

ORIGINAL ARTICLE

Development of a fluid-bed coating process for soil-granule-based formulations of *Metarhizium brunneum*, *Cordyceps fumosorosea* or *Beauveria bassiana*

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Beauveria bassiana, biocontrol agent, *Cordyceps fumosorosea*, fluid-bed coating, granules, *Metarhizium brunneum*.

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Abstract

Aim: Granule-based products of solid state fermented micro-organisms are available for biocontrol. Because liquid fermentation has several advantages, we investigated fluid-bed coating with liquid fermented biomass.

Methods and Results: Biomass containing mycelium or mycelium and submerged spores of the entomopathogenic fungi *Metarhizium brunneum*, *Cordyceps fumosorosea* and *Beauveria bassiana* were produced in liquid culture, separated and different biomass concentrations were adjusted. Based on the examined thermo-tolerance, we defined fluid-bed coating adjustments and investigated granule colonization and sporulation on granules. Granule colonization depended on the biomass concentration and strain. For *C. fumosorosea* and *B. bassiana*, concentrations of 0.003%_{dry weight} resulted in nearly 100% granule colonization, for *M. brunneum* with concentrations of 0.7%_{dry weight} in only 50%. The conidiation on granules in sterile soil was highly influenced by the moisture content. Because the granule colonization of *M. brunneum* was unsatisfactory, we pre-coated nutrients followed by coating with biomass, submerged spores or conidia. Malt extract had a positive effect on the granule colonization for biomass and submerged spores. Furthermore, aerial conidia can also be coated.

Conclusions: Fluid-bed coating of fungal biomass is suitable for the development of granules.

Significance and Impact of this Study: With this technology, cost-efficient biocontrol products can be developed.

Introduction

In Europe, agriculture based on chemical plant protection agents is currently in transition to integrated pest management (IPM) due to changes in the European legislation (Lamichhane *et al.* 2017). In the European Directive 2009/128/EC, the implementation of the principles of IPM in which non-chemical methods must be preferred is obligatory. Furthermore, a significant reduction of active substances approved in the European Union (EU) is occurring (Santin-Montanya *et al.* 2017). Especially for soil-dwelling pest insects, for example, in potatoes or corn no chemical pesticides are available or registered in

Germany for application in the field (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2020, accessed 11 May 2020). Therefore, biocontrol agents (BCAs) get an important role in IPM practice (Lamichhane *et al.* 2017). In the EU, seven strains of *Beauveria bassiana*, one of *Cordyceps fumosorosea* and one of *Metarhizium anisopliae* are approved as active substance (European Commission, accessed 11 May 2020). The *B. bassiana* strain 147 and NPP111B005 is formulated as granule for direct application between the rachis of palm trees (SANTE-2016-10424 Rev 1). On the EU database of active substances, *Isaria fumosorosea* the Apopka strain 97 is listed as approved. This Apopka strain 97, which was formerly

described as *Paecilomyces fumosoroseus*, has been indicated as *Cordyceps javanica* after genetic reanalysis by Kepler *et al.* (2017). The approved *M. anisopliae* strain BIPESCO5 or F52, originally named as Ma43, is registered as granule-based products under the product names, for example, GranMet GR^{TN}, BIO1020^{TN}, Taenur-e^{TN}, Met 52^{TN} or TickEx G^{TN} and should be used for soil application. The *M. anisopliae* products contain 3–3 g active substance per kg product, which is comparable to 10¹⁰ CFU per kg. This strain was reclassified as *Metarhizium brunneum* (Rehner and Kepler 2017). Since 2017 the product Attracap^{TN} (*M. brunneum* strain CB15-III) is allowed to be used in Germany under emergency situation (Article 53 of the Regulation (EC) No 1107/2009). Recommended application rates for granule-based products of entomopathogenic fungi are 122 kg ha⁻¹ in fruits (Met 52^{TN}) for control of, for example, black vine weevil, 30–50 kg of Melocont^{TN} with three applications per year for control of white grubs or for control of wireworms, the larval stages of Click beetles, 30 kg of the *M. brunneum*-based product Attracap^{TN}. In Europe, the prices per kg product range between 10 and 40 €. Because of the high product costs per ha, alternative production and formulation strategies are needed to reduce product costs and finally to make biocontrol more attractive.

In general, after application of granule-based formulations in the soil, conidia can directly infect the insects or conidia, microsclerotia or mycelium must grow out from the carrier and resporulate to produce the infectious conidia (Jackson and Jaronski 2009). The infection of the insects by entomopathogenic fungi starts by contact of the fungal spores with the cuticle (Ortiz-Urquiza and Keyhani 2013). There, the conidia produce a germ tube and penetrate the insect. This penetration starts mostly at the openings of the cuticle, such as the stomata. However, they can also penetrate the cuticle by a combination of enzymes and mechanical pressure of the germ tube. After successful penetration, the fungus grows in the insects' hemolymph and forms blastospores. So the fungus spreads throughout the target organism. After the death of the insect, the fungus sporulate on the surface of the insect under wet or in the insect under dry conditions (Arthurs and Thomas 2001b).

The industrial production of granules of the strain Ma43 is based on solid state fermentation, whereas the product Attracap^{TN} is based on liquid fermentation. The selection of the production system highly depends on the fungal capacity to grow and sporulate in liquid or on solid state culture. Because the production in solid state fermenters requires a longer fermentation time, has a higher risk of contamination and an industrial scale-up is more complicated, we decided to focus only on liquid fermentation. In liquid fermenter, fungi can produce

mycelium (Rombach 1989), mycelial pellets (Reinecke *et al.* 1990), microsclerotia (Behle and Jackson 2014), submerged conidia (Jenkins and Thomas 1996) or blastospores (Kleespies 1993). Often, it is a mixture of different growing stages. The current downstream processing for the fermented biomass includes separation, concentration and filtration-based purification followed by formulation of the fungal propagules. Depending on the desired application strategy, the formulation strategy has to be selected. In our study, we selected fluid-bed coating because it is a suitable method to produce granules (Stephan and Zimmermann 2001).

For this, the sprayed liquid evaporates and forms a film on the substrate. Such a product is very uniform and not dusting, which is very important for later approval of the product. In summary, the overall goal was to develop and to optimize a granule-based formulation technology for entomopathogenic fungi based on liquid fermented biomass. Temperature adjustments, biomass concentration and type of biomass as well as additives were investigated to optimize the conidiation on the surface of the granule. Conidiation is the fundamental requirement for these granules to achieve an efficient pest control in the field.

