Field efficacy evaluation of *Beauveria brongniartii* against *Melolontha melolontha* in potato cultures

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An efficacy study with *Beauveria brongniartii* (Sacc.) Petch, commercialised under the name Melocont®-Pilzgerste, for the control of *Melolontha melolontha* L. was conducted in potato fields of Oberhofen im Inntal, Tyrol, Austria. Results from these trials demonstrated that Melocont®-Pilzgerste is an effective agent for a sustainable control of *Melolontha melolontha* L. if used as part of an intelligent Integrated Pest Management (IPM) strategy. By correlating mortality data for *Melolontha* and *Beauveria* densities in the soil, a median lethal concentration (LC_{50}) of 1.1×10^5 cfu *Beauveria* per gram soil dry weight was calculated. Furthermore, the dose of Melocont®-Pilzgerste calculated to kill 50 % *Melolontha* larvae (LD_{50}) was 56 kg ha^{-1}. This corresponds to the recommended application dose of 50 kg ha^{-1} per year. These results underline the postulated efficacy of *B. brongniartii* for the control of the European cockchafer and demonstrate the benefits resulting from the use of the product as required for the registration in the European Union. The applied methods were validated and translated into standardised guidelines for the efficacy evaluation and quality control of fungal biological control agents (Risk Assessment Fungal Biocontrol Agents Standards).

Keywords: fungal biocontrol agent, entomopathogen, field trials, standard protocol.

The larvae of *Melolontha melolontha* L. and other scarab beetles feed on the roots of a wide range of economically important plants causing significant damages in grassland, arable crops and forests. The estimated affected area in Central and Northern Europe is well above 100 000 hectares and the economical damage caused by scarab larvae in Europe is several billion Euro (Strasser 2004). The majority of the damage is caused by the European cockchafer (*M. melolontha*). It is most common in sandy soil environments with low annual precipitation and has a 3 to 5 year life cycle.

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In a typical four year life cycle *imagines* emerge from the soil in April for feeding and mating. After an egg maturation period the females deposit their eggs in the original breeding grounds and die thereafter. Eggs usually hatch by June and the grubs pass three larval instars ($L_1 - L_3$) over a period of three years. During this time, larvae overwinter below the freezing level of the soil and move into the rhizosphere as soon as soil temperatures rise to above 8 °C in the spring. The main feeding period during which the most damage occurs is the second year after hatching. In the summer of the subsequent year, the third larval instar (L_3) pupate and, after a metamorphosis period of nine weeks, hatch as adult beetles. The adult beetles overwinter in the soil and emerge in the fourth year.

The control of *M. melolontha* has a long history in Austria and involved strategies such as the collection of adult beetles by farmers and growers as well as mechanical treatment. Detailed records of *Melolontha* outbreaks go back to Zweigelt (1928) and have been continued until today. This extensive data allows for an exact prediction of cockchafer flight in a specific region, providing excellent conditions to apply IPM strategies. Due to the lack of environmentally sustainable substances able to control larvae in the soil, chemical control has been limited to the *imagines*, resulting in a significant need for alternatives. *Beauveria brongniartii* (Sacc.) Petch has been known as a pathogen of *M. melolontha* for more than 100 years and occurs naturally in *Melolontha* populations. It has a very narrow host range and exclusively infects *Melolontha* spp. under natural conditions in Europe (Kessler 2004, Traugott *et al.* 2005). Its potential for the biological control of *Melolontha* spp. has been recognized as early as the late 19th century, when Giard (1893) and Le Mould (1893) conducted first inoculation trials with *B. brongniartii*. More recently, experience with various formulations of *B. brongniartii* has been gathered in France, Germany, Italy and Switzerland (Zelger 1996, Zimmermann 1998, Strasser 1999, Keller 2000, Copping 2001).

