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Media Composition Influences Growth, Enzyme Activity and Virulence of the Entomopathogen *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae)

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Abstract.- Three isolates of *Metarhizium anisopliae* were cultured on six media having different compositions on the basis of chitin, carbon and nitrogen contents. The effect of nutrition on growth and virulence was studied by measuring colony growth, spore yield, germination rate, spore bound Pr1, lipase activity and virulence of inoculum produced by different media against 2nd instar larvae of diamondback moth (*Plutella xylostella*). Chitin peptone nutrition media produced the highest colony growth but maximum conidial yield was observed for medium having high C/N ratio. Highest lipase activity (3.63 µmol/ml/min) was observed for spores produced by High C/N medium for isolates M408 while highest Pr1 activity (2.1µmol/ml/min) was observed on CPN medium for M460. Conidia from medium C/N ratio proved to be the most virulent for all the three isolates with Median survival time (ST_{50}) values of 1.45, 1.62 and 1.72 days for M408, M440 and M460, respectively. Median survival time values for different nutrient media proved to be positively correlated with spore bound Pr1 and lipase activity having correlation coefficient values of 0.42 and 0.67, respectively.

Key words: Chitin, Lepidoptera, Plutellidae, entomopathogenic fungi, microbial control, biocontrol program.

INTRODUCTION

Diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) is a major pest of cabbage, broccoli and canola. Each year, farmers worldwide spend more than $1 billion to control this pest, primarily by using chemical insecticides (Henrik *et al.*, 2000). As a result, natural enemies are sacrificed (Xu *et al.*, 2004) and many populations of diamond back moth have become resistant to conventional insecticides (Shelton *et al.*, 1993; Tabashnik, 1994). In addition, *Bacillus thuringiensis* resistant field populations have been detected in several regions, such as the Hawaii (Tabashnik *et al.*, 1990), Central America and Asia (Syed, 1992; Ferré and van Rie, 2002). Alternative control measures being investigated for diamondback moth include the use of entomopathogenic fungi (Cherry *et al.*, 2004; Muhammad *et al.*, 2005; Wright, 2004). More than 750 fungi from 90 species have been described as a pathogen against insects. Isolates of *Zoophthora radicans*, *Paecilomyces fumosoroseus* (Altre *et al*., 1999), *Metarhizium anisopliae*, *Fusarium* sp., and *Beauveria bassiana* (Vandenberg and Ramos, 1997; Vandenberg *et al.*, 1998; Shelton *et al.*, 1998; Ibrahim and Low, 1993) can infect diamondback moth. *Metarhizium anisopliae* Sorokin (Hypocreales; Clavicipitaceae) is one of the most promising fungal species currently being investigated as a biocontrol agent against diamondback moth, whiteflies and other insect pests (Altre *et al.*, 1999; Vandenberg, 1996).

Like all microorganisms, entomopathogenic fungi have specific biological characteristics that influence their activity in the environment (Parker *et al.*, 2003). To select fungal pathogen for insect control, it is necessary to study simple characteristics that are required to kill the target insects in both field and greenhouse conditions. According to Moore and Prior (1993) relevant characteristics were identified as good mass production features such as high sporulation on artificial media, high virulence against the target organisms and the ability to withstand the environment in which the pest is occurring. Fungal isolates with rapid germination and hyphal growth rates have an advantage as biological control agents

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because host infection can potentially occur much more quickly (Hajek and St. Leger, 1994; Varela and Morales, 1996). Lane *et al.* (1991) evaluated the influence of carbon- and nitrogen-limited media on the production and quality of blastospores of the deuteromycete *Beauveria bassiana*. These authors noted that the germination rate and survival of *B. bassiana* blastospores following storage was associated with appropriate concentrations of nitrogen and carbon in the culture medium. The production of blastospores that germinate more rapidly has the potential to increase infectivity.

