Germination, Radial Growth and Virulence to Boll Weevil of Entomopathogenic Fungi at Different Temperatures

Ana Laura Nussenbaum,
Maricel Angulo Lewylle and Roberto Eduardo Lecuona

Laboratorio de Hongos Entomopatógenos, IMYZA, INTA,
CC 25, 1712 Castelar, Buenos Aires, Argentina

Submitted: Sep 12, 2013; Accepted: Oct 19, 2013; Published: Oct 30, 2013

Abstract: The effect of temperature on germination, radial growth and virulence of four B. bassiana and M. anisopliae isolates virulent to the boll weevil, Anthonomus grandis, were determined at different temperatures. The optimum temperature for germination and radial growth was 27°C for both fungi species. M. anisopliae isolates managed to germinate at higher temperatures, while B. bassiana isolates failed to germinate at 35°C. With regard to radial growth rate, the B. bassiana isolates grew faster at lower temperatures, whereas the M. anisopliae ones grew faster at higher temperatures. At 35°C only M. anisopliae isolates managed to grow. None of the isolates of both species grew at 38°C. The virulence against boll weevil decreased at 35°C for B. bassiana. For M. anisopliae isolates the median survival time increased at suboptimum temperatures, but the final mortality percentage remained the same. In conclusion, it is important to evaluate the tolerance at higher temperatures when choosing an isolate for development as a microbial control agent. Based on the temperature tolerance, M. anisopliae may be a possible candidate for a boll weevil control and therefore it could be included in an Integrated Pest Management program on cotton crops.

Key words: Beauveria bassiana · Metarhizium anisopliae · Thermotolerance · Microbial control · Anthonomus grandis

INTRODUCTION

Cotton is the most important non-edible crop in Argentina. In fact, the agro-industrial complex based on the cotton stem has historically represented one of the principal sources of income and employment in the northeast and northwest of the country [1]. The largest cotton growing area is located primarily in Chaco, Santiago del Estero, Santa Fe, Formosa and Corrientes provinces, where the climate is semitropical. For instance, the medium temperature in Chaco has been found to be 28°C, with a maximum of up to 44.8°C in summer [2].

The main pest affecting cotton in Argentina is the boll weevil, Anthonomus grandis Boheman (Coleoptera: Curculionidae). The most serious damage to cotton crops is caused by adults that feed and lay their eggs in the flower buds and bolls, resulting in the falling of the squares, fruit abscission and reduced linter production and quality [3].

Entomopathogenic fungi, in particular Beauveria bassiana (Balsamo) Vuillemin and Metarhizium anisopliae (Metschnikoff) Sorokin (Hypocreales), are being developed as alternatives to chemical pesticides for the control of boll weevil [3]. Instead of chemical pesticides, entomopathogenic fungi are strongly influenced by the abiotic (e.g. temperature, UV irradiation and humidity) and biotic (e.g. ecology, physiology and life cycles) factors [4]. These factors affect the pathogen’s survival and ability to infect, the host’s susceptibility and resistance and the progress of the infection within the host [5]. For successful development as microbial control agents, entomopathogenic fungi have to be adapted to...
the environmental conditions. In particular, temperature is an important environmental factor affecting the efficacy of these fungi and the temperature of the area of application needs to be considered [6, 7].

In vitro, the velocity of mycelia development and, therefore, the rapidity of the evolution of infection, depend on temperature [8]. In general, optimum values fall between 20 and 30°C (e.g. 25°C for B. bassiana and 27-28°C for M. anisopliae). However, some fungal strains have a different behavior than that expected from the species [9]. Three broad groups of M. anisopliae strains were distinguished using temperature: those able to germinate at 5°C (cold-active), those able to germinate at 37°C (heat-active) and those unable to germinate at either of these temperatures (meso-thermoactive) [10].

For a successful development of a mycoinsecticide against adults of A. grandis, the temperature ranges found in the agricultural ecosystem must be considered, in particular the extreme environmental temperature in the north of Argentina, where the pest develops [11]. Therefore, knowledge of fungal growth according to temperature must be considered for isolates selection.

In a previous study, we screened 28 native isolates of M. anisopliae and 66 of B. bassiana against adults of A. grandis and selected the four most virulent isolates [12]. However, no assays about the effect of temperature on these isolates have been performed. The aims of the present study were: to determine the effect of temperature on In vitro radial growth and conidia germination of the four most virulent isolates of B. bassiana and M. anisopliae; and, to compare the virulence of these entomopathogenic fungi against adults of boll weevil at different temperatures of incubation.

