

# The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity

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**Ambient pH regulates the expression of virulence genes of *Metarhizium anisopliae*, but it was unknown if *M. anisopliae* can regulate ambient pH. Mutants of *M. anisopliae* altered in production of oxalic acid were evaluated for the interrelationship of ambient pH, buffering capacity added to media, growth, and generation of extracellular proteases and ammonia. Wild-type and acid-overproducing mutants [Acid(+)] grew almost as well at pH 8 as at pH 6, but acid-non-producing [Acid(-)] mutants showed limited growth at pH 8, indicating that acid production is linked to the ability to grow at higher pH. Production of ammonia by *M. anisopliae* was strongly stimulated by low levels of amino acids in the medium when cells were derepressed for nitrogen and carbon. Likewise, although *Aspergillus fumigatus* and *Neurospora crassa* produced some ammonia in minimal media, addition of low levels of amino acids enhanced production. Ammonia production by *A. fumigatus*, *N. crassa* and *M. anisopliae* increased the pH of the medium and allowed production of subtilisin proteases, whose activities are observed only at basic pH. In contrast, protease production by the Acid(+) mutants of *M. anisopliae* was greatly reduced because of the acidification of the medium. This suggests that alkalization by ammonia production is adaptive by facilitating the utilization of proteinaceous nutrients. Collectively, the data imply that ammonia may have functions related to regulation of the microenvironment and that it represents a previously unconsidered virulence factor in diverse fungi with the potential to harm tissues and disturb the host's immune system.**

Keywords: *Metarhizium anisopliae*, *Aspergillus fumigatus*, *Neurospora crassa*, protease production, pH

## INTRODUCTION

*Metarhizium anisopliae* is an insect pathogen that penetrates the proteinaceous cuticular barrier of insects. *Aspergillus fumigatus* invades the proteinaceous lung and mucous membranes and occasionally causes opportunistic systemic infection. Attributes put forth as contributing to the virulence of these fungi include secretory hydrolases, particularly proteinases, that could facilitate penetration of host barriers. Studies of bacterial pathogens and the opportunistic human pathogen *Candida albicans* have shown that many of the genes required for virulence are regulated in response to environmental signals indigenous to the host niche (Mekalanos, 1992). These signals include pH, which regulates the expression of genes essential to the virulence and survival of *C. albicans* during infection

(De Bernardis *et al.*, 1998). Regulation of gene expression by pH also occurs in the opportunistic saprophyte *Aspergillus nidulans* (Caddick *et al.*, 1986) and in *M. anisopliae* (St Leger *et al.*, 1998); in the latter species the genes regulated by pH include the cuticle-degrading subtilisin proteases and other putative virulence factors (St Leger *et al.*, 1998). The corollary, that pathogenic fungi may alter their surrounding microenvironment as a mechanism of adaptation and survival in the host niche, has not been studied extensively. The exception is *C. albicans*, which acidifies media in a carbohydrate-dependent fashion, allowing production of an aspartyl protease (Tsuboi *et al.*, 1989). Insect cuticle infected with *M. anisopliae* demonstrated an increase in pH compared to unaffected cuticles (St Leger *et al.*, 1998). This suggests that *M. anisopliae* produces one or more basic compounds. Base production has not

been extensively studied in fungi. However, acid-neutralizing activity of bacteria and the ciliate *Tetrahymena pyriformis* correlates with production of the alkaline compound ammonia (Takahashi *et al.*, 1997; Larsen *et al.*, 1988) and ammonia is known to have diverse roles in fungal physiology. As a nutrient source, ammonia regulates nitrogen metabolism (Arst & Cove, 1969; ter Schure *et al.*, 1995). As a metabolite, ammonia resulting from protein degradation determines the choice of morphogenetic pathways in *Dictyostelium discoideum* (Schindler & Sussman, 1977; Schaap *et al.*, 1995) and inhibits sporulation in *Coprinus cinereus* and *Saccharomyces cerevisiae* (Moore *et al.*, 1987). Ammonia also mediates communication between yeast colonies by inhibiting growth of the facing parts of colonies (Palkova *et al.*, 1997).

From the context of host-pathogen interactions ammonia is a highly toxic agent, which has been shown to inhibit growth in cell lines (Doyle & Butler, 1990) and is the toxin responsible for immunosuppression, pathology and death of sheep infested with *Lucilia cuprina* larvae (Guerrini, 1997). *Helicobacter pylori*, the major causative agent of human active gastritis, survives the gastric acidity by producing ammonia, which also acts as a toxin (Chen *et al.*, 1997).

Several researchers have examined the relationship between acid production and virulence in fungi, with contrasting results. Thus while mutants were used to demonstrate a role for oxalic acid in pathogenicity of the plant pathogen *Sclerotinia sclerotiorum* on *Phaseolus vulgaris* (Godoy *et al.*, 1990), oxalic acid hyperproduction in mutants of the entomopathogenic fungus *Beauveria bassiana* did not affect virulence (Bidochka & Khachatourians, 1993). Likewise some strains of *M. anisopliae* produce a carbohydrate-dependent acidifying activity but are not altered in pathogenicity compared to acid non-producers (Robert & Al-Aidroos, 1985). In this study we investigated the influence that *M. anisopliae*, *A. fumigatus* and *Neurospora crassa* have on the pH of their environment and how these pH changes influence production of proteases.