In addition, these fungi produce an array of hydrolytic enzymes thought to be utilized in cuticle penetration (Charnley and St. Leger, 1991). Lipases, chitinases and proteolytic enzymes are important factors involved in the initiation of infection process by entomopathogenic fungi (Samuels and Paterson, 1995). These enzymes are involved in penetration of the host integument and subsequent infection (Ferron, 1978). Lipids and proteins are integral to the insect cuticle and improvements in protease and lipase production by *M. anisopliae* can be highly desirable for its use as a bioinsecticide.

This paper describes the influence of six different nutrient media, having different carbon nitrogen ratios on three isolates of *M. anisopliae*. We evaluated the effect of each medium on germination, growth, sporulation and virulence of each isolate. Additionally, protease and lipase activity was investigated as a function of culture condition.

MATERIALS AND METHODS

Plutella xylostella

Adults of *P. xylostella*, were obtained from the stock cultures on *Brassica campestris* L.kept in greenhouse of the Engineering Research Center of Biological Control, South China Agricultural University, Guangzhou, Guangdong province, China. Plants were grown in plastic pots having a diameter of 15-cm. Sufficient slow release fertilizer (N: P: K=13:7:15, Shenzhen Batian Ecotypic Engineering Co., LTD. Xili Shenzhen, China) was added as required to maintain normal plant growth.

The newly molted second instar larvae of *P. xylostella* were gently removed from the host plant leaves using a fine camel hair brush (No.00) and put on a piece of fresh *B. campestris* excised leaves (100-150 cm^2).

Fungal strains

For all assays, *M. anisopliae* isolates (M408,M440 and M460) originally isolated from soil (Liu, 2006), maintained in tubes containing Sabouraud dextrose agar (SDA) and deposited in the Engineering Research Center of Biological Control, South China Agricultural University, were cultured on Potato dextrose agar (PDA) and incubated at 26±2°C for 10 days. Conidia were harvested with deionized water containing 0.03% Tween 80 and sieved using filter paper into sterile vials. Conidia were counted using a compound microscope and a hemocytometer (0.0625m m^2; Fuchs-Rosenthal Merck Euro Lab, Darmstadt, Germany) to calibrate a suspension of 1×10^6 conidia/ml. Spore viability was determined before preparation of suspension by spreading 0.2 ml of a 1×10^4 conidia/ml suspension on PDA and estimating the number of germinated propagules after 24 hrs of incubation at room temperature. Spores were considered viable when the germ tube lengths correspond to the width. The viability of conidia was assessed immediately before each experiment was started and percentage germination was estimated to > 95% for all experiments.

Culture conditions

Culture media representing disparate carbon and nitrogen sources and ratios were used in this study. They included: (1) high C/N (75 : 1) medium consisting of 9.1% glucose and 1% peptone; (2) intermediate C/N (35 : 1) medium consisting of 4% glucose and 1% peptone (= SDA); (3) low C/N (10:1) medium consisting of 0.6% glucose and 1% peptone;(4) nutrient-poor medium consisting of 2% peptone (5) 'osmotic stress' medium (OSM) consisting of 8% glucose, 2% peptone, 5.5% KCl and (6) chitin peptone nutrient (CPN) consisting of 1% chitin, 0.5% peptone, 0.2% yeast extract. Yeast extract and peptone have C/N ratios of 3.6: 1 and 8: 1, respectively and represented different carbon and nitrogen sources (Wyss *et al.,* 2001). All the media were prepared using 2% agar except CPN, with 1.8% agar, and OSM, which required 5.5% agar to

solidify. Media were sterilized at 121°C at 15 psi for 25 min and 15ml poured into 9-cm diameter Petri dishes.

Germination assay

The germination speed of inoculum from the different media for each isolate was assessed by inoculating with 10 µl of conidial suspension (1×10^6conidia ml^{-1}) in Petri dishes having SDA. Petri dishes were incubated at 26±2°C and 75±5% RH for 96 h. After 96 hrs, three separate fields were observed for germination at 40X of magnification for each treatment and 100 conidia were observed randomly in each field. Conidia with germ tubes equal to or greater than the width were considered to have germinated.

