

Thermophilic fungi: an assessment of their potential for growth in soil

A K RAJASEKARAN and R MAHESHWARI*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

MS received 5 March 1993; revised 25 May 1993

Abstract. An attempt has been made to forecast the potential of thermophilic fungi to grow in soil in the laboratory and in the field in the presence of a predominantly mesophilic fungal flora at usual temperature. The respiratory rate of thermophilic fungi was markedly responsive to changes in temperature, but that of mesophilic fungi was relatively independent of such changes. This suggested that in a thermally fluctuating environment, thermophilic fungi may be at a physiological disadvantage compared to mesophilic fungi. In mixed cultures in soil plates, thermophilic fungi outgrew mesophilic fungi under a fluctuating temperature regime only when the amplitude of the fluctuating temperatures was small and approached their temperature optima for growth. An antibody probe was used to detect the activity of native or an introduced strain of a thermophilic fungus, *Thermomyces lanuginosus*, under field conditions. The results suggest that although widespread, thermophilic fungi are ordinarily not an active component of soil microflora. Their presence in soil most likely may be the result of the aerial dissemination of propagules from composting plant material.

Keywords. Thermophilic fungi; compost; aerial dissemination; spores; *Thermomyces lanuginosus*; autecology.

1. Introduction

Among the eukaryotes, only thermophilic fungi have the exceptional ability to grow at high temperatures of 50°–60°C (Cooney and Emerson 1964). Some thirty species of thermophilic fungi are currently known, most of which originally were isolated from composts (Tansey and Brock 1978). Their prevalence in composts is explained on the basis that prolonged elevated temperatures, humid and aerobic conditions, and supply of carbohydrate and nitrogen in the mass of organic matter favours the development of thermophilic microflora (Cooney and Emerson 1964). However, it is not certain if composts can be considered as 'natural' sites where thermophilic fungi may have evolved. Therefore natural habitats of thermophilic fungi have been searched, the soil being the principal among them. Although numerically far less than in composts, soils from virtually any place, when analysed by plating technique, have yielded thermophilic fungi (Apinis and Pugh 1979; Gochenaur 1975; Huang and Schmitt 1975; Maheshwari *et al* 1987). As the opacity of soil precludes *in situ* detection of fungal growth by light microscopy, indirect approaches were taken to verify whether their presence in soil was due to growth *in situ* or due to aerial dissemination of spores from foci of growth elsewhere and their fall-out from the atmosphere. Eggins *et al* (1972) used an immersion perfusion technique to screen fungi growing in soil and reported widespread occurrence of thermophilic fungi in soil, remaining dormant in shaded soil but becoming active in sun-heated

* Corresponding author.

Abbreviation used: FA, Fluorescent antibody.

soil. Jack and Tansey (1977) studied development of thermophilic fungi in pure cultures in Petri dishes which had been sealed and then buried in field soil. All species tested germinated and sporulated under these conditions, leading the authors to the view that the extent and duration of elevated temperatures reached in sun-heated soil are sufficient for thermophilic fungi to complete life cycle in soil. However, inasmuch as thermophilic fungi occur in soil amidst a vast majority of mesophilic microflora and microfauna comprising potential competitors, antagonists and predators, the above opinion based on axenic cultures has remained doubtful.

To assess the growth of thermophilic fungi in soil at usual temperature, we have taken different experimental approaches. The influence of temperature on respiratory rates of mycelia of thermophilic and mesophilic fungi was compared to forecast their behaviour under fluctuating soil temperatures. The growth of a mixed population of mesophilic and thermophilic fungi was studied in soil plates at diurnally changing temperature in a programmed incubator to predict their competitive ability under natural conditions. An immunofluorescence technique was used to visualize the behaviour of spores of a thermophilic fungus in soil. The results are presented in this paper.

2. Materials and methods

2.1 Organisms

The following fungi, taken from our laboratory collection, were used in different experiments: *Thermomyces lanuginosus*, *Penicillium duponti* and *Sporotrichum thermophile* (thermophilic fungi); *Aspergillus niger*, *Aspergillus phoenicis*, *Penicillium notatum* and *Trichoderma viride* (mesophilic fungi).

