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Nutrition influences growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae*

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Abstract

Nutrition influenced growth, sporulation and virulence of the insect pathogenic fungus, *Metarhizium anisopliae*. Virulent conidia were produced on susceptible insect hosts, 1% yeast extract, 2% peptone, osmotic stress medium (OSM) and CN 10:1 medium. Several strain independent markers were identified that could be used to predict the virulence of *M. anisopliae* conidia. Virulent conidia typically had high levels of spore bound Pr1, an important cuticle degrading protease, and high germination rates. We also show for the first time that virulent conidia have an endogenous CN ratio below 5.2:1. Real time PCR revealed that virulent conidia from insects contained significantly higher levels of transcripts of *pr1 A* and other pathogenicity-related genes than inoculum from artificial media. Of the artificial media studied, 1% yeast extract medium yielded the most virulent conidia, these had higher levels of transcripts of these pathogenicity-related genes than the least virulent conidia from the high conidia yielding CN 35:1 medium (=SDA), however, the levels were significantly lower than those in insect-derived conidia. Our study shows for the first time that the passaged inoculum is virulent irrespective of the original culture medium or insect host. Virulent conidia were consistently produced on OSM even though growth and sporulation were poor. We postulate that starvation conditions, whether in vivo or in vitro, results in de-repression of Pr1 and that elevated levels of this enzyme enhance fungal virulence.

Keywords: *Metarhizium*; Nutrition; Virulence; Markers; Pr1; CN ratio; Starvation

1. Introduction

Conidia constitute the infective unit of insect-pathogenic fungi like *Metarhizium anisopliae* [1]. Inoculum produced on naturally infected arthropods is usually highly infective to susceptible hosts whereas that produced on artificial media can lose virulence [1]. Exactly why fungi become attenuated is unclear. For *M. anisopliae,* and other fungal biocontrol agents (BCAs), to be commercially viable, mass production has to be done on artificial substrates. This requires an understanding of the relationship between nutrition and virulence, which currently remains obscure. Nutritional studies to date reveal tenuous links between virulence and spore endogenous reserves [2] and surface carbohydrates [3]. Specific conidial traits have been identified which are considered to be good indicators of virulence including spore size, adhesion, and germination speed [4–7]. However, these traits are peculiar to some species or strains of fungi and, consequently, have limited value for quality control (i.e., to ensure the inoculum is virulent) of insect pathogenic fungi in general. In contrast, all

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entomogenous fungi are dependent on the production of cuticle-degrading enzymes (lipases, chitinases, proteases) to help penetrate the host cuticle [1]. One much studied protease, Pr1, is an important virulence determinant which is induced by insect cuticle, de-repressed under starvation conditions and repressed in the presence of excess nutrients [8,9].

Fungal pathogenicity is not determined by one single factor but is dependent on a coordinated interplay between many, disparate pathogenicity determinants. This study provides the first detailed analysis of the relationship between nutrition and virulence of conidia produced in vitro and in vivo. Most studies usually focus on inoculum produced on artificial media and describe its influence on either fungal growth or virulence. In this study, we have tried to address the influence of nutrition on both growth and virulence determinants of *M. anisopliae*. Whilst optimising nutritional conditions for the mass production of highly virulent conidia, it is important not to compromise inoculum yield. Our study shows that nutrition influences the carbon and nitrogen composition of conidia, germination rate and levels of spore bound Pr1 and that these parameters can to some extent predict the virulence of the inoculum. The significance of these findings as regards the production and quality control of inoculum in commercial systems is discussed.

2. Materials and methods

2.1. Fungi

Two isolates of *M. anisopliae* V245 and V275 from Finnish soil and *Cydia pomonella*, respectively, were passaged through *Galleria melonella* larvae and isolated on Oatmeal dodine agar. Single spore colonies were transferred to Sabouraud Dextrose Agar (SDA) and these 1st subcultures were used in subsequent studies.

