

## EFFECT OF SOME MICRO-ENVIRONMENTAL FACTORS ON CONIDIAL GERMINATION AND MYCELIAL GROWTH OF THE ENTOMOGENOUS FUNGI, *METARRHIZIUM FLAVO-VIRIDE* AND *M. ANISOPLIAE* (DEUTEROMYCOTA: HYPHOMYCETES)

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### Abstract

The effect of pH, water potential and temperature on conidial germination and subsequent mycelial growth of two species of *Metarrhizium* was examined on agar gradient plates. The pH, water potential and temperature did not act independently. Optimum temperature for germination and growth was 30 °C at which the *M. flavo-viride* strains were more tolerant than *M. anisopliae* at increasing KCl concentration (water potential) and decreasing pH. Below optimum temperature germination and growth were higher in *M. anisopliae* than the *M. flavo-viride* strains and vice versa above optimum. There was poor growth and no growth in the *M. flavo-viride* strains and *M. anisopliae*, respectively, at 37 °C. The lowest extremes of pH for germination were 3.0 for *M. flavo-viride* and 3.5 for *M. anisopliae*.

### Résumé

ADU-MENSAH, J.: *Effet de quelques facteurs micro-environnementaux sur la germination conidiale et la croissance mycélienne de mycète entomogène Metarrhizium flavo-viride et M. anisopliae (Deuteromycota: Hyphomycetes)*. L'effet de pH, de potentiel d'eau et de température sur la germination conidiale et la croissance mycélienne subséquente de deux espèces de *Metarrhizium* étaient étudiées sur les plaques de gradient de gélose. Le pH, potentiel d'eau et température n'ont pas agi de façon indépendante. La température optimum pour la germination et la croissance était 30 °C à laquelle les races *M. flavo-viride* étaient plus tolérantes que *M. anisopliae* à l'augmentation de la concentration de KCl (potentiel d'eau) et la diminution de pH. Au-dessous de la température optimum, la germination et la croissance étaient plus élevées en *M. anisopliae* qu'en race de *M. flavo-viride* et vice versa au-dessus de l'optimum. Il y avait une croissance pauvre et croissance nulle respectivement dans les races *M. flavo-viride* et *M. anisopliae* à 37 °C. Les plus bas extrêmes de pH pour la germination étaient 3.0 pour *M. flavo-viride* et 3.5 pour *M. anisopliae*.

### Introduction

Locusts and grasshoppers are the most serious pests of agriculture in the Sahelian region of Africa (Geddes, 1990). Chemical application is the methods of control, however, rising costs, environmental hazards, pest resistance to chemicals and sec-

ondary pest outbreaks have combined to provide a strong argument for long term investment in non-chemical control.

*Metarrhizium flavo-viride* Gams & Roszypal and *M. anisopliae* (Metschn.) Sorokin are important pathogens of locusts and grasshoppers (Prior et

*al.*, 1992). Several research organizations are developing oil formulations of fungal bio-pesticides for ultra-low-volume (u-l-v) application by conventional spray equipment. However, in the environments where these insects must be controlled, pathogens encounter high temperatures and low relative humidities (Marcandier & Khachatourians, 1987; Moore *et al.*, 1993) Pathogens must also tolerate the pH of the cuticular surface of host insects (Gottlieb, 1978), which is derived from toxic acids (Gershon & Shanks, 1978) and other compounds, e.g. nutrients (Smith & Grula, 1982).

*In vitro* studies on separate effects of temperature and water activity on conidial germination and mycelial growth have established high moisture requirements (0.0 Mpa to -2.8 Mpa) for several entomogenous fungi, including *M. anisopliae* (Gillespie & Crawford, 1986; Moorhouse, 1990), with optimum temperatures between 20 °C - 30 °C (Roberts & Campbell, 1977; Soares & Pinnock, 1984). However, there is no information on the effect of these variables acting simultaneously.

In this study, gradients of KCl and pH were constructed at right angles to each other at different temperatures to study the joint effects of water potential, pH and temperature on conidial germination and subsequent mycelial growth on two important species of fungal pathogens of locusts and grasshoppers.

### Experimental

*Metarrhizium flavo-viride* strains IMI 330189, IMI 324673 and *M. anisopliae* IMI 168777/ii originally isolated from the grasshoppers, *Ornithacris cavroisi* in Niger, *Zonocerus elegans* in Tanzania, and *Schistocerca gregaria* in Ethiopia, respectively, were obtained from Commonwealth Agricultural Bureau (C.A.B.), International Mycological Institute (IMI), United Kingdom. These were subsequently maintained on Molisch's agar (Speare, 1920) slants for storage at 10 °C. Conidia used in the study were

from 14-16-day old growth from plates inoculated with actively growing mycelial plugs and incubated at  $25 \pm 1$  °C in the dark. Conidia were harvested by placing five drops of sterile distilled water on the mycelium and gently scrapping the top layer with sterile loop into sterile distilled water in a McCartney bottle containing 20 sterile glass beads. The bottle was vigorously shaken to dislodge conidia into suspension.