Materials and methods

Fungal strains

Metarhizium brunneum strain JKI-BI-1339 (formerly described as *M. anisopliae* and named as Ma43, F52 or BIPESCO 5) was isolated 1971 in Austria from *Cydia pomonella* by Müller-Kögler. Based on this strain, several products like GranMet, Met52 or BIO1020 were developed by various companies. The *Cordyceps fumosorosea* strain JKI-BI-1496 was isolated 1971 in Germany from *C. pomonella* by Müller-Kögler and the *Beauveria basisana* strain B.b. 007 was isolated in Georgia from soil of high mountains of Caucasus and is deposited under IMI # 501799 at CABI Genetic Recourse and under B.b. 007 at the culture collection of entomopathogenic fungi at JKI.

Cultivation of fungi and preparation of biomass suspension

All entomopathogenic fungi were stored at –80°C in cryo-tubes (Microbank, Pro-Lab Diagnostics, Ontario, Canada) and routinely cultured at 25°C on malt peptone agar (MPA) containing 3% (w/v) malt extract (Merck, Darmstadt, Germany), 0.5% (w/v) peptone from soybean (Merck) and 1.8% (w/v) agar-agar (Roth, Karlsruhe, Germany). For preparation of the liquid culture, 100 ml of malt peptone broth (MPB = MPA without agar-agar)

was autoclaved at 121°C for 20 min in 300-ml Erlenmeyer flasks, inoculated with 1×10^8 conidia suspended in 0.1% (v/v) Tween 80 (Merck) of a 2-week-old culture from MPA and incubated for 72 h on a horizontal shaker (Novotron, 50 mm deflection, Infors, Bottmingen, Switzerland) at 25°C and 150 rev min⁻¹. In malt peptone broth, JKI-BI-1496 and B.b. 007 formed mycelium and submerged spores, whereas JKI-BI-1339 was only growing as mycelium.

For JKI-BI-1496 and B.b. 007, submerged spores (blastospores and/or submerged conidia) were partly separated after fermentation by filtration through three layers of muslin gauze. Spores can be formulated for spray applications while the remaining biomass including mycelium and spores was used for the following experiments. To remove remaining residues of the culture media, the filter cake was resuspended in 100 ml of 0.9%-NaCl solution and centrifuged at 25°C and 15 433 *g* for 10 min. The supernatant was discarded and the pellet was again resuspended. After three centrifugations, the biomass in the NaCl solution was homogenized by a disperser (TP 18/10 Ultra-Turrax; Janke & Kunkel KG, Stauffen, Germany, attachment with a diameter of 17.5 mm) at maximum speed of 20 000 rev min⁻¹ for 5 min. Thereafter, the residual moisture content was determined by a moisture determination balance (Ma30; Sartorius, Göttingen, Germany) and the dry matter of the suspension was adjusted with 0.9%-NaCl solution to final fungal biomass concentrations of 0.3, 0.03, 0.003 and 0.0003% and for further experiments with JKI-BI-1339 additionally to 0.7%. These biomass concentrations were used for coating millet in a fluid-bed drying system.

Pretreatment of the millet grain

Millet grain for food consumption (Alnatura, Bickenbach, Germany) was autoclaved at 121°C for 20 min and dried in a drying cabinet at 100°C up to a water content lower than 5%.

Thermotolerance

The thermotolerance was investigated under wet and dry heat. For experiments under wet heat conditions, 900 µl of the biomass suspension containing 0.03% biomass was filled in 1 ml reaction tubes. These tubes were transferred in a water bath with temperatures of 25, 50 and 70°C for 6 min. Before and after incubation, the tubes were homogenized on a vortex mixer for a short time. Because the biomass contained a mixture of mycelium and spores, the viability of the micro-organisms was determined by the most probable number (MPN) method. The principle of the MPN method is that suspensions of micro-

organisms will be diluted up to a concentration of lower than one viable propagule per well. Consequently, fungal growth within this well is caused by one viable propagule. 120 µl of MPB was pipetted in 96-well microtiter plates. 30 µl of the temperature-treated samples was added to the MPB in four replications. In all, 16 subsequent dilution steps (1 : 5) were made. The microtiter plates were incubated for 7 days at 25°C. Based on the number of turbid wells, the MPN was calculated with the help of a computer program (Most Probable Number Calculator ver. 4.04 © 1996, Albert J. Klee – Risk Reduction Engineering Laboratory, United States Environmental Protection Agency, Cincinnati, OH). The experiment was repeated six times time independently.

For dry heat conditions, 900 µl of each biomass concentration was filled in 1 ml Eppendorf tubes. In all, 30 millet grains were added in each tube and mixed using a vortex mixer. Thereafter, the millet grains were placed on a filter paper in a petri dish followed by an incubation for 6 min in drying cabinets at temperatures of 25, 50 and 70°C. The six minutes are equivalent to the duration of the coating procedure in the fluid-bed dryer. After incubation, the millet grains were transferred on wateragar (1.8% (w/v) agar-agar) and incubated at 25°C. After 1-week incubation, the rate of millet grains covered with fungus was determined. This experiment was repeated six times time independently with three replicates.

Fluid-bed coating

For the fungal strains JKI-BI-1496 and B.b. 007, biomass concentrations of 0.3, 0.03, 0.003 and 0.0003% were used. For the fungus JKI-BI-1339, initially the concentrations of 0.03, and 0.3% and in an additional experiment concentrations of 0.3 and 0.7% biomass were compared. In all, 20 ml of each biomass suspension was sprayed on 100 g of sterile millet in a laboratory fluid-bed dryer (Strea-1; Aeromatic-Fieldler AG, Bubendorf, Switzerland, nozzle diameter: 1 mm, Container volume: 16.5 l). The flowrate for the suspension was 3.3 ml min⁻¹. This ensured a constant spray on the millet with a pressure of 1.5 bar over a period of 6 min. The inlet temperature of the fluid-bed dryer was set to 50°C. The volume flow of the drying air was set to 130 m³ h⁻¹. After drying three times, 20 granules were transferred on water agar for each concentration. After an incubation of 1 week at 25°C, the number of granule colonization was determined visually. This experiment was repeated six times time independently.

