A STUDY OF THE LIFE CYCLE
OF NEOCOSMOSPORA AFRICANA
(VON ARX)

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A B S T R A C T

The life cycle of Neocosmospora africana was studied from single spores. These unisporous cultures developed to yield mature perithecia. The fungus was found to be homothallic.

Media containing different carbon sources were tested for the ability of the fungus to maintain growth on them. All carbon sources tested supported the growth and sporulation of the fungus. Of the different nitrogen sources tested, vegetative growth was obtained on all while sporulation occurred on all media except the one containing ammonium sulphate as a result of changes in hydrogen-ion concentration despite the heavy buffering of the medium.

Reproductive morphogenesis of the fungus was studied from surface static cultures on liquid glucose asparagine medium. Production of conidia occurred following the establishment of the mycelium. Development of perithecia in the same culture occurred much later following decline in growth rate and nutrient depletion.

The ascogonium was found to be a coiled hyphal branch. The uninucleate and binucleate cells of the ascogonium become surrounded by hyphal cells that develop from adjacent stalk cells. Growth of the perithecium is caused by the divisions and enlargement of cells of hyphae surrounding the ascogonium. Meristematic cells in the apical region of the young perithecium give rise to an ostiole lined by periphyses.

The centrum comprises the ascogonium and its descendant structures. The asci and evanescent paraphyses are borne on a

slightly convex hymenium restricted to the basal central part of the centrum. The perithecial cavity increases from the deliquescence of the inner pseudoparenchymatous walls.

The asci develop from the ascogenous system without crozier formation. Asci are double walled, cylindrical structures and are characteristically 8 - spored. Two successive mitoses in each ascospore followed by cell divii3ion synchronise with the maturation of the spores.

I. INTRODUCTION

Neocosmospora is a genus of the Nectriaceae. So far three species of this genus have been reported - N. vasinfecta, N. africana and N. ornamentata.

N. africana is an inconspicuous saprophytic member of the soil mycoflora. Apart from the works of Barbosa (8) on the laboratory culture of the fungus; and of Batista et al (11), there has been no work done on the species.

In this work an attempt has been made to study the life cycle with emphasis on cultural as well as ontogenetic studies of this organism.

The germination of the spore was studied to determine the effect of environmental factors. The carbon metabolism of the fungus has been investigated to determine the sources of carbon on which the organism can be safely cultured. Robbins (1937) classified fungi into four groups on the basis of the nitrogen sources utilised by these fungi. The nitrogen metabolism of the fungus was investigated to find out where the organism fits in this scheme. The vitamin requirement of the fungus was also determined.

The effects of the physical factors - light, temperature, and hydrogen-ion concentration on growth and sporulation were also investigated. Lilly and Barnett (57) have stated that vegetative and reproductive growth are affected by slight variations in environmental factors. These investigations were conducted to verify

this in the organism and determine the optima for the different phases of its growth.

The morphogenesis of the fungus has been investigated following closely the behaviour of the mycelium starting from a single spore. The development of sexual spores has been studied using various media and employing different techniques.

Every effort has been made to illustrate the pertinent points with the best evidence obtained. These details are reported as revealed by light microscopy.

II. REVIEW OF LITERATURE

Neocosmospora africana is an Ascomycete (Ascomycotina of Ainsworth and others, 1973) belonging to the Order Hypocreales of the Series Pyrenomycetes.

The Hypocreales is an order of fungi characterised by the presence of perithecia and sustaining or enclosing stromata, when present, that are brightly coloured. The order shows close parallelism to the Order Sphaeriales in the perithecial form and arrangement but are customarily distinguished from one another by the consistency of colour of the perithecia.

It has been contended (14) for long that the colour and consistency of the perithecium or stroma are not satisfactory as a basis of distinction of the two orders; and that the structure and mode of development of the perithecium are by far of greater value in determining true relationships. Presently, there have been rather few studies of the development of perithecium in the series Pyrenomycetes to provide enough taxonomic data to clarify this ordinal segregation.

The Hypocrealean fungi are grouped into three families - the Nectriaceae, the Hypocreaceae and the Clavicipitaceae.

The genus Neocosmospora belongs to the family Nectriaceae characterised by the following features: a true perithecium with or without a stroma and brightly coloured; ascospores are one to many - celled, ellipsoid to cylindrical in shape (14). The other genera

reported in the family include Nectria; Gibberella; Sphaerostilbe; Scoleconectria; Thyronectria.

In the genus Neocosmospora, so far only three species have been reported in mycological literature - Neocosmospora vasinfecta, N. africana, and N. ornamentata. All are saprophytic fungi or at best, facultative saprobes.

N. vasinfecta has been reported to be a mild pathogen of a wide range of tropical crop plants - having been found to be the causal organism of the false wilt of cotton (74); concerned in the etiology of gram wilt of Cicer arientinum (1); It has also been associated with infection in the destructive root rot of Cyamopsis tetragonoloba (93). Though the fungus was reported not to be directly lethal, it was found to be the cause of the drooping and flagging in some of the leaves. N. vasinfecta has been reported to be infrequently isolated from the rhizosphere microflora of diseased palms (81); in the rhizosphere of pigeon pea (1); and from diseased materials of Cyamopsis tetragonoloba (93), though no pathogenicity was established.

Neocosmospora africana was first isolated from soil samples from Rietvlei near Pretoria and identified by von Arx in 1951, and later from a soil sample he received from the Zaire in the same year. The first record of its occurrence outside Africa was in Japan in 1958 (102). It has also been reported as a member of the soil microflora in Portugal (11). Its occurrence here in Samaru has been recorded

in the rhizosphere mycoflora of Arachis hypogea (63) and there have been undocumented reports of its isolation from soils in Kano in 1968.

In all the cases, no pathgenic tendencies have been established for N. africana, the organism living saprophytically among the soil microflora. It has been reported by Batista and others (11) to produce growth promoting substances of the auxin type in the soil.

N. ornamentata, the most recently reported species of the genus, was first isolated from stored groundnuts from Portuguese Guinea (the now independent state of Guinea Bissau) and it was implicated as one of the fungi contributing to the degeneration of seed and oil quality in stored groundnuts (7).

The saprophytic nature of these three species of Neocosmospora explains their wide distribution in nature as in all saprophytic fungi and unlike parasitic fungi that are restricted in their distribution to their hosts.

Barbosa (8) succeeded in culturing the three species on semi-synthetic media. Generally, saprophytic fungi can be cultured on synthetic and semi-synthetic as well as natural media though the composition of such media varies slightly from one organism to another. The composition of a synthetic medium is precisely known while that of a semi-synthetic medium is not known because of the presence of certain substances of unknown chemical composition.

Though the composition of natural media is not known, they have been found to be the best for the cultivation of saprophytic fungi (23, 57). Synthetic media have been found to be the most useful in the laboratory studies of fungal nutritional requirements although they have been proved to be the least favourable for the laboratory cultivation of fungi.

The essential features of a culture medium that can support the growth of a fungus include the presence of a source of carbon, a source of nitrogen, essential mineral elements, available in utilisable forms, and in some cases, vitamins. Compounds that serve as carbon sources for fungi cover the whole range of organic compounds known, from simple sugars to complex polysaccharides. No single carbon source has been known to be available to all classes of fungi although certain compounds such as glucose and fructose are utilised by most fungi. The ability to utilise a compound as a source of carbon is an intrinsic feature of the organism demonstrated by the ability to secrete the necessary enzymes to digest the compound (79).

Sources of nitrogen utilised by fungi range from atmospheric nitrogen to all known nitrogen containing compounds of both organic and inorganic nature. Several workers (57) have classified fungi into four main groups based on their ability to utilise different sources of nitrogen as follows: group one - those fungi that are able to utilise atmospheric nitrogen, nitrate nitrogen, ammonium nitrogen and organic nitrogen; group two - those fungi that are able

to utilise nitrate nitrogen, ammonium nitrogen and organic nitrogen but not able to utilise atmospheric nitrogen; group three - fungi that are able to utilise ammonium nitrogen and organic nitrogen but not able to utilise nitrate nitrogen or atmospheric nitrogen; and group four - fungi able to utilise only organic nitrogen. With the exception of the few fungi able to fix atmospheric nitrogen, growth and development of fungi fail to take place in the absence of nitrogen in the medium. In addition, sporulation and conidial characteristics of many fungi are influenced by nitrogen (98). While the nitrogen supply of a fungus has a less specific effect than carbon supply on reproduction, the ratio of carbon and nitrogen has been shown to be important (47).

Not all fungi require the introduction of vitamins into the medium before they can grow. Most species of Aspergillus, Chaetomium globosum and Neocosmospora vasinfecta have been reported to be self-sufficient with respect to vitamins while Phycomyces blakesleeanus has been reported to be thiamine deficient (57).

Fungal cultures can be maintained on either solid or liquid media. In these cultures, growth of the fungus is limited by:

(i) the relatively small size of the culture vessel resulting in a barrier effect;

(ii) the release into the medium of autoinhibitory metabolites, staling substances;

(iii) the depletion of nutrients in the culture medium.

A group of mycologists and plant pathologists has subscribed to the concept of unlimited growth if these limiting factors were removed (39)

though Gottlieb (1971) has continued to question the concept as a result of his researches on the ageing of cultures.

Growth of fungal cultures is estimated by one of the following methods: visual estimate, measurement of linear growth as by measuring the diameter of culture or using the Ryan tube - suitable for solid media cultures; the measurement of the dry weight of mycelium has been the most convenient method for liquid media cultures; cell count, cell volumes and turbidity determination for unicellular fungi.

Environmental factors influencing growth and development of fungi include temperature, moisture, light, hydrogen-ion concentration, and the concentration of nutrients. Apart from these external influences, internal factors play an equally significant role in determining the rate of growth of the fungus because these external factors act only by modifying the internal environment. The internal factors include age, genetic composition and the previous history of the fungus.

Temperature affects the three different phases of growth of fungi - spore germination, vegetative and reproductive growth. For each of these phases of growth, three cardinal temperatures - minimum, optimum and maximum - exist. Similarly there is a range of hydrogen-ion concentration between which any of these phases of growth can occur. These two factors, temperature and pH, have been found to affect the morphology in certain fungi. Thermal dimorphism has been reported

(108) in the human fungal parasite, Blastomyces dermatitidis in culture. Dimorphism caused by changes in pH of the medium has been reported for the coprophilous fungus, Sordaria fimicola; chemical influences in the media have been variously reported as being responsible for the cause of dimorphism in many unrelated species and genera (57, 108). In the breaking of dormancy in certain spores, chemical influences have been implicated (23, 47, 29, 96).

Despite the concept of minimum temperatures, many fungal cells remain viable after prolonged exposure to subzero temperatures. Spores of N. vasinfecta have been reported (80) to remain viable after two years freezing in water.

Many fungi are affected by light and the effect of light on fungi fall into two main categories: morphogenetic effects in which light induces or inhibits the formation of a structure; and the non-morphogenetic effects in which light influences the rate or direction of movement, or growth of a structure, or the ability to synthesize a compound (22, 47, 53, 78). Light has been reported to influence the size and shape of spores and spore - brearing structures; some fungi have an absolute requirement for light for sporulation and these fungi belong to various classes of fungi. Fruit bodies of many fungi are initiated in the dark but develop abnormally unless exposed to light. In some fungi, light effect induces the production of alternating zones of sporing and non-sporing hyphae, due to diurnal periodicity of light and darkness. Sporulation has been

reported to be depressed or completely inhibited by light in some fungi.

In the life cycle of all true fungi, the spore germinates producing germ tube which develops into the fungal thallus. Reproductive growth closely follows vegetative growth of the fungus. Though the production of sexual spores is common in the higher fungi, it is not characteristic of all.

Different mating mechanisms are involved in sexual reproduction of many fungi. In the phycomycetes, the general impression of a common homomictic process occurring with chance cross-fertilisation was nullified by the establishment of the occurrence of a more precise determination of sexual expression in the genera Allomyces and Blastocladiella by Machlis in 1966 and was further corroborated by an increasingly sophisticated system of physiological regulation in Achlya bisexualis by Raper in 1966 (17, 83).

In sexual reproduction in the Ascomycetes, sexual mechanisms are easily understood for fungi that can be easily cultivated in vitro because in the class as a whole, dimixis and homomixis are common. Different types of ascocarp are produced in the class. But whatever the form of ascocarp, they develop in basically the same way from an ascogonium which is a specialised branch of a hypha. The nature of this ascogonium varies within the class. The ascogonium could be an oogonium or a spirally twisted branch. The occurrence of an antheridium is not universal in the class and the

various structures that have been found to function as antheridia range from spermatia to microconidia, conidia, vegetative hyphae to undifferentiated cells. The events leading from the further growth of the ascogonium to the formation of an ascogenous system and asci have been variously considered of great phyllogenetic significance in the taxonomy of ascomycetes (13; 16; 21; 24; 25; 26; 28; 38; 89; 101; 105).

MATERIALS AND METHODS

A. CULTURE OF FUNGUS

1. Origin of Culture:

The culture of *Neocosmospora africana* used in this research was obtained from Dr. C. A. Thomas of the Department of Biological Sciences, Ahmadu Bello University, ZARIA.

The fungus was subcultured on Potato Dextrose Agar and incubated at 28°C. Perithecia developed and matured in the sub-culture. From this subculture, a single ascospore was isolated and used to inoculate a fresh sterile Potato Dextrose Agar medium. The resulting unisporous culture provided the source of the inocula used in all the investigations conducted in this study.

2. Preparation and Maintenance of Pure Culture:

Use of single spore cultures was necessary in order to ensure the recovery and purification of the fungus and more essentially in the establishment of the operative mating system in the fungus.