Salt and pH gradients were constructed at right angles to one another in Sterilin<sup>c</sup> wettable square plates (10 cm × 10 cm). Each plate consisted of four layers of 15 ml malt extract agar (MEA; 20 g<sup>l</sup>-1 malt, 15 g<sup>l</sup>-1 bacto-agar (Difco) to which the following were added: Layer 1 - acid medium, 0.3 ml 1 M H<sub>2</sub>SO<sub>4</sub>; Layer 2 - alkali medium, 0.6 ml 1 M NaOH; Layer 3 - salt medium, 1.2 g or 3.6 g KCl; Layer 4 - no addition. Acid and alkali were autoclaved separately with 15 ml aliquots of agar, and plates were made aseptically on a completely flat surface. The acid medium was poured with the plate supported along one edge with a 2-mm diameter metal rod. When set (30 min), the rod was removed and the second layer was poured. After setting the plate was rotated through 90°, supported with the rod and the third layer poured. After 30 min, the fourth layer was poured with the rod removed. A polytetrafluoroethylene (PTFE) grid of 81 squares (1.1 cm × 1.1 cm) was inserted aseptically to prevent degradation of the gradient set up by diffusion of solutes (Boddy, Wimpenny & Harvey, 1989). The pH and KCl were measured at the centres of each grid square with the aid of calibration curves, using phosphate buffer solutions of pH 4, 7 and 9.2, and conductivity of solutions of known concentrations of KCl.

Plates were inoculated with 5 ml of 10<sup>4</sup> conidia ml<sup>-1</sup> in half strength MEA evenly spread across the plate and incubated at 15, 20, 25, 30 and 37 °C. Percentage germination was calculated after 24 h from a total conidia count of 24-28 from one or two fields

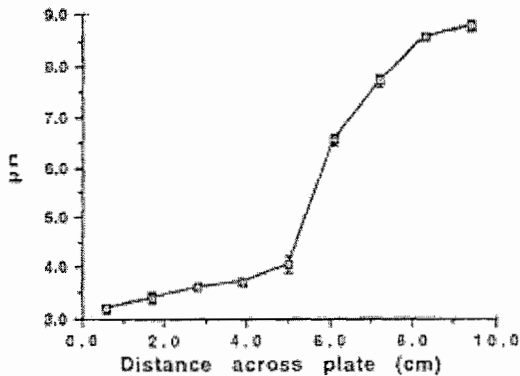


Fig. 1. pH gradient across plates

of microscope. A condom was considered to have germinated when the germ tube length was at least equal to the width of condom. Results for five plates were used to draw contour maps of percentage germination. Mycelial growth in each square was graded after 4 days using a grading score 1-4 as follows: 1 - no visible growth; 2 - individual colonies visible; 3 - patchy covering of mycelia (sparse growth); 4 - even covering of mycelia (dense growth). Scores were used to map out

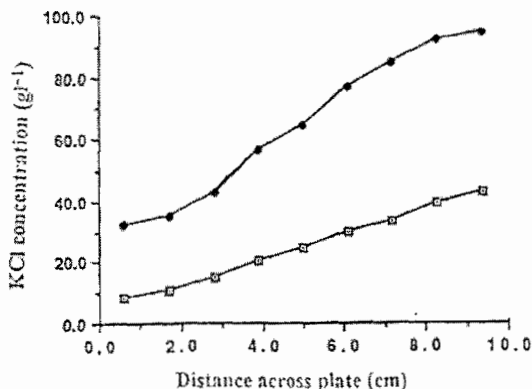


Fig. 2. Potassium chloride gradient across plates containing 1.2 g (□) and 3.6 g (●) KCl in 15 ml aliquots for the third layer.

growth.

## Results

A range of pH 3.2 - 8.9 was obtained in plates with sharp increases in pH between 5 cm and 6.1 cm in the direction of increasing pH (Fig. 1). The two sets of plates provided a range of 8.5 - 94.0 g l<sup>-1</sup> KCl with overlaps in the range of 30 - 43 g l<sup>-1</sup> KCl (Fig. 2). The highest percentage germination in both fungal species, at all temperatures studied, occurred with a combination of high pH and low KCl (Fig. 3a-d). Optimum temperature for germination and growth was 30 °C, at which temperature the *M. flavoviride* strains showed a higher tolerance to low pH and increasing KCl (Fig. 3a-d and Fig. 4a-b). There was no germination in either species at 15 °C. The effects of pH, salt and temperature were not independent. At 37 °C, there was less germination in *M. anisopliae* than *M. flavoviride* strains with higher tolerance to low pH by the latter. The lower limits of pH at which some germination was recorded was 3.0 for the *M. flavoviride* strains and 3.5 for *M. anisopliae*. Growth in *M. anisopliae* was better than *M. flavo-viride* strains below optimum temperature. The best was recorded in the latter at 20 °C, being only individual colonies in strain IMI 324673 with no growth in strain IMI 330189 in plates containing high KCl (Fig. 4a,b). At 37 °C no growth was recorded in *M. anisopliae* at all concentrations of KCl and pH studied.

