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Selection of indigenous isolates of entomopathogenic soil fungus *Metarhizium anisopliae* under laboratory conditions

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Abstract Eight native isolates of the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin were obtained by monitoring soils cultivated in a conventional manner. These isolates were compared in three areas: (a) conidial germination, (b) radial growth and sporulation and (c) ability of conidia to infect *Tenebrio molitor* larvae. All bioassays were carried out at constant temperatures of 10, 15, and 20 °C. Conidia of individual isolates demonstrated differences in germination after a 24-h long incubation at all evaluated temperatures. At 20 °C, the germination ranged from 67 to 100 % and at 15 °C from 5.33 to 46.67 %. At 10 °C, no germination was observed after 24 h; nevertheless, it was 8.67–44.67 % after 48 h. In terms of radial growth, the culture diameters and the associated production of spores of all isolates increased with increasing temperature. At 10 °C, sporulation was observed in three isolates while all remaining cultures appeared sterile. Three weeks post-inoculation, conidia of all assessed isolates caused 100 % cumulative mortality of treated larvae of *T. molitor* at 15 and 20 °C with the exception of isolate 110108 that induced 81.33 % mortality at 15 °C. At 10 °C, larval cumulative mortality ranged from 6.67 to 85.33 % depending on the isolate. Isolates 110108 and 110111 showed significantly slower outset and a much lower rate of infection at all temperatures compared to other tested isolates of *M. anisopliae*. The bioassays were carried out with the purpose to sort and select indigenous isolates of *M. anisopliae* useful as biocontrol agents in their original habitat.

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Introduction

The commonly used biocontrol agent, *Metarhizium anisopliae*, was found to be a species complex composed of nine species having convergent morphologies (Kepler and Rehner 2013) and in this sense, is understood in the presented papers. The fungus has worldwide distribution on insects as well as in the soil (Zimmermann 2007), but differences exist in the habitat from which the isolates are obtained. In temperate climate, *Metarhizium* isolates occur in soil samples from meadows (Rodrigues et al. 2005), permanent grasslands, improved field margins (Schneider et al. 2012) and agricultural soils (Meyling et al. 2011). Because of a more frequent presence in regularly disturbed soils (Quesada-Moraga et al. 2007) Meyling and Eilenberg (2007) used for *M. anisopliae* the term "agricultural" species. The presence of *M. anisopliae* in the soil is possibly most affected by the occurrence of particular insect species and the composition of the vegetation (Schneider et al. 2012).

Fungus *M. anisopliae* predominantly cycles below ground (Meyling et al. 2011) and may have evolved subtle ecological adaptations to insect parasitism in the soil (Vega et al. 2009). The fungi deposit their infectious spores there and remain in the soil for some duration of their life cycle (Medo and Cagáň 2011), and under suitable circumstances, they can develop dramatic epizootics that lead to rapid declines in host populations (Pell et al. 2010). *M. anisopliae* has great potential for use against various pests including soil-dwelling stages of thrips (Ansari et al. 2008), black vine weevil (Shah et al. 2007) or wireworms (Kabaluk et al. 2005). To increase the population of *M. anisopliae* in the soil, it was introduced directly to the roots (Bruck 2005) as a drench or addition in the growing media

(Ansari et al. 2008; Shah et al. 2007), seed treatment (Kabaluk and Ericsson 2007) or through conservation biological control (Pell et al. 2010; Shah and Pell 2003). *M. anisopliae* could colonise the roots (Hu and St. Leger 2002) and could provide growers nearly a year of residual control in covered production areas (Bruck 2005). The efficiency of the mycoinsecticide in the soil can be affected by soil texture, moisture, temperature, and a number of biotic factors (Jaronski 2010) as well as by soil components, pH and organic matter (Quesada-Moraga et al. 2007) and also by exposure to pesticides. Specifically, *M. anisopliae* is considered to be tolerant to pesticides (Klingen and Haukeland 2006).