Conidiation on the granules in soil

The biological activity of the granule is caused by the sporulation of the fungus on the granule. Therefore, in a

further experiment, the fungal sporulation on the granule under different soil moisture conditions was compared. Due to the insufficient outgrowth of strain JKI-BI-1339, this experiment was conducted only with the fungi JKI-BI-1496 and B.b. 007. For this, granules treated with the biomass concentration of 0.03% were used. For this experiment, soil (Fruhstorfer Erde Typ T; Hawita Gruppe GmbH, Vechta, Germany) was sieved through a mesh width of 13.5 and 3.0 mm and mixed with 20% (w/w) sand. After autoclaving three times at 121°C for 20 min and drying at 60°C for 2 weeks, the residual water content was approximately 3%. For each treatment, 50 ml of soil was mixed with 0, 5, 10, 15 and 20 ml of autoclaved deionized water. The residual moisture content of the soil was determined using a residual moisture meter (Ma30; Sartorius). A centrifuge tube (15 ml) was filled with soil to the mark of 1.5 ml. Here, the first coated granule was placed and the centrifuge tube was further filled with soil up to the 2.5 ml mark and another granule was put on top. This was continued for the marks of 3.5, 4.5, 5.5 and 6.5 ml. The uppermost granule was not covered with soil and was regarded as an optical indicator for the growth and sporulation of the fungus. To prevent the soil from drying, the tubes were sealed with parafilm. For each treatment with different wetness of the soil, three additional centrifuge tubes were filled. The centrifuge tubes were incubated for 7 days at 25°C. After this, the uppermost granule together with the surrounding soil was removed. The remaining content of the centrifuge tube was poured into a glass bottle. 10 ml of 0.1% (v/v) Tween 80 was filled in the emptied centrifuge tube and shaken to suspend remaining spores. The 10 ml and additional 40 ml of 0.1% (v/v) Tween 80 was filled in the corresponding bottles. The bottles were placed on a reciprocal shaker and shaken for 10 min at the highest setting. Finally, based on the specific form and shape of the fungal conidia, the spore concentration was determined using a hemocytometer. The experiment was repeated six times with three replications with the same material used for the fluid-bed drying experiments.

Optimization of granules based on strain JKI-BI-1339

Additional experiments were conducted with the strain JKI-BI-1339 to optimize the outgrowth on the granule by pre-coating nutrients within the fluid-bed coating process.

Screening of nutrients for better sporulation

In 96-well microtiter plates in half of the cavities 100 µl of single nutrient solutions of peptone, malt extract, lactose, raffinose pentahydrate (Fluka, Buchs, Switzerland), trehalose (Carl Roth GmbH), sorbitol (Sigma-Aldrich,

Buchs, Switzerland) or glucose (Merck) (all 20%, w/v) were pipetted. In the other half 50 µl of peptone (20%, w/v) plus 50 µl of lactose, raffinose, trehalose, sorbitol or glucose (all 20%, w/v) were added. Afterwards, each cavity was inoculated with 10 conidia suspended in 100 µl of deionized and autoclaved water. The microtiter plates were incubated at 25°C for 2 weeks. Within this time, the fungus was growing and sporulating on the surface of the medium. To count the number of formed conidia, 150 µl of 0.1% Tween 80 was added to each cavity. The number of conidia was determined with the help of a hemocytometer. Three time independent repetitions with three replicates were set up.

Comparison of different fungal materials and nutrients

For the production of conidia JKI-BI-1339 was mass-produced for 2 weeks on a medium containing autoclaved rice and barley in a ratio of 5 : 1 in a laboratory solid state fermenter (L-03; Prophyta, Malchow, Germany) at 25°C. Afterwards, pure conidia were harvested with a mycoharvester (5b; MycoHarvester, Ascot, UK). For the production of mycelium and submerged spores a medium containing 2% glucose, 2% yeast extract, 1.5% corn steep and 0.4% Tween 80 was selected. Submerged spores were separated from the mycelium within a Sieve-shaker using sieves with a mesh size of 400, 180 and 20 µm. Only submerged spores were able to pass the 20 µm mesh size. The remaining filtercake on the 180 µm sieve was removed and processed as described. The biomass was adjusted as described to a concentration of 0.7% g. The filtrate was centrifuged and the pellet was resuspended to a final spore concentration of 1×10^6 submerged spores or conidia per ml with a 0.9%-NaCl solution. For the pre-coating with different nutrients, 120 ml of nutrient solution (malt extract, peptone, each 20%, or malt extract + peptone, each 10%) was sprayed on 100 g millet using the above described settings. After that, 15 ml of the fungal suspension was sprayed on the nutrient-coated granule using the same settings. Deionized water was used as control. Per treatment 20 granules were placed on water agar and the number of contaminated and by entomopathogenic fungi colonized granules and the conidiation was measured after 7 days at 25°C. Granules were defined contaminated when bacterial or untypical fungal growth was visible on the granule. The experiment was repeated three times.

Statistical analysis

Data were statistically analysed with the software SAS System for Windows ver. 9.4. The Shapiro–Wilk test was applied for testing normality. The homogeneity of

Table 1 Number of living cells within a biomass suspension (0.03% dry weight) of different entomopathogenic fungi after heat treatment for 6 min

Fungal strain	Incubation temperature (°C)					
	25		50		70	
<i>M. brunneum</i> JKI-BI-1339	3.94 (± 1.05) $\times 10^6$ *	A [†]	3.94 (± 5.23) $\times 10^4$	B	7.04 (± 6.86) $\times 10^2$	B
<i>Cordyceps fumosorosea</i> JKI-BI-1496	2.65 (± 1.91) $\times 10^6$	A	6.67 (± 5.16) $\times 10^4$	B	0 \pm 0	C
<i>B. bassiana</i> B.b. 007	2.01 (± 3.60) $\times 10^5$	A	1.33 (± 2.76) $\times 10^5$	A	0 \pm 0	B

*Mean of most probable numbers and standard deviation of cells per ml of six independent experiments.

[†]Means with same letters of one strain are not significantly different (SNK, $P < 0.05$).

variance was proven by the Levene test ($P < 0.1$). For separation of means, data were compared with the Student–Newman–Keuls test (SNK) ($P < 0.05$). For analysing the influence on additives on the granule colonization and conidiation, the glim-mix procedure based on a residual likelihood was applied ($P < 0.05$). For all other experiments data showing heteroscedasticity of variance, the non-parametric U -test of Mann–Whitney or the t -test of Kruskal–Wallis was chosen. By the exact Methods in the NPR1WAY procedure (two-sided), data were compared pair wise (Wilcoxon, exact, $P < 0.05$).

Results

Thermotolerance under wet heat

Heat treatment for 6 min at 50°C resulted in a significant reduction of MPNs for JKI-BI-1339 and JKI-BI-1496. The values were two potencies lower in comparison to the 25°C treatment. For B.b. 007, no significant reduction

was measured when these two temperatures were compared. The treatment with 70°C resulted in no viability of strains JKI-BI-1496 and B.b. 007. For JKI-BI-1339, some numbers were counted even after incubation at 70°C (Table 1).