2.2. Culture media

Culture media representing disparate carbon and nitrogen sources and ratios were used in this study. They included: (1) potato Dextrose Agar (PDA); (2) high C:N (75:1) medium consisting of 9.1% glucose and 1% peptone; (3) low C:N (10:1) medium consisting of 0.6% glucose and 1% peptone; (4) intermediate C:N (35:1) medium consisting of 4% glucose and 1% peptone (=SDA); (5) nutrient poor media consisting of either 2% peptone (2P) or 1% yeast extract (1Y); (6) ''osmotic stress'' medium (OSM) consisting of 8% glucose, 2% peptone, 5.5% KCl. Yeast extract, peptone and PDA have CN ratios of 3.6:1, 8:1 and 10:1, respectively, and represented different carbon and nitrogen sources [10,11]. All the media were prepared using 2% agar except the OSM, which required 5.5% agar to solidify. Media were sterilized at 121 °C at 15 psi for 15 min and 15 ml poured into 9 cm diameter Petri dishes. Glucose, yeast extract and KCl were obtained from Sigma, while mycological peptone, agar and PDA were obtained from Difco.

2.3. Monitoring growth and sporulation

To see if there was any link with growth rate, sporulation and inoculum virulence, the above media were inoculated with a 2 mm diameter mycelial plug taken from the growing edge of a 12-day old culture grown on SDA at 25 °C in the dark. Inoculated Petri dishes were sealed in polythene bags and incubated at 25 °C in the dark. The colony diameter was measured at right angles at 3 days intervals until 15 days post-inoculation and radial growth ($mm\,d^{-1}$) calculated from the linear portions of the curves plotted from these values. Conidia from each plate were harvested at 15 days post-inoculation by scrapping with 0.05% to ensure maximum conidial harvesting. Conidial yield was determined by suspending the conidia from the whole colony in 50 ml of 0.05% aq. (v/v) Tween 80, and counting the number of spores using an improved Neubauer haemocytometer (Weber Scientific International Ltd., UK).

2.4. Determining virulence of inoculum produced on different culture media

Conidia were assayed against 4–5th instar larvae of *Tenebrio molitor*. Larvae (batch of 10) were immersed in 10 ml conidial (1×10^7 conidia/ml) suspension or 0.03% v/v aq. Tween carrier (control) for ca. 30 s and the excess moisture removed by filtering over a vacuum in a Buchner funnel. Batches of 10 larvae were incubated without food in 9 cm diameter Petri dishes lined with moist Whatman No. 1 filter paper and incubated at 25 °C in the dark. Each treatment was replicated three times with 10 larvae per replicate. Mortality was recorded daily and dead insects were transferred to Petri dishes lined with moist filter paper to encourage external sporulation of the fungus if present.

2.5. Carbon–nitrogen (CN) analysis of conidia produced on different media

Conidial CN elemental composition was determined using an automated nitrogen carbon analysis for gas solids and liquids (ANCA GSL) elemental analyser (Europa, UK) interfaced with a PDZ Europa 20/20 mass spectrometer. Conidia (1 mg) from the disparate media were wrapped in 6×4 mm tin foil discs (Elemental Microanalysis Ltd., UK) which were pre washed with acetone, stored in sealed tubes and kept

in airtight desiccators until required. Isoleucine (Sigma) ranging from 25 to 150 μg was used as a standard. Discs without conidia were used as a negative control. Each treatment was replicated and the whole procedure repeated two times.

2.6. Germination assays

The germination speed of inoculum from the different media was assessed by inoculating SDA with 10 μl conidial suspension (1×10^6 conidia/ml) then counting the number of germlings following 8 h incubation at 25 °C. Conidia with germ tubes equal to or greater than the width were considered to have germinated. For each treatment, three separate fields were observed for germination at 40× and 100 conidia observed randomly in each field.

2.7. Influence of nutrition on the activity of Pr1 and other enzymes

Pr1 bound to conidia harvested from the disparate media was quantified using the method described by St. Leger et al. [12]. Briefly, 10 mg of conidia were incubated in 1 ml of 0.1 M Tris–HCl (pH 7.95) containing 1 mM Succinyl-Ala-ala-Pro–Phe-*p*-nitroanilide (Sigma) for 5 min at room temperature. After incubation the conidia were clarified by centrifugation at 12,000*g* (in Sanyo, Harrier 18/80 centrifuge) for 5 min. The supernatant (200 μl) was transferred to wells of a microtitre plate (Dynatec) and absorbency measured using a Lab System spectrophotmeter at 405 nm . Buffered substrate was used as control.