## METHODS

**Strains.** *N. crassa* strain 2489 was obtained from the Fungal Genetics Stock Center in the University of Kansas Medical Center, KS, USA. *A. fumigatus* strain 16424 was obtained from the American Type Culture Collection, Manassas, VA, USA. It was isolated from a human patient with aspergillosis. *M. anisopliae* strains ARSEF 2575 (host: pecan weevil, *Curculio caryae*) and ARSEF 549 (host: *Galacica* sp., Lepidoptera) were obtained from the USDA Entomopathogenic Fungus Collection, Ithaca, NY, USA.

**Transfer cultures.** Standardized mycelial inocula (5 g wet wt per 100 ml) from 24 h Sabouraud dextrose broth cultures were incubated with shaking (100 r.p.m.) at 28 °C for 4 h in 20 ml minimal medium (0.02% KH<sub>2</sub>PO<sub>4</sub>, 0.01% MgSO<sub>4</sub>, 0.2 p.p.m. FeSO<sub>4</sub>, 1.0 p.p.m. ZnSO<sub>4</sub>, 0.02 p.p.m. MnMoO<sub>4</sub>, 0.02 p.p.m. CuSO<sub>4</sub> and 0.02 p.p.m. MnCl<sub>2</sub>, pH 6.5) supplemented with either insect cuticle, an amino acid, or an alternative carbon source (see Table 2). After set times, protease production,

ammonia production and pH were measured. To check for non-fungal ammonia release, un-inoculated culture media were incubated with each assay.

To determine if acid-overproducing strains of *M. anisopliae* were also altered in regulation, production or secretion of proteases, some cultures containing 1% insect cuticle were buffered at pH 4 or pH 7 with 0.1 M citric acid/sodium phosphate buffer (St Leger *et al.*, 1998). Insect cuticle was obtained from the giant cockroach (*Blaberus giganteus*) by extracting soft tissue from homogenized insects with sodium tetraborate (St Leger *et al.*, 1994).

**Growth in a nutrient medium.** The basal medium used was 1% yeast extract (Difco). Cultures (100 ml) were inoculated with  $1 \times 10^7$  spores. The pH of the medium was adjusted to 4 or 8 with 1 M HCl or NaOH. All cultures were incubated for 72 h at 28 °C, and ammonia production, pH and growth were measured over this time. In experiments to differentiate between alterations in ammonia formation due to carbon repression and changes in pH, yeast extract medium containing 1% glucose was buffered with 0.1 M citric acid/phosphate buffer at pH 4 or 7.

**Analyses.** Ammonia (NH<sub>3</sub> and NH<sub>4</sub><sup>+</sup>) was assayed by the colorimetric method of Chaney & Marbach (1962) and absorbance readings were measured in a Spectra MAX plus plate reader (Molecular Devices). For calculation of ammonia formed in yeast extract medium, the concentration (1–1.5 mmol l<sup>-1</sup>) in an uninoculated broth was subtracted from that in an inoculated broth of the same type. Dry weight was determined by filtration of 5 ml aliquots of cultures over Millipore filters of constant weight (type AP 40), washing twice with equal volumes of water, and drying at 105 °C. The identities and quantities of organic acids in spent culture media were determined by capillary electrophoresis with a Bio-Rad BioFocus 3000, employing indirect detection at 230 nm. The background electrolyte was sodium phthalate (5 mM) with cetyltrimethylammonium bromide (0.5 mM) as the osmotic flow modifier; it was prepared daily, adjusted to pH 5.6 and filtered before use. The capillary was a 50 cm × 50 µm i.d. bare silica which had been purged with 0.1 M NaOH, washed with deionized water, then purged with background electrolyte. Background electrolyte was replaced by high-pressure purging before each sample run. Samples were introduced by electrokinetic injection at 10 kV for 5 s. Run conditions of 15 kV with a polarity of – to + at 20 °C resolved standard acids (oxalic, succinic, acetic) in less than 5 min and an entire run was completed in 10 min. BioFocus Integration software was used to generate calibration curves for the standard acids and quantify the concentrations of organic acids in fungal filtrates. Samples were diluted with deionized water to bring acid concentrations within the linear response range of the calibration curves.

The concentration of oxalic acid produced by mycelium grown on agar was determined by the method of Bidochka & Khachatourians (1993). Four-day cultures and surrounding agar (2.5 cm in diameter) were homogenized in 5 ml 0.1 M HCl, centrifuged (4000 g for 20 min) and the pH of the supernatants adjusted to 5 with 1 M KOH. Oxalic acid in supernatants was quantified by a test kit using enzymic analysis (Sigma Diagnostics). Protease activity versus Suc-Ala-Ala-Pro-Phe-p-nitroanilide was measured as described previously (St Leger *et al.*, 1994).