The single spore was obtained from a culture on solid medium containing mature perithecia - mature perithecia appear brown when seen enmasse. These perithecia were scraped out from the medium into a 3 inch centrifuge tube containing sterile distilled water.

The resulting suspension of the perithecia with ascospores was centrifuged for 10 minutes at low speed (3,000 r.p.m.). This speed was found to be sufficient to cause the breaking of the perithecia, setting free the ascospores and bringing about the settling of both

ascospores and empty perithecial walls at the bottom of the tube under centrifugation. By gently shaking the tube, the ascospores were brought back into suspension with the empty perithecial walls moving up to the top and easily decanted off. A dilution method was adopted to obtain the single spore.

The final dilution containing about twenty ascospores per millilitre of spore suspension was poured into a plate of sterile solid medium and rotated gently to ensure a fairly even dispersion of the spores. Ethanol was used as a wetting agent. 0.2% of ethanol in water was found to give good results. The culture was incubated at 28^{o}C.

The spores germinated by production of germ tubes after twelve to fifteen hours of incubation and could be observed under the microscope. Isolation of the germinating single spores was done by applying a simple semi-mechanical device. This involved observing with a microscope and using a small flattened needle to remove a very small bit of agar with a single germinating spore and transplanting the spore on a fresh plate of sterile solid medium. This unisporous culture was incubated at 28^{o}C and the culture thus obtained provided the fungal material used in this research.

Isolation of a single conidium was done using a very young culture - a culture that had not shown signs of perithecium maturation was most suitable. A four to six days' old culture on solid medium was used. 10 ml. of sterile distilled water was introduced into the

culture and the dish gently rotated to allow the water to mix into the culture. By this, most of the conidia were dislodged from the short conidiophores into the water. The conidial suspension obtained was poured into a sterile test tube. A single conidium was obtained by serial dilution.

Wherever inoculation with single spores are mentioned in the text, these single spores refer to single ascospores, unless otherwise stated. All investigations conducted on solid media were inoculated with single spores. Liquid media were inoculated with a drop of standard conidial suspension prepared such that each drop contained 50 - 60 conidia. Except where otherwise stated, these concentrations were maintained.

3. Media:

Both solid and liquid media were used in most of the investigations, though in the studies of the nutritional requirements of the fungus, liquid synthetic media were exclusively employed as there was need to know with precision the composition of the media in each case. The following media were used for growing the fungus. Unless otherwise stated, all the chemicals used for the media were of analytical reagent quality.

a) Potato Dextrose Agar (PDA)

Potato		200g
Agar (Oxoid)		20g
Dextrose (Glucose)		15g
Water	to	1 litre

The peeled potato was cut into small chips and was boiled with 500 ml. of distilled water for 15 minutes and potato extract obtained by straining off the chips using muslin cloth. The extract was made up to one litre by adding more distilled water. 20g of agar was weighed out and stirred into the hot extract, heating and stirring maintained by heating on a magnetic stirrer - hotplate. 15g of D-glucose was dissolved in the medium. The medium was then charged into 250-ml "medical flats" through a funnel, filling the bottles to within one inch of their shoulders. Into each of several Mc Cartney bottles, 10ml. of medium was measured out using an automatic dispenser. The bottles were closed with loosened caps for sterilisation.

b) Malt Extract Agar (MEA)

Malt extract	(Oxoid)	20g
Agar	(Oxoid)	20g
Mycological peptone	(Oxoid)	5g
Distilled water	to	1 litre

The components were weighed out and stirred into one litre of distilled water heated on a magnetic stirrer-hotplate to get a fairly homogenous mixture. The medium was charged into "medical flats" and Mc Cartney bottles and sterilised.

c) Glucose - Asparagine Medium

D-glucose	10g
Asparagine	2g
$KH_2\ PO_4$	1g
$MgSO_4\ 7H_2O$	0.5g

Trace element soln.		2ml
Biotin		5 ug
Thiamine		100 ug
Distilled water	to	1 litre

The trace element solution was made up as shown below.

Trace element	Salt Used	Amount in mg/litre	mg. of trace element/ ml of final solution
Iron	$Fe(NO_3)_3.9H_2O$	723.5	0.2
Zinc	$ZnSO_4.7H_2O$	439.8	0.2
Manganese	$MnSO_4.4H_2O$	203	0.1

The three salts were dissolved in 600 ml of distilled water and sufficient chemically pure sulphuric acid was added to yield a clear solution and the solution was made up to one litre by adding more water. 2ml of this trace elements solution was added to a litre of the medium.

Stock solutions of the vitamins biotin and thiamine were made up in 20 per cent alcohol. Biotin solution was made in the concentration of 5 ug per millilitre; and thiamine in the concentration of 100 ug per millilitre. These solutions were stored in the refrigerator when not in use.

Agar was added at 20g. per litre of medium when a solid medium was required. 30ml of the liquid glucose - asparagine medium was dispensed into each 250 - ml Erlenmeyer flask after the adjustment of the pH of the medium to the desired level and each flask was plugged with cotton. The flasks were then sterilised by autoclaving at 1.03×10^5 Newton/metre^2 pressure for fifteen minutes.

4. Culture Vessels:

For obtaining unisporous culture, 9cm diameter petri dishes were used. In the study of spore germination, the spores (conidia and ascospores) were separately incubated in drops of liquid media on sterile glass slides and the cultures were maintained in a high humidity chamber (petri dishes with wetted filter papers) to avoid the drying out of the media. Cultures for studying the nutritional requirements of the fungus were grown in 250-ml Erlenmeyer flasks containing 30 ml of liquid medium. The developmental morphology of the fungus was also studied from all the cultures used.

5. Maintenance of Stock Culture:

After the establishment of a unisporous culture, the fungus was grown on slopes of malt extract agar (MEA) medium in Mc Cartney bottles and incubated at 28°C. After 14 days of incubation, culture bottles were tightly capped and stored at 3°C. Occasionally sub-cultures were made from the stock cultures.

6. pH of media:

The pH of all media prepared was adjusted to 6.0 (unless otherwise stated) before autoclaving by adding drops of 6N NaOH or 6N HCl to avoid unnecessary dilution before dispensing the media into bottles and flasks. The pH of each medium was determined again after autoclaving using replicate portions of media and the pH so determined was regarded as the initial pH. Where certain initial pH values were required and changes in pH occurred following autoclaving,

the volume of 1 N HCl or 1 N NaOH required for adjusting the pH to the desired point was determined. This volume of sterile acid or base was then added to the medium in the flask in a sterile chamber prior to inoculation.

In the experiments to determine the effect of hydrogen-ion concentration on growth in which there was need to maintain a constant pH in the medium throughout the period of observation and in which, naturally, the pH continued to change with growth despite the heavy buffering, the cultures were brought to the initial pH in each case every second day by adding NaOH or HCl; the volume of acid or base required was determined by harvesting replicate flasks, determining the pH of the filtrates as before with the pH meter and recording the volume of base or acid required to adjust the pH to the initial level.

7. Sterilisation of glassware and media:

Glasswares - petri dishes, pipettes, test tubes, etc. were sterilised using dry heat in a hot air oven for 3 hours at $180^{o}C$.

Sterilisation of media (both solid and liquid media) was done by steam heating in an autoclave at 1.03×10^{5} Newton/metre^{2} of pressure for 15 minutes. In the experiments involving carbon sources that breakdown on autoclaving in contact with phosphate, the media were sterilised with the aid of a microfilter using Oxoid filter membranes. Before using the microfilter, the unit was first wrapped in aluminium foil and sterilised by autoclaving. Contamination by bacterial colonies was checked by the addition of streptomycin

at 50 parts per million to the medium, when required.

8. Inoculation and incubation of cultures:

The inoculation of media was carried out in the inoculation room; sometimes the inoculation chamber was used after being sterilised. The inoculation room was sterilised with UV light for 20 minutes.

Plating of solid media was carried out in the inoculation room always.

Except where otherwise stated, solid media were inoculated with single spores and incubated at 28°C. Liquid media were inoculated with one drop each of the standard conidial suspension (a drop was standardised to contain 50 - 60 conidia), or with hyphal strands where accurrate comparative results were not involved and incubation was carried out at room temperature (unless otherwise stated).

B. MEASUREMENT OF GROWTH

The determination of the percentage germination of spores on slide cultures was carried out at 2-hourly intervals by examining each time six slide cultures microscopically. A spore was regarded as germinated if the germ tube produced was at least as long as its diameter. In making observation on each culture, three hundred spores were counted and the percentage of germinated spores based upon this total number counted. The result of each observation was calculated as the average from the results of the six readings.

Measurement of vegetative growth in both solid and liquid media was made at intervals of two days. On solid medium, vegetative growth was measured by determining the increase in cultural diameter every two days.

Growth in liquid medium was estimated as dry weight of mycelium produced. This was done by making regular periodic harvests of the mycelium from six culture vessels. These harvests were made by filtering off the mycelium using clean dry cloth, drying the cloth with the mycelium in an oven at 60^{o}C for 48 hours and determining the dry weight of the mycelium using a sensitive Mettler balance.

Determination of density of perithecia production in solid media was done by taking a number of counts of the perithecia in arbitrarily chosen microscope fields and calculating the average.

C. STAINING

In the routine observation of mycelial tissue cotton blue in lactophenol was used. In studying the developmental morphology of the fungus, fungal materials at various stages of development were fixed in certain fixatives prior to staining.

Fixatives:

(i) Acetic alcohol containing 3 parts of ethyl alcohol and 1 part of glacial acetic acid.

(ii) Lu's BAC fixative comprising 9 parts n - butyl alcohol; 6 parts acetic acid; 2 parts chromic acid (10% aqueous).

(iii) 6 parts ethyl alcohol; 1 part acetic acid; 1 part lactic acid.

HCl - Giemsa staining

Tufts of mycelium were fixed in acetic alcohol for 30 minutes. The mycelium was removed from the fixative, rinsed briefly in 70% alcohol, rinsed in two changes of distilled water and hydrolysed for 10 minutes in 1 N HCl at room temperature. Young perithecia were hydrolysed for 8 minutes at 60°C in 1N HCl.

After hydrolysis, the mycelium was placed on a clean slide and rinsed with several changes of distilled water and was next flooded with freshly prepared phosphate buffer, pH 7.2. The mycelium was teased out and the perithecia crushed with glass rod. Excess buffer was removed with absorbent paper. Two drops of fresh buffer were placed on the crushed material and a drop of undiluted Giemsa stain stock solution prepared as described by Ward and Ciurysek was added.

Staining was done for 25 - 30 minutes after which the material was rinsed with several changes of water and buffer respectively. Differentiation of nuclear detail was accomplished by absorbing excess buffer from the slide and adding a drop of aqueous 3% potassium hydroxide. The material was spread after adding a covership by tapping the coverslip with an eraser.

Composition of stain used

Giemsa stain

Giemsa stain powder	3.8g.
Glycerine	250ml.
Pure methyl alcohol	250ml.

Iron - Haematoxylin staining

The use of this stain was based on the simplified propionic-iron alum-haematoxylin stain of Henderson and Lu (1968).

Two stock solutions, A (stain solution) and B (the mordant) were needed. Solution A matured after being kept for 8 - 9 weeks when the colour changed from light-yellow to dark-brown and staining properties were much greater.

Freshly made stain was used by mixing equal volumes of both stain and mordant and allowing the mixture to stand for about 3 hours. The mixture was then filtered and used for staining the fungal material which has been fixed in Lu's BAC fixative. With ripened stain, the stain was used as both fixative and stain. The iron mordant needed was furnished by the iron mounted needles used.

Composition of stain used

Iron haematoxylin stain

Solution A - 2% (W/V) haematoxylin
Solution B - 0,5% (W/V) iron alum

Both solutions were prepared in 50% propionic acid.

Feulgen - carmine technique

The fungal material containing young perithecia was fixed for 10 minutes in acetic alcohol. Following fixation, the tissues were run down rapidly through a series of alcohols to water and washed thoroughly; treated with snail stomach fluids for 30 minutes and then rinsed in water; immersed in cold 1N HCl for 5 minutes;

hydrolysed in 1N HCl at 60°C for 5 minutes; placed in leuco basic fuchsin for one hour. The asci were teased apart with steel needles in a drop of propionocarmine, a cover slip applied, and the slide gently warmed.

Composition of stains used

Leuco basic fuchsin	0,5g
Water	100ml
Normal hydrochloric acid	10ml
Potassium metabisulphite	0,5g
Charcoal	0.5g
Filter	

Propionocarmine

Carmine powder	0.5g
45% propionic acid	100ml
Saturated ferric acetate	3 drops

For a more detailed study of the nature of the centrum, microtome sections were taken with the freezing microtome. Sections 10 microns and 12 microns thick were taken and stained with iron - haematoxylin stain and by the Feulgen - carmine technique. Iron - haematoxylin gave the best results for the study of the development of the perithecium.

PHOTOMICROSCOPY

'Wild' M20 microscope was used for most observations. Photomicroscopic equipment consisted of a Reichert (Olympus) microscope with Olympus Model PM - 7 camera attached. Photographs were taken with Ilford FP4.

IV. EXPERIMENTS AND RESULTS

EXPERIMENT 1

Spore Germination:

This experiment deals with the morphological changes which take place when the spore, is transformed to a morphologically different structure as seen with light microscopy and the environmental factors responsible for the same. Here emphasis is placed on the effect of these factors on the observed germination.

It is important to note that though the emergence of the hyphal germ tube has been used as the main criterion since it is the most obvious morphogenetic event in spore germination, it is the last point in a whole sequence of development.

Ascospores of different ages were used in the investigation, 4 weeks, 8 weeks, 12 weeks and 15 weeks old spores. Liquid glucose-asparagine medium was used and the spores were incubated on drops of medium on glass slides at various temperatures; and at different hydrogen-ion concentrations; and under different nutrient conditions.