Growth and sporulation

One micro liter freshly prepared fungal suspension (1×10^6conidia ml^{-1}) was inoculated in the centre of plates of each media type mentioned above by using a micro applicator. The plates were incubated at 25±2°C, 80±5% RH and 16:8h (L:D). There were 10 replicates for each media. The measurements of colony diameter were made every 12 hours after inoculation until 15 days. The average diameter of every colony was calculated as follows:

$$\text{Colony diameter} = \frac{(\text{long diameter} + \text{short diameter})}{2}$$

Spore production was investigated after 15 days of inoculation. Conidia from the four randomly selected plates from each treatment were dislodged by 2 washes of 5 ml 0.01% Tween 80 and agitating for 10 min. Then, the number of conidia was counted in a compound microscope using a hemocytometer.

Effect of nutrition lipase activity

Lipase activity was determined as described by Pigende *et al.* (2000). The substrate emulsion was prepared as a 1:1 mixture of olive oil (50 ml) and gum Arabic (50ml, 10% w/v).The reaction mixture contained 1 ml of spore suspension (1×10^6conidia ml^{-1}), 5ml substrate emulsion and 2 ml of 50 mM phosphate buffer (pH 6.8) and was incubated for one hour at 37^0C with shaking. The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of fatty acid released with 50 mM NaOH. One international unit was defined as enzyme activity that produced 1 µmol of fatty acid per min per ml.

Effect of nutrition on the activity of conidial Pr1 enzyme

Pr1 bound to conidia harvested from the disparate media was quantified using the method described by St. Leger *et al*. (1996), with some modifications. One milligram of conidia were incubated in 1mL of 0.1M Tris-HCl (pH 7.95) containing 1mM succinyl-ala-ala-pro–phe-p-nitroanilide for 5 min at room temperature. After incubation the conidia were clarified by centrifugation at 12,000 rpm for 5 min. The supernatant (200 µl) was transferred to wells of a microtitre plate and absorbance was measured using a spectrophotometer at 405 nm. Buffered substrate was used as control.

Virulence of inoculum from different culture media

Conidial concentration of 1×10^8 conidia ml^{-1} was prepared from the spores produced by each media according to the procedure described above. Leaves having 2nd instar diamondback moth larvae were dipped into conidial suspension of *M. anisopliae* for 20 seconds, and then removed to air dry before being transferred to 20cm diameter clean glass Petri dishes. A piece of filter paper (20 cm in diameter) was placed at the bottom of the dish having few drops of water for moisture maintenance. Leaf disks were replaced at an interval of two days except during the pupal stage. Control leaves were treated with 0.02% solution of Tween 80 prepared by using distilled water. Each treatment and control were repeated three times with a new batch of insects and new conidial suspensions, for each repetition there were four leaves with 10 DBM per leaf. All treatments and controls were assayed at one time, using randomized groups of insects from a single batch.

The insects were placed in an air-conditioned room and monitored daily until adult emergence,

and the mortalities of *P. xylostella* were recorded at 12h interval. To survey the infective mortalities, the cadavers were taken out and separately cultured at 26^0C and relative humidity >95% to help for sporulation. If the conidia spores of *I. fumosoroseus* were recovered out from a cadaver, the cadaver would be regarded as dead from infection of strain *M. anisopliae.*

Statistical analysis

Data regarding germination, growth rate, sporulation lipase activity and protease activity were subjected to two factor factorial analysis of variance (ANOVA-2) between different media and isolates followed by the Tukey's HSD test for mean comparison. Median survival time (ST_{50}) estimates were obtained by using standard Kaplan-Meier survivorship analysis, and were subjected to two-way analysis of variance (ANOVA-2) followed by the Tukey's HSD test for mean comparison. Linear regression was also performed for the calculation of growth rate, while correlation procedure was used to find any possible relationship between ST_{50} values, spore bound Pr1 and lipase activity. SAS software (Version 8.02) was used for all statistical analyses. (SAS, 2000)