2.8. Real time PCR to quantify pathogenicity-related gene expression in conidia from selected media

Insect passaged as well as conidia from intermediate C:N (35:1) and 1% yeast extract media were selected for more detailed analysis of the expression of *pr1 A* and three other genes (*ste1* – esterase, *try 1* – trypsin, *chy 1* – chymotrypsin) linked to the infection process [13]. Total RNA was extracted from the 1 mg conidia of both isolates using the Qiagen RNA-easy kit (Valencia, CA) with the DNA being removed using DNase I (Qiagen). The quantity and quality of RNA was assessed on a 1.2% formaldehyde agarose gel. The RNA (1 μg) from each sample was used to generate cDNA using the *Reverse* iT™ 1st strand synthesis kit (AB gene, UK) according to the manufacturer's protocol. The primers used for the target and internal control (small sub unit ribosomal) genes were designed using the program Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) and obtained from MWG-Biotech (Germany). The primer–sequence and annealing temperatures for these genes were as described in Table 1.

Real time quantitative PCR amplification was carried out in an I cycler (Biorad). The reaction mixture (20 μl) consisted of 2 × PCR buffer containing SYBER-Green; 100 mM KCl, 40 mM Tris–HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase, 50 U/ml, 6 mM $MgCl_2$, SYBER Green 1, 20 nM fluorescein, stabilizers, 50 ng cDNA template and 100 nM of the appropriate primer. The PCR conditions were 95 °C for 4 min; followed by 42 cycles of 95 °C for 20 s and annealing temperature according to the target gene (see above) for 30 s, one cycle at 95 °C and annealing temperature each lasting for 30 s, followed by 40 cycles of annealing temperature with each cycle lasting for 10 s. The fluorescent spectra were recorded during the elongation phase of each PCR cycle. Standard curves were also generated for house keeping and target genes in a serial dilution using V275 cDNA. Relative gene expression ratios were calculated by calibration with house keeping ribosomal gene using the iCycler iQ system software and fold changes in comparison to the gene expression in conidia from CN 35:1 media were determined.

Table 1
List of primer–sequence and annealing temperatures used for the quantitative Real time PCR of the pathogenicity-related genes

Gene	Primer	Sequence	Annealing temperature (°C)
Protease *(pr1A)*	Pr1A U	CAC TCT TCT CCC AGC CGT TC	56
	Pr1A L	TCG GCT TTG GAG GTA AGA GC	
Chymotrypsin (*chy1*)	Chy U	AGA TCC TCC TTG GCC TTT TC	59
	Chy L	GTT CGC TGG TGC TTG GAT TG	
Esterase (*ste 1*)	Ste1 U	TCT ACC ACG TTC TTC TCG CC	60
	Ste 1 L	GGC CCA GGT CCA AGG CTA CT	
Trypsin (*try 1*)	Try U	GCT GAC GAT GAA GGG GAA T	56
	Try L	GCT CTT TAT CTG CCC CTT TG	
SSU (rDNA) internal control	SRT1	CGA AAC TGC GAA TGG CTC A	Similar to that of target gene
	SRT2	CCG AAG TCG GGA TTT TTA GC	

2.9. Effect of host passage on virulence and its determinants

In order to confirm the above findings and to prove that host passage or growth on nutrient poor media enhanced virulence, conidia produced either on intermediate CN (representing low virulence) and those produced on 1% yeast extract (representing high virulence) were passed through 4th or 5th instar larvae of *Galleria mellonela* and *T. molitor*. Two different insect species were used to see if the host influenced the attributes of the inoculum (i.e., CN content, virulence). All the dead insects were transferred to moist chambers to allow fungal emergence and sporulation. Conidia were harvested from cadavers and washed with distilled water to remove any traces of insect. This inoculum was used in the following studies: bioassays to determine virulence, CN analysis, germination, determination of spore bound Pr1 and expression of *pr1 A*, *ste 1*, *try 1*, *chy 1* genes as described above.