The effect of humidity on spore germination was investigated by setting up three humidity chambers:

(i) a dessicator containing water to provide a Relative Humidity of 94% at 30°C;

(ii) a dessicator containing fused calcium cloride to provide a Relative Humidity of Zero per cent at 30°C;

(iii) a dessicator containing none of the above but only the slides with the spores to give a relative humidity of 68%.

Measurement of relative humidity was done using a Negretti and Zambra Paper Hygrometer. Each humidity chamber contained six slides on which the spores were spread. The slides were examined microscopically after 18 hours of incubation at $30^{\circ}C$ to determine the percentage germination.

The effect of oxygen on spore germination was determined using two belljars set up as follows:

(i) the first belljar enclosing the slide cultures of the spores and a small beaker containing 20ml of 3M pyrogallol; and

(ii) the second belljar (control) containing slide cultures of the spores. Observations were made after 20 hours of incubation at room remperature.

The effects of two metabolic inhibitors, potassium fluoride and sodium azide, on the changes of spore morphology leading to germination were studied by incorporating these separately in the media. Sodium azide ($Na\ N_3$) was used in the concentration of 1.0×10^{-3} M while potassium fluoride (KF) was used in the concentration of 1.0×10^{-4} M.

Ascospores and conidia showed slightly different requirements for germination. The ascospores neither germinated nor swelled in distilled water. In the absence of a carbon source in the medium, no germination of ascospores was obtained. No germination of ascospores occurred also in the absence of a nitrogen source (Table I). Complete glucose asparagine medium did not provide the best medium for the germination of the ascospores as seen from the results presented in Table I. Omission of either potassium dihydrogen

TABLE I : EFFECT OF OMISSION OF NUTRIENTS FROM GLUCOSE-ASPARAGINE MEDIUM ON ASCOSPORE GERMINATION

(Temperatures of incubation were 30°C and 35°C)

MEDIUM	% Germination after 18 hours	
	30°C	35°C
Glucose-asparagine (GA)	9	55
GA minus glucose	0	1.2
GA minus asparagine	0	0
GA minus KH_2PO_4	27	97
GA minus $MgSO_4$ $7H_2O$	29	98
GA minus salts	5.8	56
Distilled water	0	0

The composition of the media was the same except for the omission of the nutrient indicated.

orthophosphate or magnesium sulphate in the medium resulted in a high percentage of germination. The omission of one of these salts accounted for an increase of up to 42% germination above that of the complete medium. Though ascospores germinated in the complete absence of salts, the percentage as well as the total germination were found to be very low.

Ascospores required the presence of an external carbon source for germination to occur. In the complete absence of a carbon source, scarcely any germination was obtained. Total germination obtained at 35°C after 24 hours of incubation in the absence of a carbon source was 1.5%.

The need for a carbon source for the germination of the ascospores was not specific. When disaccharide or starch was provided as carbon source in the medium, germination of ascospores occurred (Table II) without any significant difference in the amount of germination obtained with the different sugars.

TABLE II : EFFECT OF DIFFERENT CARBON SOURCES ON ASCOSPORE GERMINATION

MEDIUM *	% Germination after 24 hrs. at 35°C
Glucose - asparagine	62.0
Sorbose - asparagine	59.2
Sucrose - asparagine	65.0
Maltose - asparagine	61.1
Lactose - asparagine	64.0
Starch - asparagine	39.3
Cellulose-asparagine	1.4
No carbon source	1.5

* Carbon sources were used in the quantities to furnish 4.0g of carbon per litre of medium.

A lower percentage was obtained on starch - the carbon source being responsible for the drop of about 22% germination. Provision of cellulose as carbon source proved to be as good as not having any carbon source at all in the medium. This is no doubt, a solubility effect and absence of the necessary enzymes at this stage to act on cellulose.

Production of germ tubes began after 11 hours of incubation but not earlier than this at the optimal temperature of incubation. In the presence of either potassium dihydrogen orthophosphate or

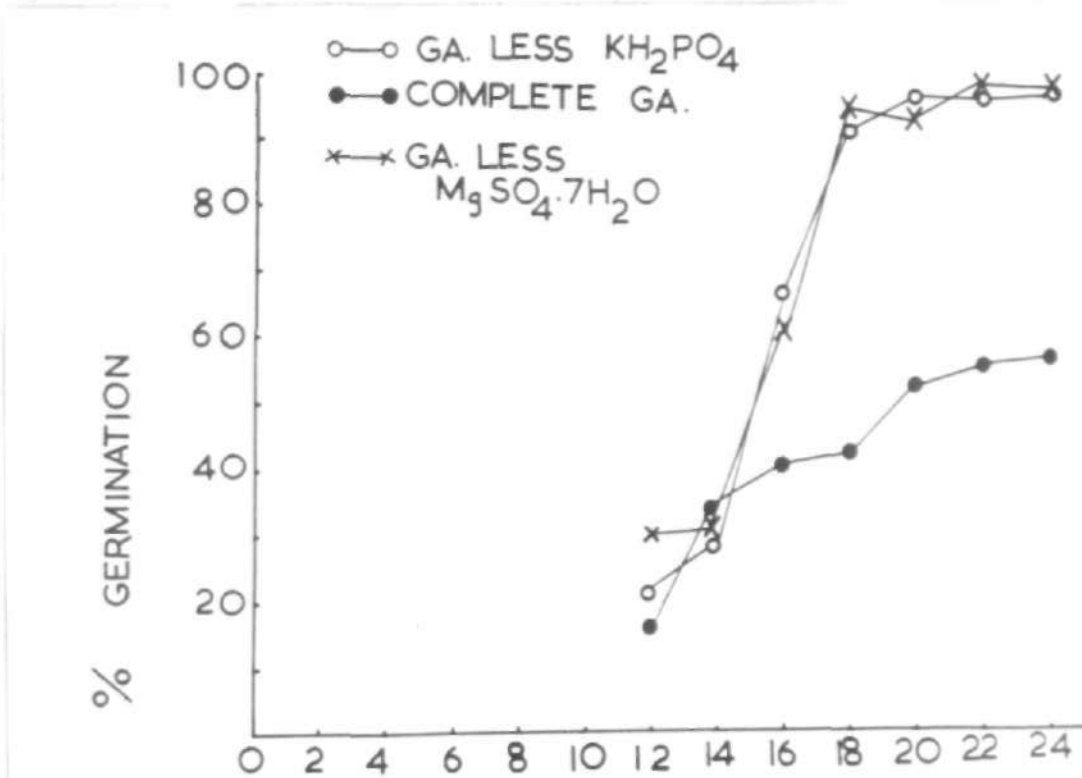


FIG.1. GERMINATION OF ASCOSPORES IN THREE DIFFERENT MEDIA.
GA = GLUCOSE ASPARAGINE MEDIUM

The percentage germination was determined from a total of 300 ascospores. Results are the averages of six replicates.

Germination of ascospores occurred over the pH range 3.2 to 9.4 with the highest percentage germination obtained between 5.0 and 6.0. This optimum did not seem to depend on the medium as the same values were obtained on the three media: complete glucose-asparagine, glucose-asparagine without KH_2PO_4, and glucose-asparagine without $MgSO_4$ $7H_2O$.

No germination of ascospores occurred in the high humidity chambers, or in the chambers containing pyrogallol to absorb any available oxygen. In the cultures containing the metabolic inhibitors, potassium fluoride and sodium azide, no germination of ascospores occurred.

Conidia also failed to germinate in the absence of oxygen, or in the presence of metabolic inhibitors. The germination of conidia was obtained under less exerting nutrient conditions. The conidia germinated in all the media (Table III) used including distilled water. Germination was observed to begin between 1 hour and 2 hours of incubation and reached almost 100 per cent after 12 hours in all the media. No conidial germination occurred at Zero per cent relative humidity and at 68 per cent Relative Humidity at 30°C. A slight germination (3 per cent) was obtained under a relative humidity of 94 per cent at 30°C after 18 hours of incubation. This germination was probably due to condensation of liquid water on the slide (Table IV).

The germination of conidia showed two pH optima in the ranges of 3.0 to 4.0 and 5.5 to 6.5. The germination of conidia began at about pH 2.0 and increased rapidly to the first optimum around

magnesium sulphate (but not both) in a concentration not exceeding 1 gram per litre, germination increased linearly reaching 97% after 18 hours of incubation (Fig. 1). The latent period of germination (i.e. the time taken for a given percentage, usually 50%, of the spores to germinate) was observed to be about 15 hours.

The need for a nitrogen source proved to be very important. No germination was recorded in the complete absence of a nitrogen source in the medium. Replacing asparagine with potassium nitrate (an inorganic source of nitrogen) led to germination in the same percentage as on asparagine. Production of germ tube was seen to require an external source of nitrogen.

When magnesium sulphate was omitted in the medium and calcium phosphate was substituted for potassium dihydrogen orthophosphate, the germination obtained was found to be similar to that obtained with potassium dihydrogen orthophosphate. Since the two salts showed the same effect, it could be inferred that the phosphate ion which was common to the two salts was the important component for germination.

No germination of ascospores was obtained below 23°C even after 48 hours of incubation. The percentage germination increased gradually from 23°C up to 26°C and rapidly to a maximum at 33°C to 35°C and dropping sharply to 41°C when no germination occurred (Fig. 2).

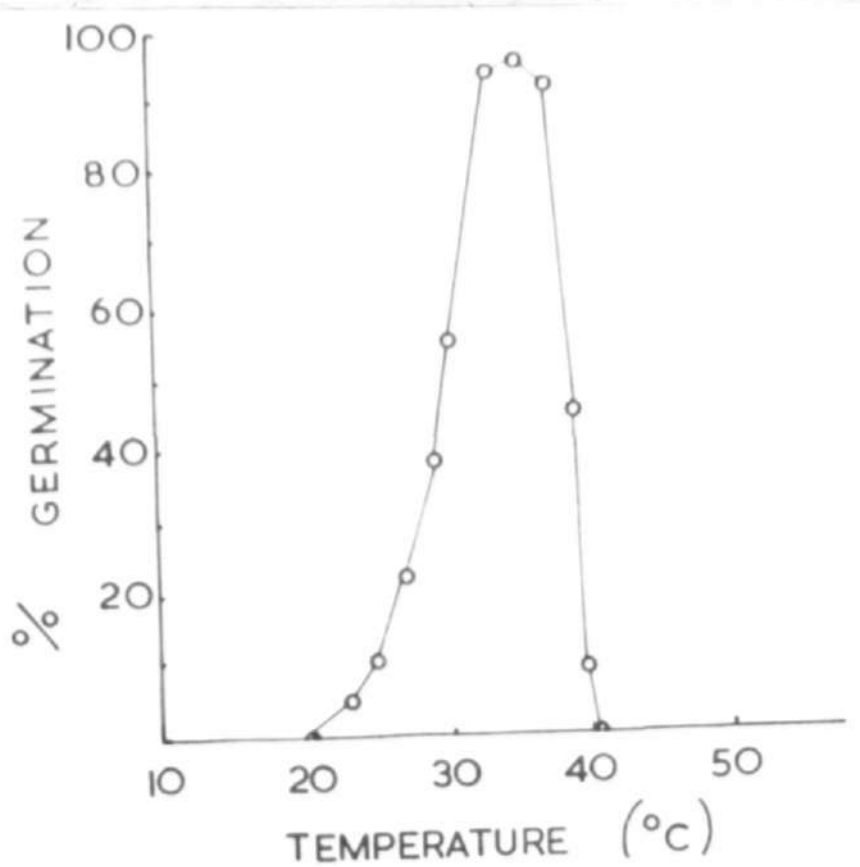


FIG. 2. EFFECT OF TEMPERATURE ON THE GERMINATION OF ASCOSPORES.

The percentage germination was determined from a total of 300 ascospores at each temperature after 18 hours incubation. Results are the averages of six replicates.

Germination of ascospores occurred over the pH range 3.2 to 9.4 with the highest percentage germination obtained between 5.0 and 6.0. This optimum did not seem to depend on the medium as the same values were obtained on the three media: complete glucose-asparagine, glucose-asparagine without KH_2PO_4, and glucose-asparagine without $MgSO_4\ 7H_2O$.