## Discussion

Optimum temperature for germination and growth was 30 °C which was within the range (25 - 30 °C) established for several entomogenous fungal genera including *Hirsute*, *Culicinomyces* and *Metarhizium* (Hall & Papierok, 1982). Similar results were reported for several strains of *M. anisopliae*, with optimum for germination marginally higher than for growth (Moorhouse, 1990). There was differences in the response to pH, KCl

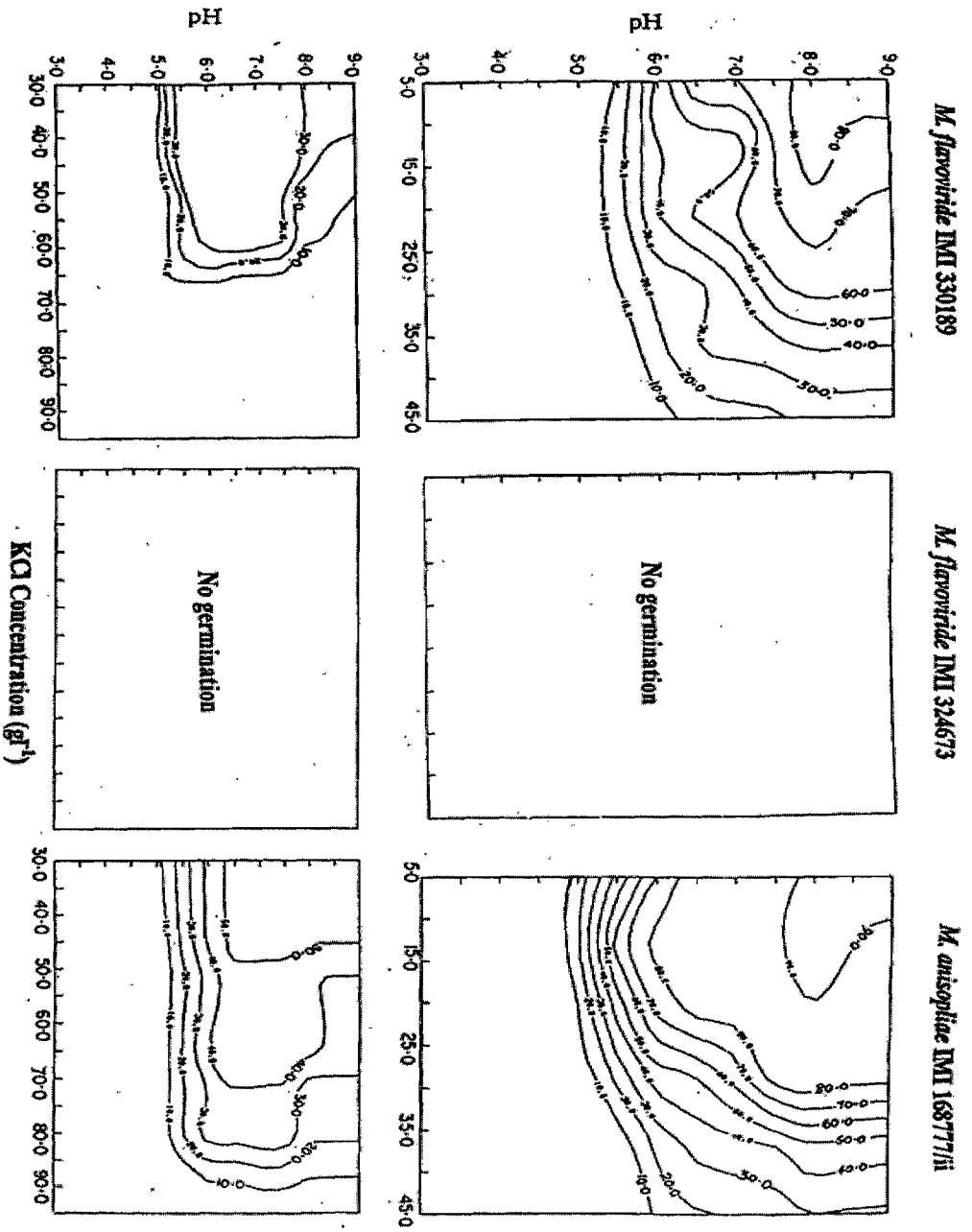


Fig. 3a. Contour lines delimiting areas of equal germination percentage of conidia of *M. flavoviride* IMI 330189, IMI 324673 and *M. ansophtiae* IMI 168777/ii after 24 h incubation at 20 °C on agar plates with gradients of pH and KCl at right angles to each other.