In Austria, *B. brongniartii* has been registered for unrestricted use (all crops) against *M. melolontha* and *M. hippocastani* L. (Austrian Plant Protection Product Register, Reg. No. 2582). To date, Melocont® -Pilzgerste (F. Joh. Kwizda GmbH) together with Betel® (Calliope SA) are the only fungal biocontrol agents (BCAs) registered for field application as an "old active compound" in several European Union Member States (i.e. in Austria, France, and Italy) (Copping 2001).

Among the obstacles for the registration of fungal BCAs on the level of the European Union under Council Directive 91/414/EEC are concerns about the environmental safety and the potential of fungal metabolites to enter the food chain. Some of these concerns have been addressed in the EC-project RAFBCA (contract no. QLK1-CT-2001-01391). Doubts about the efficacy of fungal formulations are

persistent among both growers and policy makers. This puts the promotion of fungal BCAs to a disadvantage compared to chemical pesticides, but in Austria, Melocont®-Pilzgerste is well established and is considered an effective agent. For instance, in 2004 almost 1 500 ha of land were applied with Melocont®-Pilzgerste in the province of Tyrol (Klymiuk 2004): on-farm field trials in potato plantations, known to be highly sensitive to white grub damage, were conducted.

The goals of our studies were to (i) gather dose-related efficacy data of Melocont®-Pilzgerste against *M. melolontha*, (ii) determine the *in situ* efficacy threshold for *B. brongniartii and* Melocont®-Pilzgerste, (iii) predict the long term pest reduction in a *Melolontha*-infested region, and (iv) develop and validate guidelines for a standardised efficacy evaluation of fungal BCAs against scarabaeid larvae.

Materials and Methods

Trial site

Trials were conducted in 2002 in the Kalkofen area of the municipality of Oberhofen im Inntal, Tyrol, Austria. The soil in this area is described as shallow sandy soil of low organic content derived from deposits and sediments of the adjacent Inn river. The trial field is listed as property no. 4228 in the cadastral register Oberhofen im Inntal, Tyrol, Austria. The dimensions of the field are approximately 80 m by 180 m with the geographical coordinates 11 06' 44" East, 47 18' 33" North marking the center at an elevation of 615 m above sea level.

Plot layout

The field was subdivided into 4 plots for the application of different treatments with Melocont®-Pilzgerste as well as controls. Treatments comprised the application of three different doses of Melocont®-Pilzgerste as well as sterile barley kernels. The latter is the carrier material of Melocont®-Pilzgerste and served as control. Random numbers were assigned to the different treatments as follows:

Treatment 1: 25 kg/ha Melocont®-Pilzgerste (half of recommended amount)

Treatment 2: 100 kg/ha Melocont®-Pilzgerste (double of recommended amount)

Treatment 3: 33 kg/ha sterile barley kernels (control, equals 50 kg Melocont®-Pilzgerste)

Treatment 4: 50 kg/ha Melocont®-Pilzgerste (recommended amount)

Plot sizes were determined by constraints imposed by the shape and dimensions of the agricultural field, sizes of individual plots were set at 170 m by 15 m (23 potato rows).

Application technique and agricultural data

Melocont®-Pilzgerste was applied with a Vredo sowing machine and worked into the soil by machine drilling to a depth of 20 cm. After the application of Melocont®-Pilzgerste, potatoes were planted with a row width of 65 cm to a depth of 20–25 cm. The clamp height was approximately 30 cm. These techniques are in accordance with local agricultural practices. Application and planting was done on April 22/23, 2002. Mechanical harvest of potatoes was done on August 21, 2002.