RESULTS

Germination rate of M. anisopliae *isolates in different nutritional conditions*

Germination rate of spores produced differed significantly among all the nutrient media ($F_{5,36}$ = 302.29; P <0.0001).Germination rate of spores produced from the six media studied differed significantly among the isolates ($F_{2,36}$ = 215.28; P <0.0001). Similarly there was a significant interaction effect involving fungal strains and media had types ($F_{10,36}$ = 18.26; df =10, 36; P <0.0001). In all the three isolates conidia obtained from high C/N medium had the highest germination ability of 80.93, 73.87 and 76.86% for M408, M440 and M460, respectively. Conidia produced by OSM showed the lowest germination rate for all the three isolates. M408 showed overall higher rates of germination when compared to other isolates (Fig.1).

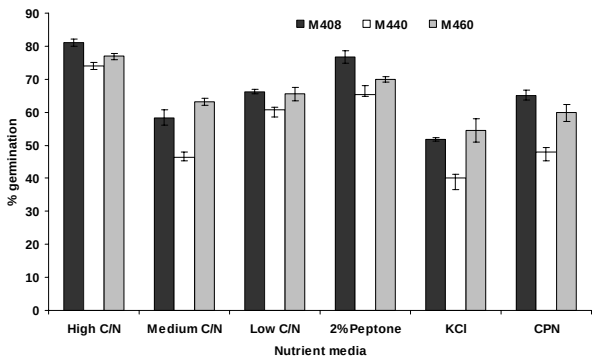

Fig. 1. Percentage germination of three *M.anisopliae* isolates under different nutritional conditions.*Bar lines shows Standard error of means.
For composition of media see Materials and Methods – Culture conditions.

Radial growth and sporulation of M. anisopliae *isolates in different nutritional conditions*

The main effect of growth medium on radial growth/day was significant ($F_{5,36}$ = 29.43; P <0.0001) *M. anisopliae* isolates also showed a significant effect on varied significantly on radial growth/day ($F_{2,36}$ = 106.91; P <0.0001) as was the interaction of two variables ($F_{10,36}$ = 2.56; P <0.0001). Average colony growth on various media showed broadest (from 2.07 to 4.17 mm/day) and narrowest (1.97 to 3.22 mm/day) range for M408 and M440, respectively. For all the isolates OSM showed the lowest growth rate and CPN medium showed the highest vegetative growth having mean values of 3.80, 3.22 and 3.46 mm/day M408, M440 and M460, respectively (Fig. 2).

The number of spores produced by *M. anisopliae* isolates differed significantly among all the nutrient media ($F_{5,36}$ = 15.45; P <0.0001). Production of spores produced from the six media studied differed significantly among all the isolates ($F_{2,36}$ = 70.21; P<0.0001). Similarly a highly significant interaction effect between the different growth media and isolates was also observed for spore production ($F_{5,36}$ = 7.91; P <0.0001). Highest conidial yield for all the isolates was obtained from high C/N medium having mean values of having mean values of 3.97, 3.4 and 3.31 $\times 10^7$ conidia mL^{-1}, for M408, M440 and M460, respectively. The lowest conidial yield was produced by 2% peptone medium for all the isolates used (Fig. 2).