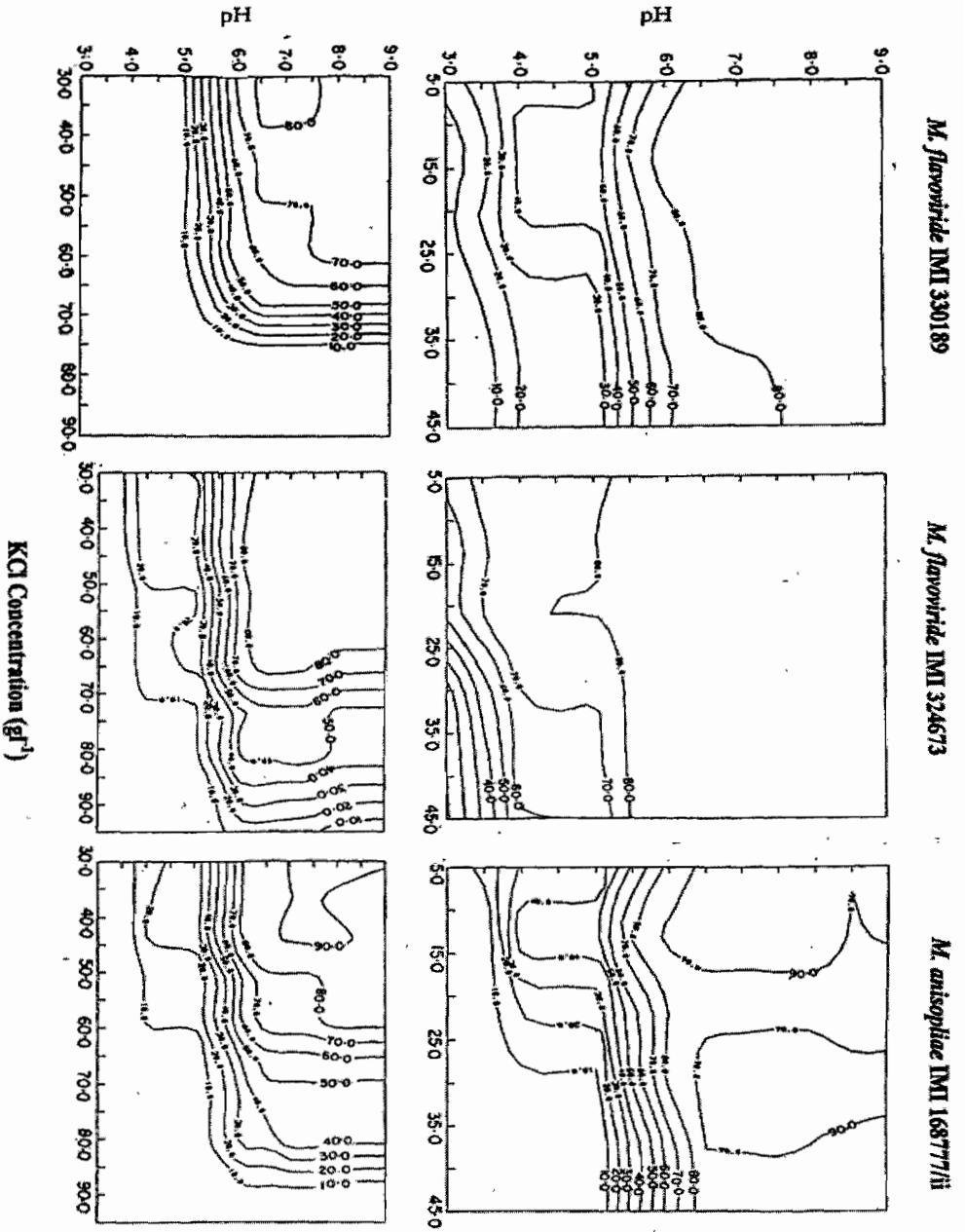


Fig. 3b. Contour lines delimiting areas of equal germination percentage of conidia of *M. flavoviride* IMI 330189, IMI 324673 and *M. anisopliae* IMI 168777/ii after 24 h incubation at 25 °C on agar plates with gradients of pH and KCl at right angles to each other.

