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Statistical experimental design to assess the influence of enzymes of nematophagous fungi *versus* helminths

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ABSTRACT

The present work used Plackett–Burman experimental design to assess the influence of enzymes of nematophagous fungi *versus Strongyloides westeri* and trichostrongylides larvae and *Platynosomum fastosum* eggs. The variables studied in the Plackett–Burman design were the proteases and chitinases of AC001 or VC4 as destructive agents of *S. westeri* and trichostrongylides larvae, and *P. fastosum* eggs. All tested enzymes had a significant effect ($P < 0.05$) on the destruction of *S. westeri* larvae. Furthermore, only VC4 and AC001 proteases showed a significant effect ($P < 0.05$) on the destruction of trichostrongylides larvae. On the other hand, chitinases of VC4 showed the highest significance ($P < 0.05$) on the destruction of *P. fastosum* eggs. It is proposed that statistical planning for the use of enzymes derived from nematophagous fungi is a viable way to elucidate some questions about their mechanism of action.

1. Introduction

Nematophagous fungi are natural enemies of gastrointestinal helminth parasites and have been studied as an important strategy to be incorporated into an integrated control system to combat the parasitic helminth forms present in the environment (Braga et al., 2008a, 2008b; Dias et al., 2012; Paz-Silva et al., 2011). Their way of 'attacking' the infective larvae and/or helminth eggs, and their production of vegetative structures (conidia and/or chlamydospores), have long been known (Barron, 1977; Gronvold et al., 1996). In more recent studies, their enzyme production has been investigated (Yang et al., 2011; Van Ooij 2011; Soares et al., 2011, 2013). According to Soares et al. (2013), proteases produced by nematophagous fungi are effective in the infection process of helminth larvae, hydrolyzing their cuticle. With regard to helminth eggs, whose main component is chitin, a number of studies have demonstrated the role of chitinases in the destruction of these eggs (Lysek, 1976; Lýsek and Stěrba, 1991). On the other hand, there is a lack of studies using statistical design to study the interaction of these enzymes together on different stages of helminths of domestic animals.

In this context, the Plackett–Burman statistical experimental design (Plackett and Burman, 1946) can be used to verify which enzyme (protease and/or chitinase) has higher significance in the destruction process of eggs and larvae of helminths. This plan provides a quick and effective way to identify important factors among a large number of variables, thus saving time and keeping convincing information about each parameter (Abdel-Fattah et al., 2005).

The aim of this study was to use a statistical experimental design to assess the interaction of enzymes of nematophagous fungi *versus Strongyloides westeri* and trichostrongylides larvae and *Platynosomum fastosum* eggs.

2. Materials and methods

2.1. Fungi

Two species of nematophagous fungi were used, *Duddingtonia flagrans* (AC001) and *Pochonia chlamydosporia* (VC4). These isolates were kept in test tubes at 4 °C containing 2% corn-meal-agar (2% CMA) in the dark for 10 days. These fungi were derived from the mycology collection of the Laboratory of Parasitology of the Veterinary Department of the Federal University of Viçosa, Minas Gerais, Brazil.

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For the production of enzymes (protease and chitinase), two different culture media were inoculated with conidia of *D. flagrans* (AC001) or *P. chlamydosporia* (VC4) obtained by the transfer of culture dishes (approximately 5 mm in diameter) in 2% CMA. The culture medium used for the production of protease contained wheat bran (5 g) supplemented with 1% casein, and 2.5 ml of liquid minimal medium containing K_2HPO_4 (5.0 g/l), $MgSO_4$ (0.10 g/l), $ZnSO_4$ (0.0050 g/l), $FeSO_4$ (0.001 g/l) and $CuSO_4$ (0.50 mg/l) (Braga et al., 2013). The culture medium used for the production of chitinase contained wheat bran (5 g) supplemented with 1% chitin and 2.5 ml of the same liquid minimal medium as described above. Enzyme extraction was performed according to Soares et al. (2010). The clear supernatant was used for the assays.

2.2. Enzyme assays

Proteolytic activity was measured by the caseinlytic method according to Soares et al. (2012), modified. A standard tyrosine curve was constructed for quantification of enzyme activity. One unit of enzyme was defined as the amount of enzyme required to liberate 1.0 μg tyrosine per min under the assay conditions.