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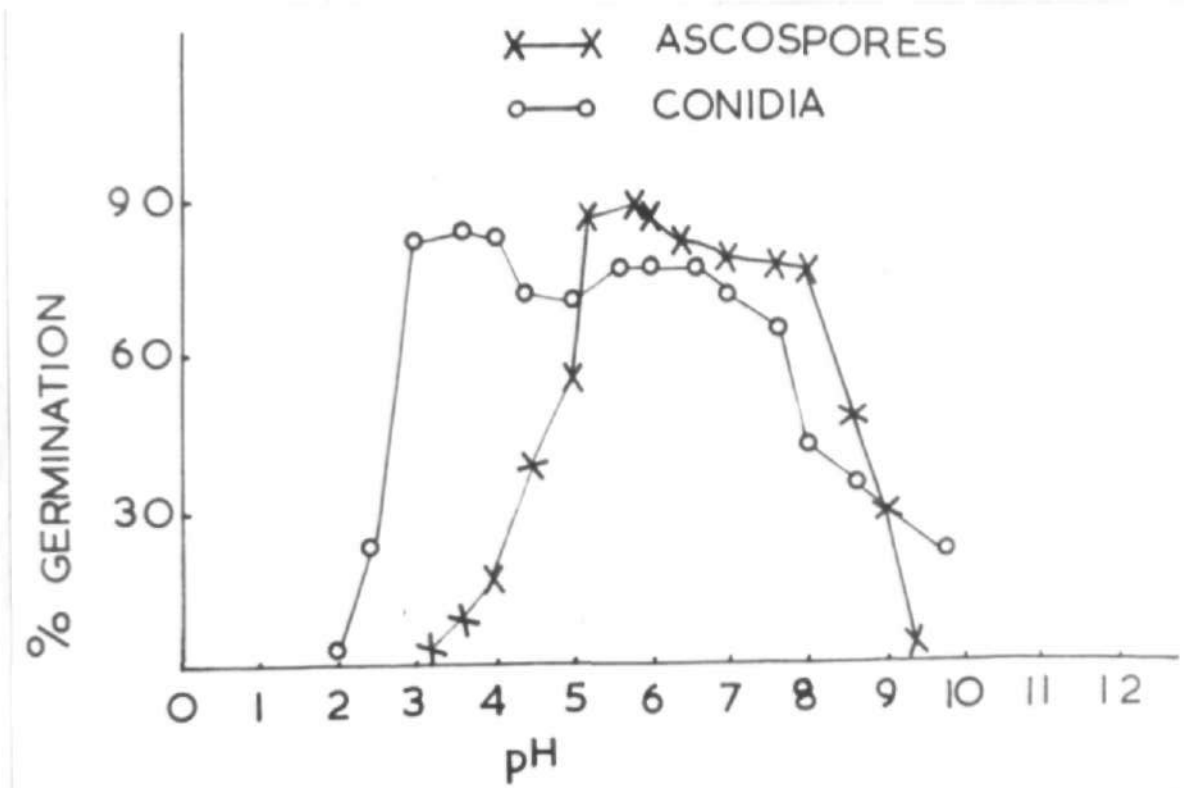


FIG. 3. EFFECT OF pH ON THE GERMINATION OF CONIDIA & ASCOSPORES.

The percentage germination was determined from a total of 300 ascospores at each pH after 12 hours (conidia) and 24 hours (ascospores) of incubation at 30°C. Results are the averages of six replicates.

TABLE III : EFFECT OF NUTRIENTS ON THE GERMINATION OF CONIDIA

MEDIUM	Percentage germination after hours of incubation					
	2	4	6	8	10	12
Complete medium (A)	1.2	16	61	92	95	98
A minus salts	13	67	88	91	97	98
A minus asparagine	6	20	83	90	94	96
A minus KH_2PO_4	1.8	41	64	92	91	96
A minus $MgSO_4$ $7H_2O$	1.1	29	79	84	93	94
A minus Glucose	4	41	67	95	98	97
Distilled water	1	43	49	79	92	93

Temperature of incubation was 35°C. The complete medium (A) was glucose-asparagine. Results are the averages of six replicates.

TABLE IV : EFFECT OF HUMIDITY ON THE GERMINATION OF CONIDIA

R. H. at 30°C	Percentage germination
0%	0
68%	0
94%	3.0

Results are the averages of six replicates.

pH 3.5 and declined slightly from pH 4.2 to pH 5.0 and then increased again to another optimum at pH 6.5. The percentage germination then declined until at pH 10.0 when no conidial germination occurred.

In both conidia and ascospores, the optima were all in the acid range and no germination was obtained above the pH of 10.0 (Fig. 3).

The results obtained from these investigations show that the two kinds of spores (conidia and ascospores) produced by the fungus were not alike in their environmental requirements for germination. The conidia are thin-walled while the ascospores are thick-walled. The need for an external carbon source and an external nitrogen source for the germination of ascospores showed that ascospore germination which involved the rupturing of the thick wall is active cell metabolism the energy for which could not be provided by the reserves within the spore. The observation that in the presence of the inhibitors sodium azide and of potassium fluoride, no germination of ascospores and conidia occurred in the presence of both carbon and nitrogen sources further showed that respiratory energy was involved in spore swelling and germ tube protrusion. Sodium azide is an inhibitor of cytochrome oxidase and potassium fluoride is an inhibitor of the enzyme enolase of the EM pathway of the glycolytic breakdown of sugars. Energy needed for reactivation of growth and synthesis within the spore comes from the carbon metabolism of the fungus while the need for a nitrogen source appeared to be a consequence of the increased synthesis of new protoplasm including enzymes in the reactivated spore.

Though ascospores have been shown in the investigations to require salts in the medium, the presence of both KH_2PO_4 and $MgSO_4\ 7H_2O$ in the same medium gave rise to more than 40 per cent decrease in germination. These results indicate that these two salts

were mutually inhibitory to the use of one another in the medium as each one used alone gave a higher percentage germination.

Using ascospores of different ages did not affect either the latent period of germination or the total germination. Test observations made with ascospores from the original stock culture (about 62 weeks old) preserved in a refrigerator gave results similar to 4 weeks old ascospores. This showed that the spores remain viable over long periods of time. This is not surprising considering that these ascospores are thick-walled and contain comparatively little water, not enough to allow the metabolic mechanisms to function at more than a minimal rate.

The conidia, on the other hand, are thinner-walled and require for the restoration of the initial active morphogenesis only water in a favourable environment to protrude a germ tube. Latent period of germination of the conidia was found to lie between 5 and 6 hours. Germ tubes produced from conidia in distilled water did not develop into a mycelium unless a suitable nutrient medium was introduced. The emergent germ tube grew without branches for up to 29 hours of incubation after which further growth in length ceased and length attained was up to 332 microns. Germ tubes emerging from ascospores developed into a mycelium by profuse branching that occurred soon after germination (Plate I).

No sharply dividing line could be drawn between germ tube growth and vegetative hyphal growth in the spores of N. africana. Manners in 1966 had tried to define the point at which a germ tube became a

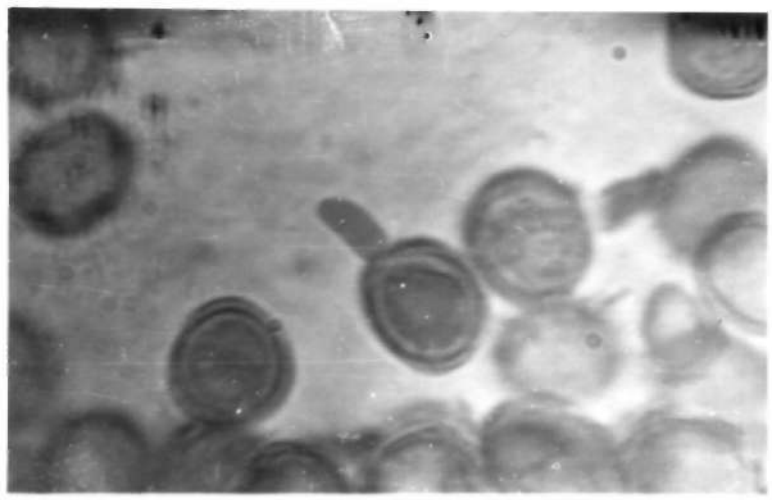


Plate Ia. Germinating ascospores after 12 hours incubation.

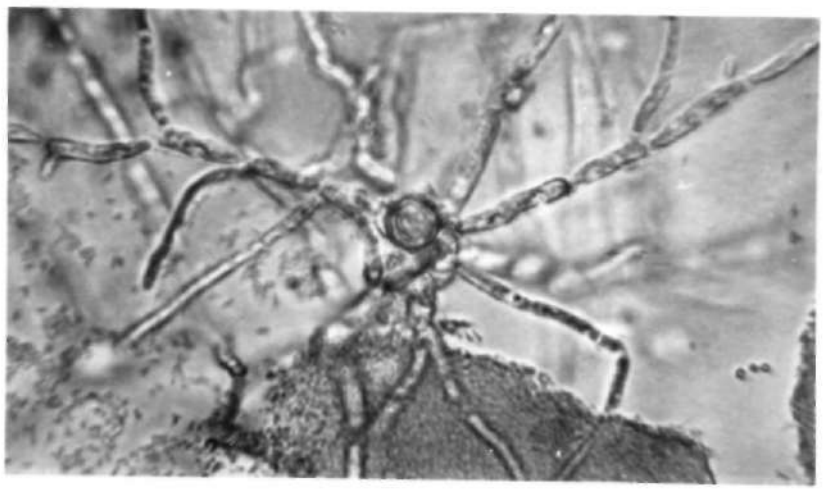


Plate Ib. Unisporous culture (20 hours old).

vegetative mycelium by postulating that a structure be considered a germ tube for as long as such a structure obtained its nutriment mainly from the spore. Based on this, it would only be practicable to determine germ tube growth only in the case of conidia which have germinated in the presence of distilled water only. This definition is not much used in practical assessment though it may be physiologically sound. In the presence of nutrients, the transition of germ tube into a vegetative hypha was not gradual.

Results of this investigation show that the ascospores germinated by producing a germ tube through any spot on the spore periphery; that the asexual and sexual spores of the fungus differed slightly in their physiologic as well as nutritional demands, the conidia being nutritionally independent whereas the ascospores showed nutritional dependence; the conidia showing double optima and the ascospores a single optimum pH for germination.

EXPERIMENT 2

Effect of Carbon Source on Growth and Sporulation:

Fungi differ in the carbon compounds on which they can maintain vegetative growth and spore production. This experiment was designed to find out the carbon compounds on which the fungus can conveniently be grown.

The medium used in the investigation was of the same composition as glucose-asparagine medium except that the nitrogen source (asparagine) was replaced throughout the investigation by potassium

nitrate (3.069g per litre of medium) because asparagine is also a source of carbon. Glucose, in subsequent media prepared, was replaced separately by the following carbon sources tested: D(+) galactose, L-sorbose, Mannitol, sucrose, maltose, Lactose, Asparagine, starch and cellulose. Carbon sources were used at the rate which supplied 4g of carbon per litre of medium. Both solid and liquid media were used.

30ml of liquid medium was used in each 250ml Erlenmeyer flask and initial pH of medium was aseptically adjusted to 6.0. Each flask was inoculated with one drop of the standard conidial suspension. The rate of growth was determined by making harvests of the mycelium from six cultures every 48 hours. The change of pH with growth was also determined every 2 days.

With the exception of cellulose, the fungus maintained good vegetative growth in liquid media on all the carbon sources tested. In solid medium, vegetative growth and production of perithecia occurred on all the carbon sources tested. Even in water-agar, some growth and sporulation occurred. The results for individual carbon sources are presented.

D-Glucose: Results obtained from both solid and liquid media cultures showed that glucose constitutes a good source of carbon for both vegetative growth and sporulation. Autoclaving glucose in contact with phosphate at pH 6.0 did not affect the rate of growth as well as the maximum amount of growth. The dry weight of the

TABLE V : EFFECT OF DIFFERENT CARBON SOURCES ON THE RATE OF GROWTH OF THE FUNGUS (Dry weight of mycelium in milligrams)

Carbon Source	Dry weight (mgm) of mycelium after days of growth						
	2	4	6	8	10	12	14
Glucose	10.9	28.5	49.6	72.7	89	92	92.8
Sorbose	8.1	16.5	26	44.7	52	55	58.1
Galactose	9.8	25	47.1	80	98.2	102	101.4
Sucrose	10.1	24	49.1	73	94	99.1	102
Maltose	10.4	22.8	41	63	80.6	79.9	86.2
Lactose	11.6	27.0	50	75.1	91.7	97.6	101.7
Mannitol	9.6	30.1	55.4	86.5	103	118	107
Starch	8.6	24.1	49.3	57.6	69.2	68.1	69.0
Cellulose	-	2.1	2.6	2.4	3.1	2.7	2.8
Asparagine	5.7	18.1	30.2	34.0	36.7	37.6	36.0

Each weight in the table is the average of 6 cultures.

TABLE VI : EFFECT OF CARBON SOURCE ON THE PRODUCTION OF PERITHECIA

Carbon Source	Number of Perithecia
Glucose	44.1
Galactose	42.1
Sorbose	47.3
Maltose	42.7
Lactose	43.1
Sucrose	42.4
Mannitol	36.5
Starch	20.1
Cellulose	18.7
Asparagine	20.2
Water-agar	2.2

The number of perithecia refers to the number of perithecia taken as the average in ten arbitrarily chosen microscope fields on each of three plates for each carbon source.

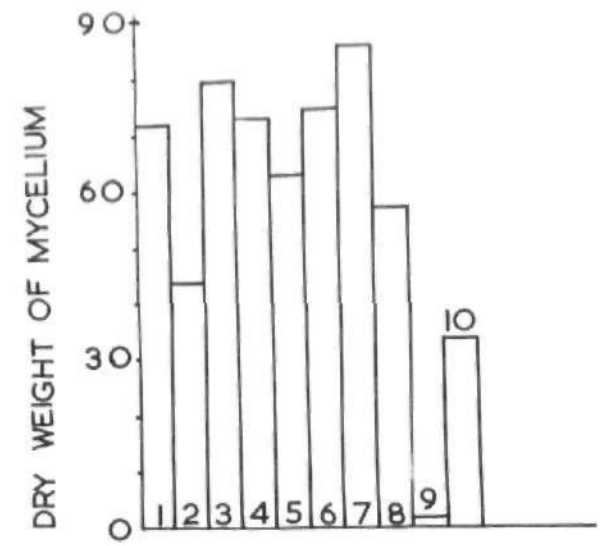


FIG. 5. MILLIGRAMS OF MYCELIUM PRODUCED ON DIFFERENT CARBON SOURCES.

1. GLUCOSE
2. SORBOSE
3. GALACTOSE
4. SUCROSE
5. MALTOSE
6. LACTOSE
7. MANNITOL
8. STARCH
9. CELLULOSE
10. ASPARAGINE

Mycelium harvests were made from 8 days old cultures. Results are the averages of six replicates.