The ultimate goal of the selection process is to find a fungal entomopathogen providing consistent insect control under field conditions (Jackson et al. 2010). However, little emphasis has been placed on understanding the ecology of individual isolates (Bruck 2010). The origin of isolates can play a role in many aspects and the most significant is thermotolerance (Rangel et al. 2005), when various strains react differently to a diverse temperature (Ment et al. 2011; Zimmermann 2007) and the associated sporulation and virulence to the target organisms (Bugeme et al. 2009; Tefera and Pringle 2003) as well as conidial germination and mycelial growth (Dimbi et al. 2004). Differences in the efficacy of various isolates to species of target organisms have been demonstrated (Ansari et al. 2008; Ansari et al. 2009).

In the presented papers, we focused on the testing and selection of indigenous isolates of *M. anisopliae* useful in biological control applied in their original habitat. We used three temperatures and three basic bioassays to demonstrate the differences among isolates in germination, radial growth, sporulation and efficacy against target organism *Tenebrio molitor*. Because it was proved that various isolates show diverse results, using of proper strains leads to a more effective control of pests and helps to avoid the failure of the treatment caused by application of unsuitable isolates.

Materials and methods

Isolates, cultivation of cultures and preparation of spores suspension

Evaluated isolates were obtained by monitoring the presence of entomopathogenic fungi in arable soils in 2010–2011 at selected locations in the South Bohemian region (Table 1). The compared isolates originate from farmland cultivated by conventional agriculture methods and were isolated using modified selective medium potato dextrose agar (PDA)-dodine (Chase et al. 1986). All isolates were identified microscopically based on morphological characteristics using taxonomic keys (Humber 1997). Eight isolates were intentionally selected according to a diverse morphotype of 21-d old central cultures formed on PDA (24 g/L PDB, HiMedia Laboratories Pvt. Ltd., Mumbai, India, and 16 g/L agar, Dr. Kulich Pharma s. r. o., Hradec Králové, Czech republic) at 20 °C and compared to isolate F52 (*Metarhizium brunneum* formerly identified as *M. anisopliae*) re-isolated from the commercial product BIO 1020® (Bayer AG, Germany).

Individual isolates were cultivated as separation lines (two separated lines on one plate) on PDA in a 90-mm Petri dish at 20 °C without access to light for 2 weeks. A conidial suspension was then prepared by washing the conidia from the surface of fully sporulated cultures using 0.05 % Tween 80 (HiMedia Laboratories Pvt. Ltd., Mumbai, India) and adjusted to final concentration of 10^6 spores per mL using a Neubauer-improved chamber.

Germination assessment

The vitality of conidia was determined using a standard test of germination. Microscope slides were first covered with a thin layer of PDA and, after drying, five drops of each isolate's suspension were placed with 1-μL inoculation loop on the PDA surface. After drying, the slides were placed in a Petri dish (90 mm) containing a filter paper wetted with 0.5-mL sterile distilled water. Incubation took place at 10, 15 and 20 °C with three replicates for each variant. Conidial vitality was assessed after 24 and 48 h. Germination was considered to have occurred if the germ tube was at least as long as the half length of the mother conidium. Percentage germination was determined by viewing 100 conidia per replicate under ×400 light microscope on one slide.

Radial growth and sporulation assessment

Using a 1-μL inoculation loop, one drop of adjusted conidia suspension (10^6 spores per mL) was placed into the centre of a Petri dish (90 mm) containing artificial medium (PDA). After the drops dried, the Petri dishes were enclosed in polyethylene bags to prevent water loss and cultivated in total darkness at temperatures of 10, 15 and 20 °C for 3 weeks. Four replications for each isolate at each temperature were evaluated. After 3 weeks, the individual cultures were measured in two perpendicular directions at right angles to each other to establish the diameter of the culture. Subsequently, each culture was separately homogenised vigorously in distilled water with 0.05 % Tween 80 in liquid volume adequate to allow us to harvest the conidia and determine the rate of sporulation. The concentration of conidia per plate was estimated in a counting chamber.