Thermotolerance under dry heat

After heat treatment, the number of colonized millet was determined. For JKI-BI-1339 and JKI-BI-1496, no significant difference of the granule colonization was obtained at temperatures of 25–70°C, whereas for *B. bassiana* strain B.b. 007 it was significantly reduced at 70°C (Fig. 1). For *M. brunneum* strain JKI-BI-1339, the granule colonization was highly depended on the biomass concentration. Only a biomass concentration of 0.3% resulted in nearly 100% granule colonization. For the strains JKI-BI-1496 and B.b. 007 even concentrations of 0.0003% biomass resulted in nearly 100% granule colonization.

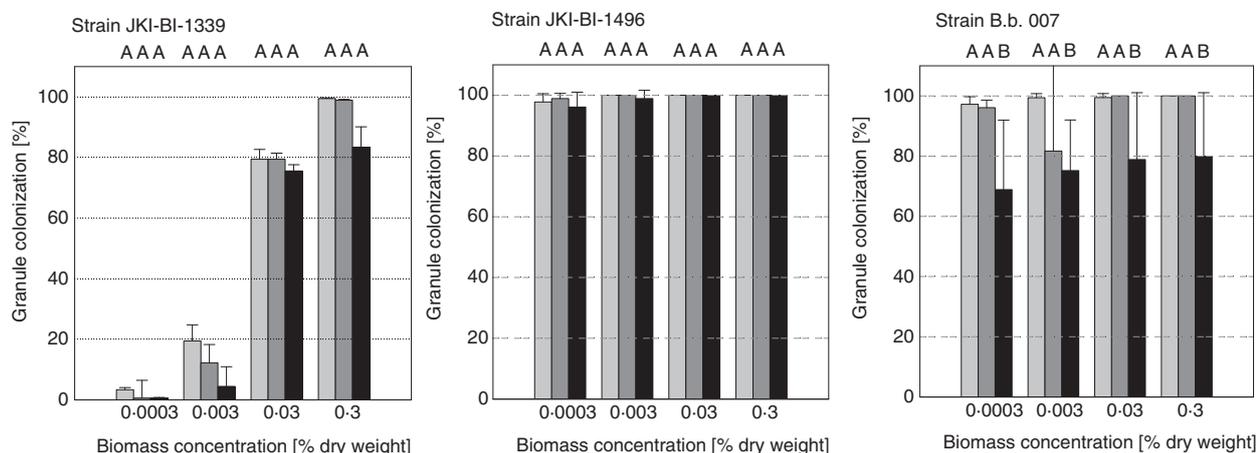


Figure 1 Effect of dry heat at 25°C (□), 50°C (■) and 70°C (■) on the granule colonization after dipping in a suspension of strain *Metarhizium brunneum*, strain JKI-BI-1339, *Cordyceps fumosorosea*, strain JKI-BI-1496, or *B. bassiana*, strain B.b.007, with different biomass concentrations. The mean values and the standard deviations are shown. Means with same letters of one biomass concentration and strain are not significantly different (U -test, $P < 0.05$, $N = 18$).

Survival rate of the fungi after fluid-bed drying

For JKI-BI-1339, two sets of experiments were conducted. In the first experiment, granules coated with a biomass concentration of 0.3% showed a granule colonization of 45% and in the second experiment of 33%. When the biomass concentration was lowered to 0.03%, the granule colonization was reduced to 8%. In the second experiment increasing the biomass to 0.7% resulted in a granule colonization of 48%. The granule colonization was not significantly improved (Fig. 2). In contrast to JKI-BI-1339 even concentrations of 0.03% of strain JKI-BI-1496 resulted in 100% granule colonization. An additional 10 times reduction of biomass resulted in a significant reduction of granule colonization. For 0.0003% only 40% of the granules were mycosed (Fig. 3). The highest granule colonization was achieved with strain B.b.007. Even for the lowest concentration of 0.0003% biomass, the granule colonization was still more than 60% (Fig. 3).

Conidiation on the granules in soil

Beside granule colonization, the conidiation is an important factor for efficient biocontrol of soil-dwelling pest insects. These experiments were only conducted with strain JKI-BI-1496 and B.b.007 with the lowest biomass concentration of 0.03% with 100% granule colonization on the granules and showed that the conidiation on the granule is dependent on the soil wetness. For JKI-BI-1496, a water content of 40% resulted in more than

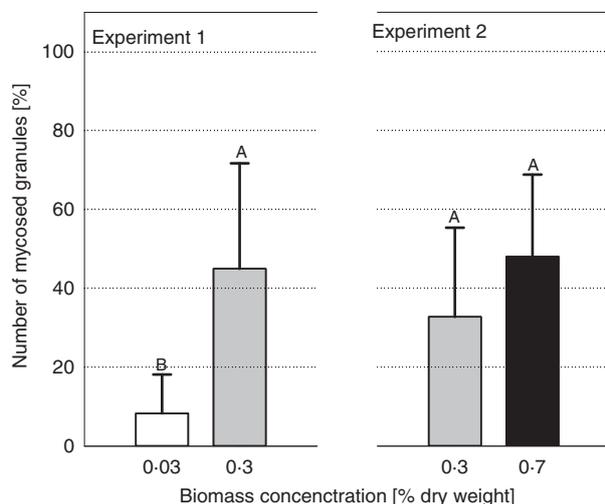


Figure 2 Influence of the biomass concentration on the granule colonization which was coated with *Metarhizium brunneum*, strain JKI-BI-1339, by fluid-bed coating. Mean value and standard deviation are shown. Means with same letters of one biomass concentration of the strain are not significantly different (*U*-test, $P < 0.05$, $N = 18$).

5×10^9 conidia per granule which was more than five times higher in comparison to a water content of 3% (Fig. 4). For B.b.007, the highest conidiation was achieved in the most wet soil with approximately 2×10^9 conidia per granule (Fig. 4).

Optimization of granules based on strain JKI-BI-1339

Because the number of colonized granules was too low for JKI-BI-1339, we proofed the potential for optimizing the fluid-bed drying process by pre-coating the granule with nutrients. In the first experiment, the combination of malt extract with peptone resulted in the highest sporulation on liquid media (Fig. 5). Based on these results, malt extract, peptone or a mixture of both was coated on the granule before the fungal biomass was applied. Additionally, we compared in this set of experiments aerial conidia, submerged spores or a mixture of mycelium with submerged spores. For submerged spores and mycelium, the addition of malt extract alone or in combination with peptone had a significant positive effect on the granule colonization which was around 20% higher than in the untreated control (Fig. 6). Peptone alone had a significant negative effect on the granule colonization and an unwanted positive effect on the number of contaminated granules. Nearly 100% granule colonization was achieved by coating the granule with conidia. For conidia, no additional positive effect was achieved by adding nutrients to the fluid-bed drying process. The results on the conidiation on the granule indicate that although the granule colonization was optimized, the conidiation was not enlarged. For none of the fungal material, higher sporulation was achieved by adding nutrients (Fig. 6).

