# Mortality of *Diatraea saccharalis* is affected by the pH values of the spore suspension of *Beauveria bassiana* and *Metarhizium anisopliae*[1]

*Tamires Doroteo de Souza*[2*], *Ariadne Costa Sanches*[2], *Maiara Alexandre Cruz*[2], *Thais Juliane do Prado*[2], *Patrice Jacob Savi*[2], *Ricardo Antônio Polanczyk*[2]

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## ABSTRACT

Fungal virulence is multifaceted and dependent on multiple factors including the pH of the spore suspension. In this study, we accessed effects of six pH values of *Beauveria bassiana,* and *Metarhizium anisopliae* medium for the growth, sporulation, and mortality on sugarcane stalk borer *Diatraea saccharalis*. The culture of fungi was performed onplates containing the PDA (Potato Dextrose Agar) medium. Virulence was tested in *D. saccharalis* larvae distributed in four replicates of 15 larvae. To evaluate the performance of the isolates, they were grown at different pH values in an artificial chitin medium to confirm the degradation capacity of the fungi at each pH. No significant difference was observed for the sporulation at pH ranged from 4 to 9 for both fungi. In the mortality assay, larval mortality was higher at pH 7 and 8 for both fungi, reaching 87% for *B. bassiana* and 81% for *M. anisopliae*.

**Keywords:** entomopathogenic fungi; sugarcane borer; microbial control.

## INTRODUCTION

The entomopathogenic fungus, *Beauveria bassiana* (Bals-Criv.) Vuill. (Hypocreales: Cordycipitaceae*)* and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) are hemibiotrophic, cosmopolitan and ubiquitous fungus in the soil (Jaronski, 2010; Vega, 2018). These organisms are widely used for the microbial control of various agricultural arthropod-pests contributing to the ecological balance in natural environments and agricultural ecosystems (Wang & Wang, 2017).

The sugarcane borer, *Diatraea saccharalis Diatraea saccharalis* Fabricius, 1794 (Lepidoptera: Crambidae), is one of the the major economically significant pest of sugarcane crops in Brazil major. Its larval stages are capable of reducing crop yields by feeding on the stalk and thereby facilitating the infection of plant pathogenic fungi through their feeding galleries, (Francischini *et al.*, 2017). The biological control of this pest has already been widely used, with the introduction of parasitoids as natural enemies such as the use of *Cotesia flavipes* (Hymenoptera: Braconidae) (Zappelini *et al.*, 2010). However, with the rapid expansion of the sugarcane crop, the production of parasitoids would be inversely proportional. Thus, the use of other control methods, such as entomopathogenic fungus, will increase the biological control of this sugarcane borer.

However, the efficiency of fungi in microbial control is strongly influenced by biotic and abiotic factors (Pell *et al.*, 2001) Among these factors, the pH presents great importance in fungi development, influencing the process of germination, growth, and virulence (Alves, 1998). Even knowing the importance of pH *in vitro* and field cultures (soil pH), as well as its influence on the persistence and efficacy of entomopathogenic fungi, information about the role of pH on fungi is still little understood (Inglis *et al.*, 2001).

Reports about the pH effect on fungi survival, ecological distribution, and virulence are contradictory. Bidochka *et al.* (1998), Groden and Lockwood (1991) observed that the inhibition of *B. bassiana* growth in the soil was affected by soil pH. Rath *et al.* (1995) verified

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[2]Universidade Estadual Paulista 'Júlio de Mesquita Filho', Jaboticabal, São Paulo, Brazil. tamiresdoroteo@gmail.com; ariadnesanches7@gmail.com; maiara_agronomia@hotmail.com ; prado.agro@yahoo.com.br; savipatricejacob@yahoo.fr; r.polanczyk@unesp.br
*Corresponding author: tamiresdoroteo@gmail.com

that the soil pH has no influence on the distribution of *M. anisopliae* isolates in a field study. In controlled conditions, few studies reported the pH influence.

For entomopathogenic fungi to be efficient microbial control agents, it is necessary to understand all the factors that may negatively affect their development, mainly the pH of the water at the time of spraying. Therefore, this study aimed to elucidate possible effects on different pH values of the culture medium for the growth, sporulation, and virulence of the fungi *Beauveria bassiana* and *M. anisopliae*.