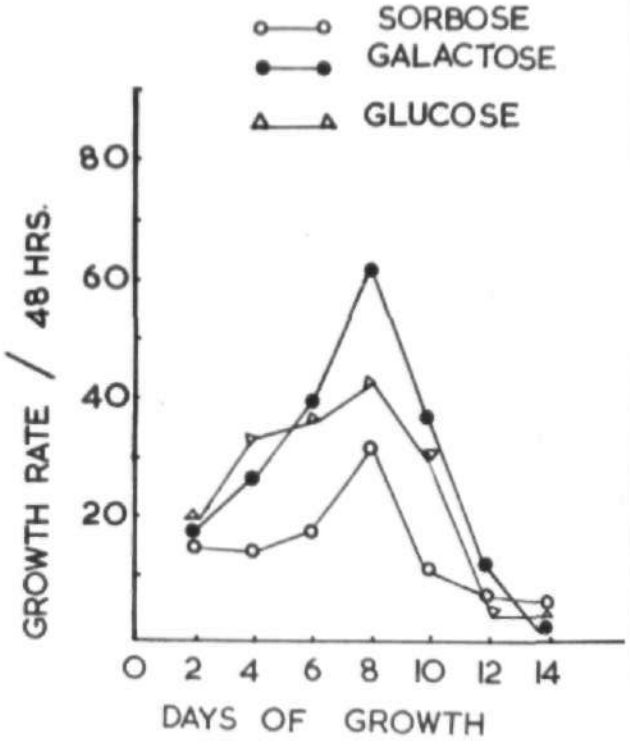


FIG. 4. GROWTH ON THREE SIMPLE SUGARS.

Growth rates determined from change in dry weight of mycelium every 2 days.

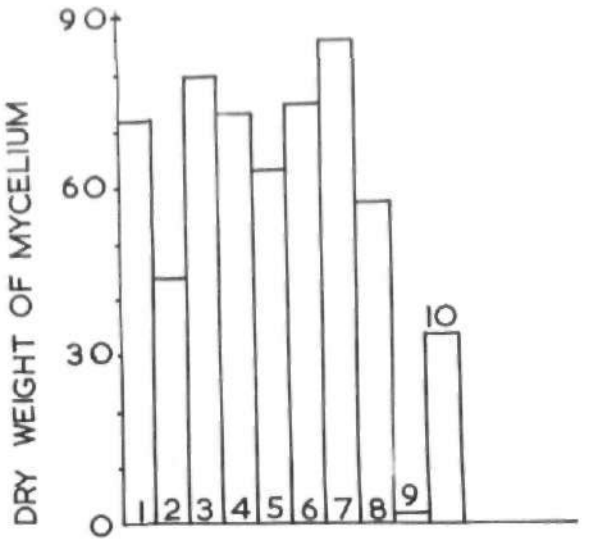


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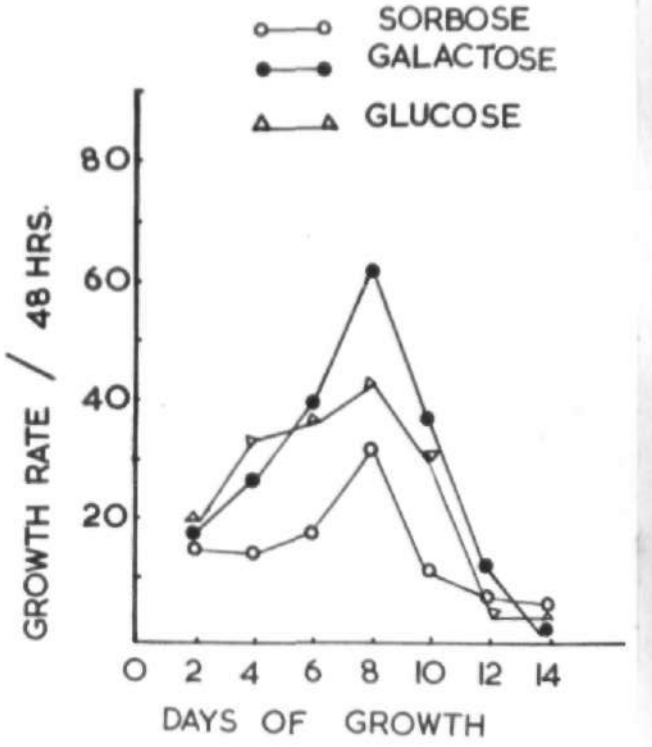


FIG. 4. GROWTH ON THREE SIMPLE SUGARS.

Growth rates determined from change in dry weight of mycelium every 2 days.

mycelium harvested from 8 days old culture on autoclaved glucose-nitrate medium was 72.7mg while a similar harvest from a filter-sterilised glucose-nitrate medium was 74.1mg. With the growth of the fungus on glucose, there was an initial drop in pH from 6.0 to 5.6 and then a gradual rise up to 7.6. The initial drop in pH is significant of the production of some organic acids in the culture which later disappeared and hence the rise in pH.

L-SORBOSE: This aldohexose and an enantiomorph of glucose was utilised for both vegetative growth and sporulation. Autoclaving the sugar in the medium caused the breakdown of the sugar forming a straw-yellow coloured solution. Vegetative growth was not as dense as in glucose but density of sporulation was highest on this sugar. The breakdown product of sorbose on autoclaving in the medium was shown by Cochrane (1958) to be 5 - hydroxymethylfurfural. Fig. 4 shows the comparison of rates of growth on the three isomers glucose, galactose and sorbose.

D(+) GALACTOSE: The growth obtained on this carbon source was heavy; rate of vegetative growth was higher on galactose than was obtained on sorbose or on glucose. Autoclaving the sugar in the medium did not affect the amount of growth obtained as both autoclaved and filter sterilised media produced 80.0mg and 78.7mg of mycelium respectively. The change in pH of the medium with days of incubation showed an upward trend from the initial pH of 6.0 reaching a peak at 7.1 and remaining fairly stable at that range.

TABLE VII : THE CHANGE IN HYDROGEN-ION CONCENTRATION FOLLOWING THE GROWTH OF THE FUNGUS ON DIFFERENT CARBON SOURCES

Carbon Source	Change in pH with days of growth							
	Initial pH	2nd Day	4th Day	6th Day	8th Day	10th Day	12th Day	14th Day
D-Glucose	5.9	5.7	5.6	6.3	7.1	7.4	7.4	7.6
L-Sorbose	5.3	5.8	6.9	7.5	7.7	7.3	6.6	6.8
D(+) Galactose	5.8	6.5	7.1	6.5	6.9	7.1	7.1	6.9
Sucrose	6.3	6.0	5.9	6.6	6.7	7.2	7.4	7.1
Maltose	6.1	6.4	6.9	7.5	7.8	7.8	7.9	8.1
Lactose	6.1	6.9	8.0	7.4	6.0	6.1	6.4	
Mannitol	5.6	6.6	6.5	6.5	6.8	6.6	6.5	6.7
Starch	5.9	6.7	8.1	8.7	8.8	8.8	8.7	9.0
Cellulose	6.2	6.8	8.6	8.9	9.1	9.0	9.0	9.1
Asparagine	6.1	6.8	8.3	8.6	8.8	8.9	8.9	8.80

Results are the averages of six replicates.

SUCROSE: Results showed that sucrose served as a good source of carbon (Fig. 6). Autoclaving sucrose in the medium did not lead to a breakdown of the sugar as seen from the comparison of results obtained with filter sterilised medium. Sucrose is a disaccharide which on hydrolysis yields two monosaccharide sugar molecules glucose and fructose. When a mixture of these two simple sugars was used as the source of carbon, 203.2mg of mycelial harvest was obtained after 8 days of incubation as opposed to 73.0mg obtained by using sucrose as the carbon source (Table VIII). The results showed that the effect of using glucose and fructose mixture was to produce a weight of

TABLE VIII : EFFECT OF MIXED CARBON SOURCES ON THE GROWTH OF THE FUNGUS
The carbon sources were used at the rate that supplied 4g of carbon per litre of medium. Results are the average of six replicates.

Carbon Source (grams)	Dry weight of mycelium (mgm)
Glucose 10g	72.7
Fructose 10g	69.3
Galactose 10g	80.0
Sucrose 9.5g	73.0
Lactose 10.008g	75.0
Glucose 5g + Galactose 5g	211.0
Glucose 5g + Fructose 5g	203.2
Sucrose 9.5g + (autoclaved)	71.2

mycelium by far in excess of what the two sugars produced when used separately. Mycelial harvests obtained from filter sterilised sucrose and autoclaved sucrose both separately contrasted with the harvest from the mixture of glucose and fructose and this showed that sucrose did not undergo any hydrolytic breakdown as a result of autoclaving.

A similar increased effect of mixed carbon sources was obtained with glucose and galactose, the component monosaccharides of the disaccharide sugar lactose, the yield from the mixed carbon sources being greater than the yield from the disaccharide or any of the simple sugars used singly (Table VIII).

Maltose was utilised by the fungus for growth and sporulation. Density of perithecial production on the three disaccharides, maltose, sucrose and lactose, showed that these sugars were equally suitable for reproduction in the fungus.

MANNITOL: This is a sugar alcohol derived by the reduction of the aldehyde group of the simple sugar, mannose. It is a naturally occurring sugar alcohol. Results of these tests showed that the highest yield of mycelial harvest was obtained on mannitol (Fig. 5, Plate IIb). Perithecium development was slow to begin in the culture and density of perithecia produced on the solid medium was not very high. Hydrogen-ion concentration changed with growth but the medium was acidic for the fourteen days of the observation unlike what obtained on the rest of the carbon sources tested in which at some stage of the growth, pH increased above 7.0.

STARCH: This is a polysaccharide - and is not soluble in water. Autoclaving makes starch colloidally soluble. It is a polymer of D-glucose. Test for starch conducted after autoclaving of the medium confirmed presence of starch. Test for starch following the periodic harvests of mycelium from the culture showed that all the starch in the medium had been completely used up after the sixth day of incubation (or else converted to some other carbohydrate). The economic coefficient of the fungus on starch obtained after six days of incubation at room temperature with the fungus grown as a surface static culture was 25 per cent. This economic coefficient is defined by the formula:

$$\frac{\text{Mycelium dry weight (gms)}}{\text{Amount of carbon compound consumed}} \times 100$$

$$= \frac{49.3}{198} \times \frac{100}{1} = 25\%$$

Such a low efficiency obtained on starch is indicative of the production of significant amounts of soluble carbon compounds (soluble metabolites).

CELLULOSE: Cellulose is insoluble and in liquid medium the particles settled at the bottom of the flask. No observable growth was obtained on cellulose in liquid medium. In solid medium, the particles settled at the bottom of the petri dishes as the hot medium cooled down and became solid. Mycelial growth on the solid medium became apparent after 8 days of incubation at 28^{o}C and the mycelium was found to develop submerged in the solid medium around the cellulose particles. The lag period of 8 days was not obtained in liquid medium. This lag period before an observable growth showed the operation of an adaptive system. The agar in the medium provided a substratum on which an initial scanty growth was maintained before growth could begin on the cellulose medium.

In liquid medium, where no alternative source of carbon was available, scarcely more than 2.8mg of mycelium was obtained after 21 days of incubation. No perithecium was produced in liquid media despite the long period of incubation over which the cultures were kept under observation.

In the cellulose-agar medium (solid), perithecia were produced submerged in the medium and not superficially on the solid medium as obtained in other media containing soluble carbon sources.

ASPARAGINE: This is the amino acid derived from aspartic acid. It is a 4-carbon compound and was found to provide carbon utilisable by the fungus. Growth of the fungus on the medium with asparagine as carbon source was accompanied by a rise in the hydrogen-ion concentration up to about 9.0. Production of perithecia was obtained in the medium.

In the medium prepared without any source of carbon, no growth was obtained. A scanty growth was also obtained on water-agar.

From the results obtained in these investigations, it has been established that the fungus is able to grow on glucose, fructose, sorbose, maltose, sucrose, lactose, starch and asparagine as sole carbon sources. The finding here is of compounds metabolised rather than a consideration of the mechanisms of the dissimilation of these compounds by the fungus; it is hoped that future researches will lead to the determination of the utilisation patterns of these carbon sources.

Cellulose was also found to be metabolised by the fungus but not in liquid medium where it was the sole source of carbon but in solid medium where in conjunction with the agar, it provided the carbon needed for growth. In the solid medium, an initial sparse growth was obtained on the agar and after 8 days of incubation, growth of the fungus on cellulose became apparent as the mycelium formed balls around the particles of cellulose precipitated in the medium. This type of utilisation of a compound as the source of

carbon with an initial lag period shows the operation of an adaptive enzyme system which takes time to develop in culture. In liquid medium with cellulose as sole carbon source no such growth occurred.

Such carbon sources as cellulose which in short term observations appear not to be utilised, prove to be good sources of material for fungal growth and sporulation after prolonged incubation. Again, such seemingly unavailable carbon sources have been utilised without any lag period when mixed with some quantity of more readily utilisable sources as a result of an adaptive enzyme system or the occurrence of some mutation in the species (Cochrane, 1958).

In view of the existence of these two concepts of utilisation of a substance, the use of mixed carbon sources was not taken as the basis for establishment of ability of the fungus to metabolise a carbon source.

N. africana is a soil inhabiting fungus. It derives energy as well as structural materials for the synthesis of cell substance from the large variety of carbon compounds in the soil. These compounds include carbohydrates and related compounds, organic acids and related compounds, polycyclic compounds and alkaloids - all produced continually from microbial metabolism into the central pool in the soil. It is from this central source that the N. africana derives its nutrients while at the same time regenerating materials by its metabolism for other lives in the soil. As a result of this complex admixture in the soil, uninterrupted utilisation of cellulose is bound to be taking place.

The condition under which the fungus lives in nature departs markedly from the single-substrate pure culture habitat. It lives in a dilute solution with more than one carbon source available or potentially available. Better utilisation of more of these carbon sources will therefore be expected. More work on this problem is therefore necessary and it is to be hoped that such future work will be greatly benefited by the accumulation of knowledge on adaptation growth in culture media and induced enzymes in many microorganisms.