Discussion

The aim of this work was to develop a technology for formulating liquid fermented biomass of entomopathogenic fungi for control of soil-dwelling insect pests. In this work, we developed granules based on fluid-bed coated biomass of three different genera of entomopathogenic fungi. In the developed system, autoclaved millet was used as core particle, on which a thin layer of fungal biomass was coated. Millet has the advantage that it is cheap, the main component is starch and the size and stability is optimal for application with common granule application technology in agriculture (H. Lehner, pers. communication). Additionally, starch in form of, for example, corn meal or wheat flour can be used as natural source of CO_2 , which is attracting soil-dwelling pest insects (Bernklau *et al.* 2004). Starch can as well be used as carbohydrate source by the

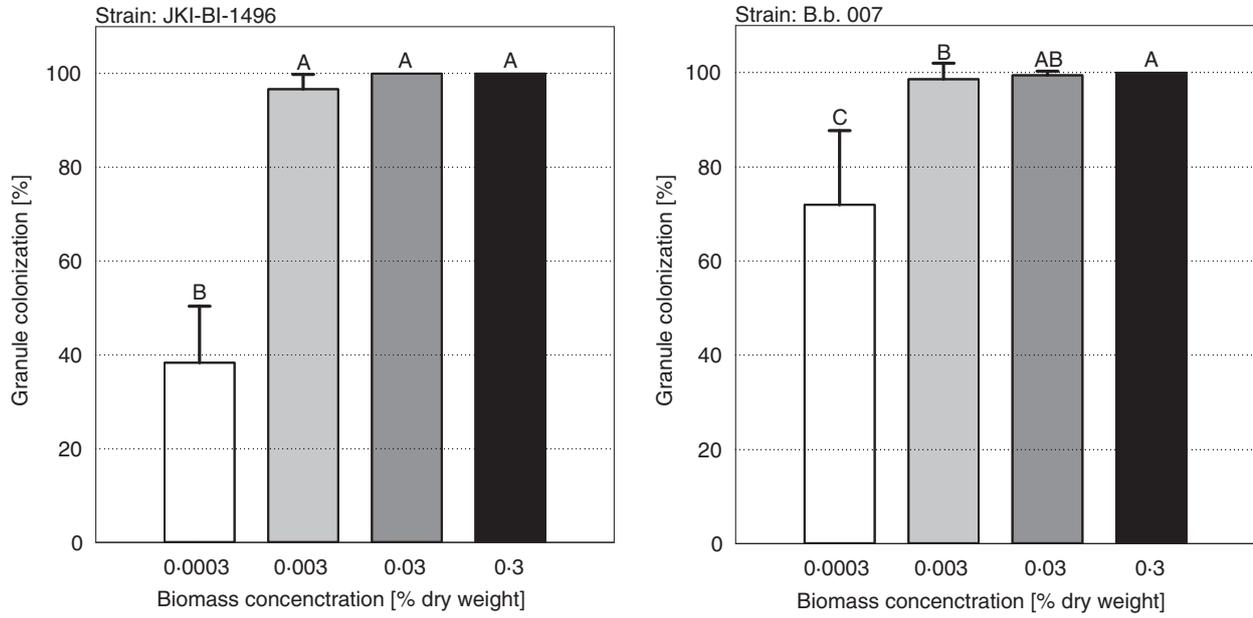


Figure 3 Influence of the biomass concentration on granule colonization which was coated with *Cordyceps fumosorosea*, strain JKI-BI-1496, or *B. bassiana*, strain B.b.007, by fluid-bed drying. Mean value and the standard deviation are displayed. Means with same letters of one biomass concentration of the strain are not significantly different (*U*-test, $P < 0.05$, $N = 18$).

entomopathogenic fungus and will be completely degraded in the soil. Based on the fungal growth on the granule, it can be expected that CO₂ will be produced

and may attract soil-dwelling pest insects which is the basis of CO₂-releasing co-formulations based on starch and *Saccharomyces cerevisiae* (Vemmer et al. 2016). Zhou

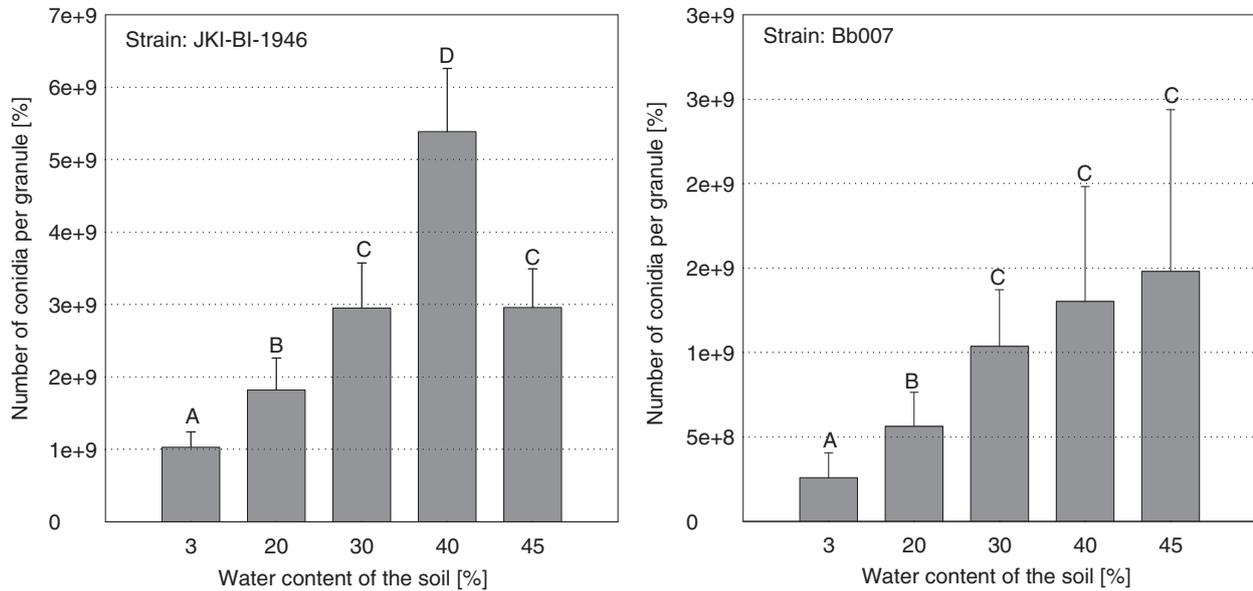


Figure 4 Fungal conidiation of *Cordyceps fumosorosea*, strain JKI-BI-1946, or *B. bassiana*, strain B.b.007, on the granule in soil with different water content. The biomass concentration of the granules was 0.03%. Mean value and the standard deviation are displayed. Means with same letters of the strain are not significantly different (*U*-test, $P < 0.05$, $N = 18$).