## MATERIAL AND METHODS

### *Obtaining and Multiplication of Fungus*

We used the *B. bassiana* (strain ESALQ 171) and *M. anisopliae* (strain ESALQ 935) fungi from the collection of Laboratory of Microbial Control of Arthropods Pests, São Paulo State University (FCAV/UNESP), Jaboticabal, São Paulo, Brazil.

The isolates were kept in dishes (9x12 cm) containing on Potato Dextrose Agar (PDA) medium for 10 days at 27 ± 5 °C 10% R.H. and 12:12 h L:D. For culture rejuvenation, an aliquot of the stock fungi culture was inoculated into 15 mL PDA medium plates. The inoculation was performed with a platinum loop transferring spores to the central point of the plates. Then, the fungi were incubated at 27 °C in an oven for 20 days (Aguirre, 2009).

### *Conidia viability*

An aliquot of the fungi matrix was placed in PDA medium and after 12 h was performed the counting of viable conidia, following the method described by Padmavathi *et al.* (2003).

### *Growth and spore production*

We evaluated six pH conditions (4, 5, 6, 7, 8, and 9) of *B. bassiana* and *M. anisopliae* medium. The pH was measured by a pH meter (MS TECNOPON®) and the adjustment was carried out using 0.5 N NaOH or 1.0 N HCl solution. To evaluate the growth on plates, 10 ul ($10^8$ conidia $ml^{-1}$) of the suspension were inoculated into dishes using 20 plates per treatment pH. The evaluation of the radial growth rate of the colony was performed on the $6^{th}$, $9^{th}$, and 12th days after inoculation. For this purpose, the shape of each colony was drew on A4 paper, on the outer face of the bottom of each dish (9x12 cm), and then each draw has scaled the area with the aid of a leaf area meter (the electronic device that measured leaf area, Licor 3100).

Spores production was evaluated 20 days after incubation. To check whether the pH influenced the fungus virulence, five plates from each treatment were randomly selected, resuspending an aliquot of each plate with a different pH for test tubes containing 10 mL of a saline mixture (0.89% w / v NaCl) and Tween 80® solution (0.1% v / v) using a magnetic stirrer. Spores were extracted from the sample and counted with a Neubauer camera to standardize concentrations for the insect bioassay adapted from Rodrigues *et al.* (2010).

### *Mortality test*

The mortality from *B. bassiana* and *M. anisopliae* isolates was evaluated in the third instar larvae of *Diatraea saccharalis*. The larvae provided byUsina São Martinho-SP, were placed in dishes, and immersed in 1 mL of spores suspended at the concentration of $10^8$ viable for each pH (water + Tween 80® adhesive spreader - 0.01%). As a control, the insects were immersed in the aqueous solution of Tween 80® adhesive spreaders (0.01%). The larvae were transferred to plastic pots for bioassays with an artificial diet used to raise the species in the laboratory (Cruz, 2007) and kept under controlled conditions (26 ± 1 °C 10% R.H. and 12:12h L:D). The mortality larvae were evaluated daily after seven days of fungus application. Each isolate was tested with 4 repetitions and 60 larvae in total.

### *Confirmation of cuticle degradation*

Two isolates tested from each fungus were cultured at different pH values (4, 5, 6, 7, 8, and 9) in a synthetic (artificial/PDA) medium of chitin. To evaluate the performance of the isolates were observed chitin degradation, that is, the formation of an halo on the plate after seven days of incubation. Ten plates were used for each pH value for each fungal isolate. The plates were stored in an incubator under controlled conditions (27 °C 14h L:D).

### *Statistical analysis*

The averages obtained for spore production were subjected to analysis of variance and when significant compared by the Tukey test (P d" 0.05). Mortality test data were submitted to Probit analysis (SAS Institute Inc 2018). The figures were made in the SigmaPlot program (version 11.0).

## RESULTS

An interaction between pH and evaluation days after contact of the fungi with the medium ($p < 0.01$), for *M. anisopliae* and *B. bassiana* was observed given the diameter off the colonies grew (Table 1). The *B. bassiana* ESALQ 171 grown up rapidly in the pH range from 5 to 7, suggesting that this isolate presents a good growth in acidic or neutral pH. However, *M. anisopliae* ESALQ 935 isolate ranged from pH 4 to 9. The diameter growth of the colonies ranged from 5.27 mm to 20.9 mm for *M. anisopliae* and from 4.94 mm to 23.5 mm for *B. bassiana*, with a higher growth rate in the pH 6 and 7 for both fungi (Table 1).