2.10. Statistical analysis

The whole study was repeated two times with each treatment replicated three times unless stated otherwise. Data was subjected to one-way ANOVA followed by the Tukey test. Wherever required, linear regression for the calculation of growth rate/day, LT_{50}, and enzyme activities was also done. For all statistical analysis SPSS 11 software was used.

3. Results

3.1. Growth of M. anisopliae on different media

Vegetative growth of *M. anisopliae* varied significantly ($P < 0.05$) on the different media ranging from 1.4 to 6.3 mm/day for V245 and 1.7–6.9 mm/day for V275, respectively (Tables 2 and 3). For both isolates, radial growth was highest on media with C:N ratio of 35 and 75:1 and was least on the high osmolarity medium (Tables 2 and 3). Conidial production of *M. anisopliae* varied significantly ($P < 0.05$) on the different media with highest yield being observed in CN 35:1 media and least in OSM (Tables 2 and 3).

3.2. Virulence of M. anisopliae conidia produced on different media

Nutrition influenced conidial virulence (Tables 2 and 3). The most virulent conidia of V245 and V275 were

Table 2
Effect of nutrition (media composition) on the growth and virulence attributes of *M. anisopliae* V245

Media	Radial growth (mm/day)	Conidial yield ($\times 10^9$)	Conidial C:N	Germination (%)	Spore bound Pr1 (μmol/ml/min)[A]	Virulence LT_{50} (days post-inoculation)
PDA	4.19^{b}	0.7^{b}	7.12^{a}	54.33^{c}	1.33^{c}	3.9^{b}
1% Yeast extract	4.51^{b}	1.6^{a}	4.70^{b}	74.66^{b}	2.92^{a}	3.72^{c}
2% Peptone	4.04^{b}	0.9^{b}	4.82^{b}	78.66^{ab}	1.49^{bc}	3.70^{c}
C:N 10:1	4.82^{b}	0.8^{b}	4.87^{b}	69.0^{b}	3.27^{a}	3.63^{cd}
C:N 35:1	6.3^{a}	1.6^{a}	6.35^{a}	47.0^{c}	0.70^{d}	4.12^{a}
C:N 75:1	5.55^{b}	0.5^{b}	7.06^{a}	67.0^{b}	0.81^{d}	3.66^{cd}
KCl	1.44^{c}	0.3^{cB}	6.49^{a}	89.33^{a}	1.79^{b}	3.51^{d}

The results are representative of two similar experiments. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey test).
[A] Pr1 activity is expressed as μmol NA ml^{-1} min^{-1} released from succinyl-$(Ala)_2$-Pro-Phe-NA.
[B] Conidial concentration is $\times 10^8$ for this treatment.

Table 3
Effect of nutrition (media composition) on the growth and virulence attributes of *M. anisopliae* V275

Media	Radial growth (mm/day)	Conidial yield ($\times 10^9$)	Conidial C:N	Germination (%)	Spore bound Pr1 (μmol/ml/min)[A]	Virulence LT_{50} (days post-inoculation)
PDA	4.1^{c}	1.5^{b}	7.1^{e}	32.6^{c}	1.5^{c}	3.93^{a}
1% Yeast extract	4.5^{c}	1.3^{bc}	4.0^{a}	93.0^{a}	3.2^{a}	3.46^{d}
2% Peptone	3.6^{c}	1.3^{bc}	4.3^{b}	87.0^{a}	3.1^{a}	3.58^{cd}
C:N 10:1	4.9^{b}	0.3^{cd}	5.1^{c}	93.0^{a}	2.5^{b}	3.66^{bc}
C:N 35:1	6.9^{a}	5.3^{a}	6.8^{d}	44.6^{b}	1.5^{c}	3.86^{ab}
C:N 75:1	6.3^{a}	1.0^{bcd}	7.3^{f}	84.3^{a}	1.5^{c}	3.89^{a}
KCl	1.7^{d}	0.9^{dB}	6.6^{d}	83.6^{a}	1.1^{d}	3.49^{cd}

The results are representative of two similar experiments. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey test).
[A] Pr1 activity is expressed as μmol NA ml^{-1} min^{-1} released from succinyl-$(Ala)_2$-Pro-Phe-NA.
[B] Conidial concentration is $\times 10^8$ for this treatment.

produced on the OSM and/or 1% yeast extract media whilst the least virulent conidia were produced on PDA and/or C:N 35:1 (Tables 2 and 3). Conidia of V275 were marginally more virulent than those of V245 when produced on the same medium (Tables 2 and 3).