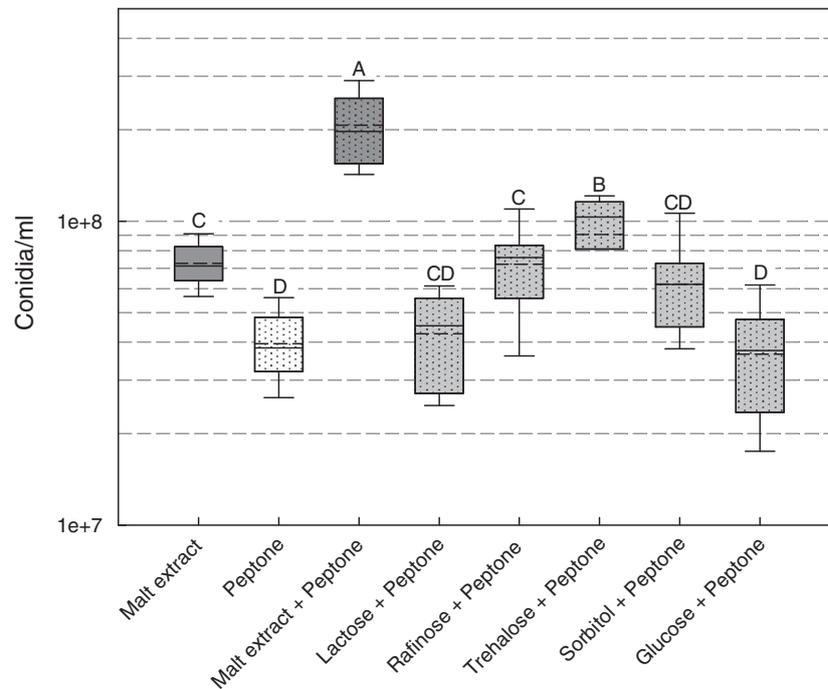


Figure 5 Fungal conidiation of *Metarhizium brunneum*, strain JKI-BI-1339, on liquid culture containing different nutrient components after 14 days incubation at 25°C. Box plot with median (black line) and mean (short dash line). Means with same letters are not significantly different (SNK-Test, $P < 0.05$, $N = 9$).

and Feng (2010) observed that millet powder can be utilized by the fungus and improved the sporulation of *Pandora nouryi* on alginate pellets. In general, fluid-bed coating offers the possibility to alter and to improve various characteristics of core particles. The challenge is to get a constant coating quality, especially during process up-scaling (Parikh 2017). Therefore, a homogeneous coating material is required. When fungi are cultivated in liquid culture, the growth behaviour extremely depends on the cultivated strain, the cultivation media and cultivation conditions (Kleespies 1993). In liquid, the *M. brunneum* strain JKI-BI-1339 is mainly growing as mycelium. Under specific conditions, it is only producing filamentous mycelium or compact pellets which were the basis for the first BIO1020TM product (Reinecke *et al.* 1990). The potential of JKI-BI-1339 to sporulate in liquid is limited and after optimization max. 3.25×10^7 blastospores per ml were formed (Kleespies 1993) which is unsuitable for commercial production. In contrast, this strain can easily be produced in solid state fermenter with high conidiation on the substrates. Based on this material, the two products Met52 GranularTM (conidia on a granular matrix) and Met52 ECTM (liquid-based formulation of conidia) are registered in a range of countries as plant protection product (novozymes, 2020).

Beauveria bassiana strain Bb.007 can be easily produced in liquid and on solid media. In liquid culture, B.b.007 is growing in the mycelial phase and as well is producing up to 1.2×10^8 submerged spores per ml. Since 2019, strain *B. bassiana* strain B.b. 007,—IMI # 501799, is registered by the Ministry of Environment Protection and Agriculture of Georgia, National Food Agency in 2019, as trade mark Bover-GE (Registration # 3142). It is expected that the market potential of B.b 007 will rise when new cost-efficient formulations will be available. *Cordyceps fumosorosea* strain JK-BI-1496 can easily be produced in liquid culture with a spore yield of up to 4×10^8 submerged spores per ml (D. Stephan, unpublished). Because of its interesting biological activity and uncomplicated production in liquid culture, this strain has a high potential for further product development.

To get a homogeneous biomass suspension, which can pass the nozzles of the fluid-bed dryer the biomass was homogenized. The MPN values indicate that biomass is still viable after homogenization. Assuming that an incubation of 6 min at 25°C is not effecting the fungal viability, our MPN numbers indicate that in 1 g biomass of JKI-BI-1339 and JKI-BI-1496 the MPN is approximately 10^{10} MPN/g_{dry weight}. For B.b.007, the value is around 10 times lower. Because in biological control living