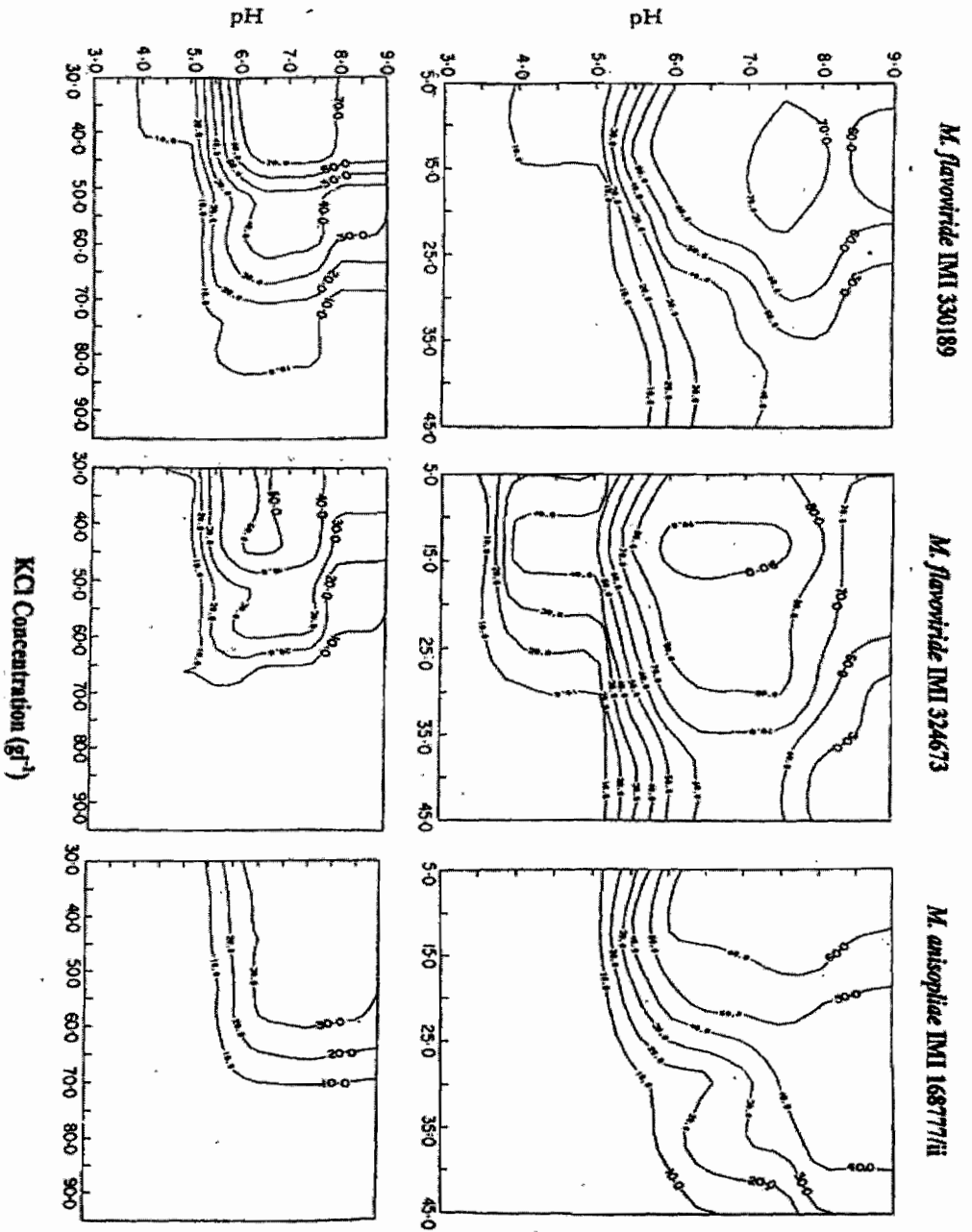


Fig. 3c. Contour lines delimiting areas of equal germination percentage of conidia of *M. flavoviride* IMI 330189, IMI 324673 and *M. anisopliae* IMI 168777/ii after 24 h incubation at 30 °C on agar plates with gradients of pH and KCl at right angles to each other.