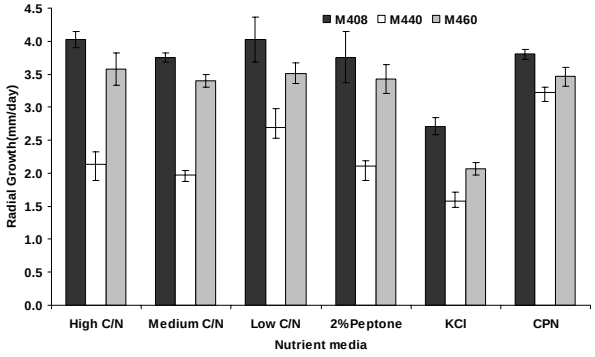

Fig.-2. Radial growth (mm/day) of three *M.anisopliae* isolates under different nutritional conditions.*Bar lines shows Standard error of means.
For composition of media see Materials and Methods – Culture conditions**.**

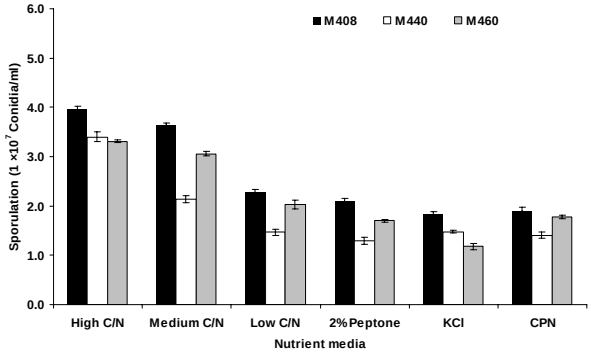

Fig.-3. Conidia production (1×10^7 conidia/mL) of three *M. anisopliae* isolates under different nutritional conditions. *Bar lines shows Standard error of means.
For composition of media see Materials and Methods – Culture conditions**.**

Effect of nutrition on lipase activity

There were significant differences among various nutrition media when compared for lipase activity ($F_{5,36}$ = 25.32; P <0.0001).Lipase activity of *I. fumosoroseus* isolates varied significantly on different media within each isolate ($F_{2,36}$ = 110.46; P <0.0001). Interaction effects between the different growth media and isolates also proved to be significantly different when compared for radial growth/day ($F_{10,36}$ = 41.16; P <0.0001). The highest lipase activities for all the isolates was observed for spores produced by medium-C/N (35:1) media showing mean values of 3.63, 3.21 and 3.24 µmol/ml/min, respectively for M408, M440 and M460. For M408 lowest lipase activity (2.98µmol/mL/min) was observed from the spores yielded by OSM. For Isolates M440 and M460 lowest lipase activity was observed from spores produced by 2% peptone medium (Fig. 4).

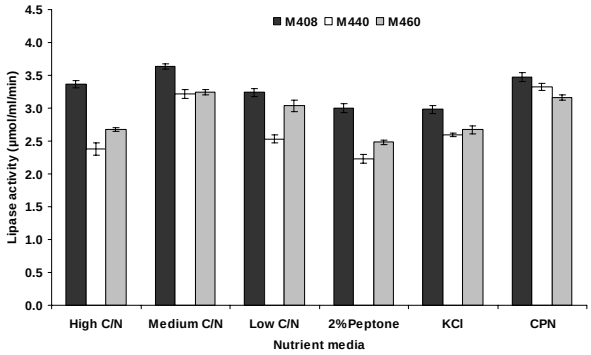

Fig. 4. Spore bound lipase activity (µmol/ml/min) of three *M.anisopliae* isolates under different nutritional conditions.*Bar lines shows Standard error of means
For composition of media see Materials and Methods – Culture conditions.

Effect of nutrition on Pr1 activity

Pr1 activity of spores produced differed significantly among all the nutrient media ($F_{5,36}$ = 58.62; P <0.0001).Germination rate of spores produced from the six media studied differed significantly among the isolates ($F_{2,36}$ = 25.77; P <0.0001). Similarly there was a significant interaction effect involving fungal strains and media types ($F_{10,36}$ = 15.27; P <0.0001). CPN medium proved to be the most active inducer of Pr1 activity showing the highest Pr1 activity for all the isolates whereas lowest Pr1 activity was observed from the spores produced by OSM. Lower spore bound Pr1 activity was observed for M460 when compared to other isolates (Fig. 5).