2.2 Measurement of respiration

Thermophilic and mesophilic fungi were grown at 50° and 30°C, respectively. All media incorporated 2% glucose as the carbon source. Cultures were grown in 500 ml Erlenmeyer flasks containing 150 ml medium on a gyratory shaker (240 rpm). The mycelia were harvested by filtration at the time when the growth reached half the maximal biomass. *T. lanuginosus* and *T. viride* were grown in a glucose-asparagine medium (Maheshwari and Balasubramanyam 1988) and used after 16 and 24 h, respectively, *P. duponti* was grown for 24 h in the above medium supplemented with 0.01% (w/v) yeast extract. *A. phoenicis* was grown for 18 h in a mineral salt solution. Washed mycelia were suspended in 50 mM, pH 5.6, sodium-potassium phosphate buffer and used for Warburg manometry as described previously (Prasad *et al* 1979). Respiration was measured in the presence of added glucose (2%, w/v).

2.3 Soil plating

Soils were collected from different places in India: Bangalore, Karnataka; Kota, Rajasthan; Pachmarhi, Madhya Pradesh; and Darjeeling, West Bengal. Approximately 500 mg soil was sprinkled on solidified YpSs agar (Cooney and Emerson

1964) which contained streptomycin sulphate (50 mg l^{-1}) and rose bengal (60 mg l^{-1}). Each sample of soil was replicated six times. Incubation was for up to 1 week at constant temperature or at a fluctuating temperature regime in a programmable incubator (Model 124L Plant Growth Chamber, Controlled Environment Ltd., Winnipeg, Canada).

2.4 Terminology

Visibly distinct fungal colonies, isolated on soil plates, were categorised on the basis of their temperature requirements for growth on YpSs agar: (i) thermophilic, by its ability to grow at $48^{\circ}\text{--}50^{\circ}\text{C}$ but not at 25°C , (ii) thermotolerant, by its ability to grow at temperatures from 25° to 50°C , and (iii) mesophilic, by its growth at 25°C but not at $48^{\circ}\text{--}50^{\circ}\text{C}$. The temperature tests were not done when the cultures could be identified correctly by morphological characters.

2.5 Preparation of fluorescent-labelled antibody for immunofluorescence microscopy of *T. lanuginosus*

Spores of *T. lanuginosus* strain RM-B were incubated in a glucose-asparagine medium at 50°C for 9 h on a shaker. Washed germlings were suspended in sterile 0.85% NaCl at $2 \text{ mg wet wt ml}^{-1}$ and used as the antigen. Male rabbits were injected intravenously with 1 ml of the fungal suspension at intervals of 1 week for 4 weeks. The rabbits were rested for two weeks after the final injection before blood samples were collected from the ear vein. Antibody production was determined by agglutination test with spores. The maximum agglutination titer was 1/2048.

A fluorescent antibody probe was prepared by coupling fluorescein isothiocyanate to goat anti-rabbit immunoglobulin (IgG). For indirect immunofluorescence microscopy, $100 \mu\text{l}$ of 1:50 diluted antibody was placed over the sample on cover slips. After incubation at room temperature for 1 h, the sample was rinsed in 5 changes of 0.1 M, pH 6.8, sodium-potassium phosphate buffer containing 0.9% NaCl. The sample was treated with 1:50 diluted fluorescein-labelled anti-rabbit IgG. After incubation for 30 min, the sample on the cover slip was again rinsed in the buffer-saline solution, mounted in 70% glycerol and viewed under UV light using a Leitz Ortholux microscope.

2.6 Detection of *T. lanuginosus* in soil

The growth of *T. lanuginosus* in soil was studied by a modification of the buried-Slide technique (Schmidt and Bankole 1963). Clean cover slips (20 by 20 mm) and those with *T. lanuginosus* spores adhering to them were buried in soil at different sites in a field. The spores were adhered to the cover slips by gently warming a smear of an aqueous suspension after checking that this procedure did not affect their viability. At least 20 cover slips were buried per site at a depth of approximately 3 cm in loosened soil in shaded and exposed areas. Replicate cover slips were also placed in soil in Petri dishes in the laboratory to monitor germination. At intervals, the cover slips were removed, rinsed gently in sterile water and examined microscopically using phase-contrast or UV optics.