No significant difference was observed for the sporulation of *B.assiana* and *M. anisopliae* with the pH ranges from 4 to 9 (Table 1). In the virulence assay, the larval mortality was 81% due to *M. anisopliae* (Fig. 1A) and 87% due to *B. bassiana* (Figure 1B).

## DISCUSSION

The production stage of the entomopathogenic microorganisms is of great importance for the use of these agents as bioinsecticides. However, the production of conidia can be affected by determinant factors, as temperature, radiation, and pH, reducing efficiency as a bioinsecticide (Trumper *et al.*, 2004; Song *et al.*, 2014). The results obtained in the present study showed that the pH of the medium may be highly determinant for the efficacy of the entomopathogens since the tested isolates had a different behavior both in the germination in the plate and in contact with the cuticle of *D. saccharalis*.

The differences in the pH medium as regards the germination speed of the two isolates tested showed that these fungi require an ideal range and the non-adaptation of this parameter can alter the physiological process reducing their efficiency. In a study by Sautour *et al.* (2001), the conidia of *Penicillium chrysogenum* presented optimum pH for germination in the range of 3.5 to 6.5. On the other hand, when they evaluated another parameter did not notice a significant difference in sporulation. This result indicates that in all the pH ranges, there is the elongation of the germinative tubes, but with a lower speed for each pH value, resulting in the difference of germination. That is, the development of the entomopathogens needs a higher germination speed so that the differentiation of the apical compartment occurs in a reproductive structure, called phialide, which is responsible for the mitotic division, allowing the production of conidia (Roncal *et al.*, 2002; Roncal and Ugalde, 2003).

In general, our studies suggest that for the induction of sporulation, growth and sporulation, the *B. bassiana* isolate needs an optimum pH of 5 to 7 and the *M. anisopliae* isolate needs 6 to 8. Our results coincide with the optimum pH range of 5 to 8.5 for the growth of *M. anisopliae* and *B. bassiana* defined by Galani (1988). Contrary to ours, Inglis *et al.* (2001) observed that variations in the pH of the medium did not interfere in the growth of *M. anisopliae* isolates, showing, even more, the need for studies in this aspect.

**Table 1**: Synthesis of the analysis of variance for the growth of the fungus colony diameter (mm). Interaction between pH and evaluation days after contact of the fungus with the medium (PDA), and colony growth with the effect of pH on the sporulation of Beauveria bassiana and Metarhizium anisopliae fungi

| **Factors** | **Value of F to Diameter of the colony (mm)** | |
|---|---|---|
| | ***Beauveria bassiana*** | ***Metarhizium anisopliae*** |
| pH | 34.35** | 10.57** |
| Days | 10.50** | 17.52** |
| pH x Days | 59.43** | 46.62** |
| CV (%) | 0.41 | 0.25 |

| | **Growth of the fungus colony diameter in days (mm)** | | | | | |
|---|---|---|---|---|---|---|
| **pH** | ***Beauveria bassiana*** | | | ***Metarhizium anisopliae*** | | |
| | **6 (days)** | **9 (days)** | **12 (days)** | **6 (days)** | **9 (days)** | **12 (days)** |
| 4 | 6.97 dC | 10,13 eB | 11.53 eA | 5.20 fC | 8.51 eB | 10.20 fA |
| 5 | 7.53 cC | 10.83 cB | 12.13 dA | 8.76 dC | 12,24 dB | 14,34 dA |
| 6 | 8.99 bC | 11.23 bB | 13.56 cA | 9.21 bC | 13.33 cB | 15.26 cA |
| 7 | 9.94 aC | 13.43 aB | 22.53 aA | 12.89 aC | 18.16 aB | 20.13 aA |
| 8 | 7.43 cC | 10.53 dB | 17.86 bA | 9.07 cC | 15.63 bB | 18.43 bA |
| 9 | 4.46 eC | 6.26 fB | 9.43 fA | 7.75 eC | 8.17 fB | 10.83 eA |

| **pH values** | **Number of conidia/cm³ for *Beauveria bassiana*** | **Number of conidia/cm³ for *Metarhizium anisopliae*** |
|---|---|---|
| 4 | $1.0 \times 10^8$ | $1.8 \times 10^8$ |
| 5 | $1.8 \times 10^8$ | $2.5 \times 10^8$ |
| 6 | $4.8 \times 10^8$ | $4.0 \times 10^8$ |
| 7 | $7.2 \times 10^8$ | $6.6 \times 10^8$ |
| 8 | $6.8 \times 10^8$ | $6.1 \times 10^8$ |
| 9 | $3.7 \times 10^8$ | $5.3 \times 10^8$ |

**: significant ($p < 0.01$); CV%: coefficient of variation. The averages followed by the same lower case letters in the column and uppercase letters in the row do not differ significantly by the Tukey test at 5% probability.