EXPERIMENT 3

Effect of Nitrogen Sources on Growth and Sporulation:

Fungi have been classified according to their ability to utilise different sources of nitrogen (57). Despite the admitted imperfection of the classification, it has been found very useful in preparing medium and in discovering the causes of failure of some fungi to grow on certain media.

The purpose of this experiment was to determine the utilisation of different sources of nitrogen by the fungus.

The basal synthetic medium used was glucose-asparagine. Asparagine was replaced in other media prepared separately by the nitrogen sources - potassium nitrate (3.069g); ammonium sulphate (2.005g); Glycine (2.279g); Glutamic acid sodium salt (5.681g); Urea (0.892g); and Casamino acid (4.25g). Solid media cultures were also used to determine the ability of the fungus to sporulate on the nitrogen sources. Initial pH was in each case adjusted to 6.0. Each

30ml of liquid medium in 250ml Erlenmeyer flask was inoculated with one drop of the standard conidial suspension. Solid media were inoculated with single spores. Observations were made at 2 days intervals. Growth rate in liquid media was determined over 14 days of incubation while the density of perithecia production was determined by making counts in 21 days old cultures..

TABLE IX : EFFECT OF DIFFERENT NITROGEN SOURCES UPON THE RATE OF GROWTH OF THE FUNGUS

	Dry weight (mgm) of mycelium after days of growth						
	2	4	6	8	10	12	14
Asparagine	10.9	28.5	49.6	72.7	89	90.7	92
Ammonium Sulphate	6.8	15.6	19.5	20.5	21.0	19.7	21.5
Glycine	9.8	22.5	43.0	60.5	76.0	85.1	87.0
Potassium Nitrate	11.5	22.0	45.7	66.5	81	89.5	91.5
Urea	14	28.5	50.6	93.5	101.5	104.8	107
Glutamic Acid	10.8	24.5	46.0	74.0	95.0	101.1	100.6
Casein Hydrolysate	8.9	21.5	31.0	43.5	51.5	61.0	63.5

Dry weight in milligrams. These compounds were used at the rate which supplied 0.425g of nitrogen per litre of medium. Each weight in the table is the average of six cultures.

The fungus was found to utilise nitrate nitrogen, ammonium nitrogen as well as organic nitrogen for vegetative development. No growth occurred in the absence of a nitrogen source in the medium. Results for each nitrogen source are presented in Table IX.

Asparagine is an amide of the monoamino dicarboxylic acid - aspartic acid. It is a source of organic nitrogen and is readily found free in plants as a source of reserve nitrogen. Vegetative growth on asparagine was dense and production of perithecia was high (Table X). With growth in liquid medium, there was an initial drop of the hydrogen-ion concentration from 6.0 to 5.6 and then a rise up to 7.6 (Fig. 7).

TABLE X : EFFECT OF NITROGEN SOURCE ON THE PRODUCTION OF PERITHECIA WITH GLUCOSE AS CARBON SOURCE

Nitrogen Source	Number of Perithecia *
Asparagine	46.3
Ammonium Sulphate	Nil
Glycine	44.7
Potassium nitrate	45.1
Urea	46.8
Glutamic Acid	48.0
Casein hydrolysate	49.4

* The number of perithecia refers to the number of perithecia taken as the average in ten arbitrarily chosen microscope fields on each of three plates for each nitrogen source.

AMMONIUM SULPHATE: There was a short period of growth in this medium which was terminated by the fourth day. With the start of growth, there was a steady drop in the pH from 6.0 to 2.8 and the cessation of growth was as a result of this change in pH. The uptake of ammonium ions from the medium resulted in the increased acidity. This high acidity was only fungistatic and not fungicidal as was

revealed by inoculating fresh glucose-asparagine medium with a tuft of fungal tissue from a 28 days old culture on ammonium sulphate that had stopped growing after 4 days of incubation.

Microscopic examination of the slimy mycelium showed that the morphology of the fungus was affected by this change in acidity following growth in the medium. The mycelium developed much branching with large swollen cells. These swollen cells looked like chlamydospores but their walls remained thin.

After 14 days of incubation, the medium was analysed for ammonium which was found to be present. This showed that the termination of growth was not as a result of the exhaustion of the nitrogen source. Addition of sterile drops of 1N NaOH to adjust the pH to 6.0 led to renewed growth which was again short-lived because the pH dropped again to 2.5 after two days of growth. No perithecia were produced in these media in which the pH changed so drastically.

Perithecial initials developed in the flasks in which there was a daily adjustment of the pH to 6.0 by aseptic addition of NaOH but further development of these initials into mature perithecia was stopped when the adjustment of pH was stopped.

In solid medium, the fungus did not produce the usual filamentous growth. A small thick leathery stroma measuring about 33mm in diametor (Plate IIa) and consisting of swollen cells was produced. No perithecia or initials were formed in solid medium.

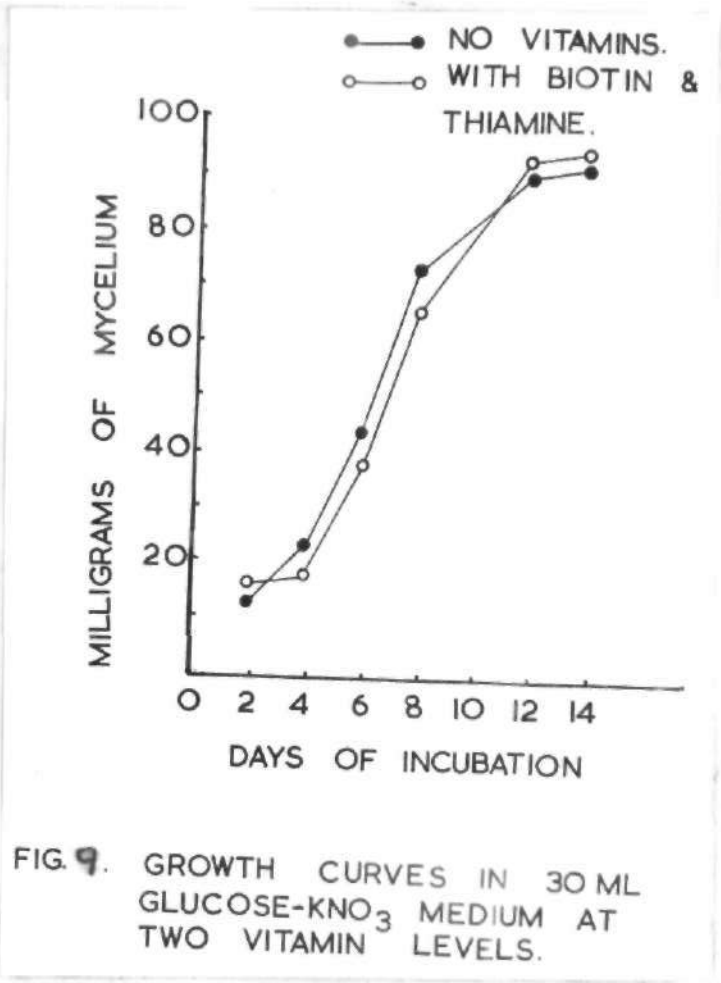


FIG. 9. GROWTH CURVES IN 30 ML GLUCOSE-KNO_3 MEDIUM AT TWO VITAMIN LEVELS.

Results are the averages of six replicates.

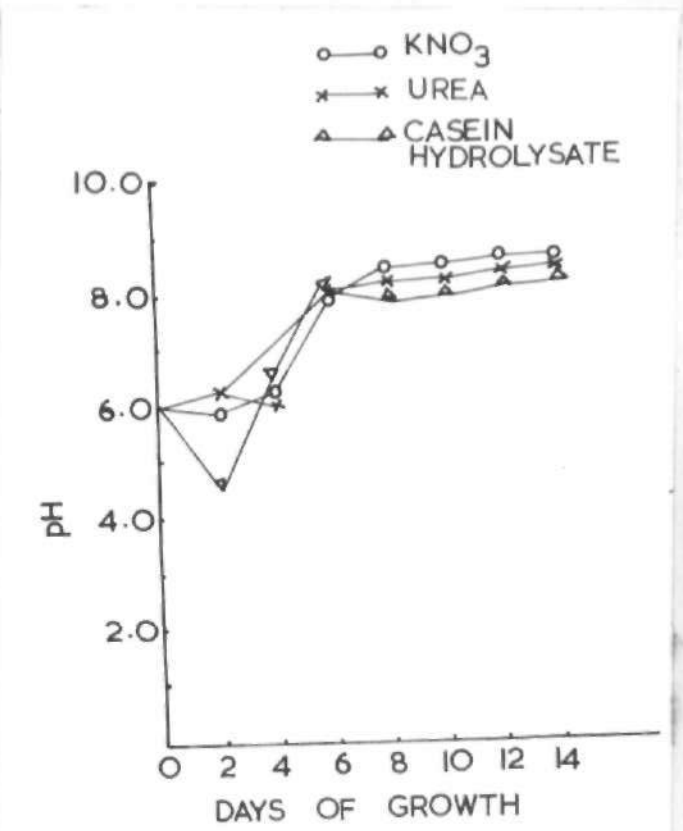


FIG. 8. CHANGE OF pH WITH GROWTH ON DIFFERENT NITROGEN SOURCES

Results are the averages of six replicates.

GLYCINE is a monoamino monocarboxylic acid. Vegetative growth and production of perithecia occurred readily on both solid and liquid media. Density of perithecia produced was high.

POTASSIUM NITRATE is a salt and provided inorganic nitrogen in the culture. Results showed that the fungus maintained good growth and produced perithecia on this inorganic nitrogen source. pH of medium rose with growth up to 8.6 (Fig. 8).

UREA: Of all the nitrogen sources tested, mycelial growth was found to be greatest on urea as source of nitrogen. Autoclaving urea in the medium led to a breakdown of the nitrogen source; as a result the autoclaved medium was not used in the investigation. A fresh medium was prepared and filter-sterilised using Oxoid membrane. With growth of the fungus, the pH rose gradually from 6.0 to 8.5.

GLUTAMIC ACID: This amino acid proved to be a very good source of nitrogen supporting dense mycelial development and perithecium formation. There was a steady rise in the pH with growth from 6.0 to 9.3.

CASEIN HYDROLYSATE: This was used as Casamino acid (Difco). In liquid medium, vegetative growth was poor but density of perithecia was very high on the poor growth. In solid medium, casein hydrolysate proved to be the best nitrogen source for the production of perithecia of all the sources of nitrogen tested.

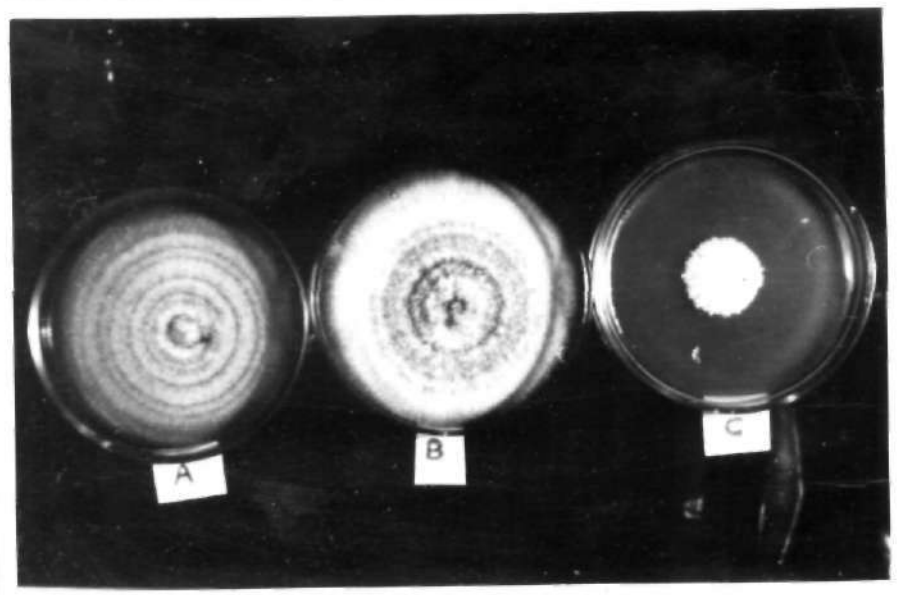


Plate IIa. Cultural morphology of the fungus on three different media A (MEA), B (PDA) and C (Glucose Ammonium Sulphate) 15 days old cultures.

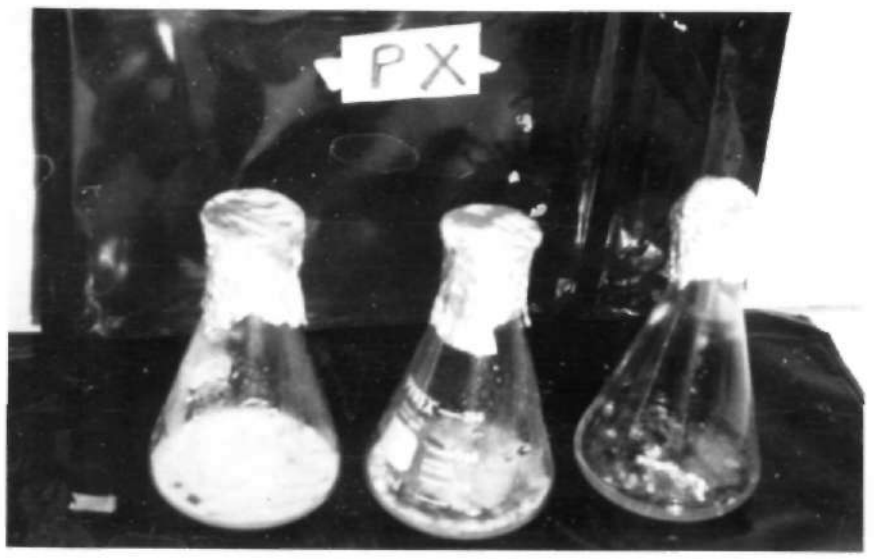


Plate IIb. Growth of the fungus on three different carbon sources from left to right, Mannitol, Galactose and Sorbose. 8 days old cultures.