2.7 Soil temperature

Soil temperature in the field was measured using a thermistor probe (Yellow Springs Co., Yellow Springs, Ohio, USA) connected to a battery-powered strip-chart recorder.

2.8 Sampling of air-borne spores

A volumetric, rotating-arm impaction device (Model 82 Rotorod Sampler, Sampling Technologies Inc., Los Altos, California, USA) was used to sample air-borne spores, 2 feet above the ground. The collector rods were coated with a jelly mixture containing 4% gelatin, 50% glycerine and 0.1% Tween 20. After use, the rods were placed in sterile water to remove spore deposit and aliquots of aqueous suspension were plated on YpSs agar at 50° C for isolation and identification of thermophilic fungi.

3. Results

3.1 Effect of temperature on respiratory activity of fungi

To determine whether mesophilic and thermophilic species can maintain their metabolic rate during temperature fluctuations of the environment, the respiratory rates of fungal mycelia were measured over a range of temperature. These data are given as Arrhenius plot of the logarithm of respiratory rate (Q_{O_2}) against the reciprocal of the absolute temperature (figure 1). The maximal Q_{O_2} of thermophilic fungi (*T. lanuginosus* and *P. duponti*) and of mesophilic fungi (*T. viride* and *A. phoenicis*) occurred, respectively, at 50° C and 30°-40° C. The Q_{O_2} of mesophilic fungi was higher than that of thermophilic fungi over a wide range of temperatures.

3.2 Competitive growth of mesophilic and thermophilic fungi at fluctuating temperature regimes

The type of fungus—mesophilic or thermophilic — that would grow from propagules in soil when it is plated on YpSs agar and incubated under a fluctuating temperature regime was determined. It was first ascertained that the soils tested contained both types of fungi. The control soil plates were incubated at constant 28° C or at constant 48° C. Incubation at 28° C yielded colonies of mesophilic fungi of which the following were dominant species: *Aspergillus* spp. (mostly black aspergilli), *Mucor* sp. and *Penicillium* spp. Incubation at 48° C yielded one or more species of the following thermophilic fungi in every soil tested: *Chaetomium thermophile* La Touche, *Humicola grisea* Traaen, *Malbranchea pulchella* Saccardo and Penzing var. *sulfurea* (Miehe) Cooney and Emerson, *Sporotrichum thermophile* Apinis, *Thermoascus aurantiacus* Miehe, *Thermomyces lanuginosus* Tsiklinsky and *Torula thermophila* Cooney and Emerson. Among the thermophilic fungi, *T. lanuginosus* was the most common in soil plates. Quantitation of fungal colonies on the soil plates incubated at 48° or 28°C was not done, but there was an obvious