**Calculations.** The expected pH at different concentrations of ammonia was calculated from the Henderson-Hasselbach equation using a pK<sub>a</sub> value of 9.27 and NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub> ratios at

each pH from Emerson *et al.* (1975):  $\text{pH} = \text{pK} + \log(\text{NH}_3/\text{NH}_4^+)$ .

**Mutagenesis of *M. anisopliae* strain 2575.** The alkylating reagent ethyl methanesulphonate (EMS) was used in a procedure adapted from that of Al-Aidroos & Seifert (1980). One millilitre of a spore suspension ( $1 \times 10^7$  conidia  $\text{ml}^{-1}$ ) was added to 9 ml potassium phosphate buffer (pH 7.0, 0.05 M) containing 100  $\mu\text{l}$  EMS (Sigma). Treatments were carried out at 28 °C for 7.5 h in a shaking water bath, which gave 10% survival. Samples (1 ml) were removed and dispensed into buffer containing 10% (w/v) sodium thiosulphate. After incubation for 45 min, spores were diluted to  $1 \times 10^3$   $\text{ml}^{-1}$  and 1 ml samples were dispersed on selection media (1% yeast extract, 2% agar, 0.01% bromocresol purple; adjusted to pH 4.5, 6.3 or 7.0 with 0.1 M HCl), which were subsequently incubated at 27 °C. Colonies that lacked, or had reduced or increased zones of surrounding blue colour were removed and screened twice on solid pH-detection medium before critical study in liquid culture.

**Protease activity.** Protease degradation in solid media was measured by clearing zones (produced by degradation of milk proteins) in pH-indicator medium (0.1% yeast extract, 2% agar, 0.01% bromocresol purple; adjusted to pH 5.2) containing 1% skimmed milk. *M. anisopliae* produces a similar range of subtilisins and trypsin-like proteases in cultures containing skimmed milk as it does in those containing insect cuticle (St Leger *et al.*, 1987, 1994).

**pH measurements on agar plates.** pH gradients through bacterial colonies have been measured using microelectrodes (Robinson *et al.*, 1991). Similarly, the pH of fungal colonies and the surrounding agar was measured using a PHR-146 micro pH electrode (Lazar Research Laboratories). Images of Petri dishes were generated using an HP Scanjet 6200cse flatbed scanner at 300 d.p.i. The generated files were imported into Corel Draw 7 and the colours adjusted to compensate for scanning distortion. Finally the individual images were collated in Powerpoint 97.

## RESULTS

### pH changes in solid media

The ability of colonies of *M. anisopliae* wild-type strain 2575 to neutralize environmental pH during growth on solid media containing yeast extract was followed using a dye indicator that changes from yellow (below pH 5.2) through dark red to purple to blue (at pH 6.8 and above) (Fig. 1) and by direct measurements of the agar surface using a microelectrode (Fig. 2). When exploiting an acidic medium (pH 4.5, yellow colour) the colonies had an overall alkalinization effect, reflected in the appearance of a transitory dark red/purple zone around cultures up to 3 d old, which became blue in older cultures (Fig. 1). At an initial pH of 5.0 the transition was from yellow to blue and no intermediate red colour was observed. These colour changes reflected formation of a pH gradient (Fig. 2). The centre of the colonies (10 mm diameter) had a pH of 6.95 and the periphery of the colonies had a pH of 7.05–7.15. The maximum distance affected by each colony was approximately 25 mm, with the pH decreasing with increasing distance from the colony. When 4 d plates were stored at 4 °C the blue colour faded over 48 h, indicating that the agent

responsible is probably volatile and requires to be replenished if the alkalinization effect is to be maintained.