Results of this investigation showed that the fungus utilised ammonium nitrogen, organic nitrogen as well as nitrate nitrogen for growth and sporulation. The fungus did not grow in the absence of nitrogen in the medium though the medium was in contact with atmospheric nitrogen. Based on this finding, the fungus will be placed in Group Two of Robbins (1937) - fungi able to utilise ammonium nitrogen, nitrate nitrogen and organic nitrogen but unable to utilise atmospheric nitrogen.

EXPERIMENT 4

Influence of Vitamins on Growth and Sporulation:

This experiment was designed to determine any vitamin def-iciency in the fungus.

Vitamin-free chemicals were used. Potassium nitrate was substituted for asparagine in the basal glucose-asparagine medium. To remove traces of vitamins from the liquid medium, the medium was boiled with activated charcoal and filtered.

For solid media, purified, vitamin-free agar was used to solidify the medium. Agar was purified by leaching with aqueous pyridine solution as follows: 45g of agar was placed in a 2 litre flask and 500ml of distilled water and 50ml of pyridine were added and allowed to stand for 24 hours. A piece of 6mm glass tubing of sufficient length (about 50cm) was inserted to admit air to the flask. The flask was inverted to allow the pyridine to drain. The agar was washed three times with distilled water and twice with 95% ethanol and the alcohol left to stand over the agar for 10 hours



Plate III. The auxoautotrophy of the fungus. 3 cultures grown in glucose-asparagine meding. Left to right: without vitamin, with biotin only, with thiamine only. 8 days old cultures incubated at room temperature.

before draining. The agar was dried between clean cloth and 20g of this purified agar was used to solidify one litre of medium. Media were prepared as follows for this investigation:

a) glucose - potassium nitrate medium containing no vitamins.

b) glucose - potassium nitrate medium containing 100ppm of thiamine.

c) glucose - potassium nitrate medium containing 5ppm of biotin.

d) glucose - potassium nitrate medium containing thiamine (100ppm) and biotin (5ppm).

Results obtained from growing the fungus under different vitamins showed that the fungus is self-sufficient with respect to vitamins. Cultures grown without vitamins in the medium grew as fast as the cultures grown in the presence of both biotin and thiamine in the medium. (Table XI) (Plate III). The growth curves obtained by growing the fungus with two different vitamins in liquid glucose-potassium nitrate medium confirmed this auxoautotrophy (Fig. 9).

Varying the concentration of biotin between 50ppm and 200ppm of medium did not affect the amount of growth obtained though the density of perithecia produced was significantly lowered at 200ppm in solid medium (Plates IV and V) (Table XII). With increased thiamine in the medium up to 500ppm, there was no effect on growth (Plate IV) and sporulation (Table XII). The result of increased biotin concentration of depressing sporulation in the fungus without



Plate IV. Effect of varying concentration of thiamine. Left to right: 100 ppm, 200 ppm, 500 ppm. 8 days old cultures at room temperature.



Plate V. Effect of varying concentration of biotin. Left to right: 50 ppm, 100 ppm, 200 ppm. 8 days old cultures at room temperature.

TABLE XI : EFFECT OF VITAMINS ON THE GROWTH OF THE FUNGUS

MEDIUM	Dry weight of 8-days old culture (mgm)
A (no vitamins)	72.4
B (with thiamine only)	73.1
C (with biotin only)	68,7
D (both biotin and thiamine)	71.2

Results are the average of six replicates.

TABLE XII : EFFECT OF VITAMIN CONCENTRATION ON GROWTH AND SPORULATION

Vitamin	Concentration ppm	Dry weight of 8 days old culture (mgm)	Number of Perithecia
None	-	72,4	45.2
Biotin	50	68.9	43.8
Biotin	200	70.9	27.6
Thiamine	200	73.7	42.0
Thiamine	500	69.8	43.1

The results are the averages of six replicates. Dry weight measurements were obtained from cultures on liquid medium; density of sporulation expressed as number of perithecia were obtained from cultures on solid medium.

a corresponding depression of vegetative growth suggests variations in the biosynthetic ability of biotin for the two phases of growth.

It is known that variations in the biosynthetic ability of some auxoautotrophs may occur during the life cycle of the organisms (Fries, 1965); in particular the very first stage of development, the germination and outgrowth of the spore seems to differ in this respect from the fully developed mycelium. Thus in typically self-

sufficient species as Penicillium digitatum (Fergus, 1952) and Aspergillus nidulans (Strigini and Morpurgo, 1961) initial growth may be strongly promoted by added vitamins. Also the germinated spores of Myrothecium verrucaria continue their growth very slowly unless the medium contains biotin, whereas the fully developed mycelium exhibits no requirement for biotin (Mandels, 1965). In Neocosmospora africana, no such differential biosynthetic ability with respect to biotin and thiamine obtains.

Furthermore, the requirements for a certain growth factor depends on the composition of the nutrient medium at any particular temperature within the biokinetic zone. For example, Pellicularia koleroga can utilise thiamine or biotin for growth on sucrose but requires thiamine exclusively for growth on glucose (Mathew, 1952). Pythium butleri was reported by Robbins and Kavanagh (1938) to become thiamine-heterotrophic as soon as the mineral salt concentration exceeds a certain level. In the test organism, N. africana, the use of the carbon sources glucose, galactose, sorbose, maltose, lactose, starch, mannitol, asparagine and the nitrogen sources urea, glycine, ammonium sulphate, potassium nitrate, casein hydrolysate, asparagine etc. does not affect the need for either biotin or thiamine.

There does not seem to be any obvious correlation between the ecology and the vitamin requirements of a fungus. The work of Cook and Lochhead (1959) maintains that there are certain indications that fungi living near the roots in the rhizosphere are more

vitamin-dependent than other soil fungi. N. africana usually grows in the rhizosphere of the groundnut but has not proved from cultural studies to be vitamin-dependent; perhaps a verification of more soil inhabiting fungi would be necessary to determine this observation.

EXPERIMENT 5

Effect of Light on Growth and Sporulation:

This experiment was designed to illustrate the variable effects of visible light and ultraviolet light. In some fungi, exposure to light is essential to spore formation whereas in others abundant sporulation occurs in the complete absence of light (22, 47).

Malt Extract Agar (MEA) was used in the investigation. All the petri dishes were inoculated with single spores. The cultures were set up under the following conditions:

(i) In continuous total darkness.

(ii) In continuous light-illumination in the chamber measured 166 lux on a Megatron D - 3 Light Meter.

(iii) Under alternating periods of 12 hours light and 12 hours darkness.

(iv) Grown under alternating light (ultra-violet light) and darkness. Exposure to UV light was limited to 3 minutes every 48 hours and was carried out in the inoculation room by removing the lid of the petri dish and holding the dish 10 inches from the source (Philips TUV 30W fluorescent tube) of ultra-violet light.

(v) Cultures grown in continuous light with light supplied by six overhead fluorescent bulbs. Light intensity measured above 3,000 lux.

Results obtained from these cultures showed that production of perithecia occurred in all the conditions tested. Production of perithecia in concentric rings was obtained in all the light conditions tested but the intensity of the zonation varied from one condition to the other. Zonation of perithecia was best defined in Group Three - cultures exposed to alternating 12 hours light and 12 hours darkness.

TABLE XIII : EFFECT OF LIGHT ON CULTURAL MORPHOLOGY OF THE FUNGUS

GROUP	Culture diameter (mm)	Colour of Perithecia	Number of Perithecia
I	64	Orange	45.6
II	66	Orange	44.9
III	64	Orange	46.2
IV	48	Black	26.7
V	68	Cream White	38.9

The pigmentation of the perithecia was also most pronounced in these cultures. With maturation of the culture the colour of the culture changed from creamy white to orange and to brown. Maturation of the perithecia begins from the centre of the culture and progresses gradually to the periphery. Between two consecutive zones of perithecia, a zone of dense hyphal growth occurred with scattered perithecia.

Cultures exposed to illumination above 3,000 lux did not develop the characteristic orange pigmentation, though still pigmented perithecia were produced (Table XIII). On removal of cultures from

the bright chamber to light of 166 lux, orange colour developed in all the perithecia after 36 hours.

Cultures exposed to intermittent UV light showed slower growth rate and produced black perithecia (Table XIII). On cessation of UV light treatment and exposing the culture to 12 hours of low light intensity (166 lux), the black perithecia changed to orange-coloured perithecia. Subsequent zones of perithecia produced after restoring the culture to mild light did not appear black before changing to orange. When some of the cultures exposed to UV light for some days were left in the dark and UV light treatment was discontinued, and the culture examined 8 days after the discontinuation of light treatment, it was found that the zones of perithecia formed during UV light treatment remained black whereas the zones formed after the cessation of UV light treatment but still in the dark were orange-coloured.

These results showed that vegetative growth was not adversely affected by exposure or non-exposure to visible light but exposure to UV light greatly diminished growth. Formation of perithecia in concentric zones in culture was also found to be a light-independent phenomenon.

In many ascomycetes, light has a stimulatory effect on reproduction but may or not be essential. Light is essential for the initiation of apothecia in Pyronema confluens (Carlile and Friend, 1956); in Ascobolus magnificus (Yu, 1954) and in the initiation of perithecium in Pleurage setosa (Callaghan, 1962).

In N. africana as shown from these photomorphogenetic studies, the extent of perithecium production on MEA was not affected by exposure or non-exposure to moderate light intensities but there were marked differences between illuminated and non-illuminated cultures as regards the distribution pattern of the perithecia within the petri dish. A similar situation has been noted for Sordaria fimicola (Ingold and Dring, 1957)

Zonation of perithecia was most intense in alternating 12 hours light and 12 hours darkness while still occurring in all the cultures grown. It is obvious then that for such light-enhanced zonation, it is not necessary for light to be essential for sporulation; it is just sufficient that light should be stimulatory. Thus, in all the cultures, there was none with a uniform distribution of perithecia. The perithecia were variously concentrated into narrow zones of intense sporulation. The conclusion that the effect of light is stimulatory is based on the observation of more intense zonation of sporulation in the culture in alternating 12 hour light and 12 hour darkness, each intense zone followed by areas of sparse sporulation clearly showing that for some time there has been a surpassing of the fungus spore producing capacity.

Carlile (1965) reported that differences in colour between fungi grown in the light and those maintained in darkness are common, and are often the result of a stimulus to carotenoid production by by light. In Pyronema omphalodes, absolute necessity for carotenoid

production has been demonstrated (47). Though this necessity for light in carotenoid synthesis has been reported for many fungi, this importance diminishes in the various pigmented fungi. In Phycomyces blakesleeanus light merely increases carotenoid production (22); in Neurospora crassa, the conidia contain carotenoids even when the fungus has grown in darkness though synthesis of carotenoids in the mycelium requires light (Zalokar, 1954). In Neocosmospora africana, the production of carotenoids in the perithecia has been found to occur even in the absence of light, though under continuous high light intensities there was poor carotenoid development - a phenomenon brought about by either a depression of carotenoid biosynthesis by light or due to photodestruction.

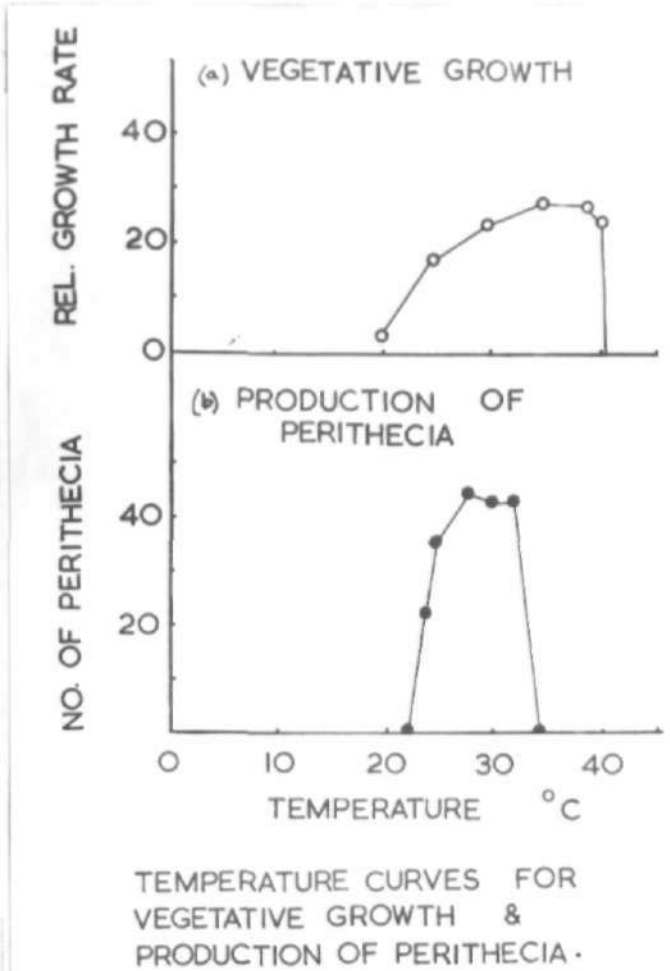
EXPERIMENT 6

Effect of Temperature on Growth and Sporulation:

The purpose of this experiment was to find out the effect of different temperatures on growth and sporulation of the fungus, to determine the optimum and to compare this with the optimum for other phases of growth in the fungus.