Quantification of *Beauveria brongniartii* in soil

Soil samples were drawn from every plot at the time of harvesting using a soil corer (inner diameter 1 cm). A total of 30 cores to a depth of 30 cm were taken per plot. Cores were split into a 0–10 cm and a 10–30 cm fraction, and blended to a pooled sample for each plot and depth fraction. Pooled soil samples were mixed thoroughly, air-dried, and sieved through a 2 mm sieve. Ten gram sub-samples from each depth (three replicates) were added to 40 mL 0.1 % (w/v) Tween®80, shaken at 150 rpm for 30 min and then treated in an ultrasonic bath for 30 seconds. *Beauveria*-selective agar plates (Strasser *et al.* 1996) inoculated with 50 µL of these soil suspensions and dilutions thereof, respectively, were incubated for 14 days at 25 °C and 60 % RH (four replicates per sub-sample). Colonies of *B. brongniartii* are given as colony forming units (cfu) per gram soil dry weight. More detailed information on the quality control of fungal biological control agents (BCAs), the isolation procedure and field efficacy of fungal BCAs, respectively, can be found in the appendices: Risk Assessment of Fungal Biocontrol Agents Standards (RAFBCA Standards).

Grub sampling

The average pre-application larval density of *M. melolontha* in the Kalkofen area was determined by digging 50 cm × 50 cm sample holes to a depth of at least 50 cm and manually screening the removed soil for *M. melolontha* larvae.

Four months after the Melocont®-Pilzgerste applications, immediately prior the potato harvest, the larval density of *M. melo-*

lontha was assessed again. In each plot 10 holes, evenly distributed over the length of a plot, with a size of 25 cm × 50 cm with a depth of at least 50 cm were dug at the clamp of the potato rows. The soil from these holes was placed on plastic tarps and was manually screened for *M. melolontha* larvae. Each larva was counted, their larval stage as well as their condition (alive/dead) was recorded. Larvae killed by *B. brongniartii* were densely covered with white to creme coloured mycelium and/or showed reddish staining of their cuticle caused by oosporein.

Harvest assessment

Potato tubers recovered from each plot were counted and classified as "healthy tubers" or "tubers damaged by white grub feeding". The number of healthy tubers was then split into "marketable size tubers" and "non-marketable size tubers". Tubers in each category were separately collected and pooled over each plot (treatment). The pooled harvest fractions were weighed, the biomass of each fraction and its percentage in relation to the total biomass of collected potatoes was recorded.

Statistical analyses of data

Beauveria density in the soil: the amount of *B. brongniartii* propagules per gram soil dry weight was determined for each replicate, the median value with upper and lower quartiles was calculated for each soil sample. Comparisons of medians were performed with a Mann-Whitney-U-test (comparison of 2 medians) or a Kruskal-Wallis One Way ANOVA on Ranks (comparison of 3 or more medians), followed by Dunn's pair wise comparison method if differences were found. Correlations between the applied amount of Melocont^{R}-Pilzgerste and the observed *Beauveria* densities were analysed with a Spearman rank order test. All tests were done with Sigmastat 3.0 for Windows.

Efficacy of Melocont^{R}-Pilzgerste against *M. melolontha*: the *B. brongniartii* induced *M. melolontha* mortality rate was calculated as percentage of the total number of larvae found in each sample and was averaged over each treatment. Means were statistically compared using Oneway ANOVA followed by Bonferroni *post hoc* comparisons when data passed a normality test (Lilliefors correction), otherwise a Kruskal Wallis Oneway ANOVA on Ranks followed by Dunn's multiple comparison was used.

The relationship between *B. brongniartii* density in the soil (soil depth: 10-30 cm) and the mortality of *M. melolontha* was described

by the calculation of a dose-response curve using the statistical software Graphpad Prism 4 for Windows. Minimum and maximum values of the curve were set to 0 and 100 percent mortality, respectively. LC_x-values (LC_{10}, LC_{50}, LC_{90}) were calculated from the obtained curves by customizing the software as recommended in the regression manual (Motulsky & Christopoulos 2004).

Analysis of harvest data: the ratio between healthy and damaged tubers in each plot was calculated as percentage of the total number of collected tubers. SigmaStat 3.0 for Windows was used to run a Kruskal Wallis Oneway ANOVA on Ranks on the fraction of tubers in each category.