3.3. Endogenous carbon–nitrogen (CN) analysis of conidia produced on different media

The CN ratio of the conidia produced on different media varied significantly ($P < 0.05$) between treatments. V245 and V275 conidia had similar CN profiles. For example, the CN ratios were significantly higher in conidia produced on C:N 35:1, PDA, C:N 75:1 and OSM but were comparatively lower in 1% yeast extract, 2% peptone and C:N 10:1 (Tables 2 and 3). The highest CN ratios were in PDA and C:N 75:1 and the least in 1% yeast extract.

3.4. Germination assays

Germination speed of conidia produced on different media varied significantly ($P < 0.05$) between treatments and between the two isolates (Tables 2 and 3). However, the influence of media on germination was strain dependant. At 8 h post-inoculation significant germination was observed for V275 but ranged between 2% and 10% for V245, therefore V245 germination was assessed at 10 hpi. The germination rate for both isolates was lowest on PDA and C:N 35:1 (Tables 2 and 3). The highest germination rates for V275 (>90%) were noted for conidia produced on 1% yeast extract and C:N 10:1, while those of V245 (89%) were noted for conidia produced on OSM (Tables 2 and 3).

3.5. Influence of nutrition on the activity of Pr1 and other enzymes

Spore bound Pr1 varied significantly ($P < 0.05$) depending upon the medium on which conidia were produced (Tables 2 and 3). Highest activities for both isolates were observed for conidia produced on 1% yeast extract and C:N 10:1. V275 also appeared to have more spore bound Pr1 when produced on 2% peptone. V275 generally produced more Pr1 than V245 except for conidia produced on the C:N 10:1 and OSM (Tables 2 and 3).

3.6. Effect of host passage on virulence

Conidia produced on *T. molitor* and *G. mellonella* as well as 1% yeast extract media were significantly more virulent than those produced on intermediate C:N media (Table 4). Conidia of *M. anisopliae* V245 recovered from *T. molitor* were significantly more virulent than that produced on either 1% yeast extract or intermediate CN media (Table 4). Conidia of V275 recovered from insects did not significantly differ from inoculum produced on 1% yeast extract media but differed significantly with those produced on intermediate CN media (Table 4). No significant differences in virulence were observed for each strain whether they were passaged through *Galleria* or *Tenebrio* (Table 4).

3.7. Effect of host passage on conidial carbon and nitrogen ratio

The CN ratio of conidia of both V245 and V275 recovered from mycosed cadavers (i.e., passaged inoculum) was significantly lower than conidia produced on media with intermediate CN but varied non-significantly with conidia produced on 1% yeast extract (Table 4).

3.8. Effect of host passage on conidia germination

Conidia of V245 and V275 recovered from mycosed insects germinated the fastest (Table 4). V275 produced on 1% yeast extract medium was equally fast whereas that of V245 germinated more slowly (Table 4). However, the slowest germination was recorded for conidia

Table 4
Effect of host passage on the conidial C:N, germination, spore bound Pr1 and virulence of *M. anisopliae*

Source of conidia	Conidial C:N		Germination (%)		Spore bound Pr1 (μmol/ml/min)[A]		Virulence LT_{50} (days post-inoculation)	
	V245	V275	V245	V275	V245	V275	V245	V275
C:N 35:1	6.35^{a}	6.84^{a}	48.66^{c}	47.33^{b}	1.08^{e}	1.67^{d}	4.22^{a}	3.96^{a}
1% Yeast extract	4.47^{bc}	4.05^{c}	80.33^{b}	93.33^{a}	1.94^{c}	2.43^{b}	3.65^{b}	3.47^{b}
C:N 35:1 passed through *Galleria mellonella* larvae	5.12^{b}	4.66^{bc}	91.66^{a}	91.0^{a}	2.64^{a}	2.69^{a}	3.56^{bc}	3.40^{b}
1% Yeast extract passed through *G. mellonella* larvae	5.08^{b}	4.64^{bc}	93.0^{a}	92.33^{a}	2.71^{a}	2.59^{ab}	3.61^{bc}	3.43^{b}
C:N 35:1 passed through *Tenebrio molitor* larvae	4.92^{b}	4.47^{bc}	92.66^{a}	93.33^{a}	2.75^{a}	2.61^{ab}	3.44^{c}	3.44^{b}
1% Yeast extract passed through *T. molitor* larvae	5.15^{bc}	4.38^{bc}	92.0^{a}	94.0^{a}	2.75^{a}	2.75^{a}	3.42^{c}	3.32^{b}