MATERIALS AND METHODS

Insect, Fungal Cultures and Conidial Suspensions:
The boll weevils were collected from rural areas of Santa Fe province, Argentina (28° 5’ 06.11” S; 59° 14’ 37.53” W; Florencia). Insect colonies were reared in the laboratory at 27 ± 1°C, 50 ± 10% humidity, 12:12 photoperiod and fed with an artificial diet [13]. The In vitro assays were carried out using 24-h old red adults that were fed on a diet without fungicides. Once the assays were performed, the insects were maintained at a controlled room environment with 50 ± 10% humidity and a 12:12 photoperiod. The artificial diet was replaced daily.

Two isolates of B. bassiana (Bb 23 and Bb 301) and two of M. anisopliae (Ma 20 and Ma 50) were obtained from the Entomopathogenic Fungi Laboratory culture collection (IMYZA, INTA, Castelar, Argentina). This study was carried out using the selected fungal isolates because of their high virulence against the boll weevil. These isolates were obtained from different sources (insects and soil) and all of them were isolated in Argentina [12].

The isolates of B. bassiana were cultured on Complete Medium Agar (CMA), which is composed of (g/l): KH₂PO₄, 0.4; Na₂HPO₄, 1.4; SO₄Mg, 0.6; KCl, 1; NH₄NO₃, 0.7; glucose, 10; agar, 15; and yeast extract, 5; at 27°C. The isolates of M. anisopliae were cultured on Potato Dextrose Agar (PDA, Oxoid) at the same temperature.

Conidial suspensions were obtained from each fungal isolate. For B. bassiana, cultures developing on CMA medium were used for this purpose. Conidia were collected from one-week old fungal cultures and suspended in Tween 80 (0.05%). For M. anisopliae, the conidial suspensions were prepared in Tween 80 (0.05%) from one-week old cultures in sterile polypropylene bags with 90 g of parboiled rice and 45 ml of distilled water [14]. Concentrations of all suspensions were quantified with an improved Neubauer chamber. Viability of the conidia was assessed by a germination test prior to the experiment and found to be > 95% [15].

Experimental Performance: To test the conidial germination of B. bassiana and M. anisopliae isolates at different temperatures of incubation, conidial suspensions were adjusted at 2 x 10⁷ conidia / ml using improved Neubauer chamber. Aliquots of 100 µl were spread over the surface of Petri plates containing CMA or PDA plus 0.002% (w/v) benomyl for B. bassiana or M. anisopliae isolates, respectively. The benomyl was a formulated powder with 25% active ingredient (Punch Química SA, Argentina). The low concentration of benomyl in the medium allows germination to be monitored for longer periods of time because it inhibits the growth of germ tubes without adversely affecting germination [16]. The plates were incubated at 23, 27, 30 and 35°C for 24 h. Four replicates (or plates) were done for each temperature and fungal isolation. After incubation, one drop of lactophenol blue (Sigma, USA) and a coverslip were placed on the plates and germination was observed at 400 x magnification in an optical microscope. For each plate,
four microscopic fields, each containing a minimum of 100 conidia, were evaluated and the conidial germination percentage for each plate was calculated.

Radial growth of the fungal isolates was evaluated at different temperatures. Aliquots of 5 µl of each conidial suspension (10^5 conidia / ml) were inoculated with a calibrated loop on the surface of the culture medium (one drop on the center of each plate). The initial inoculum diameter size was 4 mm. Cultures were incubated at 23, 27, 30, 35 and 38°C. Colony growth at a given temperature was assessed by cross-measuring the diameters of all fungal colonies. The inoculations were performed in six independent replicates for each treatment (temperature x isolate). Colony diameters were taken daily during the first week and subsequently a few data were collected until the 15th day. Special attention was given to the ability to sporulate at different temperatures.

For virulence evaluation, boll weevil adults between 1 and 3 days old were submerged into conidial suspensions at a concentration of 5 x 10^5 conidia/ml. Control was performed with sterile distilled water containing Tween 80 (0.05 %). Once the insects were inoculated, they were incubated at different temperatures in controlled chambers. The temperatures evaluated were 27, 30 and 35°C. Humidity was maintained at 50 ± 10 % by placing containers filled with water inside the chambers. Each treatment and the control were replicated 3 times with 20 boll weevil adults per replicate. Since the 6th day, mortality was recorded daily for 14 days. Dead insects were placed in humid chambers to confirm fungal infection.

Statistical Analysis: The percentage of conidial germination was calculated for the different isolates at different temperatures as a percentage of the four plates, as follows: Conidia germination percent = total of conidia germinated x 100 / total of conidia.