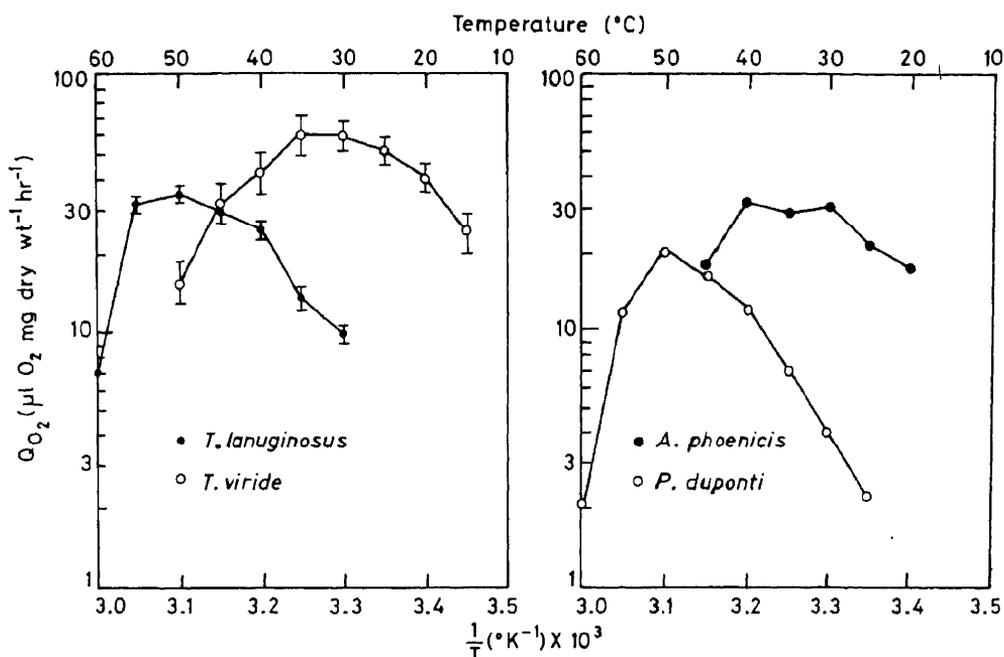


Figure 1. Arrhenius plots of respiratory rates of mesophilic and thermophilic fungi at different temperatures. The data for *T. lanuginosus* (thermophilic) and *T. viride* (mesophilic) are average values \pm SE from three experiments. The data for *P. duponti* and *A. phoenicis* are average values of triplicate Warburg flasks from a single experiment.

excess of mesophilic fungi over thermophilic fungi showing that soil contained higher number of mesophilic propagules.

Incubation at fluctuating temperature regimes, from 24° to 48° C, 32° to 48° C and from 36° to 48°C (figure 2) yielded mesophilic fungi or a thermotolerant fungus identifiable as *Aspergillus fumigatus*. At the fluctuating temperature regime, from 36° to 48°C, the thermophilic fungus *H. grisea* var. *thermoidea* was also present in some soil plates. By contrast, at the fluctuating temperature regime of 40° to 48° C, only thermophilic fungi appeared in soil plates; *T. lanuginosus* being the most common.

It was determined whether increasing the number of propagules of a thermophilic fungus in soil can improve its recovery in soil plates at the temperature regimes which were unproductive in the above experiment. Approximately 10⁸ spores of *T. lanuginosus* were thoroughly mixed with 50 g of garden soil and a portion of it was plated. Approximately 2-4 times more colonies of *T. lanuginosus* were recovered from the enriched soil than from the control, but again when incubation temperature varied between 40°C (minimum) and 48° C (maximum) only. The results showed that temperature of environment was an important factor determining growth of thermophilic fungi in soil plates.

Growth of *T. lanuginosus* was also tested in pure culture at the temperature regimes which were unfavourable in soil-plate experiment. Spores seeded on YpSs agar and incubated at any of the fluctuating temperature regimes developed colonies. This showed that the temperature of the fluctuating cycle *per se* can support germination and development of *T. lanuginosus* provided it is present alone.

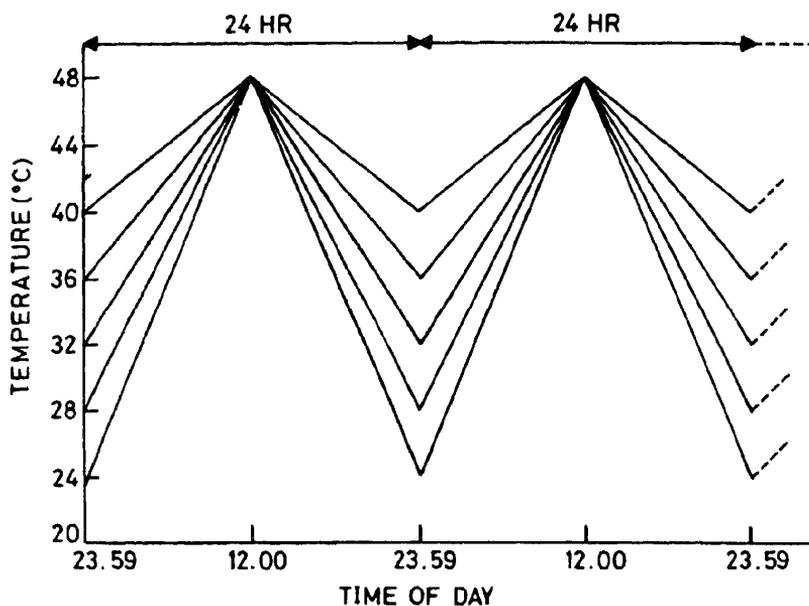


Figure 2. Diagram of five diurnally fluctuating temperature regimes used to programme controlled environment chamber for study of the competitive growth of a mixed soil fungal flora on nutrient media.