The results are representative of two similar experiments. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey test).

[A] Pr1 activity is expressed as μmol NA ml^{-1} min^{-1} released from succinyl-$(Ala)_2$-Pro-Phe-NA.

Table 5
Fold changes in the expression of pathogenicity-related genes encoding cuticle-degrading enzymes in un-germinated conidia of *M. anisopliae*

Source of conidia	Fold changes in gene expression							
	pr1 A		*try 1*		*chy 1*		*ste 1*	
	V245	V275	V245	V275	V245	V275	V245	V275
1% Yeast extract	2^{b}	6^{b}	2.5^{b}	1^{c}	1.1^{d}	1^{d}	-1.2^{c}	-1.1^{c}
C:N 35:1 passed through *Galleria mellonella* larvae	20^{a}	17^{a}	$>100^{a}$	$>100^{aa}$	13^{bc}	15^{ab}	1.2^{b}	3^{ab}
1% Yeast extract passed through *G. mellonella* larvae	34^{a}	26^{a}	$>100^{a}$	$>100^{a}$	12^{bc}	12^{abc}	5^{a}	2^{b}
C:N 35:1 passed through *Tenebrio molitor* larvae	20^{a}	18^{a}	$>100^{a}$	99^{ab}	10^{c}	17^{a}	2^{ab}	3^{ab}
1% Yeast extract passed through *T. molitor* larvae	35^{a}	23^{a}	$>100^{a}$	84^{ab}	18^{abc}	6^{c}	2^{ab}	1^{b}

Fold changes are expressed in comparison with gene expression levels observed in ungerminated conidia produced on CN 35:1 media. The results are representative of two similar experiments. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey test).

of V245 and V275 produced on intermediate CN media (Table 4).

3.9. Effect of host passage on spore bound Pr1

Spore bound Pr1 was significantly higher in conidia produced on mycosed cadavers than conidia produced on artificial media (Table 4). Spore-bound Pr1 production was significantly different for conidia of the two strains produced on artificial media. Conidia of V275 produced on 1% yeast extract had similar Pr1 production to the passaged inoculum whereas that of V245 exhibited slightly less Pr1 production (Table 4). Least spore bound Pr1 production was observed in conidia produced on intermediate CN media (Table 4).

3.10. Real time PCR analysis of cuticle-degrading enzymes in conidia

Expression of *pr1 A*, *ste 1*, *try 1* and *chy 1* genes was significantly higher in conidia produced on mycosed cadavers than conidia produced on artificial media irrespective of the strain or insect host or original medium on which the fungi were grown (Table 5). Conidia produced on 1% yeast extract media had significantly higher expression of *pr1 A* than conidia produced on intermediate CN media. For all other genes, these two treatments varied non-significantly with each other (Table 5).

4. Discussion

Conidial yield of *M. anisopliae* is dependent upon the fungal strain and nutrition and does not appear to be linked with radial growth as an increase in radial growth did not resulted in simultaneous increase in conidial yield. Maximum yields, for both strains were achieved in a 35:1 CN medium similar to SDA. Similarly, the mycoherbicide, *Colletotrichum truncatum*, produced the highest yields in 30:1 CN medium and not at higher or lower CN ratios [14]. In contrast, highest yields were observed for *Beauveria bassiana* and *M. anisopliae* in PDA which has a CN content of 10:1 [11,15]. Similarly, spore production of *M. anisopliae*, *B. bassiana* and *Paecilomyces fumosoroseus* was highest in a broth medium with CN ratio of 10:1 [16]. Although there is a tendency for more fungal spores to be produced on carbon rich media, the threshold varies with species and the nature of the CN source [11,17].