Radial growth rates (RGR, velocity in mm / day) were calculated from the regression slope of colony diameter versus time during the linear growth phase, because radial measurements (from the 3rd to the 14th day) fit a linear model (Colony diameter = RGR * time + b) [17]. This was used as the main parameter to evaluate the influence of temperature on fungal growth. The RGR per isolate and per temperature was expressed as the mean RGR of the six replicates.

The RGR and germination percentage from different exposure temperatures and fungal isolates were compared by means of ANOVA. Tukey’s tests were performed for post hoc comparisons (p < 0.05).

The mortality percent (number of adults dying of fungal infection x 100 / total of adults) was calculated for each treatment. These data at different temperatures for each isolate were compared by means of one-way ANOVA and Tukey’s tests (p < 0.05). The median survival time (ST50) and 95% confidence intervals for adults receiving each treatment were calculated based on Kaplan-Meier survival distribution function.

RESULTS AND DISCUSSION

The percentage of conidial germination for all isolates was above 97 % at 23, 27 and 30°C, except for Bb 301 at 30°C, where germination was 94.51% (Table 1). No significant differences in M. anisopliae germination were observed among the temperatures of incubation (F(4,12) = 1.56, p < 0.25; F(4,12) = 1.45, p < 0.28), possibly because of the greater variability in the data obtained for this species. On the other hand, B. bassiana isolates showed significant differences in germination at different temperatures (F(3,12) = 179.59, p < 0.001; F(3,12)= 2833.2, p < 0.001). Both Bb 23 and Bb 301 achieved the highest germination at 27°C and Bb 301 also achieved it at 23°C. Other works studied the germination of conidia at different temperatures and found that the optimum temperature range is between 25 - 30°C, but this varies with the strain and species of fungi [7, 18, 19].

M. anisopliae isolates were more thermotolerant than B. bassiana ones. Only the M. anisopliae isolates managed to germinate at 35°C and the conidia germination percentage at this temperature was high (100 and 96.4 % for Ma 20 and Ma 50, respectively). Similarly, other studies showed that the upper thermal limits of M. anisopliae were around 37°C for conidial germination [20] and 37- 40°C for hyphal growth [17, 21]. Additionally, Glare & Milner (1991) [18] and Tefera & Pringle (2003) [19] found that the tolerance of thermal stress for M. anisopliae isolates was higher than for B. bassiana or Paecilomyces lilacinus. However, some isolates of M. anisopliae did not grow at 35°C, or the germination declined strongly at this temperature [9]. McCammon & Rath (1994) [11] determined the germination rates of 122 isolates from 16 M. anisopliae var. anisopliae strains over a range of temperatures (2.5 - 37°C) and separated them with a canonical analysis in three groups: the cold-active strains (which germinated at 5°C); the heat-active strains (which germinated at 37°C) and meso-thermoactive strains (which germinated at neither 5 nor 37°C).
Table 1: The percentage of conidial germination (±SD) of the *B. bassiana* and *M. anisopliae* isolates incubated at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Isolates</th>
<th>23°C</th>
<th>27°C</th>
<th>30°C</th>
<th>35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bb 23</td>
<td>97.26 (0.40)</td>
<td>100.00 (0.00)</td>
<td>98.11 (0.48)</td>
<td>0.00 (0.00)</td>
<td>D</td>
</tr>
<tr>
<td>Bb 301</td>
<td>99.63 (0.63)</td>
<td>100.00 (0.00)</td>
<td>94.51 (0.70)</td>
<td>0.00 (0.00)</td>
<td>C</td>
</tr>
<tr>
<td>Ma 20</td>
<td>99.82 (0.21)</td>
<td>100.00 (0.00)</td>
<td>98.68 (2.33)</td>
<td>100.00 (0.00)</td>
<td></td>
</tr>
<tr>
<td>Ma 50</td>
<td>99.39 (0.64)</td>
<td>100.00 (0.00)</td>
<td>97.85 (2.62)</td>
<td>96.43 (4.55)</td>
<td></td>
</tr>
</tbody>
</table>

Means followed by different letters in each line (isolate) differed significantly (Tukey’s test, p <0.05).