Chitinase activity was measured according to Braga et al. (2013), modified. The amount of reducing sugars released was determined by absorbance at 550 nm. A standard curve of *N*-acetylglucosamine was constructed by varying its concentration. One unit of chitinase was defined as the amount of enzyme required to liberate 1.0 μmol *N*-acetylglucosamine per min under the assay conditions.

2.3. Obtaining eggs and larvae

2.3.1. Trichostrongylides

Feces of domestic ruminants (sheep) infected with trichostrongylides were collected. Fecal samples of 20 g each were mixed with 8 g vermiculite and 20 ml distilled water, and incubated for 8 days at 25 °C in the absence of light. Then, larvae were recovered from cultures in hemolysis tubes through Baermann apparatus. The larvae were identified according to Ueno and Gonçalves (1994) and Van Wyk et al. (2004).

2.3.2. S. westeri

To obtain infective larvae of *S. westeri*, coprocultures of positive feces from naturally infected young horses were performed. Next, these larvae were classified according to the criteria established by Soulsby (1982). Only larvae of *S. westeri* were identified.

Table 1
High (+1) and low (−1) levels (presence or absence) of the four analyzed variables (proteases and chitinases of *Duddingtonia flagrans* (AC001) and *Pochonia chlamydosporia* (VC4)) in the Plackett–Burman statistical design.

Variables	High level (+1)	Low level (−1)
VC4 chitinases	1	0
VC4 proteases	1	0
AC001 chitinases	1	0
AC001 proteases	1	0

2.3.3. P. fastosum

Adult parasites were collected from the bile ducts of one female cat with a history of sudden death. Parasites were dissected to obtain eggs. Egg viability was analyzed by light microscopy using a 10× objective lens according to Urquhart et al. (1998). Subsequently, the eggs were washed 10 times in distilled water, and, for each wash, centrifuged at 1000 *g* for 5 min. The supernatant was discarded after each centrifugation. The eggs were kept in a solution containing 0.005% streptomycin sulfate and 0.1% chloramphenicol (Araújo et al., 1995).

2.4. Statistical experimental design

The Plackett–Burman experimental design was used to scan some variables about their respective influences on the destruction of *S. westeri* and trichostrongylides larvae, and *P. fastosum* eggs. The studied variables were proteases and chitinases of *D. flagrans* (AC001) and *P. chlamydosporia* (VC4). These variables were analyzed on two levels: −1 for lower level and +1 for higher level (Table 1), where +1 = presence of the enzyme in question and 0 = absence of the enzyme in question. The response used was based on the number of larvae and/or viable eggs of helminths studied.

Next, the software Minitab Release 15 was used to perform the analyses. The number of experiments was $n+1$ according to the Plackett–Burman experimental design, where n is the number of variables. The experimental design, where each line represents one experiment and each column represents an independent variable, can be seen in Table 2. The analysis of the studied factors is shown in Table 3 and the ANOVA statistical analysis are shown in Tables 4–6. The experiment was performed in triplicate, and the mean of these values was used as the response Y (number of larvae or eggs).

The experiments were performed as follows: in a sterile tube, 20 μl of crude enzyme (approximate enzymatic activity for each enzyme: 0.427 U/ml VC4 chitinase, 13.56 U/ml VC4 protease, 0.446 U/ml AC001 chitinase and 54.26 U/ml AC001 protease) were

Table 2
Matrix of the Plackett–Burman experimental design used to scan the enzymes (proteases and chitinases) of the nematophagous fungi *Duddingtonia flagrans* (AC001) and *Pochonia chlamydosporia* (VC4) regarding their respective influences on the destruction of *Strongyloides westeri* and trichostrongylides larvae and *Platynosomum fastosum* eggs.

Runs	VC4 chitinases	VC4 proteases	AC001 chitinases	AC001 proteases	*S. westeri* larvae	Trichostrongylides larvae	*P. fastosum* eggs
1	1.0	0	1	0	15.0	48.0	32.0
2	1.0	1	0	1	8.0	32.0	20.0
3	0.5	1	1	0	16.0	35.0	22.0
4	1.0	0	1	1	12.0	30.0	15.0
5	1.0	1	0	1	10.0	27.0	20.0
6	1.0	1	1	0	9.0	28.0	21.0
7	0.5	1	1	1	8.0	25.0	25.0
8	0.5	0	1	1	12.0	32.0	29.3
9	0.5	0	0	1	24.0	38.0	36.0
10	1.0	0	0	0	25.0	49.0	31.0
11	0.5	1	0	0	26.0	33.0	37.0
12	0.5	0	0	0	56.3	70.3	65.0