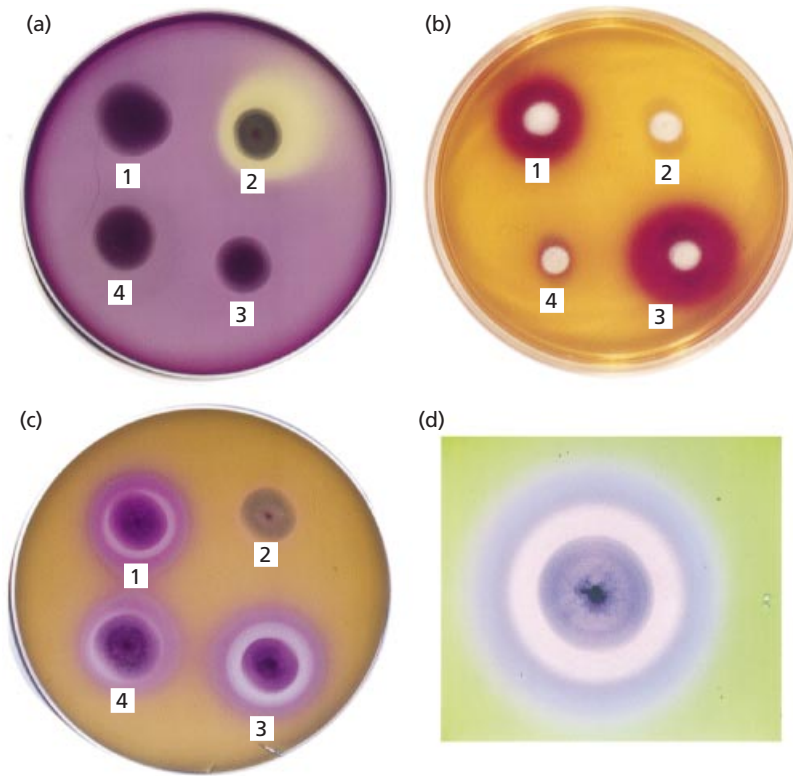
From a population of 1814 surviving colonies of EMS-treated conidia of strain 2575, six mutants were identified that lacked, or had reduced or increased zones of surrounding blue colour. These were removed and screened twice on solid pH-detection medium at pH 4.5, 6.3 or 7.0. Two broad classes of mutants were obtained. In contrast to the wild-type strains 549 and 2575, the four Acid(+) mutants [Acid(+)1, 2, 3 and 4] produced an overall acidification of yeast extract medium at pH 6.3 (Fig. 1a, colony 2). Irrespective of whether the initial pH of the medium was 4.5 or 6.3, the centre and peripheries of the colonies of Acid(+)1 had similar pH values, of 4.6 and 4.3, respectively (Fig. 2). The two Acid(−) mutants [Acid(−)1 and 2] resembled the wild-type strains in producing large red/blue zones at pH 4.5 (Fig. 1b). The centre and peripheries of the colonies of Acid(−)1 had pH values of 7.1 and 7.5, respectively, and the maximum distance of elevated pH was 30 mm (Fig. 2). Production of oxalic acid by wild-type and mutant strains on agar medium at pH 5.0 was determined enzymically. Recovery of oxalic acid from cultures of the wild-type strain after 4 d growth was  $20 \pm 4$   $\mu\text{g ml}^{-1}$ , as compared to the  $113 \pm 8$  and  $7 \pm 2$   $\mu\text{g ml}^{-1}$  from cultures of the Acid(+)1 and Acid(−)1 mutants respectively.

Colonies of wild-type strains 549 and 2575, and the Acid(+) mutants of 2575 grown on yeast extract plus glucose at pH 6.3 (purple colour) showed an overall acidification of the medium, with a yellow halo extending approximately 12 mm from the edge of the colonies, in keeping with the generation of organic acids from carbohydrates. In contrast, the Acid(−) mutants produced no halo (data not shown).

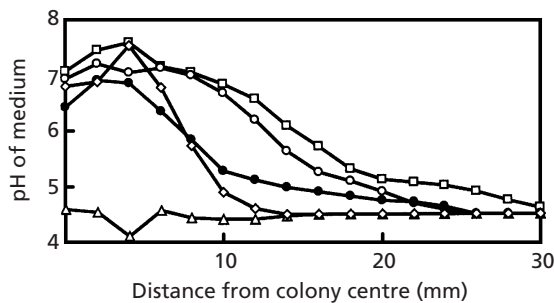
### Protease production

Protease degradation in solid media was determined by measuring clearing zones in pH indicator medium containing skimmed milk (Fig. 1c, d). Wild-type strains and Acid(−) mutants of *M. anisopliae* produced proteolytic clearing zones slightly smaller than the zone of alkalinization (blue halo). Acid(+) strains, which did not result in an overall alkalinization of the agar, likewise did not produce proteolytic clearing zones (Fig. 1c). To determine if these results may be more generally applicable to fungi, strains of *A. fumigatus* and *N. crassa* were tested. *A. fumigatus* produced a proteolytic clearing zone within the zone of alkalinization resembling that of *M. anisopliae*. *N. crassa* produced a thin net-like mycelium on the surface of the agar and a smaller-diameter zone of protein degradation within the alkaline zone it generated (data not shown).

To determine if the failure of *M. anisopliae* Acid(+) strains to produce protease in agar is due to a single mutation causing acid overproduction, the Acid(+)1 strain was subjected to a second round of mutagenesis



**Fig. 1.** pH changes in agar media during growth of *M. anisopliae*. Strains 2575 (1), Acid(+)-1 (2), Acid(-)-1 (3) and 549 (4) were point-inoculated onto 1% yeast extract, 2% agar with 0.01% bromocresol purple at pH 6.3 (plate a) or pH 4.5 (plate b), and incubated for 3 d (plate b), 4 d (plates a and c) or 5 d (plate d). Agar in plates (c) and (d) contained 1% skimmed milk as well as 0.1% yeast extract (pH 5.2) and 0.01% bromocresol purple to detect protease activity as clearing zones (produced by degradation of milk proteins). Plate (d) (detail) shows the extensive clearing zone within the zone of alkalization produced by the Acid(-) mutant. Bromocresol purple in agar changes colour from yellow (below pH 5.2) to dark red (approx. pH 6) to blue (above pH 6.8). Fig. 1(d) was adjusted using Corel Draw 7 to optimize contrast between the clearing zone and pH colour change.