Table 2: Linear radial growth rates (RGR; mm d⁻¹) from each *Beauveria bassiana* and *Metarhizium anisopliae* isolates during 14 d incubation at 23°, 27°, 30°, 35° and 38°C.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Bb 23</th>
<th>Bb 301</th>
<th>Ma 20</th>
<th>Ma 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>T°C</td>
<td>RGR (DE)</td>
<td>R²</td>
<td>RGR (DE)</td>
<td>R²</td>
</tr>
<tr>
<td>23</td>
<td>3.64 (0.12) B, a</td>
<td>0.996</td>
<td>3.44 (0.13) B, ab</td>
<td>0.994</td>
</tr>
<tr>
<td>27</td>
<td>4.98 (0.08) A, a</td>
<td>0.999</td>
<td>4.55 (0.12) A, b</td>
<td>0.999</td>
</tr>
<tr>
<td>30</td>
<td>3.73 (0.06) B, a</td>
<td>0.996</td>
<td>2.56 (0.07) C, b</td>
<td>0.989</td>
</tr>
<tr>
<td>35</td>
<td>0.11 (0.18) C, a</td>
<td>0.44</td>
<td>0.13 (0.07) D, a</td>
<td>0.610</td>
</tr>
<tr>
<td>38</td>
<td>0 C</td>
<td>0 D</td>
<td>0 D</td>
<td>0 D</td>
</tr>
</tbody>
</table>

Linear radial growth rates were estimated as the slope of the following function: Colony diameter = RGR x time + b. Rate data are means of six replicates.

Means followed by different lowercase letters in each line (temperature) differed significantly (Tukey’s test, p <0.05). Means with different uppercase letters in each column (isolate) differed significantly (Tukey’s test, p < 0.05).

The radial growth fit the linear model for all isolates and at all temperatures of incubation. The coefficient R² was higher than 0.9, except for the *B. bassiana* isolates at 35°C, whose slopes tended to zero (Table 2). No radial growth was found at 38°C for the isolates. All RGR of the isolates were significantly affected by temperature (*F*<sub>Bb 23</sub> (4, 23) = 2549.84, p < 0.01; *F*<sub>Bb 301</sub> (4, 24) = 2885.60, p < 0.01; *F*<sub>Ma 20</sub> (4, 25) = 534.59, p < 0.01; *F*<sub>Ma 50</sub> (4, 25) = 160.70, p < 0.01)

The highest RGR was registered at 27°C for all isolates and this parameter decreased at higher or lower temperatures. An exception to this was the isolate Ma 20, in which no significant differences were found between its RGR at 27 and 30°C. In addition, at these temperatures the colony grew faster. The isolates of *B. bassiana* grew faster than the isolates of *M. anisopliae* at 23 and 27°C (*F*<sub>Bb</sub> (3, 19) = 41.39, p < 0.001; *F*<sub>Ma</sub> (3, 19) = 164.36, p < 0.001). The most affected isolate for low temperatures was Ma 50, whose RGR was the lowest at 23°C (RGR = 2.71 ± 0.21). Bb 301 grew at a slower rate than the other isolates at temperatures higher than 30°C (*F*<sub>Bb 301</sub> (3,18) = 145.78; p < 0.001). None of the *B. bassiana* isolates grew from 35°C, whereas the *M. anisopliae* isolates managed to grow at 35°C at a slower rate (*F*<sub>Ma 50</sub> (3,20)= 12.97; p<0.001). Finally, none of the isolates grew at 38°C (RGR = 0). These results are in agreement with the germination findings. Others reported that most of the *B. bassiana* isolates were more cold-active, whereas *M. anisopliae* isolates were more thermotolerant [6, 19, 22, 23]. Milner et al. (1991) [16] found for *M. anisopliae* isolates that there was no mycelial growth at 35°C, and that rapid growth occurred between 20 and 30°C, with the most growth at 30°C. On the other hand, Ekesi et al. (1999) [7] found that the upper limit was 35°C, while Ouedraogo et al. (1997) [6] observed that most of the isolates grew between 11 and 32°C, some of them managed to grow between 8 to 37°C, but neither grew at 40°C.

With regard to sporulation, conidial production was observed for all isolates at 23, 27 and 30°C. Only one plate of Bb 23 and only one of Bb301 managed to sporulate at 35°C, but none of the *M. anisopliae* isolates was able to sporulate at this temperature. Other authors found the optimum temperature for sporulation was 25°C [19]. The lack of *M. anisopliae* isolates sporulation at 35°C may have been caused by obscurity incubation of the plates. *M. anisopliae* species require light hours to sporulate than the other isolates at temperatures higher than 30°C (*F*<sub>Bb</sub> (3,18) = 145.78; p < 0.001). None of the *B. bassiana* isolates grew from 35°C, whereas the *M. anisopliae* isolates managed to grow at 35°C at a slower rate (*F*<sub>Ma</sub> (3,20)= 12.97; p<0.001). Finally, none of the isolates grew at 38°C (RGR = 0). These results are in agreement with the germination findings. Others reported that most of the *B. bassiana* isolates were more cold-active,
Fig. 1: Mortality percentage of the boll weevil at different temperatures with conidial suspensions of $5 \times 10^8$ conidia/ml of two isolates of B. bassiana (Bb 23 and Bb 301) and two of M. anisopliae (Ma 20 and Ma 50) isolates. Lower letters represent significant difference between temperatures for this isolate (ANOVA and Tukey test, $p<0.05$).