Efficacy on *Tenebrio molitor* larvae

The efficacy of an individual isolates was tested on the larvae of *T. molitor*. The bioassay arenas used were multi-well plates

Table 1 Geographical location of soil sampling sites including acreage and crops cultivated in sampling seasons

Locality (no.)	Code of isolate	Acreage (ha)	GPS coordinates	Altitude (m.a.s.l.)	Crop	
					2010	2011
2	110102	10.36	N48 47.967 E14 50.117	481	Rye	Wheat
5	110105	12.20	N49 03.717 E15 06.053	613	Clover	Clover
6	110106	10.75	N49 04.340 E15 01.936	530	Wheat	Poppy
8	110108	104.97	N48 56.106 E15 30.880	486	Clover	Clover
10	110110	17.72	N49 02.842 E13 57.107	565	Oat	Wheat
11	110111	10.03	N49 05.524 E14 02.312	474	Oat	Wheat
12	110112	13.30	N49 03.127 E14 53.249	445	Wheat	Triticale
13	110113	7.01	N49 05.514 E14 50.197	422	Wheat	Barley

Basic characteristics of conventional farm sites where different isolates of entomopathogenic fungi *M. anisopliae* selected for this study were isolated

with 6.5-mL wells. At the bottom of each well was a piece of sterile filter paper (2×2 cm) wetted with 0.150 mL of sterile distilled water. Larvae of the same size (approximately 2 cm) were surface sterilised in 0.05 % NaClO and rinsed three times with pure sterile distilled water and placed on filter paper to dry. Larvae were then immersed for 2–3 s into the suspension of conidia (10^6 spores per mL) and placed on filter paper to remove excess slurry (Goettel and Inglis 1997). The treated larvae were placed individually into the prepared wells. For each variant (isolate), 25 larvae were used in three repetitions (total of 75 larvae per isolate). In the control variant, the larvae were immersed for 2–3 s into a solution of 0.05 % Tween 80 and put on a filter paper to remove excess slurry—25 larvae prepared in triplicate. All prepared plates were enclosed into plastic bags (to prevent drying). Each variant was incubated in the dark at 10, 15, and 20 °C. Larval condition was evaluated under binocular microscope daily for 3 weeks to ascertain living, dead and infected individuals. "Cumulative mortality" includes mortality and infection of individuals.

Statistical analysis

Percentage data were normalised using arcsine transformation, while sporulation of the centre culture was based on data transformed to $\log_{10}(x+1)$. Radial growth data were not transformed. Data were subjected to analysis of variance (ANOVA) using software for statistical analysis. Differences among means were compared using Tukey's test ($P<0.05$).

Results

Effect of temperature on germination

Germination was assessed after 24 and 48 h (Table 2). Twenty-four-hour post-inoculation, no germinating spores were observed at 10 °C. At 15 and 20 °C, germination was 5.33–46.67 % and 67–100 %, respectively. The lowest values in both evaluations were observed in isolate 110108, while the highest values in isolate 110112 at 15 °C and isolate F52 together with isolate 110110 at 20 °C. Significant interactions occurred between temperature and isolate ($P<0.05$). After 48 h, germinating spores were observed even at 10 °C in the range from 8.67 to 44.67 %. Much higher germination was found at 15 °C, where the range of values increased to 65.67–98.67 % and at 20 °C, where observed germination was greater than 95 %. The lowest germination was detected in isolate 110108 across all temperatures. Significant differences ($P<0.05$) between isolates were found.

Effect of temperature on radial growth and sporulation

The size of the cultures, as well as spore production, rose as the temperature did (Table 3). At 10 °C, the size of the cultures ranged between 3.25 and 11.63 mm. The smallest cultures were formed by isolates 110108 and 110111. Sporulation was poor at this temperature as just three of the isolates noticeably produced spores—110102, 110112 and F52. The others did not sporulate or the sporulation could not be established because of the resolving power of the counting chamber. Significant differences ($P<0.05$) were found among culture sizes and spore production.