3.3 Autecology of *T. lanuginosus* by immunofluorescence microscopy

Immunofluorescence was used to study the autecology of *T. lanuginosus* in soil. This fungus was chosen because it is the most ubiquitous thermophilic fungus in soil (Maheshwari *et al* 1987). First, the specificity and the efficacy of the fluorescent antibody (FA) preparation was tested. *T. lanuginosus* mycelium treated with pre-immunized serum did not fluoresce. The FA reacted strongly with strain RM-B and five other strains of the same fungus, showing that the antibody was not strain-specific. The FA reacted also with mycelia of *A. niger* grown in axenic culture but not with those of the four other fungi tested: *P. duponti*, *S. thermophile*, *P. notatum* and *T. viride*. The results showed that FA was sufficiently, but not absolutely, specific to *T. lanuginosus*.

In order to learn whether the FA preparation would detect *T. lanuginosus* if present in soil, spores were adhered to glass cover slips and incubated under non-sterile garden soil in Petri dishes kept in incubators at 30°, 50°C or at a temperature regime which fluctuated between 40° and 48°C. At 30°C, spores remained dormant up to three weeks when the observations were terminated. In contrast, at 50°C spores germinated in 4 h (figure 3A). The hyphal growth on cover slips reacted positively with the FA preparation and produced a bright apple-green fluorescence (figure 3B). The wall of the germinated spore did not react in immunofluorescence although the antiserum agglutinated the ungerminated spores. Presumably, the antigen from the spore wall was lost during germination. Spores germinated also at the fluctuating temperature regime (figure 3C) and the germ tubes gave a positive reaction with FA (not shown). The results showed that *T. lanuginosus* can grow in soil and maintain its antigenicity.