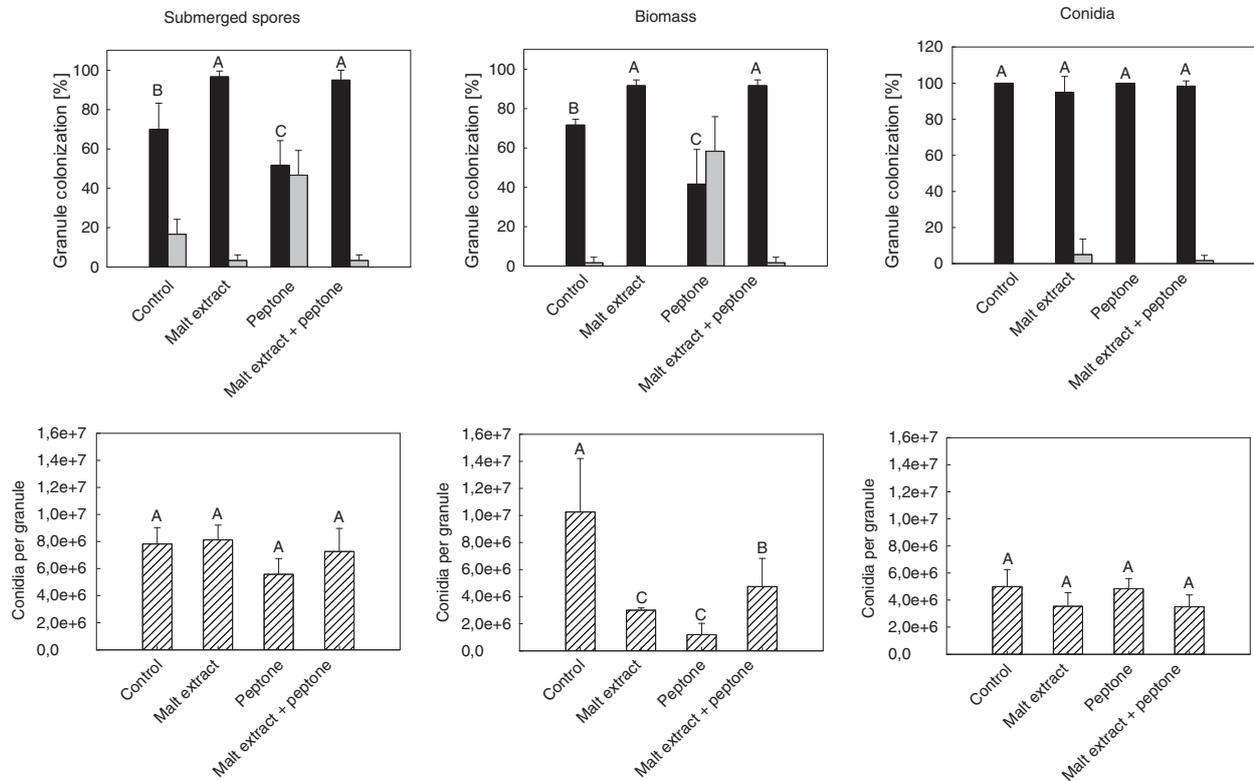


Figure 6 Influence of pre-coating with different nutrients on the granule colonization (■), contamination (▨) and conidiation (▩) of granule formulations based on submerged spores (left), biomass (middle) and conidia (right) of *Metarhizium brunneum*, JKI-BI-1339. Mean value and standard deviation are displayed. Means with same letters are not significantly different (Glimmix, $P < 0.05$, submerged spores, biomass: $N = 3$, conidia: $N = 6$).

organisms are applied in the field, the organism has to survive the thermal and dehydration stress within the formulation process. Our results confirmed that liquid fermented fungi are sensitive for thermal stress. When fungal biomass suspended in water was heated for only 6 min, even at temperatures of 50°C the viability for strains JKI-BI-1339 and JKI-BI-1496 was reduced to 4%, compared to an incubation at 25°C. This thermal sensitivity within a formulation process correspond to results of Stephan and Zimmermann (2001) and Horacek and Viernstein (2004). We confirmed that strains differ in their thermotolerance (Rangel *et al.* 2005). The *B. bassiana* B.b.007 tolerated temperatures of 50°C but only for JKI-BI-1339 limited growth was detected at 70°C. Under the microscope, only mycelium was seen but it cannot be excluded that in very low concentrations, for example, microsclerotia were formed under oxidative stress (Georgiou *et al.* 2006), which are a melanized structure, that possibly can survive better than mycelium itself (Willets 1971).

The second set of thermal experiments was more adapted to the fluid-bed coating conditions. Millet was

covered by a thin but undefined film of biomass suspension and was incubated under different temperature conditions. During this incubation phase, water was able to evaporate and consequently to cool the desiccating biomass with a clear effect on the granule colonization on the millet. Under these conditions for none of the strains any negative effect of an incubation temperature of 50°C was detected and 70°C only effected B.b. 007 significantly. The cooling effect of evaporating water is used in several drying systems. Stephan and Zimmermann (1998) demonstrated that submerged spores of *Metarhizium acridum* can be spray-dried even under high inlet temperatures without loss of viability. When different biomass concentrations were tested, only for JKI-BI-1339 the granule colonization declined independently of the temperature treatment. Because the MPN of the biomass suspension of JKI-BI-1339 and JKI-BI-1496 was nearly the same, the results indicate that the biomass of these two strains has a different desiccation tolerance. It is expected that the fragmented mycelium is more desiccation sensitive than the remaining submerged spores, which were partly in the biomass of strain JKI-BI-1496 and B.b.007.

Drying of mycelium for getting formulations for bio-control was conducted by several authors (Mc Cabe and Soper 1985; Rombach 1989; Pereira and Roberts 1990, 1991; Krueger *et al.* 1992). But in contrast to our experiments, all of the authors described slow drying processes. Our results of the thermal experiment were transferred to the fluid-bed coating system. Again, for JKI-BI-1339 the granule colonization was low. It was not possible to achieve colonization rates of more than 50% by enlarging the biomass concentration to a maximal concentration of 0.7% dry weight which was the maximal pump- and sprayable concentration. On the other side, for the two other strains concentrations of 0.003% dry weight was efficient enough to get nearly 100% colonized granules. We know from other entomopathogenic fungi, that a production of 1.5%_{dry weight} fungal biomass in liquid culture is realistic (Bernhardt, pers. comm., (Ortiz-Urquiza *et al.* 2010). Based on our fluid-bed coating settings and results, 1 l of fermentation culture of JKI-BI-1496 or B.b.007 with 1.5%_{dry weight} fungal biomass would be enough to produce 25 kg granule with around 100% granule colonization. For that, the authors considered fluid-bed coating as an economically interesting technique. Although (Gotor-Vila *et al.* 2017) achieved good results with freeze-drying of *Bacillus amyloliquefaciens*, this drying process was considered as too time-consuming and cost-intensive. Based on their positive results, they preferred a combination of spray drying and conventional fluid-bed drying technique. Reddy *et al.* (2014) treated oil-coated granules with conidia of entomopathogenic fungi. This is an easy and suitable system for lipophilic conidia but not suitable for hydrophilic biomass. Several authors described formulations containing a mixture of growing substrate with conidia (Ricaño *et al.* 2013; Zhang *et al.* 2019), microsclerotia with clay (Behle and Jackson 2014; Behle and Goett 2016) or in combination with attractants like pheromones (Kabaluk 2014; Todd Kabaluk *et al.* 2015) or yeast (Brandl *et al.* 2017). Whenever growing substrate is a part of the granule formulation, the whole granule has to be dried to get a storable product. The same applies for other formulations, for example, alginate pellet formulations. As a result, the formulation costs are expected to be higher.