Radial growth at 15 °C was faster. At day 21, the size ranged from 14.75 to 31.13 mm and the smallest culture formed was by isolate 110111. The number of spores produced ranged between 4.23×10^7 and 4.43×10^8 spores per culture. The largest cultures were formed at 20 °C where the diameter reached from 32.38 to 50 mm and the spore production increased to 1.20×10^8–1.88×10^9 spores per culture. The largest cultures were formed by isolate F52 at temperatures of 10 and 15 °C. The worst growth parameters were obtained for isolates 110108 and 110111. At 15 as well as 20 °C,

Table 2 Germination of individual isolates after 24 and 48 h period at given temperatures (mean % ± SD)

Code of isolate	24 h			48 h		
	10 °C	15 °C	20 °C	10 °C	15 °C	20 °C
F52	0±0.00	42.33±4.50 a	100±0.00 a	39.67±1.89 a	94.33±1.70 ab	100±0.00 a
110102	0±0.00	36.67±2.87 a	99.33±0.94 a	41.33±2.05 a	95.67±1.25 ab	100±0.00 a
110105	0±0.00	16.33±3.40 bc	98.33±1.70 a	38.33±3.68 a	97.00±1.63 ab	100±0.00 a
110106	0±0.00	19.67±3.09 b	98.00±2.16 a	37.67±2.49 a	90.67±1.70 b	100±0.00 a
110108	0±0.00	5.33±2.62 d	67.00±5.10 c	8.67±2.05 b	65.67±1.25 c	95.33±2.05 b
110110	0±0.00	43.33±2.05 a	100±0.00 a	42.33±3.09 a	98.67±0.94 a	100±0.00 a
110111	0±0.00	10.00±1.63 cd	81.67±4.64 bc	12.33±1.24 b	73.67±4.78 c	95.67±1.25 b
110112	0±0.00	46.67±2.87 a	95.33±3.86 ab	44.67±2.05 a	93.67±1.25 ab	100±0.00 a
110113	0±0.00	44.00±3.27 a	99.00±1.41 a	43.00±2.45 a	98.33±1.25 a	100±0.00 a

Means in a single column that are followed by the same letter are not significantly different, $P<0.05$ (ANOVA, Tukey test)

differences among isolates were demonstrable ($P<0.05$) for both assessed indicators.

Effect of temperature on efficacy on *T. molitor* larvae

Markedly visible differences in the cumulative mortality of *T. molitor* larvae treated with different isolates among the assessed temperatures were observed (Fig. 1). Irrespective of the isolate, at 10 °C cumulative mortality appeared first on day 17 of the bioassay in the range from 1.33 to 5.33 % except for isolates 110108, 110111, 110113 and untreated control that showed no changes in larval vitality. There were no significant differences in cumulative mortality levels ($P>0.05$) after 17 d, whereas at the end of the bioassay, day 21, results observed were significantly different ($P<0.05$). The cumulative mortality of the treated larvae ranged from 6.67 to 96 %. The highest cumulative mortality observed was by isolate F52, and the lowest by isolates 110108 and 110111.

A faster onset of cumulative mortality was observed at 15 °C, namely on larvae treated with isolate 110105 (1.33 %) and 110110 (2.67 %) after 6 d of bioassay, but no significant differences were detected ($P>0.05$). As early as day 11, isolates 110102 and 110113 caused 100 and 98.67 % cumulative mortality of *T. molitor* larvae, respectively, while the cumulative mortality observed for other isolates was very inconsistent (0–92 %). By the end of the bioassay, significant differences were observed ($P<0.05$); all isolates showed 100 % cumulative mortality with the only exception being isolate 110108 which caused 81.33 %. Vitality of the larvae was not affected by temperature during bioassay since mortality of untreated larvae (control) was 0–4 %.

The most rapid increase in cumulative mortality was observed at 20 °C where, after 4 d, cumulative mortality caused by both of the isolates 110102 and 110110 reached 2.67 %. By day 7, the worst cumulative mortality of 21.33–28.00 % showed isolates 110106, 110108 and 110111. The other isolates demonstrated 97.33–100 % cumulative mortality

Table 3 Radial growth and sporulation of individual isolates after 3-week cultivation on PDA (mean % ± SD)