**Fig. 2.** pH at the surface of the agar, plotted against distance from the centre of the fungal mycelium (10 mm diameter), for *A. fumigatus* (◇), and *M. anisopliae* strains 549 (●), 2575 (○), Acid(+)-1 (△) and Acid(-)-1 (□). The initial pH of the medium was 4.5.

with EMS. From a population of 3100 surviving colonies, 13 mutants were identified that resembled wild-type 2575 in not acidifying yeast extract medium at pH 7.0. These strains also produced proteolytic clearing zones similar to the 2575 wild-type strain, confirming that overproduction of acid and non-production of proteases are linked characteristics.

To determine if the failure of *M. anisopliae* Acid(+)- strains to produce protease in agar is due only to acidification of the medium, or if additional pH-independent changes in regulation of production occur,

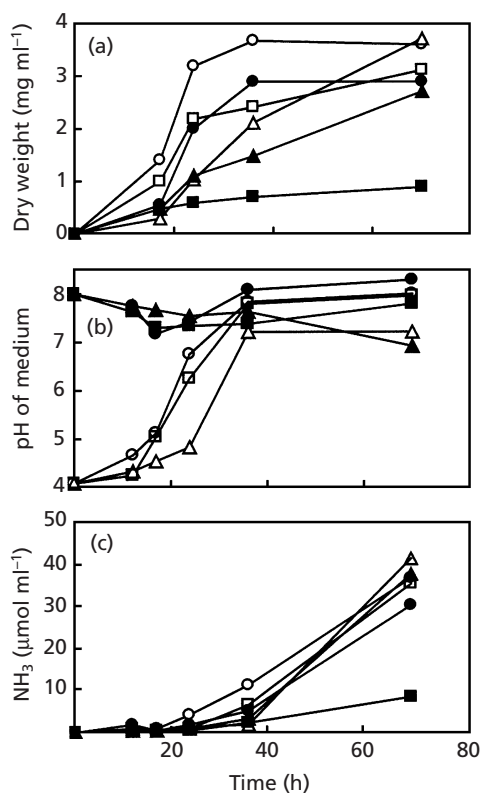
the wild-type and the Acid(+)-1 mutant were assayed for enzyme activity 24 h after exponentially growing mycelium was transferred to buffered (pH 4 or pH 7) liquid minimal medium containing 1% insect cuticle as sole carbon and nitrogen source. Wild-type and Acid(+)-1 strains produced similar levels of protease activity against the subtilisin substrate Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide both at pH 7 [wild-type,  $115 \pm 7$  nmol  $\text{min}^{-1}$ ; Acid(+)-1,  $123 \pm 8$ ] and at pH 4 [wild-type,  $10 \pm 2$ ; Acid(+)-1,  $10 \pm 1$ ]. This suggests that regulation of protease production is not altered in Acid(+)-1 compared to the wild-type.

#### Ammonia and acidic metabolites in nutrient medium

Wild-type *A. fumigatus*, *N. crassa* and *M. anisopliae*, as well as mutants of *M. anisopliae* selected on pH indicator medium, were evaluated for interrelationship of pH, mycelial dry weight, and generation of organic acids and ammonia (Figs 3 and 4).

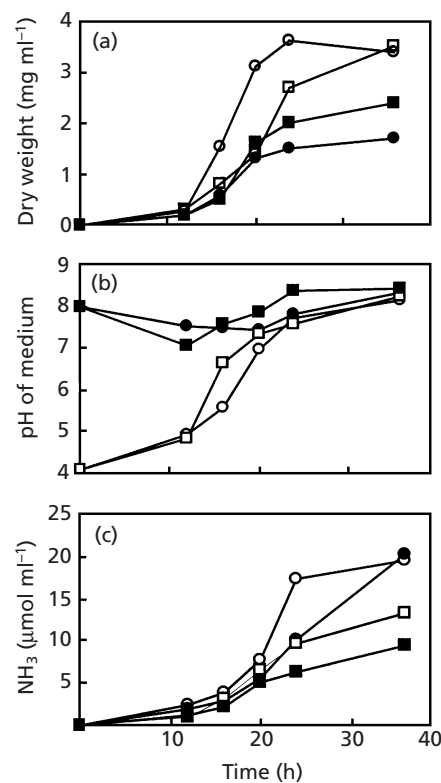
The pH of yeast extract medium changed sharply during growth by the three fungal species. In medium with the pH initially adjusted (with NaOH) to 8, the pH decreased initially to around 7.3 but rose after fungal growth was nearly complete, and attained a value above the initial pH. In the case of growth in medium adjusted (with HCl) to pH 4, the pH rapidly increased, again reaching a value above 8.