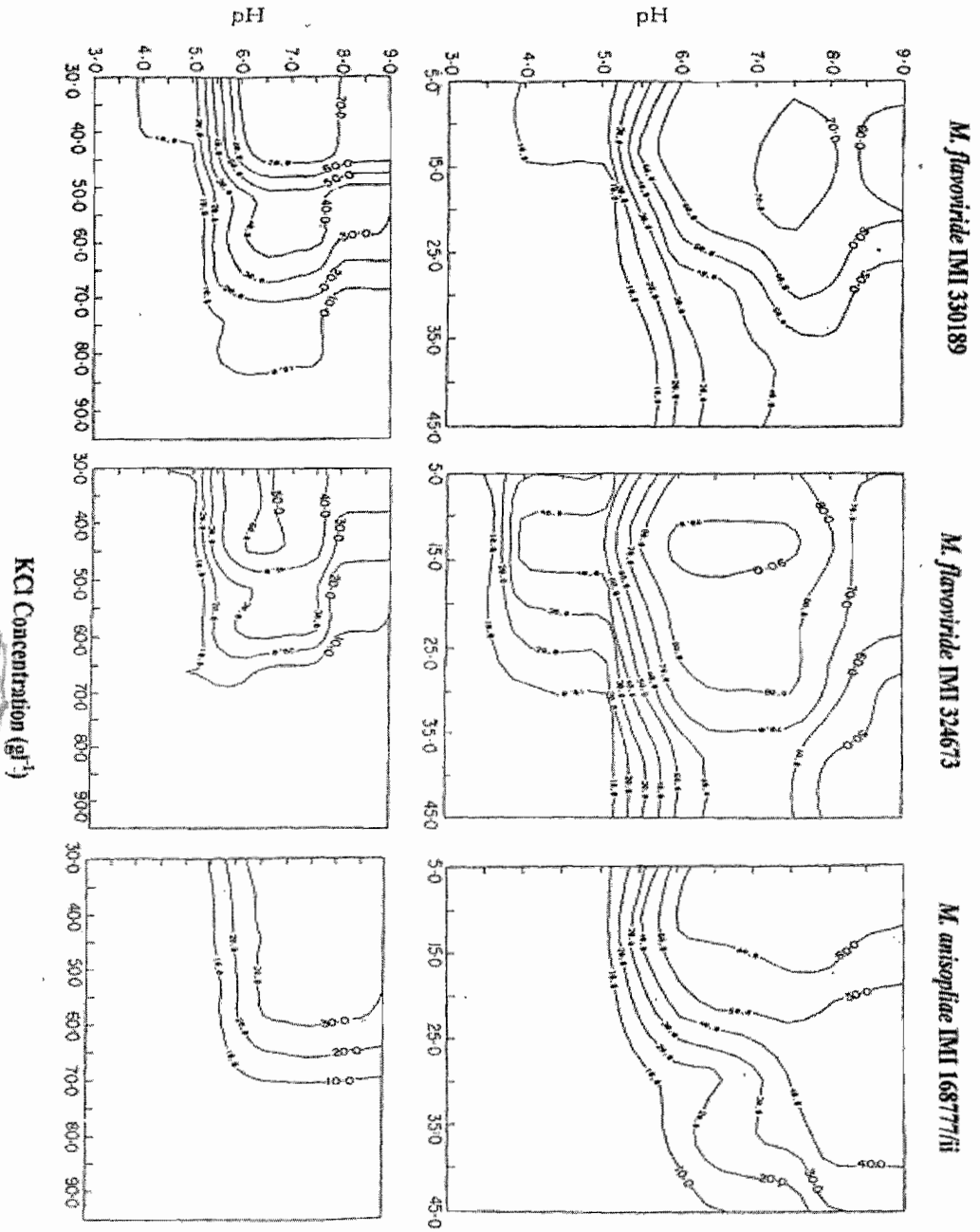


Fig. 3d. Contour lines delimiting areas of equal germination percentage of conidia of *M. flavoviride* IMI 330189, IMI 324673 and *M. anisopliae* IMI 168777/i after 24 h incubation at 37 °C on agar plates with gradients of pH and KCl at right angles to each other.

