SECTION - C

CHAPTER III

EFFECT OF INHIBITORS AND STIMULATORS ON SPORANGIOSPORE GERMINATION
INTRODUCTION

A cell inhibited by an antibiotic is in a dynamic state: interaction between an antibiotic and a specific binding site in a sensitive cell triggers a chain of reaction which leads ultimately to inhibition of normal growth and cell division. Germination of spore is prevented by polyene antibiotics; if germinated spores of *Aspergillus niger* were placed into filipin-containing medium, a portion of the germtube near its tip swelled and finally bursted (Gottlieb et al., 1960). The effect of antibiotics on spore germination of fungi has been studied by many workers (Dhanvantari, 1968; Patil and Rao, 1972; Cheema and Jeyaranjan, 1971; Kushwaha and Agrawal, 1975; Valaskova, 1963). Gupta (1958) stated that streptomycin was more inhibitory than penicillin to *Protomyces macrosorius*. Polynoxin-D is also a strong inhibitor of spore germination in *Mucor rouxii*; it affects chitin synthesis (Bartnicki and Lippman, 1972). The antibiotics, like griseofulvin and cyclohexamide affect greatly the morphology of germtubes and metabolism of spores (Issac and Milton, 1967). Thakre and Johri (1974) have studied in detail the effect of antibiotics on the swelling and germination of spores of thermophilic fungi. The thermophiles may have special organellar membrane system responsible for their high temperature resistance.
Spore swelling is inhibited by sodium azide, fluoride, malonate and 2,4-dinitrophenol in *Penicillium strovenatum* (Gottlieb and Tripathi, 1968) while Martin and Nicolas (1970) have reported the inhibition of spore germination in *Penicillium notatum* and *Trichoderma lignorum* by sodium azide. Generally all the respiratory inhibitors checked spore germination of *Rhizopus arrhizus* (Skundaryo, 1966). Lysine inhibited the growth of fungus *Rhizopus nigricans* (Stocks and Ward, 1962). Growth of many fungi on the contrary is stimulated by glutamic acid and peptone (Sumner, 1970; Nicolas and Villanueva, 1965).

There are numerous records of the effects of volatile metabolites on fungi. Several workers (Mixon and Curl, 1967; Gilbert et al., 1969; Hora and Baker, 1970; Hutchinson, 1971) have shown that the growth of soil inhabiting fungi can be stimulated by gaseous products from higher plants. The production of biologically active volatile metabolites by culture of *Trichoderma viridae* has been reported by Bilai (1956) and Dennis and Webster (1971). Volatile substances produced by bacterial species greatly inhibit growth and sporulation of fungi (Moore Landecker and Stotzky, 1972). A particularly interesting aspect of volatiles has been in the sphere of *in vitro* and *in vivo* inhibition of fungal spores (Robinson and Garrett, 1969; Balis and Konyeas, 1968). Singhai
(1973) also added valuable information to this aspect of volatile inhibitors. Volatile metabolites which are emitted from fungi may inhibit germination of their own spores (Allen, 1955; Carlile and Sellin, 1963; Robinson and Park, 1966) or spores of other fungal species (Robinson et al., 1968; Tyner, 1966). The vapours of p-chloro-n-cresol and MgCl₂ inhibited spore germination (Alieja and Strizeczyk, 1968). Spore germination of Agaricus bisporus (Losel, 1964) and Agaricus campestris (Mc-Teague et al., 1959) may be stimulated by volatiles secreted by their own mycelium. Volatiles from alfalfa hay increase germination and respiration of fungi (Menzies and Gilbert, 1967; Owens et al., 1969), stimulate growth of Sclerotium rolfsii (Linderman and Gilbert, 1968) and inhibit Verticillium dahliae in soil (Gilbert and Griebel, 1969). The spores also contain a volatile self-inhibitor, recently identified as methyl ferulate (Macko et al., 1971), which is removed from the spores by soaking them in water. Sclerotium fructicola exudes a compound which in high concentration inhibited but in a low concentration acted as a stimulator of spore germination (Ende and Cools, 1967).

In view of the information described above it was of interest to evaluate the role of antibiotics, metabolic inhibitors, stimulators and volatiles on the swelling and germination phases of the sporangiospores of R. rhizopodiformis and to compare it with the pattern known in the literature for mesophilic forms.
MATERIALS AND METHODS

The culture of *R. rhizopodiformis* was maintained as described previously. Swelling and spore germination were studied under the influence of following substances. The influence of antibiotics (suresofungin, erythromycin, griseofulvin, nystatin and pentone DG-15) and metabolic inhibitors (sodium arsenate, azide, flumoxide, fluoroacetate, malonate and 2,4-dinitrophenol) was studied in glucose asparagine broth; the former were supplied in 25 to 200 ppm and the later in 0.0003 to 0.003 M concentrations. The effect of inhibitors of germination (ammonium chloride, copper sulphate, lysine, phosphoric acid and puromycin) and stimulators (ascorbic acid, boric acid, ethylacetate, glutamic acid and peptone) was studied in the concentrations ranging from 10 to 1000 ppm; the broth without any substance served as control.