The mechanism of early alkalization of the pH of the



**Fig. 3.** Changes in mycelial dry weight (a), pH of medium (b) and production of ammonia (c) during growth of *M. anisopliae* 2575 (○, ●), Acid(+)<sub>1</sub> (△, ▲) or Acid(-)<sub>1</sub> (□, ■). Each initial pH was adjusted by the addition of 1 M HCl to pH 4 (open symbols) or 1 M NaOH to pH 8 (filled symbols). The results are mean values of three experiments. Variation about the means was 7% or less in each case.

medium was investigated by following the concentration of ammonia in the medium. Increasing concentrations of ammonia correlated with the increasing medium pH in cultures of *M. anisopliae*, *N. crassa* and *A. fumigatus*. Using 9:1 as the ratio of  $\text{NH}_4^+$  to  $\text{NH}_3$  at pH 8.3 (Emerson *et al.*, 1975), the calculated pH of 3 d cultures of *M. anisopliae* wild-type strain based on ammonia production ( $680 \mu\text{g ml}^{-1}$ ) is 10.3. Consequently, production of ammonia is more than sufficient to account for the rise in pH, and presumably additional more acidic components are present in cultures, and/or the cultures contain buffering agents. An assessment of organic acids detected by capillary electrophoresis indicated that the wild-type *M. anisopliae* produced acetic, succinic and oxalic acids in yeast extract medium (Table 1). Traces of other acidic metabolites were present; however, these did not closely match the standards and were not positively identified. The Acid(+)<sub>1</sub> mutant produced 30-fold higher levels of oxalic acid compared to the wild-type 2575, but other organic acids were not detected. Such enhanced production of oxalic acid may account for the final pH of Acid(+)<sub>1</sub> cultures being lower than that of wild-type or



**Fig. 4.** Changes in mycelial dry weight (a), pH of medium (b) and production of ammonia (c) during growth of *A. fumigatus* (○, ●) and *N. crassa* (□, ■). Each initial pH was adjusted by the addition of 1 M HCl to pH 4 (open symbols) or 1 M NaOH to pH 8 (filled symbols). Results are mean values of three experiments. Variation about the means was 6% or less in each case.

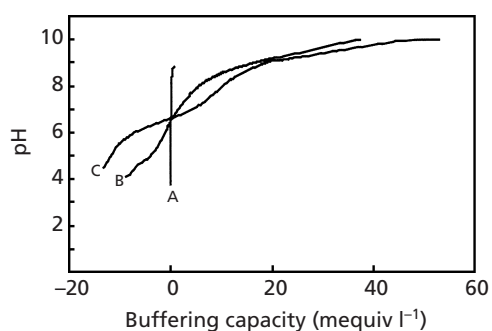
**Table 1.** Composition of acidic metabolic products of wild-type and mutant strains of *M. anisopliae* cultured in yeast extract for 3 d

Cultures (100 ml) were inoculated with  $1 \times 10^7$  spores. The identities and quantities of organic acids in spent culture medium were determined by capillary electrophoresis.

<i>M. anisopliae</i> strain	Oxalate (mg ml <sup>-1</sup> )	Succinate (mg ml <sup>-1</sup> )	Acetate (mg ml <sup>-1</sup> )
Wild-type	0.23	0.19	0.06
Acid(+) <sub>1</sub>	7.50	0	0
Acid(-) <sub>1</sub>	0.12	0.15	0

Acid(-)<sub>1</sub> cultures. The Acid(-)<sub>1</sub> mutant produced negligible levels of oxalic acid.

Titration curves of water, yeast extract and spent yeast extract media from a culture of the *M. anisopliae* wild-type strain culture are shown in Fig. 5. Unlike water, yeast extract medium has a significant buffering action



**Fig. 5.** Buffering capacity of 1% yeast extract medium that has supported growth of *M. anisopliae* 2575 for 2 d (C), as compared with unused yeast extract medium (B) and water (A). Mean values of two experiments.

over the entire pH range. After 2 d growth of wild-type or Acid(+) strains (not shown) the buffering capacity of the medium increased over much of the range, as shown by the flatter portions of the graph.

### Transfer experiments

Exponentially growing mycelia of *M. anisopliae*, *N. crassa* and *A. fumigatus* were transferred from Sabouraud dextrose broth to minimal medium or minimal medium supplemented with carbon sources (Table 2). Only trace levels of ammonia were formed by *M. anisopliae* over a 4 h period in minimal medium (minus carbon and nitrogen), indicating that ammonia production does not respond to carbon and nitrogen limitation alone. Cells grown with glucose or glycerol as carbon sources, or with  $\text{NaNO}_3$  as a nitrogen source, also failed to produce ammonia. However, ammonia accumulated in media supplemented with low concentrations (1 mM) of amino acids. Peak ammonia production occurred with 1 mM L- or D-methionine and 10 mM L-glutamate, but ammonia production was greatly decreased at higher amino acid concentrations (Table 2). In contrast, both *A. fumigatus* and *N. crassa* produced ammonia in un-supplemented minimal medium, indicating that extra-cellular nitrogenous nutrients are not required for ammonia formation. However, supplementing minimal medium with 1 mM D-methionine resulted in a 2.5-fold

**Table 2.** Ammonia production and pH changes produced by *M. anisopliae*, *A. fumigatus* and *N. crassa*

Mycelial inocula from Sabouraud dextrose cultures were incubated for 4 h in minimal medium with the potential inducer (initial pH 6.5), then ammonia and pH were measured.