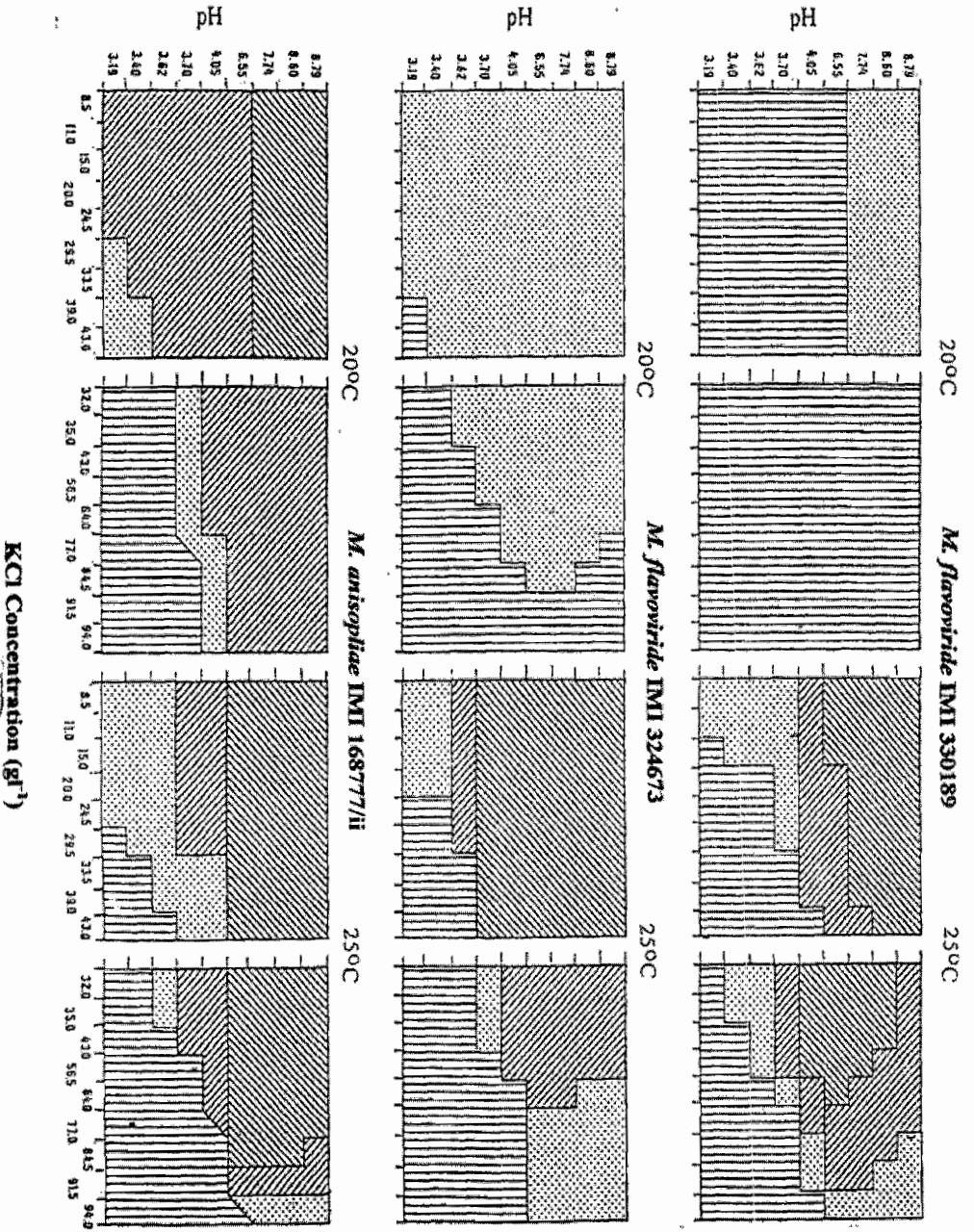


Fig. 4a. Mycelial growth of *M. flavoviride* IMI 330189, IMI 324673 and *M. anisopliae* IMI 168777/ii after 4 days at 20 °C, 25 °C, and 30 °C, 37 °C respectively on agar plates with gradients of pH and KCl at right angles to each other: [diagonal lines] even covering of mycelia (dense growth); [horizontal lines] patchy covering of mycelia (sparse growth); [white] only individual colonies visible [no hatching] no growth