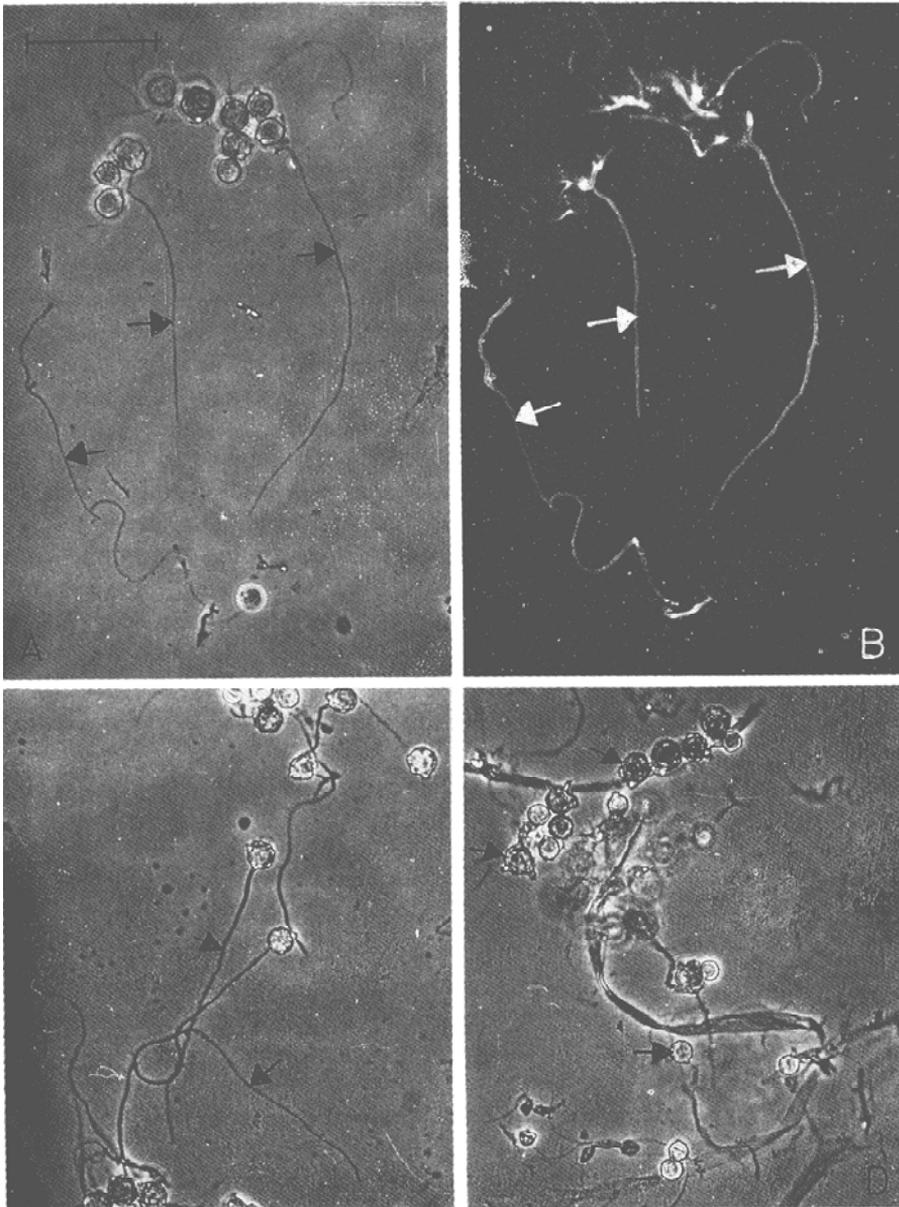


Figure 3. Microscopic examination of *T. lanuginosus* spores on cover-slips. **(A)** Germinating spores (↑) on cover-slip placed in contact with soil in Petri dish in a constant 50° C incubator for 4 h and stained with fluorescent-antibody. Phase contrast microscopy. **(B)** Same as viewed under UV showing fluorescence of hyphal growth (↑) **(C)** Cover-slip bearing spores of *T. lanuginosus* in contact with soil in Petri dish in an incubator operating on a diurnally fluctuating thermal regime (min. 40° C, max. 48° C) for 4 h. Germination of spores (↑) has occurred. Phase contrast microscopy. **(D)** Phase contrast microscopy of cover-slip bearing *T. lanuginosus* spores (↑) buried in soil in the field for 8 days and treated with a fluorescent-antibody. Dormant spores of *T. lanuginosus* and unidentified growth are seen. The hyphal growth did not show fluorescence when viewed under UV light. All micrographs are approximately of same magnification. Bar length is 50 μm.

Based on the above results, an attempt was made to detect *T. lanuginosus* in a field. Samples of soil from the experimental sites were plated to confirm the presence of *T. lanuginosus*. Phase-contrast microscopy showed mycelial growth on cover slips which had been buried in field for over three weeks. On some cover slips sporulating mycelia of *Alternaria* sp. and of an actinomycete were present which did not react with FA. Mycelia which reacted positively with FA were not found.

The concentration of the resident population of *T. lanuginosus* in soil could be too low for successful competition and detection. Therefore, spores of *T. lanuginosus* strain RM-B were adhered on cover slips and introduced into shaded and exposed areas in the field. These spores remained dormant for up to three weeks when observations were terminated. However, some hyphal growth was observed but it was not produced from spores of *T. lanuginosus* adhering to the cover slips (figure 3B). This fungal growth reacted negatively with FA in most samples. In a few cover slips, however, the hyphal fragments stained with FA but since reproductive structures were absent, these could not be identified as belonging to *T. lanuginosus*. The diameter of the hyphal fragments appeared too wide and the cell wall exceptionally thick and as a result these hyphae were not considered to be *T. lanuginosus*. The above experiments with the buried cover slips were done during June-September when the moisture levels in the soil seemed favourable for microbial activity as evidenced by decomposing substrates in the field. The soil temperature during the experiments varied between 22°–28° C.

3.4 Detection of spores of thermophilic fungi in atmosphere

Sampling of air in the field by Rotorod Sampler yielded thermophilic fungi. *T. lanuginosus* and three unidentified fungi were recovered, showing the presence of thermophilic fungi in air.