Virulence of inoculum from different culture media:

There were significant differences in virulence to 2nd instar *P. xylostella* larvae associated with the type of growth medium from which the spores had been obtained (F = 87.02; df =5; P <0.0001). Virulence of *M. anisopliae* isolates varied significantly on different media within each isolate

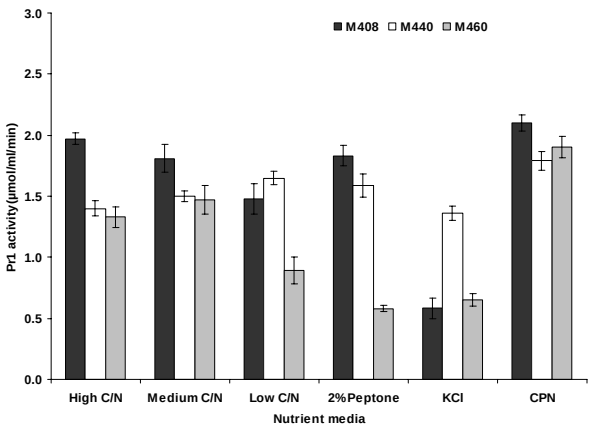

Fig5. Spore bound Pr1 activity (µmol/ml/min) of three *M.anisopliae* isolates under different nutritional conditions.*Bar lines shows Standard error of means.
For composition of media see Materials and Methods – Culture conditions.

(F =87.02; df =2; P <0.0001). Interaction effects between the different growth media and isolates also proved to be significantly different when compared for virulence against *P. xylostella* larvae (F =28.90; df =10; P <0.0001). Conidia from nutrient poor media (2% peptone) proved to be the least virulent for all the three isolates with ST_{50} values of 1.89, 1.89 and 1.81 days for M408, M440 and M460 respectively. Conidia produced by Medium-C/N (35:1) media proved to be the most aggressive inoculum for all the isolates showing ST_{50} values of 1.45 , 1.72 and 1.12 days, respectively for M408, M440 and M460, respectively Isolate M460 was most virulent against 2nd instar *P. xylostella* larvae when compared to other two isolates (Table I).

Correlation between virulence and spore bound lipase and Pr1 activity of M. anisopliae *isolates*

Data regarding the any relationship between the virulence and spore bound Pr1 and lipase activity has been presented in the form of a correlation matrix (Table II). The analysis of correlation yielded a correlation value of 0.42 (P =0.0145) between ST_{50} and spore bound Pr1 activity similarly a positive correlation effect (0.67) was observed between ST_{50} values and spore bound lipase activity (P <0.0001). A positive correlation (0.51) was also observed between spore bound Pr1 and lipase activities on different nutrient media.

Table I.- Mean virulence (± SE; n = 3) of three *Metarhizium anisopliae* isolates from various culture media.

Medium[1]	ST_{50} of *M. anisopliae* isolates (days)		
	M408	M440	M460
High C/N	1.54 ±0.14 de	1.65 ±0.09 cd	1.85 ±0.37 b
Medium C/N	1.45 ±0.09 e	1.62 ± 0.01 cd	1.72±0.04 c
Low C/N	1.50 ± 0.17 de	1.67 ±0.03 cd	1.84 ±0.05 b
2% peptone	1.89 ±0.26 b	1.89 ±0.03 b	1.81 ±0.07 bc
KCl	1.84 ±0.08 b	2.03 ±0.34a	1.66 ±0.21 d
CPN	1.67 ±0.12 cd	1.83 ±0.11b	1.93 ±0.07 ab

Mean followed by different letters are significantly different from each other (Tukey's HSD test: P<0.05)
[1]For composition of media see Materials and Methods – Culture conditions**.**