Results

Beauveria density in the soil

In the 10–30 cm soil layer where most of the feeding damage of *Melolontha melolontha* occurs, the densities of *Beauveria* increased with the amount of applied Melocont®-Pilzgerste (Tab. 1). A Spearman rank order test revealed a highly significant correlation ($r = 0.848$, $p < 0.001$).

Tab. 1. – *Beauveria brongniartii* density in the potato clamp at different soil depths. Median values with $(Q_{75}\text{-}Q_{25})/2$ as a measure of variation.

Treatment	*B. brongniartii* density [cfu g^{-1} dry weight soil] Soil depth: 0–10 cm	Soil depth: 10–30 cm
33 kg ha^{-1} sterile barley	$1.27 \times 10^2 \pm 3.00 \times 10^2$	$1.92 \times 10^3 \pm 6.09 \times 10^2$
25 kg ha^{-1} Melocont®-Pilzgerste	$5.21 \times 10^3 \pm 1.89 \times 10^3$	$6.99 \times 10^4 \pm 7.49 \times 10^3$
50 kg ha^{-1} Melocont®-Pilzgerste	$1.32 \times 10^5 \pm 2.61 \times 10^4$	$8.95 \times 10^4 \pm 2.57 \times 10^4$
100 kg ha^{-1} Melocont®-Pilzgerste	$2.22 \times 10^5 \pm 6.25 \times 10^4$	$1.79 \times 10^5 \pm 3.36 \times 10^4$

A scattered indigenous population of *Beauveria* was observed in the control plot with densities of 1.9×10^3 cfu g^{-1} dry weight in the top soil, and 1.3×10^2 to 3.7×10^3 cfu g^{-1} dry weight in the deeper soil layers (10 cm to 30 cm).

M. melolontha grub density and mortality rate

An average pre-application density of 40 *M. melolontha* larvae per square meter was detected for the entire Kalkofen area. The *Melolontha* infestation rate observed at the time of the harvest was between 18 and 63 larvae per square meter, which equals an average of between 2.1 and 5 grubs per sample. The mortality rates of white grubs killed by *B. brongniartii* were between 38 % (50 kg/ha Melo-

cont®-Pilzgerste) and 63 % (100 kg/ha Melocont®-Pilzgerste) in the *Beauveria*-treated plots, differing significantly ($p < 0.05$, Bonferroni) from the control plot (Tab. 2).

Tab. 2. – *Melolontha melolontha* infestation rate for trial plots and *Melolontha* mortality caused by *Beauveria brongniartii*. The values are averages ± standard deviations derived from at least ten sampling holes of each treatment. N expresses the total number of grubs found in each plot.

Treatment	*M. melolontha* larve [m^{-2}]	*Beauveria*-killed *M. melolontha* larvae [%]	N
33 kg ha^{-1} sterile barley	18.81 ± 13.56	3.33 ± 10.54	21
25 kg ha^{-1} Melocont®-Pilzgerste	44.59 ± 20.98	43.30 ± 25.77	45
50 kg ha^{-1} Melocont®-Pilzgerste	51.69 ± 35.63	37.94 ± 34.65	44
100 kg ha^{-1} Melocont®-Pilzgerste	63.52 ± 49.51	63.89 ± 29.92	50

Correlation between *B. brongniartii* density in the soil and *M. melolontha* mortality