Supplement	<i>M. anisopliae</i>		<i>A. fumigatus</i>		<i>N. crassa</i>	
	$\text{NH}_3$ ( $\mu\text{mol ml}^{-1}$ )	pH	$\text{NH}_3$ ( $\mu\text{mol ml}^{-1}$ )	pH	$\text{NH}_3$ ( $\mu\text{mol ml}^{-1}$ )	pH
None	$0.01 \pm 0$	6.44	$1.96 \pm 0.16$	7.03	$2.07 \pm 0.11$	6.23
<b>L-Methionine</b>						
1 mM	$0.85 \pm 0.04$	6.50	$3.31 \pm 0.21$	6.83	$2.15 \pm 0.09$	6.31
10 mM	$0.75 \pm 0.05$	6.58	$2.78 \pm 0.04$	7.15	$1.25 \pm 0.07$	6.28
100 mM	0	6.45	$0.01 \pm 0$	6.67	$0.03 \pm 0$	6.28
<b>D-Methionine</b>						
1 mM	$1.23 \pm 0.08$	6.41	$4.89 \pm 0.35$	7.01	$3.49 \pm 0.26$	6.41
10 mM	$0.82 \pm 0.02$	6.53	$2.67 \pm 0.17$	7.00	$0.77 \pm 0.13$	6.30
100 mM	0	5.9	$0.01 \pm 0$	7.2	0	6.21
<b>L-Glutamate</b>						
1 mM	$2.10 \pm 0.13$	6.82	$3.44 \pm 0.16$	7.44	$2.55 \pm 0.15$	6.91
10 mM	$5.73 \pm 0.07$	7.65	$3.72 \pm 0.35$	7.53	$3.86 \pm 0.27$	7.52
100 mM	$1.51 \pm 0.01$	7.71	$0.70 \pm 0.05$	7.65	$2.33 \pm 0.16$	7.03
<b>Glucose</b>						
1 mM	0	6.56	$0.66 \pm 0.04$	7.11	$0.82 \pm 0.05$	6.29
10 mM	$0.01 \pm 0$	5.87	$0.03 \pm 0.01$	4.85	$0.02 \pm 0$	5.57
100 mM	0	5.31	0	4.41	$0.02 \pm 0$	5.05
<b>Glycerol</b>						
1 mM	$0.01 \pm 0$	6.04	$1.23 \pm 0.05$	6.97	$1.44 \pm 0.25$	6.17
10 mM	$0.01 \pm 0$	6.17	$1.78 \pm 0.07$	7.19	$0.81 \pm 0.03$	5.94
100 mM	$0.01 \pm 0$	6.02	$0.46 \pm 0.02$	6.62	$0.75 \pm 0.10$	5.67
<b><math>\text{NaNO}_3</math></b>						
1 mM	$0.01 \pm 0$	6.41	$1.32 \pm 0.15$	7.17	$1.18 \pm 0.15$	5.85
10 mM	$0.01 \pm 0$	6.31	$0.97 \pm 0.08$	6.90	$1.37 \pm 0.75$	5.81
100 mM	$0.01 \pm 0$	6.10	$0.74 \pm 0.02$	6.74	$2.18 \pm 0.16$	5.93

(*A. fumigatus*) or 1.7-fold (*N. crassa*) increase in ammonia levels. Higher concentrations of amino acids or glucose blocked ammonia formation. Growth on glucose led to lowering of the pH of the medium, indicating that acidification can result from organic acids released during glycolysis. To distinguish between a decrease in ammonia formation due to glucose, and inhibition due to fall in pH, the levels of ammonia produced in buffered yeast extract medium in the presence and absence of glucose were compared with those produced in unbuffered medium. *M. anisopliae*, *A. fumigatus* and *N. crassa* all produced similar levels of ammonia at pH 4 and 7, indicating that the decrease was due to the glucose itself rather than low pH (data not shown).

## DISCUSSION

Chemical mutagenesis resulted in several *M. anisopliae* mutants that were altered in production of oxalic acid. Wild-type and Acid(+) *M. anisopliae* grow almost as well at pH 8 as at pH 6. This is consistent with previous observations that *M. anisopliae* can grow over a pH range of 2.5–10.5 (Hallsworth & Magan, 1996). Thus when yeast extract serves as the nutrient source, the pH of the medium does not increase to the point where growth is prevented. Acid(–) mutants showed limited growth at pH 8, indicating that acid production is linked to the ability to grow at higher pH. This implies an unexpected role for organic acid production in pH homeostasis, and the flexibility of growth observed in this fungus. However, attempts to produce revertants of Acid(–) mutants by EMS treatment failed, even though we screened more than 8000 survivors (unpublished data). Further research will be required to confirm that these putative pleiotropic characteristics involve a single mutation. Presumably, accumulation of oxalic acid in the cytoplasm could help regulate cytoplasmic pH. Oxalic acid is produced in the cytoplasm of *Aspergillus niger* with little or no participation of the tricarboxylic acid cycle (Strasser *et al.*, 1994).