Code of isolate	10 °C		15 °C		20 °C	
	Number of spores per culture	Colony size (mm)	Number of spores per culture	Colony size (mm)	Number of spores per culture	Colony size (mm)
F 52	$5.23\pm0.33\times10^{6}$ a	11.63±0.48 a	$2.66\pm0.11\times10^{8}$ c	31.13±0.78 a	$1.24\pm0.09\times10^{9}$ b	49.63±1.49 a
110102	$2.16\pm0.23\times10^{5}$ b	10.88±0.33 b	$2.63\pm0.10\times10^{8}$ c	24.88±0.78 b	$1.16\pm0.10\times10^{9}$ b	44.25±1.79 d
110105	0±0.00 d	9.50±0.50 c	$3.36\pm0.14\times10^{8}$ b	22.50±0.50 c	$3.89\pm0.45\times10^{8}$ d	46.25±0.66 bc
110106	0±0.00 d	9.13±0.33 cd	$3.53\pm0.16\times10^{8}$ b	25.00±1.00 b	$1.58\pm0.02\times10^{9}$ a	50.00±0.50 a
110108	0±0.00 d	3.25±0.43 e	$6.63\pm0.27\times10^{7}$ d	15.88±0.60 e	$3.01\pm0.21\times10^{8}$ e	37.00±1.12 e
110110	0±0.00 d	11.00±0.00 ab	$4.23\pm0.10\times10^{7}$ f	22.63±0.48 c	$1.28\pm0.09\times10^{8}$ f	44.75±1.30 cd
110111	0±0.00 d	3.25±0.43 e	$4.28\pm0.16\times10^{7}$ f	14.75±0.66 e	$1.20\pm0.06\times10^{8}$ f	32.38±0.48 f
110112	$6.00\pm1.54\times10^{4}$ c	8.75±0.43 d	$4.43\pm0.18\times10^{8}$ a	30.38±0.48 a	$1.88\pm0.09\times10^{9}$ a	47.00±0.50 bc
110113	0±0.00 d	9.75±0.43 c	$5.47\pm0.18\times10^{7}$ e	19.13±0.78 d	$6.60\pm0.58\times10^{8}$ c	37.88±1.62 e

Means in a single column that are followed by the same letter are not significantly different, $P<0.05$ (ANOVA, Tukey test)

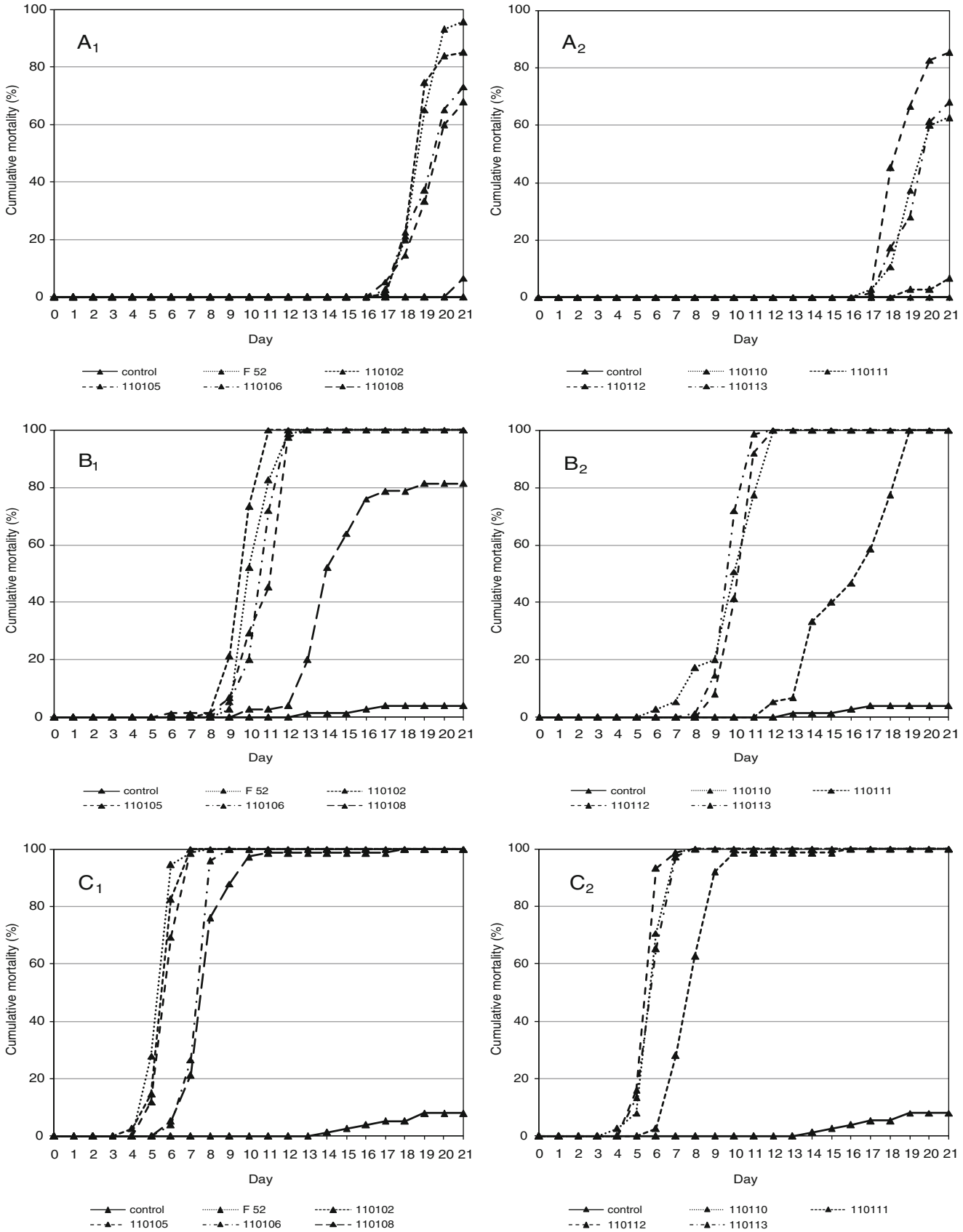
together with 0 % mortality in the untreated control ($P<0.05$). All isolates brought about 100 % cumulative mortality compared with 8 % mortality in untreated control at the end of the bioassay. In comparison with other isolates, 110108 and 110111 demonstrated a slower rise in cumulative mortality during the bioassay.