When comparing mortality of *M. melolontha* to the *B. brongniartii* density in the soil layer where most of the larvae fed on potato tubers (soil depth 10 cm to 30 cm), a highly significant correlation between the two data sets was calculated (Spearman r = 0.612; $p < 0.001$). Thus, a higher density of *Beauveria* density in the soil was accompanied by a higher mortality of *Melolontha* larvae. More detailed information on this relationship was obtained from the calculation of the dose mortality curve (Fig. 1). This allowed the calculation of LC_{50}-values ("concentration" of *Beauveria* required to kill 50 % of *Melolontha* larvae) and LD_{50}-values ("dose" of Melocont®-Pilzgerste that kills 50 % of larvae) and hence a prediction of mortality based on a given *Beauveria* density. The LC_{50}-value was 1.1×10^5 cfu *Beauveria* per gram soil dry weight with a 95 % confidence interval (CI) from 7.3×10^4 to 1.7×10^5 cfu g^{-1} soil dry weight (Fig. 1, Tab. 3). The according LD_{50} of Melocont®-Pilzgerste (dose-mortality curve not shown) was estimated at 56.5 kg ha^{-1} (CI 25.8–123.5 kg ha^{-1}).

Table 3. LC_x values (concentration to cause x % mortality) with 95 % confidence limits calculated with sigmoidal regression curve shown in Fig. 1.

$R^2 = 0.4079$	LC_x-value [cfu g^{-1} dry weight soil]	95 % - Confidence limits [µM]
LC_{01}	1.14×10^3	1.50×10^1 – 8.61×10^4
LC_{10}	1.24×10^4	1.56×10^3 – 9.84×10^4
LC_{50}	1.11×10^5	7.35×10^4 – 1.68×10^5
LC_{90}	9.95×10^5	1.08×10^5 – 9.14×10^6
LC_{99}	1.12×10^7	1.12×10^5 – 1.12×10^9

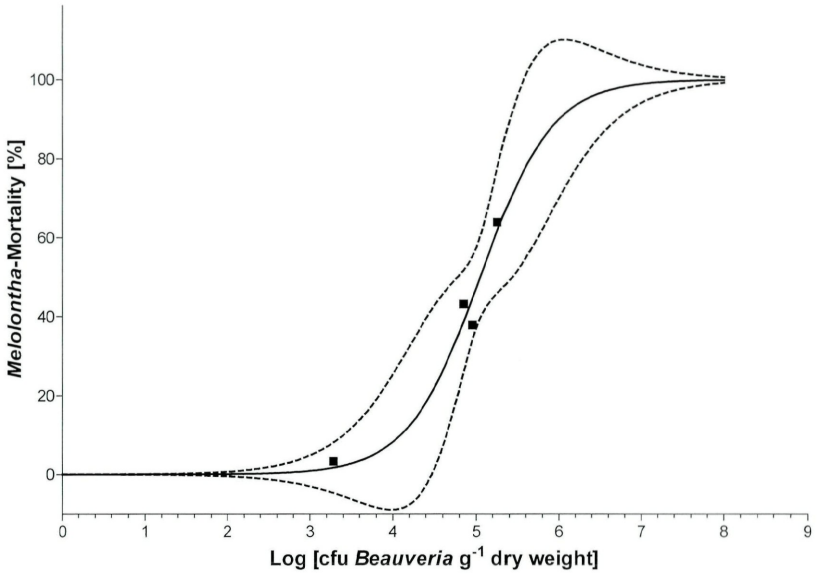

Fig. 1. Dose-response relationship between *Melolontha* mortality and *Beauveria* density in the soil of Kalkofen, area of the municipality Oberhofen im Inntal (Austria). Solid squares show empirical means, solid line shows sigmoid regression curve with 95 % confidence limits (dotted lines; $R^2 = 0.408$).

Quality of potato harvest

Between 19.9 % and 31.5 % of potato tubers were undamaged. None of the treatments with Melocont®-Pilzgerste had a direct effect by significantly reducing the feeding damage on potatoes. The total harvest in the trial field was 36 t ha^{-1}. At a sale price of 0.31 € per kilogram of potatoes this equals an economic loss of more than 6 000 € ha^{-1}.