In contrast to growth, protease production was reduced by acidification of the medium in Acid(+) mutants, and these traits co-reverted, indicating that they involve a single mutation. In addition, wild-type and Acid(+) mutants produced very similar levels of protease activity in buffered liquid culture containing insect cuticle, demonstrating that regulation of protease production was not altered in the mutants. Tests with solid media permitted simultaneous evaluation of changes in the pH of the medium and in protease production, and their interrelations. Our results show that alkalization of the medium is closely related to the degradation of extracellular proteins in wild-type and Acid(–) mutants. This suggests that alkalization is adaptive, facilitating the utilization of a nutrient source, and is linked to our observation that extracellular enzyme activities are only produced at the pH at which they are active (St Leger *et al.*, 1998). Similar observations with *A. fumigatus* and *N. crassa* suggest that this phenomenon may be widespread.

The capacity to elevate pH in their locality confirms that fungi produce one or more basic compounds. The pH at the margin of colonies on solid media is greater than the pH at the centre. This presumably reflects greater metabolic activity at growing hyphal points, including oxidative deamination of amino acids leading to ammonia accumulation and diffusion. Analysis of end-products in liquid culture revealed that ammonia was produced during growth on yeast extract at levels sufficient to account for the rise in the pH of the medium. Ammonia production by some fungi during growth on protein as sole carbon source is believed to result from dissimilation of amino nitrogen produced in excess of that required for growth (Jennings, 1989). Growing *M. anisopliae* in the presence of low levels of single amino acids also resulted in high levels of ammonia formation. Complete catabolism of glutamate liberates two ammonia molecules per molecule of glutamate, which could explain the elevated ammonia levels compared with growth on methionine. However, increasing the abundance of amino acids decreased the formation of ammonia, suggesting that in nature, continual formation of ammonia requires release of low levels of amino acids from a protein source. This is contrary to the idea that ammonia is a by-product of excess amino nitrogen during amino acid catabolism and implies either induction of catabolite repressible enzyme(s), presumably deaminases, or regulation of the enzyme activity via substrate inhibition. Regulation of amino acid oxidase in *N. crassa* is inducible by amino acids and would release ammonia (Sikora & Marzluf, 1982). However, *N. crassa* and *A. fumigatus* also produced ammonia in minimal medium, again contradicting the 'superabundance of nitrogen' theory and implying that the source was internal nitrogenous reserves. Ammonia production by the three fungal species also declined or ceased altogether when amino acids were supplemented with glucose. Glucose may have repressed production of deaminases. It is also possible that the higher growth rate with glucose allowed more complete utilization of ammonia released from amino acid catabolism. In addition, the fungi may have switched from catabolizing amino acids to catabolizing sugars for the production of nitrogen-non-containing polymers, thereby decreasing free ammonia production.

Previous studies with bacteria (Mekalanos, 1992), *C. albicans* (De Bernardis *et al.*, 1998) and *M. anisopliae* (St Leger *et al.*, 1998) have focused on pH as an environmental cue in the host niche, serving to regulate the differential gene expression needed to survive within that niche. However, filamentous fungi are also able to modify the environmental pH, and these modifications may have a function related to exploitation of resources rather than just being by-products of metabolic activity. Ammonia production by *M. anisopliae* is tightly regulated by amino acids, which as protein degradation products could serve as signals for the presence of proteinaceous nutrients in the environment. Generation of ammonia by *N. crassa* or *A. fumigatus* is more loosely

regulated and occurs whenever they are in nutrient-deprived conditions, presumably from degradation of endogenous compounds. Given that ammonium is linked with nitrogen catabolite repression (Arst & Cove, 1969), there are presumably additional regulatory mechanisms that allow protease production in the presence of ammonia.

Most of the ammonia produced by fungi will be transformed to ammonium in solution. However, at higher pH a larger proportion of the ammonium becomes toxic unionized ammonia; the approximate ratios of  $\text{NH}_4^+$  to  $\text{NH}_3$  will be 1800:1 at pH 6, and 9:1 at pH 8.3. Ammonia is very toxic because it is lipid soluble and raises intracellular pH, thus inhibiting protein synthesis and enzyme activity (Doyle & Butler, 1990). Measurement of the pH of insect cuticle infected with *M. anisopliae* demonstrated an overall increase compared to unaffected cuticles, but the precise pH at sites of infection by *M. anisopliae* and *A. fumigatus* remains to be determined. Further research will indicate whether acid/base production by *M. anisopliae* and *A. fumigatus in vivo* is a factor in their virulence to animal hosts. As regards saprophytes such as *N. crassa*, proteases can work as important factors in scavenging nutrients during saprotrophy.

The results of this study suggest that ammonia may have functions related to regulation of the microenvironment and may represent a previously unconsidered virulence factor in diverse fungi with the potential to harm tissues and disturb the host's immune system.

## ACKNOWLEDGEMENTS

This research was supported, in part, by grant 9602033 of the US Department of Agriculture, by a grant from the Noble Foundation, OK, USA and by a grant from the Coffee Federation of Colombia (CENICAFE).

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Received 18 March 1999; revised 17 May 1999; accepted 8 June 1999.