bassiana* as an endophyte: a critical review on associated methodology and biocontrol potential. BioControl 62, 1–17. https://doi.org/10.1007/s10526-016-9769-5.
Moonjely, S., Barelli, L., Bidochka, M.J., 2016. Insect pathogenic fungi as endophytes. Adv. Genet. 94, 107–135. https://doi.org/10.1016/bs.adgen.2015.12.004.
Muratoglu, H., Demirbag, Z., Sezen, K., 2011. The first investigation of the diversity of bacteria associated with *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Biologia 66, 288–293. https://doi.org/10.2478/s11756-011-0021-6.
Padhi, S.K., Tripathy, S., Mohanty, S., Maiti, N.K., 2017. Aerobic and heterotrophic nitrogen removal by *Enterobacter cloacae* Cf-S27 with efficient utilization of hydroxylamine. Bioresour. Technol. 232, 285–296. https://doi.org/10.1016/j.biortech.2017.02.049.
Pava-Ripoll, M., Posada, F.J., Momen, B., Wang, C., St Leger, R., 2008. Increased pathogenicity against coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) by *Metarhizium anisopliae* expressing the scorpion toxin (AaIT) gene. J. Invertebr. Pathol. 99, 220–226. https://doi.org/10.1016/j.jip.2008.05.004.
Polenogova, O.V., Kabilov, M.R., Tyurin, M.V., Rotskaya, U.N., Krivopalov, A.V., Morozova, V.V., Mozhaitseva, K., Kryukova, N.A., Alikina, T., Kryukov, V.Y., Glupov, V.V., 2019. Parasitoid envenomation alters the *Galleria mellonella* midgut microbiota and immunity, thereby promoting fungal infection. Sci. Rep. 9, 4012. https://doi.org/10.1038/s41598-019-40301-6.
Posada, F., Aime, M.C., Peterson, S.W., Rehner, S.A., Vega, F.E., 2007. Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). Mycol. Res. 111, 748–757. https://doi.org/10.1016/j.mycres.2007.03.006.
Ramirez, J.L., Dunlap, C.A., Muturi, E.J., Barletta, A.B.F., Rooney, A.P., 2018. Entomopathogenic fungal infection leads to temporospatial modulation of the mosquito immune system. PLoS Negl. Trop. Dis. 12 https://doi.org/10.1371/J.pntd.0006433 e0006433.
Ríos-Moreno, A., Garrido-Jurado, I., Raya-Ortega, M.C., Quesada-Moraga, E., 2017. Quantification of fungal growth and destruxin a during infection of *Galleria mellonella* larvae by *Metarhizium brunneum*. J. Invertebr. Pathol. 149, 29–35. https://doi.org/10.1016/j.jip.2017.06.007.
Ríos-Moreno, A., Quesada-Moraga, E., Garrido-Jurado, I., 2018. Treatments with *Metarhizium brunneum* BIPESCO$_5$ and EAMa 01/58-Su strains (Ascomycota: Hypocreales) are low risk for the generalist predator *Chrysoperla carnea*. J. Pest. Sci. 91, 385–394. https://doi.org/10.1007/s10340-017-0905-5.
Serebrov, V., Maljarchuk, A., Shrernshis, M., 2007. Spontaneous variability of *Metarhizium anisopliae* (Metsch.) sor. strains as an approach for enhancement of insecticidal activity. Plant Sci. (Sofia) 44, 236–239.
Soderhall, K., Ajaxon, R., 1982. Effect of quinones and melanin on mycelial growth of *Aphanomyces* spp. and extracellular protease of *Aphanomyces astaci*, a parasite on crayfish. J. Invertebr. Pathol. 39, 105–109. https://doi.org/10.1016/0022-2011(82)90164-1.
Sorokan, A.V., Benkovskaya, G.V., Blagova, D.K., Maksimova, T.I., Maksimov, I.V., 2018. Defense responses and changes in symbiotic gut microflora in the Colorado potato beetle *Leptinotarsa decemlineata* under the effect of endophytic bacteria from the genus Bacillus. J. Evol. Biochem. Physiol. 54, 300–307. https://doi.org/10.1134/S0022093018040063.