Nutrition influenced not only spore production but also spore quality. Germination and virulence of V245 and V275 were poor if produced on CN 35:1 but exactly why germination should be better at CN ratios higher and lower than 35:1 is unclear. Conidia of *C. truncatum* also germinated more rapidly and were more virulent if produced in CN 10:1 media [14]. The rapid germination was linked to the relatively high protein content of this inoculum [14]. In our study, we did not discriminate between the different endogenous reserves but noted that virulent conidia of *M. anisopliae* had relatively low CN ratios and high germination rates. It is possible that the high nitrogen levels in *M. anisopliae* correspond to storage and structural (e.g., cytoskeletal, membrane) proteins as well as enzymes that facilitate rapid germination. Conidia produced in OSM were an exception; their rapid germination may have been due to other reasons, such as high polyol content [2,18].

The most aggressive inocula of *M. anisopliae* V245 and V275 were produced on insect hosts, OSM, 2% peptone and 1% yeast extract. These disparate substrates probably require different pathways for the utilisation of nutrients and subsequently regulation of sporulation and virulence genes. Indeed, earlier studies show that nitrogen compounds that support growth were less favourable for spore germination and, since different amino acids stimulated particular stages of growth and sporulation, a complex nitrogen source was required to optimise these processes [19]. Presumably, yeast extract, peptone and the insects used in our studies possess nutritional components not present in simple, traditional, inexpensive mycological media. We postulate that starvation conditions whether in vivo or in vitro

results in de-repression of Pr1 and that elevated levels of this enzyme enhances fungal virulence. Pr1 is without doubt an important virulence determinant. Mutants lacking the *pr1* gene are less pathogenic and those over-expressing Pr1 are hypervirulent [9,12]. Nutrient deprivation triggers increased Pr1 transcription and rapid secretion of this enzyme [12,20]. Furthermore, Pr1 production far exceeds synthesis of other proteins when *M. anisopliae* differentiates infection structures [21]. It is tempting to speculate that conidia with low levels of endogenous reserves would rapidly exhaust these reserves and starvation would induce Pr1 and other virulence determinants. This may explain why conidia from mycosed insects were aggressive since they had low carbon content (ca. 30–50% lower than conidia from artificial media) and subsequently fewer endogenous reserves (e.g., glycogen, lipid).

High levels of transcripts of the pathogenicity-related enzymes in conidia would accelerate production of these enzymes and result in faster germination and infection. The cuticle-degrading enzymes Pr1 and Pr2 have already been shown to release peptides that induce more Pr1 [22]. Presence of cuticle-degrading enzymes in the conidial cell wall suggests that enzymes were secreted during conidiation and the level of activity appeared to be correlated with the amount of transcripts in the cell. Higher levels of enzyme activity were detected on conidia from infected *Manduca sexta* larvae than those from SDA suggesting that environmental conditions in which conidia develop pre-adapts them for the pathogenic life style [12]. Our study shows for the first time that the passaged inoculum is virulent irrespective of the original culture medium or insect host. The insect host clearly provides the nutrition and development cues for production of virulent conidia.

We have shown that CN ratios below 5.2:1 are a good indicator of virulence and recommend it as a tool for quality control, particularly if used with other parameters such as spore bound Pr1 and high germination rates. The virulent conidia produced on OSM were an anomaly since these had CN ratios similar to those of the less virulent conidia produced on CN 35:1. The high salt levels did influence *M. anisopliae* physiology since it resulted in conidia that were paler and less hydrophobic than those produced on the other culture media and infected insects [3]. Again this phenomenon was independent of strain.

Bio-manufacturers of *M. anisopliae* or any other fungal biocontrol agent must take into account several factors when developing inexpensive media for the mass production of these agents. Culture media must not only maximise spore yield but also enhance qualities such as desiccation tolerance, stability as a dry preparation and virulence. Much attention has focused on manipulating nutritional conditions during growth and sporulation towards accumulation of appropriate endogenous reserves so that the newly formed conidia possessed the above qualities [23]. Our study has helped identify specific quality control markers (conidial CN, germination speed and spore bound pr1) that could 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 and prolonged shelf life.

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