Discussion

Efficacy of *Melolontha* control and crop protection

The observed *M. melolontha* mortality of up to 64 % after a single application of Melocont®-Pilzgerste is remarkably high for a biological product intended for long-term protection. Studies on the efficacy of *B. brongniartii* in grassland reported lower *M. melolontha* mortalities in the first year. Strasser (1999) considered a field prevalence of mycosis of >20 % sufficient for the suppression of cockchafer larvae in grassland. Kessler *et al.* (2004) reported for *Melolontha* in Swiss pastures mortality rates similar to those rates as

found in the present study. Nevertheless, a direct comparison of results is not admissible due to the differences in the applied methods. The mortality rate reported by Kessler *et al.* (2004) combines the observed field mortality with a projected mortality of larvae following major disturbance and incubation under different conditions in the laboratory. In contrast, the mortality rates determined in this study refer to pure field data and does not diagnose infected *M. melolontha* larvae. But the simple counting of mummified cadavers and alive larvae in the field allows the calculation of the cumulative mortality rate over the period of several months. While this method may result in lower efficacy rates than those determined with the method used by Kessler *et al.* (2004), we consider the pure field mortality rate to be of higher reliability for an *in situ* assessment of efficacy: infection of larvae which might occur in the laboratory are excluded. Despite the high *M. melolontha* mortalities observed in the treated plots, the short-term goal of preventing feeding damage on potatoes could not be reached with a single application of high doses of Melocont®-Pilzgerste. This makes the need for long term IPM strategies for the control of *M. melolontha* in highly sensitive crops evident.

Previous studies have shown that under ideal conditions mechanical soil treatment such as drilling or harrowing can have an efficacy of up to 90 % (Pötsch *et al.* 1997). These mechanical control measures are only efficient if the larvae are present in the top soil at the time of treatment and are most effective against white grubs in the first larval stage (Strasser 2001). A further study conducted in spring 2003 in the area adjacent to the former fields confirmed that the planting of potatoes at the time when most of the larvae are present in the top soil resulted in a four-fold higher yield of healthy potatoes (data not shown). Consequently, it can be concluded that mechanical treatment together with the application of Melocont®-Pilzgerste at the right time allows a successful cultivation of sensitive high-valuable crops even in infested areas. Our statistical approach has shown that solid dose/mortality curves can be obtained and effectively used by directly processing field data. To our knowledge, this is the first attempt to establish LC_{50}/LD_{50}-values for a fungal BCA by linking field efficacy of the product to application rates and BCA densities in soil. The results from these models confirm the validity of previously published recommendations with regard to both the amount of BCA to be applied (Inglis *et al.* 2001) and the concentration of *Beauveria brongniartii* required to ensure epizootics in *M. melolontha* populations (Ferron 1979). The LD_{50} value of 56 kg ha^{-1} Melocont®-Pilzgerste corresponds well to the recommended rate of one application of 50 kg ha^{-1} or two applications of 30 kg ha^{-1} per year (Strasser & Pernfuß 2005). Ferron (1979)

considered a concentration of 2×10^4 *Beauveria brongniartii* spores per gram dry weight soil as threshold value to ensure epizootics in *M. melolontha* populations. This would correspond to a mortality rate of approximately 15 % in our model. Assuming that the principle of "inoculation biological control" (Eilenberg *et al.* 2001) is appropriate, this rate should be sufficient to induce epizootics, leading to a multiplication of *Beauveria* in soil and, thus, to an increased pest mortality.

A comparison of data from different studies demonstrated that the *Beauveria* levels are more than 10 times higher where the *Melolontha* abundance is high (Laengle, 2005). In field studies focused on non-target effects and *B. brongniartii* persistence, *B. brongniartii* density was found to decrease by 50 % within 400 to 450 days of application in the absence of *M. melolontha* (Laengle, 2005). This confirms the finding of Kessler *et al.* (2004) that the reproduction of *B. brongniartii* in the soil mainly depends on the presence of *M. melolontha* and is therefore a true "inoculation biological control" agent (Eilenberg *et al.* 2001).