DISCUSSION

Germination rate is an important virulence determinant factor that can be affected by nutritional conditions (Fragues *et al.,* 2001). Conidia originated from media having medium C/N ration had the highest germination speed all the isolates of *M. anisoplia*, Comparatively higher rates of germination were observed on media having medium to low ratios of carbon and nitrogen (Fig.1). Lane *et al.* (1991) found that *Beauveria bassiana* blastospores produced on nitrogen limited media also accumulated higher concentations of lipids and glycogen but had an enhanced, rather than lessened, germination rate on leafhoppers (*Nephottetix virescens*) wings. These findings reveal that the fungal environment and/or fungal organism can interact with the culture conditions and strongly influence the germination rate of blastospores and conidia. Additional experiments are needed to determine whether these increased proteinaceous reserves are actually involved in enhancing the germination rate of blastospores of *M. anisopliae* and if they are, how they carry out this function.

Radial growth of fungal strains changes not only with fungal species and isolates but also with the C/N ratio of the media used for production of conidia (Safavi *et al.,* 2007). All the *M. anisopliae* isolates used in this study showed maximum colony growth rate for chitin peptone nutrition medium but conidial yield was lowest in case of this media for all the isolates. Therefore, colony conidial yield is

not definitively correlated with colony growth rate. High conidial yield in case of all three *M. anisopliae* was observed for high C/N medium although maximum colony growth rate was not observed for this media. Similar results have been produced in some isolates of *B. bassiana* and *M. anisopliae* using potato dextrose agar (PDA; C/N ratio 10:1) medium (Wyss *et al.,* 2001). Also, a liquid medium with C/N ratio of 10: 1 resulted in the maximum sporulation in the entomopathogens *B. bassiana, M. anisopliae* and *Paecilomyces fumosoroseus* (Vega *et al.,* 2003). Likewise, evaluation of 33 carbon sources on 11 fungal biocontrol agents revealed their role in spore germination, hyphal growth and sporulation (Sun and Liu, 2006).

The presence of proteins, chitin, lipids and waxes on insect cuticle would require a sequential action of appropriate hydrolytic enzymes. These enzymes would facilitate the early stages of fungal infection. Additionally some of these catabolic enzymes may be important in the invasion of fungi into the haemocoel or body cavity. Cuticle-degrading Pr1 enzyme and lipases have been determined as a pathogenicity determinant in most entomopathogenous hyphomycetes, with an established role in virulence towards insect hosts (St. Leger *et al.,* 1988, 1996; Goettel *et al.,* 1989) and it has been suggested that Pr1 enzyme releases peptides that induce further Pr1 production (Paterson *et al*., 1994a). The presence of Pr1 in the conidial cell wall shows that this enzyme is secreted during conidiation; the level of activity appeared to be correlated with the amount of transcripts in the cell (St. Leger *et al.,* 1996). Analysis of spore-bound Pr1 and lipase activity data with ST_{50} values to investigate any relationship between them revealed some interesting results. ST_{50} values for different nutrient media proved to be positively correlated with spore bound Pr1 and lipase activity having correlation coefficient values of 0.42 and 0.67 respectively. Although Pr1 activity in isolate M460, except in CPN and high C/N, was lower than for two other *M. anisopliae* isolates, ST_{50} values for this isolate were remarkably low, except on 2% peptone medium, than in M408 and M460, indicating that cell-wall Pr1 is not the only virulence determinant in *M. anisopilae.* A possible reason for such enzymatic activity pattern can be the repression of carbon and nitrogen previously suggested for *M. anisopliae* (St. Leger *et al*., 1988; Paterson *et al.,* 1994a, b; Screen *et al*., 1997, 1998).

Our study has helped to identify specific factors (germination, growth, conidia production, lipase and Pr1 activity) which can help in the development of inexpensive media for the mass production of virulent inoculum. However, further work is needed to determine the relationship between endogenous reserves and the desired attributes of virulence of fungal pathogens.

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