The effect of nutrients described above were carried out by dispensing 10 ml of glucose asparagine broth (without microelements) in 100 ml Büchi flasks. After autoclaving, each flask was inoculated by suspension to provide a final concentration of app. 2 x 10⁵ spores per ml. The flasks were incubated at 45 °C in a rotatory shaker. Samples were withdrawn aseptically at 4.30 and 6.0 hr for study of swelling and germination of sporangiospores.
The effect of volatile compounds on the spore germination and mycelial growth was studied by providing dilutions ranging from 25 to 100%. The effect of volatile compounds on spore germination was studied by modified cellophane agar disc technique (Schuepp and Green, 1964). In this method the bottom half of Petriplate was covered with sterile cellophane after dispensing 10 ml of desired volatile compound. The paper was allowed to draw out of the margins where it was fixed with the plate by using a cello-tape. A U-tube was then placed over the cellophane paper, on which a cavity slide was kept having a drop of spore suspension in the nutrient medium. The assembly was covered with a bottom lid of the Petriplate and fixed with cello-tape. The plates were incubated at 45°C and swelling and germination counts were made after 4.30 and 6.0 hr, respectively.

The effect of volatile substances on growth was studied in 100 ml Erlenmeyer flasks with side tubes. Each flask received 30 ml of medium and after autoclaving they were inoculated by an agar disc of 6 mm taken from the edge of a 4-day old colony; for each treatment two flasks were inoculated. A day after inoculation 5 ml of the volatile substance (to be tested) was taken in the culture tube and this was connected to the flasks with the help of rubber tubing. Mouth of flasks and tubes were twined with polythene paper in order to minimize evaporation of the volatile; the tubes were kept erect in a stand. The whole assembly was kept at 45°C for 3 days. A separate set of flasks without volatiles served as
control. The mycelial weight was obtained by filtering the content of the flasks through preweighed filter paper discs, drying and reweighing them.

RESULTS

**Antibiotics:**

All the antibiotics inhibited swelling and germination of sporangiospores of *Rhizopus rhizopodiformis* (Fig. 11). The inhibition of spore germination was more pronounced than its swelling phase. Aureofungin and pentene DG-15 (200 ppm) were able to bring about complete inhibition of spore germination while for erythromycin, nystatin and griseofulvin this concentration was only partially inhibitory; nystatin was not effective at lower concentrations. The normal trend was a gradual lowering of the germination rate with a progressive increase in the concentration of antibiotics. In case of pentene DG-15, lower concentrations were more active in reducing the size of swollen spores and the length of germ tubes.

**Metabolic inhibitors:**

Sodium arsenate, azide, fluoride, fluoroacetate and malonate were strongly inhibitory as they did not allow
Fig. 11. Effect of antibiotics on swelling and germination of sporangiospores of *R. rhizopodiformis*.

A - Aureofungin
B - Emysin
C - Griseofulvin
D - Nystatin
E - Pentene DG-15
germination of the spores (Fig. 12). Only 4% spores germinated when azide was added at 0.003 M concentration; sodium fluoride and fluoroacetate showed only moderate inhibition at this concentration. Sodium arsenate and malonate were comparatively less effective at the lower concentration (0.0003 M). In case of 2,4-dinitrophenol, concentration up to 0.0006 M increased the swelling and germination of sporangiospores while higher concentration reduced per cent swelling and germination.

**Germination inhibitors and stimulators:**

Only 32% swelling and 14% germination was observed when ammonium chloride was added at a concentration of 1000 ppm (Fig. 13). Copper sulphate was stimulatory at lower concentration (10 ppm) but inhibited swelling and germination of the sporangiospores at higher concentrations. Puromycin, lysine and phosphoric acid were very effective in inhibiting the germination of spores. There was no germination when puromycin was added at a concentration of 1000 ppm although some swelling of the spores was observed; in case of lysine and phosphoric acid only 2-3% spores germinated at this concentration. In general, concentrations higher than 500 ppm were strongly inhibitory to the sporangiospores.

At higher concentration (1000 ppm), all the known stimulators of spore germination reduced the per cent germination of sporangiospores (Fig. 14). At higher
Fig. 12. Effect of metabolic inhibitors on sporangiospore swelling and germination of *R. rhizopodiformis*.

A - Sodium arsenate
B - Sodium azide
C - Sodium fluoride
D - Sodium malonate
E - 2,4-dinitrophenol
Fig. 13. Effect of known germination inhibitors on sporangiospore swelling and germination of R. rhizophiiformis.

A - Ammonium chloride  
B - Copper sulphate  
C - Lysine  
D - Phosphoric acid  
E - Puromycin
Fig. 14. Effect of known germination stimulators on swelling and germination of sporangiospores of \textit{R. rhizopodiformis}.