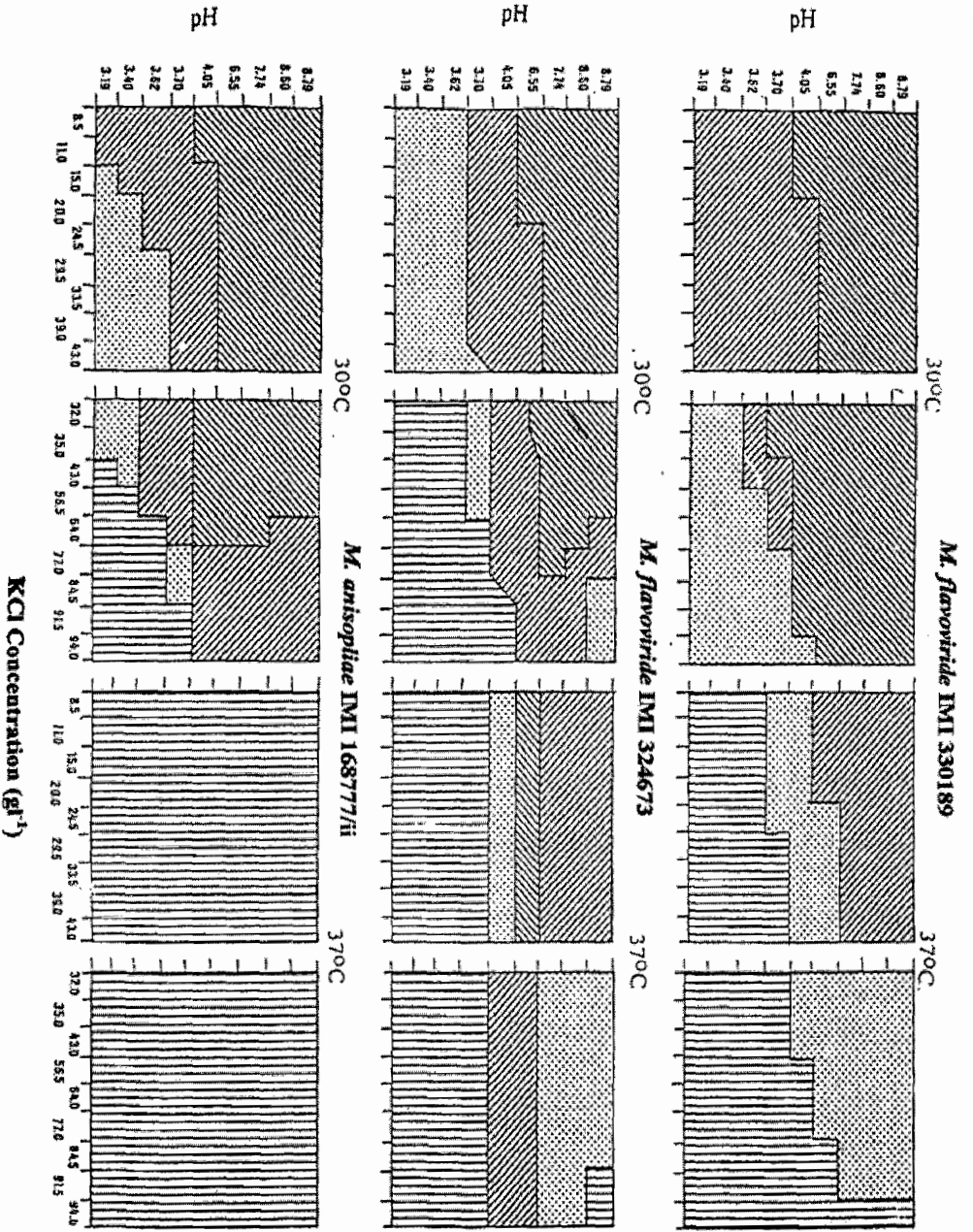


Fig. 4b. Mycelial growth of *M. flavoviride* IMI 330189, IMI 324673 and *M. anisopliae* IMI 168777ii after 4 days at 20, 25, 30 °C, 37 °C respectively on agar plates with gradients of pH and KCl at right angles to each other: [diagonal lines] even covering of mycelia (dense growth); [horizontal lines] patchy covering of mycelia (sparse growth); [no hatching] only individual colonies visible [no hatching] no growth.

and temperature by the two species of entomogenous fungi. Temperature extremes appeared to exert a greater influence than water potential. The suppression of germination in the *M. flavoviride* strains by increasing KCl at 20 °C may be due to low temperature *per se* as a limiting factor, while at 37 °C high temperature suppressed germination and growth in *M. anisopliae* (Fig. 3d and 4b).

The lowest pH (3.0) at which some germination was possible in the *M. flavo-viride* strain was lower than the *M. anisopliae* strain (pH 3.5), giving a wider range of tolerance to pH for conidial germination to the former. However, both fungal species may be described as facultative acidophiles with a wide range of pH for maximum growth (pH 4.05 - 8.79). This is typical of most fungi with optimum for growth in the range of pH 5 - 7) but growing well above 7 (Brock, Smith & Madigan, 1984). The effect of pH on fungal growth is variable, with low pH affecting enzyme systems and high pH involved in metal solubility, permeability and surface phenomena of the cell wall (Cochrane, 1958). For many fungi the ability to modify the immediate environment makes growth possible at pHs which do not permit the initiation of growth.

In general, moisture requirements for germination and mycelial growth in entomogenous fungi are high (Gillespie & Crawford, 1986, Moorehouse, 1990). The source of moisture for conidial swelling, which is the initial stage of the infection process, may be the micro-climate of the insect cuticle, especially the arthrodial membranes (Prior *et al.*, 1992). Results of the present study may be useful in the screening for pathogens with low water requirements for germination and subsequent penetration of the host insect.

All the three strains were originally isolated from locusts and grasshoppers in tropical Africa (Abraham *et al.*, 1991), yet differences in temperature profile and tolerance to water stress have been

established. No apparent link has been established between origin and temperature profile for fungal pathogens. For example, differences in temperature profile were established between *M. anisopliae* strain 100-82 and 101-82, although both originated from the same host species in France (Moorhouse, 1990). Similarly, Hall (1977) and Gillespie (1984) found no correlation between temperature optimum and region of isolation for *Verticillium lecanii* and *M. anisopliae* strains, respectively. Once applied in the field adverse environmental effects may be encountered. The *M. flavoviride* strains would have the edge over *M. anisopliae* IMI 168777/ii in ultra-low-volume (u-l-v) oil formulations for field application in the hot and arid conditions where locusts and grasshoppers are pests, other factors such as virulence to target hosts being the same.

The use of fungal pathogens may constitute an important pest management component for the suppression of locust and grasshopper populations below the Economic Injury Level (EIL). Several fungal strains have been isolated and characterized. For each isolate, a clear definition of the set of micro-environmental conditions necessary for germination and growth, which are the initial steps in the infection process, is important. Gradient plates make it possible to study three micro-environmental factors acting simultaneously. The decay of the pH and KCl gradients in the plates due to lateral diffusion can be minimized by the insertion of PFTE grid. Buffers can be used to improve the situation further and to reduce the sharp change in pH over the middle region of plates. However, buffers must be carefully selected since many fungi are not tolerant to cations and anions present in many buffer solutions, e.g. phosphate (Cochrane, 1958).

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