Discussion

Isolates of *M. anisopliae* were obtained by monitoring soils managed in conventional agriculture. For the bioassay, eight of them were chosen that revealed different culture morphotypes. It was assumed that we would observe some kind of variability among these isolates as reported by De Croos and Bidochka (1999) and Talaei-Hassanloui et al. (2006), who described the differences in the morphology and the qualitative characteristics among the isolates of *M. anisopliae* and *Beauveria bassiana*. The differences in morphology are most probably connected with the *M. anisopliae* complex consisting of nine taxa (Bischoff et al. 2009; Kepler and Rehner 2013). Our isolates can correspond to one or several of the genetic clusters or species in the *M. anisopliae* complex, and some of the ecological and biological parameters that we evaluated could be specifically associated with individual taxa or species. Nevertheless, we demonstrated that although the selected isolates had significant morphological diversity, only two of them demonstrated essential differences in the examined characteristics.

It has been confirmed many times that *M. anisopliae* grows in a wide temperature range, between 5 and 40 °C (Hallsworth and Magan 1999) and the optimal temperature for growth is generally considered between 20 and 25 °C (Dimbi et al. 2004; Zimmermann 2007). However, we chose three temperatures based on the climate in the original environment of the isolates for practical purposes in our evaluation. The average yearly temperature in the South Bohemia region, where the isolates originate from, has been around 8 °C over the last 5 years (CHMI 2012). The presented results indicate that isolates 110108 and 110111 had low germination (less than 12.5 %) at 10 °C (after 48 h) and never reached higher values than the other isolates in subsequent evaluations. It could therefore be generalised that the primary selection based on the germination of the spores assessed at 10 °C for the acknowledged environmental conditions could be reliably applied. The reason for this is that it is necessary to respect the origin of the isolate because as Fernandes et al. (2008) ascertained, there are isolates of *M. anisopliae* where no germination was observed after 15 d at 5 °C and those that never reached 20 % germination. De Croos and Bidochka (1999) found demonstrable differences in cold activity among isolates when only some of the isolates grew at 8 °C, one which did not grow at 15 °C at all, all of them grew at 22 °C and the production of conidia depended on the temperature as well. Tefera and Pringle (2003) found that the germination of *M. anisopliae* conidia was highly inhibited at 15 °C. For our purposes, the higher temperatures of 15 and 20 °C were useful for more specific selection of the proper isolate potentially usable for biological control in this area. The germination achieved at 15 and 20 °C was high after 48 h (with the exception of the two above mentioned isolates 110108 and 110111 at 15 °C) and, because of the evenness of the values, this parameter could not be used for more detailed selection (at least in this case).