- A - Ascorbic acid
- B - Boric acid
- C - Ethylacetate
- D - Glutamic acid
- E - Peptone
concentration (1000 ppm) all the known germination stimulators except ethylacetate reduced the spore germination percentage. Glutamic acid was able to stimulate swelling of spores up to 500 ppm and germination up to 100 ppm. Boric acid, on the other hand, increased swelling and germination of spores at 50 ppm concentration.

Volatile compounds:

Different volatiles showed varied effects on growth of the fungus (Fig. 15). Inhibition of spore germination and fungal growth was noted in the presence of ammonia, methanol, acetone and benzene when they were supplied at lower concentration (25%). The more concentrated series strongly inhibited germination of spores and growth of the fungus which gradually declined at lower concentrations. At 25% level, amylalcohol and butanol were stimulatory but at 50% concentration swelling and germination of spores was almost comparable to controls; the dry wt. of the mycelium, however, decreased. Undiluted amylalcohol (100%) resulted in decreased dry wt. although butanol was not as effective. Ethanol stimulated spore germination and growth up to 50% concentration but higher concentrations were inhibitory; similar effect was shown by isopropyl alcohol. The effect of ethylacetate was unlike other volatiles since spore germination and dry wt. of the mycelium increased up to a concentration of 75%; the dry wt. of fungus decreased at higher concentration but the values
Fig. 15. Effect of volatile compounds on sporangiospore swelling, germination and mycelial growth of *R. rhizopodiformis*.

A - Acetone
B - Ammonia
C - Amyl alcohol
D - Benzene
E - Butanol
F - Ethanol
G - Ethylacetate
H - Isopropyl alcohol
I - Methanol
were still higher than those observed in controls (without ethylacetate).

DISCUSSION AND CONCLUSION

The germination of spores is comparable to any other metabolic process which is influenced by several factors. The inhibition of spore germination and fungal growth by antifungal polyene antibiotics is well illustrated in the literature (Thirumalacher et al., 1961, 1964, 1969; Trinci and Gull, 1970). The inhibition of spore germination of thermophiles by antifungal antibiotics was quite strong though higher concentrations alone were effective in bringing about complete check. The swelling phase in *R. rhizopodiformis* was comparatively less sensitive to the antibiotics. In case of this fungus about 10% of the spores germinated at a concentration of 200 ppm of nystatin while in *Aspergillus* species no swelling and germination occurred at 50 μ units per ml (Stanley and English, 1965). Effect of higher concentration of aureofungin on the spores of *R. rhizopodiformis* resembled with the mesophilic species of *Alternaria* (Singh et al., 1973); conidial germination of two species of *Cystospora* was inhibited by very low concentration of aureofungin. The results showed that pentene DG-15 completely checked spore germination of higher concentration. The metabolic inhibitors also inhibited spore swelling and germination in the fungus.
**R. rhizopodiformis.** Inhibition of spore germination in *Rhizopus arrhizus, Penicillium atroventum, P. notatum* and *Trichoderma lignorum* by sodium azide, fluoride, arsenate and 2,4-dinitrophenol is well documented (Ekundayo, 1966; Gottlieb and Tripathi, 1968; Martin and Nicolas, 1970), though similar studies with thermophilic fungi are almost completely lacking (Thakre and Johri, 1973, 74).

The experimental results indicate that some volatile compounds act as an inhibitor while few are stimulatory. The spore germination and mycelium growth of *R. rhizopodiformis* was inhibited by ammonia at very low concentration while in *Aspergillus clavatus* ammonia and urea have been used as nitrogen source during conidial germination (Robinson *et al.*, 1974). Ammonia is also known to repress the formation of several proteins involved in the nitrogen metabolism of *Aspergillus nidulans* (Cove, 1966; Arst and Cove, 1969; Cohen, 1972) and inhibits the active transport system for acidic amino acids (Robinson *et al.*, 1973a, b). Ethanol increases germination and growth of fungi at low concentration by being utilized as carbon source and also increases the efficiency of utilization of glucose in many fungi (Fries, 1973). Robinson and Park (1966) reported that germination of spores of *Rhizopus stolonifer* is induced by low concentration of ethanol. Wheeler (1972) has stated that mycelium growth and maturation of sclerotia was delayed by addition of ethanol at 0.5 to 3% to *Sclerotium rolfsii* but lower concentrations
stimulated growth. Inhibition of spore germination has also been attributed to acetaldehyde (French, 1962) or to a mixture of acetaldehyde and ethanol (Glen and Hutchinson, 1969). Butanol stimulated growth in \textit{R. rhizopodiformis} as seen in some microorganisms (McLee \textit{et al.}, 1972). Acetone acted as an inhibitor for \textit{R. rhizopodiformis} but the acetone extracts of soil with microorganisms stimulated the formation of asexual bodies at very low concentration (Ayers, 1971). Ethyl acetate extracted from \textit{Fusarium oxysporum} retarded the germination of sporangiospores of \textit{Cunnighamella elegans} (Robinson and Garrett, 1969) though spore germination and fungus growth was stimulated by this volatile in case of \textit{Rhizopus rhizopodiformis}. These results indicated that the effect of one volatile at different concentration can be different in respect of germination and growth phases.