Table 3: Median survival time (ST$_{50}$) (Andersen 95% confidence intervals (CI)) of Anthonomus grandis inoculated by dipping adults in suspensions with $5 \times 10^8$ conidia/ml with different isolates of B. bassiana and M. anisopliae at three incubation temperatures.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>20°C</th>
<th>27°C</th>
<th>35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bb 23</td>
<td>13 (10.75 - 15.24)</td>
<td>8 (7.20 - 8.79)</td>
<td>n.o.</td>
</tr>
<tr>
<td>Bb 301</td>
<td>9 (8.34 - 9.66)</td>
<td>6</td>
<td>n.o.</td>
</tr>
<tr>
<td>Ma 20</td>
<td>8 (7.47 - 8.53)</td>
<td>6 (5.71 - 6.30)</td>
<td>8 (7.57 - 8.43)</td>
</tr>
<tr>
<td>Ma 50</td>
<td>n.o.*</td>
<td>8</td>
<td>8 (7.29 - 8.71)</td>
</tr>
<tr>
<td>Control</td>
<td>n.o.</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
</tbody>
</table>

*n.o. = 50% mortality was not observed in the bioassays

In relation to the virulence, the majority of the control insects survived to the end of the assessment period (20 d) (Figure 1). Only three adults died at 35°C in one control replicate. The mortality percentage of the adults treated with B. bassiana isolates did not show significant differences between 20 and 27°C. But, at 35°C the mortality decreased significantly and about 90% of the adults survived ($F_{Bb23}(2,6) = 52.86$, $p < 0.001$; $F_{Bb301}(2,6) = 135.71$, $p < 0.001$). The incubation temperature did not affect the mortality of the adults treated with M. anisopliae isolates ($F_{Ma20}(2,6) = 2.40$, $p < 0.17$; $F_{Ma50}(2,6) = 4.83$, $p < 0.06$). The most virulent isolate was Ma 20 at 35°C with 95 ± 5% of mortality in adults.

The ST$_{50}$ values ranged from 8 to 13 days at 20°C, from 6 to 8 days at 27°C and 8 days at 35°C (Table 3). The lowest ST$_{50}$ (6 days) was found at 27°C for the isolates Ma 20 and Bb 301. At 20°C the ST$_{50}$ increased for each isolate as compared with the same isolates at 27°C. Finally, at 35°C B. bassiana isolates did not kill 50% of the adults and therefore the ST$_{50}$ could not be calculated, whereas M. anisopliae isolates took 8 days to kill half of the adults at the same temperature. Similarly, M. anisopliae isolates were more effective against three fruit flies species at 30°C, but at the lower temperature of 20°C, the onset of the disease was delayed but did not affect total mortality [9].

Our findings, in agreement with previous studies [25, 26], suggest that conidial thermotolerance of these fungi varies greatly among natural strains. The inhibition in germination and radial growth of the B. bassiana isolates at 35°C was reflected in low pathogenicity at this temperature. Consequently, the ability of an isolate to germinate under given environmental temperature regimes is a critical determinant of efficacy [11]. However, the relationship between thermotolerance as measured by in vitro growth and disease development needs to be first established in each particular system [6]. Samuels et al. (1989) [8] demonstrated that rapid germination and growth of M. anisopliae was correlated with higher virulence toward Nilaparvata lugens (Hom.: Delphacidae). On the other hand, Tefera y Pringle (2003) [19] found no consistent relationship between virulence, conidia germination, vegetative growth and sporulation, indicating that there may be other factors governing virulence of the isolates. For instance, high temperature and solar UV-B radiation are the principal factors limiting entomopathogenic fungi under field conditions. These factors cause both the conidial inactivation and delay in the germination of the survivors [22]. Therefore, tolerance to UV-B radiation should be investigated for selecting fungal candidates for cotton boll weevil control. On the other hand, not only is the natural tolerance important, but formulation development must also consider the use of temperature and UV-B protectant to maximize biocontrol efficacy.

ACKNOWLEDGMENTS

To the Laboratory of the Entomopathogenic Fungi staff for assistance; to Instituto Nacional de Tecnología Agropecuaria and Fondo para la Investigación Científica y Tecnológica, Agencia Nacional de Promoción Científica y Tecnológica (FONCYT-ANPCyT), Argentina, for funding this research.

REFERENCES