But granule colonization is just one quality criteria. The fungus has to respore in the soil to infect target insects. Conidiation of fungi depends on the environmental conditions like nutrient resources and humidity (Arthurs and Thomas 2001a; Nuñez-Gaona *et al.* 2010). Jackson and Jaronski (2012) demonstrated that microsclerotial granules with higher moisture levels produced more conidia immediately after drying and granules with low moisture produced more conidia after 12 months storage. In our experiments, we compared different water

contents in a sterile soil substrate mixed with sand by adding water. For agricultural soils, the water availability described as field capacity and the available water capacity are the important parameters which are highly influenced by the loam content. Therefore, further experiments with different types of grown soil have to follow. Our results underline that conidiation takes place on the granule in the soil as well as on pure water-agar (data not shown). Consequently, the nutrient source of millet is sufficient for sporulation. This corresponds to results of Pandey and Kanaujia (2008).

Additionally, in our system, sterile soil was used to avoid contamination effects which is far away from the reality in the field. Anyhow, our results indicate that JKI-BI-1496 has a clear optimum at 40% water content. Additional experiments with JKI-BI-1339 indicate that for this strain the optimum is lower than 27% water content (data not shown). The results underline differences between strains.

For the *C. fumosorosea* strain JKI-BI-1496 and the *B. bassiana* strain B.b.007, the results were acceptable so that further experiments in the field can follow. For the *M. brunneum* strain JKI-BI-1339, the fungal growth on the granules was too low so that further optimization steps followed. Sharma *et al.* (2020) demonstrated that millet as a granular carrier improved the efficacy of entomopathogenic fungi under field conditions. The reason is unknown. Kim *et al.* (2020) confirmed that millet-based solid cultured granules of *M. anisopliae* were effective against soil-dwelling pest insects. Conidiation is highly influenced by the composition of nutrients. Nitrogen and carbohydrate sources are important ingredients and their ratio as well as their quantity is influencing the growth and sporulation on strain level (Gao *et al.* 2007; Uzma and Gurvinder 2009). Soy-peptone is a commonly used nitrogen source. Li and Holdom (1995) compared different sugars in terms of mycelial growth and sporulation of *M. anisopliae* with best effects of D-Mannose, maltose and D-Glucose. Because of the bee toxicity of mannose (Staudenmayer 1939), this nutrient is not suitable for the development of granules in agriculture and was not included in our studies.

Our results confirm that malt extract with the main component maltose resulted in high sporulation. In contrast, D-glucose was not sufficient in our experiments. As already described by Ottow and Glathe (1968), malt extract can be used as a single nutrient component for fungal growth. In our experiments, best sporulation was obtained with a combination of malt extract and peptone. Further optimization steps should follow because several aspects, for example, C/N ratio of nutrients are important factors influencing fungal growth and sporulation (Safavi *et al.* 2007). When the granule was pre-coated with malt extract, peptone or a combination of

both the granule colonization rate was influenced. Pre-coating with malt extract alone or in combination with peptone resulted in a significant higher granule colonization when fungal biomass or submerged spores were used. These results indicate that there is a potential to optimize the coating process by adding specific nutrients to the process. The fluid-bed coating process was not done under sterile conditions. Therefore, the additional information about risk of contamination was important. Especially, when peptone was pre-coated alone, the contamination rate was more than 40%. Micro-organisms compete with nutrients and peptone seems to support bacterial growth (Hibbing *et al.* 2010). These bacterial contaminations were not further investigated but the results show us an important hint what may occur in an unsterile soil. Sugar components are known to protect micro-organisms within a drying process (Stephan and Zimmermann 1998; Stephan *et al.* 2016; Gotor-Vila *et al.* 2017; Bisutti and Stephan 2020). Therefore, it is likely that malt extract has as well a protective effect during the drying process. Aerial conidia are amenable to simple drying techniques with better storability (Bidochka *et al.* 1987; Lane *et al.* 1991) and therefore it is likely, that conidia survive the coating process better than submerged spores or mycelium. Granules coated with conidia were colonized up to 100% even without any pre-coating. These results demonstrate that for coating of the granule as well conidia can be used. However, it must be taken into account that aerial conidia are produced on solid substrates. It is contra productive to suspend these nearly dry conidia in water and dry them again. In our experiments, none of the pretreated granules resulted in a higher conidiation after coating submerged spores, mycelium plus submerged spores or conidia. Steyaert *et al.* (2010) stated that the transition from fungal mycelium to spore is determined by the interplay of environmental cues, whereby one factor alone is not necessarily sufficient to evoke change. For the genus *Trichoderma*, the relative ratio of carbon and nitrogen has a strong influence on conidiation and growth. Gao *et al.* (2007) have shown that for *Trichoderma* higher amounts of nitrogen favored mycelial growth whereas nitrogen limitation favored conidiation when sucrose was used as carbon source. Possibly, the higher numbers of colonized granules and its conidiation can be explained by the carbohydrate and nitrogen availability. But it is not explaining the differences between conidia and liquid fermented biomass. It should also be mentioned that the conidiation was measured after 1-week incubation. It cannot be excluded that within 1-week incubation the fungus did not reach the maximal level of conidiation. That possibly explains the low numbers of conidia per granule in comparison to JKI-BI-1496 and B.b.007. The idea of the pre-coating experiments was to add

nutrients. Additionally, nutrients like sugars can be used as protectants within the drying process (Stephan and Zimmermann 1998; Horaczek and Viernstein 2004; Stephan *et al.* 2016). This could possibly be achieved by coating a mixture of biomass with nutrients.

In conclusion, the results clearly indicate that liquid fermented biomass of entomopathogenic fungi can be the affective ingredient of granules for biocontrol of soil-dwelling pest insects. Fluid-bed coating is an efficient process to coat thin layers of fungal material on the surface of granules. In further experiments, the efficacy of this type of granule has to be tested in the field. Finally, the economic and technical feasibility of the described process has to be verified.

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Conflict of Interest

The authors declare no conflict of interest on any of data published in this manuscript.

Author contributions

D.S.: conceived and designed the analysis, contributed data analysis tools, supported the statistical analysis, wrote the paper, coordinated all experiments, the paper was written and was circulated and accepted by all other co-authors; T.B.: collected the data, contributed data analysis tools, involved in writing the first draft version and creating the graphs; M.B.: collected the data, contributed data analysis tools, supported the literature search and writing the discussion; C.S.: collected the data, performed the analysis; J.S.: conceived and designed the analysis, collected the data, performed the analysis; N.M.: collected the data, contributed data analysis tools, performed the analysis. J.P.: conceived and designed the analysis; collected the data; performed the analysis.

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