4. Discussion

The Arrhenius plots of Q_{O_2} of mycelia as a function of temperature indicate that the metabolic rate of mesophilic fungi is affected less by the changes in temperature than that of thermophilic fungi (Prasad *et al* 1979; present study). The biochemical basis of this difference in the thermal responses of mycelia of mesophilic and thermophilic fungi has not been investigated; nevertheless this behaviour may have implications for growth in natural conditions. If mycelia in soil environment respond similarly (figure 1), then the fungi which can maintain optimal metabolic rate over a broad range of temperatures may be expected to have a competitive advantage over those which lack this ability. Mesophilic fungi, rather than thermophilic fungi, would appear to be better adjusted to soil where temperatures vary both spatially and temporally.

This inference is corroborated by the results of soil plating at the fluctuating temperatures. Mesophilic fungi developed in soil isolation plates when the minimum temperature was varied from 24°–36°C to a maximum of 48°C. Thermophilic fungi appeared in soil plates only when the incubation temperature fluctuated to a small extent (between 40° and 48°C) approaching the average optimum growth temperature of 46°C of thermophilic fungi (Cooney and Emerson 1964). Although soil in some places does heat up to 40°–50°C or more (data

provided by Meteorological Survey of India, Pune; Stotzky 1974), the important point is whether adequate moisture is available when soil temperatures conducive for thermophile growth are attained. Cooney and Emerson (1964) emphasized moisture as one of the factors of crucial importance in development of thermophilic microflora.

The thermophile *T. lanuginosus* is one of the most common fungi occurring in soil (Maheshwari *et al* 1987). However, immunofluorescence microscopy did not detect the growth of either the indigenous or of the introduced strain of this fungus on cover slips buried in the field. Because of the problems encountered in observing and identifying propagules in soil itself, we used glass cover slips with or without spores of *T. lanuginosus* adhered to it as the support. It may, however, be that mycelia of *T. lanuginosus* grow in between crevices of soil particles and glass cover slips did not provide a suitable surface which acted as a barrier for diffusion of water and nutrients for initiation of growth. This problem was minimized by using the buried cover slips during the monsoon season with moisture levels in soil favourable for microbial activity in the field. Since *T. lanuginosus* spores placed on cover slips in soil did germinate at 50° C in an incubator, it seems reasonable that low temperature rather than wrong surface, nutrient limitation or contact with inhibitory substances was the chief reason for the failure of spores to germinate in field conditions.

The results of all three experimental approaches used in this study suggest that thermophilic fungi are not a normally active component of microflora in the soil. In as far as is known thermophilic fungi are poorly represented also in thermal soils from volcanic regions (Cooney and Emerson 1964). How then may we explain the world-wide distribution of *T. lanuginosus* and other species of thermophilic fungi in soils (Ellis and Keane 1981; Maheshwari *et al* 1987)? It is significant that species of thermophilic fungi obtained from sampling of air, 6 km from a waste compost system in Bombay (Thakur 1977), were the same as those from over 200 soil samples from several places in India (Apinis and Pugh 1979; Maheshwari *et al* 1987). The presence of air-borne propagules of thermophilic fungi in temperate (Evans 1972; Hudson 1974; Hughes and Crossier 1973), subtropical or tropical countries (Hedger 1975; Sandhu and Singh 1985; Thakur 1977; present study) suggests that their wide distribution in soil is a result of the dissemination of their propagules from composts.

It may be asked if 'natural' composts existed before man where thermophilic fungi might have evolved? Cooney and Emerson (1964) suggested that nests of an old group of birds, the Megapodidae, or the incubator birds, which have probably existed since 50 to 60 million years (Grzimek 1984), as possible natural sites of occurrence of the thermophilic fungi. These birds occur in Australia and islands of the south western Pacific, and incubate their eggs inside large mounds (composts) which they construct out of forest litter wherein the microbial activity produces respiratory heat that warms them up to 33°-50° C for several months (Seymour and Bradford 1992). Probably thermophilic fungi evolved from their mesophilic counterparts in such thermal, humid and aerobic environment.

Acknowledgements

We thank Professor (Emeritus) Alfred S Sussman, University of Michigan, for his

valuable comments on this manuscript and suggestions on future work. This work was supported by the Department of Science and Technology, New Delhi.

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