Due to these biological constraints the short term protection of potatoes is not possible with *B. brongniartii* based on Melocont®-Pilzgerste. The damage threshold for *M. melolontha* infestation in potato fields is considered to be approximately 3 larvae per square meter. In grassland this threshold is as high as 30 larvae per square meter (Pötsch *et al.* 1997, Strasser 2001). In a heavily infested area with more than 50 larvae per square meter, even an annual efficacy of 50 % will not reduce the pest population below the damage threshold for potato before 4-5 years. In grassland, however, this result could be achieved within one season. Summarizing, Melocont®-Pilzgerste is an effective agent for a sustainable control of *M. melolontha* if used as part of an intelligent IPM strategy. Previous reports about the efficacy of the product (Keller 2000, Strasser 1999, Zelger 1996) have been confirmed by this study.

Socio-economic implications and consequences for the European registration process

A precondition for the effective use of long term biological control is that growers believe in the product. It is important to educate farmers with regard to the handling and application, and perhaps even more importantly, to provide knowledge about the potential and limitations of biological control agents. For instance, the efficacy of biocontrol agents is frequently compared to that of chemical pesticides: an efficacy of 60–70 %, as found in this study, would be considered insufficient. Due to these issues a close dialogue between advisors and growers should ensure that control strategies are per-

formed effectively. In this context, an easy to use advisory guidlines would be helpful for users. An example has been set up for *Agriotes* sp. (Elateridae) in Canadian potato farming (Vernon *et al.* 2004), and could serve as a basis for *Melolontha* control. The model must include simple methods for the detection of the *M. melolontha* infestation rate in a specific field to enable the calculation of the infestation threshold.

The efficacy data required for the European registration of a plant protection product must make it possible to "evaluate the nature and extent of benefits that accrue following use of the preparation, where they exist in comparison to suitable reference" (Council Directive 91/414/EEC, Council of European Union 1991), Annex III A section 6 established by Commission Directive 93/71/EEC (European Commission 1993). "If no suitable reference product exists, the plant protection product must be shown to give a defined benefit in terms of the level, consistency and duration of control or protection or other intended effects under the agricultural, plant health and environmental (including climatic) conditions in the area of proposed use" (COM 814, Bull EU 12/03, proposal for establishing Annex VI B of Dir. 91/414/EEC, European Commission 2003). Both of these conditions have clearly been met. The efficacy of *B. brongniartii* for the control of *Melolontha* sp. has been thoroughly documented in this and in previous studies (Keller 2000, Keller *et al.* 1997, Strasser 1999, Zelger 1996). *B. brongniartii* is the only available agent that can provide long-term protection against *Melolontha* species. It provides farmers with a real alternative after chemical pesticides, such as methyl bromide and chlorpyriphos, have been phased out in the European Union due to unacceptable side effects (Butt *et al.* 2001b, Strasser *et al.* 2000b).

One obstacle for the registration, however, may be that according to Directive 93/71/EC (European Commission 1993) efficacy studies are required to be conducted under the regulations of EPPO Standard PP 1/152. The EPPO document requires that all efficacy studies are conducted as a randomized block design, which has proven not to be feasible for efficacy studies with white grubs in soil (Strasser *et al.* 2005).

Experience gathered during the planning and conduction of this study has shown the need for standardised guidelines specific to the efficacy evaluation of fungal BCAs both in the laboratory and in the field to improve quality of data. Such guidelines were developed as part of the RAFBCA project (EC project no. QLK1-CT-2001-01391). Easily applicable protocols for the quality control and efficacy evaluation of fungal BCAs, as well as the monitoring of the fungal inoculum density in the soil as required for the efficacy evaluation are published in this issue of Sydowia (see appendices A, B and C).

The presented standards will help to promote the registration of reliable fungal BCAs in the European Union and will assist in future research to design sustainable and efficient IPM strategies.

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