Nevertheless, the vitality of the spores was closely connected with subsequently evaluated criteria of radial growth and sporulation. *M. anisopliae* might have evolved due to both rapid vegetative growth and quick sporulation and also, fungal isolate has a greater effect on it than fungal species (Sun et al. 2002). As mentioned above, all of the isolates in our bioassay grew at 10 °C, but just some of them produced spores. On the contrary, the sporulation at 15 and 20 °C reached values 4.23×10^{7}–1.88×10^{9} spores per culture, respectively. Growth, as well as sporulation, is a very important step in the planning of commercial production because isolates unable to grow and sporulate are of little commercial value. With the importance of these steps in mind, isolates 110102 and 110112 were chosen as the most suitable. However, before a pathogenicity bioassay is carried out, a final selection cannot be made because, as suggested by Rath et al. (1995a), not all of the isolates of *M. anisopliae* exhibit pathogenicity or high virulence to a specific host. The pathogenicity of the spores can be considered as the key characteristic that indicates the potential for practical use of the isolate. Comparably to previous assays, there were differences in efficacy among isolates. Ansari et al. (2009) found significant differences in the efficacy between the isolates of *M. anisopliae* against *Agriotes lineatus* larvae in controlled conditions (22±1 °C; 60–70 % RH) 3 weeks after treatment with conidial suspension (10^{8} spores per mL). Only two of the ten isolates of *M. anisopliae* in their study caused 90 and 100 % cumulative mortality of the larvae of *A. lineatus* and the virulence of the other isolates ranged between 10 and 70 %. In our study, the cumulative mortality of *T. molitor* larvae after 3 weeks at 20 °C was 100 % regardless of the isolate and 100 % cumulative mortality was reached at 15 °C as well (except for isolate 110108) even when the concentration of suspension per ml was 100 times lower than what Ansari et al. (2009) used. The greatest differences in efficacy were observed at 10 °C where the satisfactory results (more than 80 % cumulative mortality) were found only in three isolates, including the above mentioned isolates 110102 and 110112. Although the tested isolates caused high cumulative mortality even at low temperatures, the results reported by Rath et al. (1995b) that in field conditions, the efficacy on host organisms could be slower, have to be taken into


A1
A2
B1
B2
C1
C2
Cumulative mortality (%)
Day
control
F 52
110102
110105
110106
110108
110110
110111
110112
110113

◀ **Fig. 1** Mean percentage cumulative mortality of *T. molitor* larvae at different temperatures, during the 3-week bioassay. Data show the mean of three replicates with 25 larvae per replicate for each isolate. The course of cumulative mortality is expressed using two graphs for each of the tested temperatures—graphs A_1, A_2 at 10 °C; graphs B_1, B_2 at 15 °C; graphs C_1, C_2 at 20 °C

consideration. In vitro sporulation could be by as much as 232 times higher than in vivo (Sun et al. 2002). Moreover, the presence of *M. anisopliae* in the soil is influenced with the soil components, pH, and organic matter (Quesada-Moraga et al. 2007) which in high content meant lower occurrences of *M. anisopliae* (Medo and Cagáň 2011). Bruck (2005) notes that the *M. anisopliae* population responded favourably to the rhizosphere microclimate and Hu and St. Leger (2002) found that factors in the rhizosphere could promote the biological activity and persistence of *M. anisopliae*.

We can conclude that the results gained at temperatures close to the average ambient regional temperature of strain origin could be the essential indicators for primary selection. Our results suggest that the pathogenicity is connected to the vitality of the spores, but good germination does not necessarily mean good efficacy. It is important to combine all the presented assays to find the most suitable isolates and even according to economic efficiency which is indicated by high sporulation and radial growth. Based on all these evaluations, the isolates 110102 and 110112 were chosen as the most appropriate for the potential use in biological control and will be further assessed for this purpose.

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