Stein, L., 2011. Heterotrophic nitrification and nitrifier denitrification. In: Ward, B., Arp, D., Klotz, M. (Eds.), Nitrification. ASM Press, Washington, DC, pp. 95–114.
St Leger, S.R., Joshi, L., Bidochka, M.J., Roberts, W.D., 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc. Natl. Acad. Sci. U. S. A. 93, 6349–6354. https://doi.org/10.1073/pnas.93.13.6349.
Sun, C.-H., Yu, J.-Q., Hu, D.-G., 2017. Nitrate: a crucial signal during lateral roots development. Front. Plant Sci. 8 https://doi.org/10.3389/fpls.2017.00485.
Vega, F.E., 2018. The use of fungal entomopathogens as endophytes in biological control: a review. Mycologia 110, 4–30. https://doi.org/10.1080/00275514.2017.1418578.
Vey, A., Fargues, J., 1977. Histological and ultrastructural studies of *Beauveria bassiana* infection in *Leptinotarsa decemlineata* larvae during ecdysis. J. Invertebr. Pathol. 30, 207–215. https://doi.org/10.1016/0022-2011(77)90221-X.
Voronina, A.G., 1997. Entomophthorales fungi and biological products with epizootic and toxigenic action. Zashita rasteniy 5, 12–13 (in Russian).
Wan, W., He, D., Xue, Z., 2017. Removal of nitrogen and phosphorus by heterotrophic nitrification-aerobic denitrification of a denitrifying phosphorus-accumulating bacterium *Enterobacter cloacae* HW-15. Ecol. Eng. 99, 199–208. https://doi.org/10.1016/j.ecoleng.2016.11.030.
Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267. https://doi.org/10.1128/aem.00062-07.
Wei, G., Lai, Y., Wang, G., Chen, H., Li, F., Wang, S., 2017. Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality. Proc. Natl. Acad. Sci. U. S. A. 114, 5994. https://doi.org/10.1073/pnas.1703546114.
Xie, T., Wang, Y., Yu, D., Zhang, Q., Zhang, T., Wang, Z., Huang, B., 2019. MrSVP, a secreted virulence-associated protein, contributes to thermotolerance and virulence of the entomopathogenic fungus *Metarhizium robertsii*. BMC Microbiol. 19, 25. https://doi.org/10.1186/s12866-019-1396-8.
Yakutina, O.P., Nechaeva, T.V., Smirnova, N.V., 2015. Consequences of snowmelt erosion: soil fertility, productivity and quality of wheat on Greyzemic Phaeozem in the south of West Siberia. Agric. Ecosyst. Environ. 200, 88–93. https://doi.org/10.1016/j.agee.2014.10.021.
Yaroslavtseva, O.N., Dubovskiy, I.M., Khodyrev, V.P., Duisembekov, B.A., Kryukov, V.Y., Glupov, V.V., 2017. Immunological mechanisms of synergy between fungus *Metarhizium robertsii* and bacteria *Bacillus thuringiensis* ssp. morrisoni on Colorado potato beetle larvae. J. Insect Physiol. 96, 14–20. https://doi.org/10.1016/j.jinsphys.2016.10.004.
Zacharuk, R.Y., 1973. Penetration of the cuticular layers of elaterid larvae (Coleoptera) by the fungus *Metarhizium anisopliae*, and notes on a bacterial invasion. J. Invertebr. Pathol. 21, 101–106. https://doi.org/10.1016/0022-2011(73)90118-3.
Zhang, H., Jennings, A., Barlow, P.W., Forde, B.G., 1999. Dual pathways for regulation of root branching by nitrate. Proc. Natl. Acad. Sci. U. S. A. 96, 6529. https://doi.org/10.1073/pnas.96.11.6529.