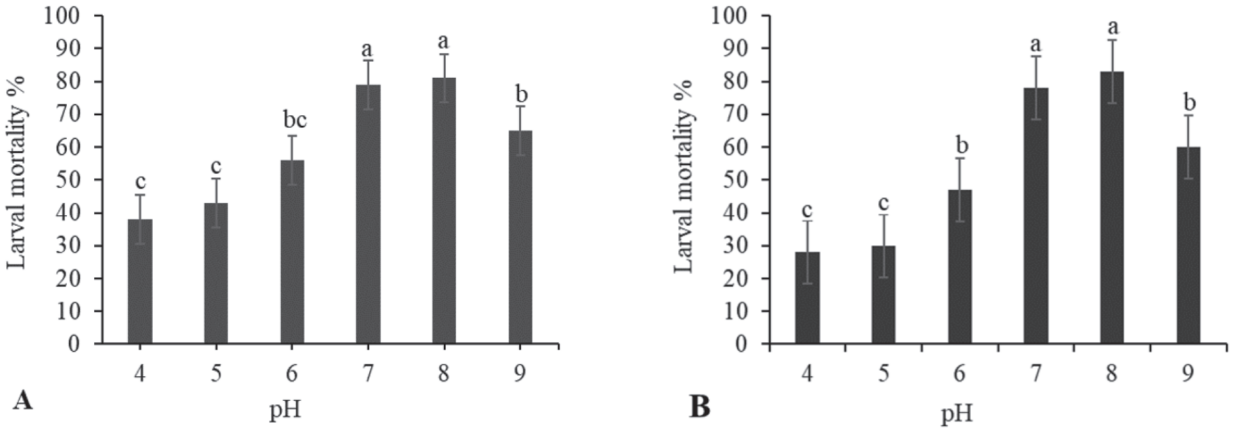


**Figure 1**: A- Effect of pH on the mortality of Metarhizium anisopliae in third instar larvae of Diatraea saccharalis. B- Effect of pH on Beauveria bassiana mortality in Diatraea saccharalis larvae after seven days incubated at 27 °C, total mortality. Treatments with the same letter show no statistical differences according to Tukey's test at 5%.

Mortality in *D. saccharalis* was directly affected by the pH range fungal development and can be explained by the fungal infection process in the host cuticle that is mediated by enzymes that are directly influenced by the pH of the microorganism (Caddick *et al.*, 1986). Some enzymes produced by fungi, such as proteases, play an important role in the infection process in insects, directly affecting the virulence of fungi. In *M. anisopliae*, virulence is regulated by pH in the cuticle of the insect, as these proteases are involved in the hydrolysis of the cuticle, facilitating the penetration of hyphae (St. Leger *et al.*, 1998).

Considering the larval mortality of *D. saccharalis*, the best pH conditions were 7 and 8, differing from the plate growth range. This difference may be related to the activity of fungal enzymes in contact with the cuticle of *D. saccharalis*. In addition to proteases, entomopathogenic fungi produce chitinases in the infection process, which also has an optimal pH of 5 to 8 (St. Leger *et al.*, 1998). Possibly, this value of 8 in pH for a higher percentage of dead bollworm is justified by the adequate activity of enzymes with protease and chitinase activity involved in insect penetration events.

The formation of chitin by microorganisms varies according to the medium, having several variables for suitability (Synowiecki and Al-Khateeb, 2003). The degradation of the insect cuticle was entirely linked to the degradation of chitin by fungal microorganisms. In our bioassays, we verified the chitinase activity of the two fungal plate isolates for each pH value (data observed during the evaluation of the experiment). We observed that the values of 7 and 8 of the pHs for *B. bassiana* and *M. anisopliae*, respectively, showed the highest degradation of chitin in the medium confirming the results when tested on *D. saccharalis*. Once again, we confirm that the pH directly influences the mortality of the fungi facilitating or not the degradation of the cuticle of the insects.

These results show that in agricultural practice when water is used to mix the fungus application in the field, care must be taken with the pH of the spray suspension to better control the fungus the desired pest.

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