Potato dextrose agar (PDA) was used in the experiments and inoculation was with single spores. Temperatures tested range from $0^{\circ}C$ to $45^{\circ}C$.

No growth was observed at temperatures below $14^{\circ}C$. At $15^{\circ}C$, growth was very slow and after 14 days of incubation, only about 4mm of culture diameter was attained. Increasing rates of vegetative growth were obtained from $20^{\circ}C$ to $35^{\circ}C$ (Fig. 10) with an optimum lying between $28^{\circ}C$ and $34^{\circ}C$.



TEMPERATURE CURVES FOR VEGETATIVE GROWTH & PRODUCTION OF PERITHECIA.

FIG. 11. Vegetative growth was measured from culture diameter and number of perithecia was determined from solid medium.

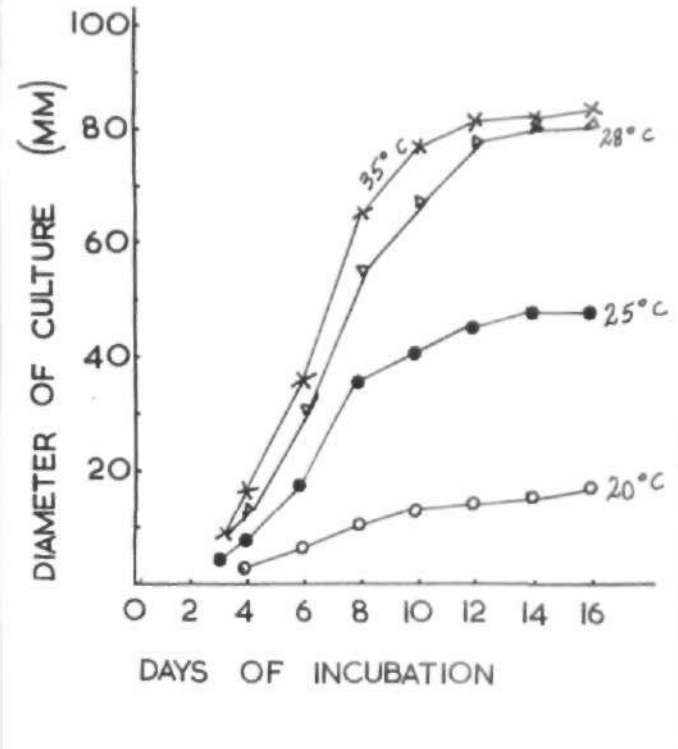


FIG. 10. EFFECT OF TEMPERATURE ON GROWTH.

Growth was measured by the increasing diameter of culture in mm.

Above 35°C, vegetative growth sharply declined. And at 39/40°C, the growth of the culture did not extend beyond 4mm in the second day of incubation after which the culture died out. No growth occurred above 40°C.

Determination of relative growth rate over the period of most active growth showed that the optimum was in the range of 28° - 35°C for vegetative growth.

Production of perithecia was observed over a wide range of temperature between 24°C and 33°C. At 34°C and above, no perithecia were produced in culture. Production of perithecia was most abundant between 28° and 32°C (Table XIV).

TABLE XIV : EFFECT OF TEMPERATURE ON PRODUCTION OF PERITHECIA IN CULTURE

Temperature of Incubation	Number of Perithecia
22°C	Nil
24°C	23.7
25°C	36.2
28°C	44.9
30°C	43.6
32°C	43.8
34°C	Nil

The results are the average of six replicates.

Microscopic examination of the culture incubated at 35°C showed a dense production of conidia and no perithecia. Transferring a culture that had been incubated for 6 days at 35°C to an incubator at 30°C resulted in a development of the normal type of mycelium with conidia and perithecia produced. Even in the new temperature (30°C), no perithecium was formed in the mycelium previously developed under 35°C.

The optimal temperatures of the different morphogenetic phases of the life cycle of the fungus have been found to be: germination of spores (30 - 35°C); vegetative growth (28 - 35°C); and production of perithecia (28 - 33°C). A closer look at these optima shows that the production of perithecia has a narrower optimum than any of the other phases. The range of temperature permitting vegetative growth (15 - 40°C) was also found to be narrower than the range permitting production of perithecia (24 - 33°C).

The shape of the temperature curve for vegetative growth resembles that for reproductive growth (Fig. 11); that is, the optimum in both cases is nearer to the maximum than to the minimum. The rate of growth increases from the minimum up to the optimum is assumed to be the effect of temperature on the many chemical metabolic processes which control growth and that the eventual rapid fall in growth after the optimum temperature is reached is due to the effect of high temperature on the physical state of the cytoplasm (Ainsworth and Sussman, 1966).

In Neocosmospora africana, this effect is not reversible since the fungus is unable to survive at temperatures above the the maximum

for growth (41°C) for 5 days. In most fungi, the effect is reversible as the fungi survive at temperatures somewhat above the optimum and to resume growth and sporulation on being transferred to more favourable conditions. The relatively narrow range of temperature permitting reproduction suggests that this phase involves some chemical and physical processes which are not necessary for vegetative growth and which are more exacting in their temperature requirements than are those which suffice for vegetative phase.

EXPERIMENT 7

Effect of Hydrogen-ion Concentration on Growth and Sporulation:

Earlier experiments with carbon sources and nitrogen sources have shown that pH of the culture medium changes during the growth of the fungus. This experiment was designed to determine the most favourable pH for growth and sporulation.

Liquid glucose-asparagine was used in the investigation. The pH of the medium was checked every 48 hours of growth and adjusted to the initial pH. Growth of the fungus over the pH range 1.0 to 12.0 was tested.

Vegetative growth did not occur below pH 3.0. Growth was obtained within the range 3.0 to 9.8. At pH 10.0 and above, no growth of the fungus was obtained. Vegetative growth was found to be greatest in the range 5.5 to 6.5.

Solid glucose-asparagine medium was used in determining the effect of pH on the production of perithecia. Results obtained from this investigation is to be viewed with caution because it was

not possible to carry out the periodic adjustment of pH in solid media while growth of the fungus on solid media was followed by a continued changing in the pH of the media. Production of perithecia in solid medium was obtained in the cultures at 5.0 to 8.4. The determination of an optimum was difficult as high density of perithecia was recorded at 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 (Table XV).

TABLE XV : EFFECT OF HYDROGEN-ION CONCENTRATION ON GROWTH AND SPORULATION

Hydrogen-ion Concentration	Dry weight of mycelium (in mgm)	Number of Perithecia
3.0	28.9	Nil
3.5	26.4	Nil
4.0	46.3	Nil
4.5	45.7	Nil
5.0	53.4	40.3
5.5	76.6	46.2
6.0	83.5	44.1
6.5	81.0	39.7
7.0	70.3	43.2
7.5	61.2	38.8
8.0	63.8	21.9
8.5	50.0	13.8
9.0	39.1	Nil
9.5	21.2	Nil
10.0	Nil	Nil

Results in the above Table XV show the dry weight of mycelium obtained from liquid medium and the numbers of perithecia obtained from counts in the solid media cultures.

Vegetative growth occurred over a wider range of pH than production of perithecia. It is seen from above results that pH is a critical factor in the production of perithecia by the fungus in culture.

pH acts in several different ways to affect growth and sporulation such as influencing enzyme action, altering metal solubilities, modifying surface reactions, preventing or facilitating the entry of vitamins, organic acids or minerals into the hypha. The poor growth obtained at certain pH values is likely due to changing of the composition of the medium. It is known that various anions and cations may combine to form insoluble compounds at certain pH values (56). Magnesium and phosphate ions are compatible in acidic solutions but as the concentration of hydrogen ion is decreased, these ions combine to form an insoluble compound the solubility of which becomes less as the pH is increased (57). This low pH is brought about either by an initial low pH or low pH resulting from the metabolism of the fungus in which acids are formed from neutral metabolites like carbohydrates.

With most media in which no adjustment to initial pH was performed except on glucose-ammonium sulphate medium, the change in pH caused by the formation of bases resulted in a sharp decline of growth. This change in pH was not the sole factor responsible for this decline; other factors as nutrient depletion and ageing of culture were also implicated.

V. THE MORPHOLOGY OF NEOCOSMOSPORA AFRICANA

Apart from the work of Doguet in 1956, there has been no work on the developmental morphology of this fungus. This work showed that the perithecium arose from a vegetative hypha which developed several filaments that branched and became entangled together to form a nodule, and this gave rise to the perithecium (31). This study was designed to determine the life cycle.

Germinating single spores were transferred to fresh plates of solid media and incubated at 28°C constant temperature. Fresh liquid media were also inoculated with single spores and incubated at room temperature.

Small blocks of material to be studied were cut out of the agar, killed and fixed in acetic alcohol. The blocks were aspirated to afford a more rapid penetration of the fixative. Sections were cut at a thickness of 12 u using a Leitz freezing microtome and were mordanted in iron alum. These were then stained in haematoxylin and in HCl - Giemsa stain.

Studies of perithecium development and ascus were made on fresh materials. Squash mounts were made in HCl - Giemsa stain or in Feulgen carmine or in 2% haematoxylin or in 1% aceto-orcein (49, 60, 79, 89). Haematoxylin gave the best results and was used almost exclusively.

Ascospores and conidia were capable of germination immediately upon maturity when placed in a suitable environment. The conidia were mainly single celled and less commonly, two-celled (Plate VIb). Ascospores were single celled and uninucleate. Both types of spores germinated by means of germ tubes; conidia began germination when placed in a favourable medium or in distilled water within 2 hours, ascospores did not germinate in distilled water but in a nutrient medium within 12 hours of incubation at 28°C.

Ascospore germinated by a single germ tube (Plate Ia) emerging at any point on the spore surface. The splitting of the two walled spore was a result of internal changes, the sequence of which could not be followed. The outer wall of the germ tube was not continuous with the inner wall of the ascospore but with the cytoplasm. Extension growth of the germ tube continued and septa soon developed in the germ tube which became transformed into a vegetative hypha. The hypha proceeded to branch profusely (Plate Ib) soon giving rise to a well developed mycelium.

Measurement of hyphal extension growth on solid medium (MEA) using the micrometer scale showed that each hyphal tip advanced at a rate of 80 - 83 u per hour at room temperature (varying between 24°C and 27°C). Cultures arising from single spores were visible to the naked eye as whitish dots after 24 hours of incubation at room temperature. Cultures from single conidia resembled cultures from single ascospores in all respects except for the initial

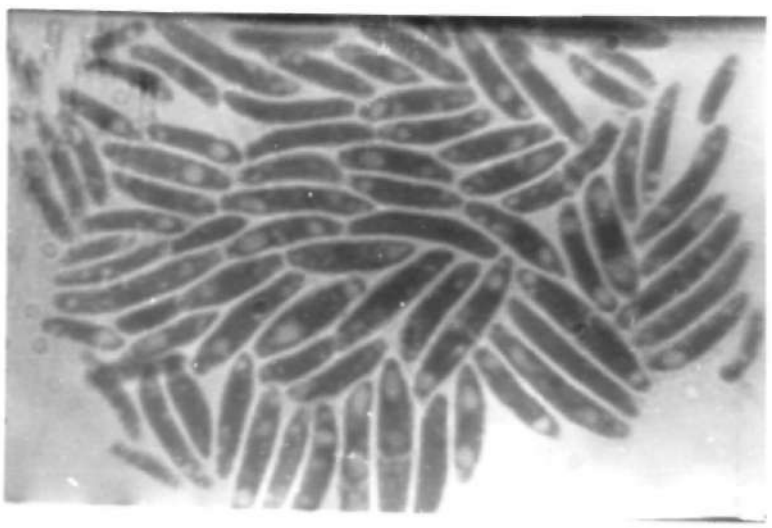


Plate VIa. Conidia on a conidiophore. x 1500

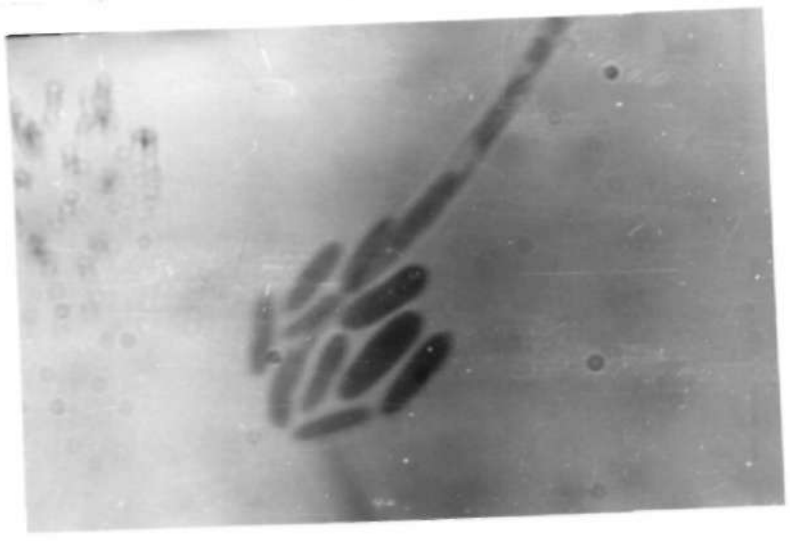


Plate VIb. Conidial suspension showing single celled and 2 celled conidia with two or more oil droplets. 1200

faster rate of germination of the conidia under the same conditions of incubation. 2-celled conidia germinated by producing two germ tubes while single celled conidia germinated by one germ tube production.

Mycelial production was best in MEA and PDA. The mycelium produced varied from a small, thick, leathery stroma on glucose-ammonium sulphate (Plate IIa) to a dense fluffy mycelium on PDA (Plate VII). Growth was slow even at best averaging 2mm per day.

The conidial stage (Cephalosporium sp.) was produced abundantly. Conidiophores and conidia formed in 2 - 3 days old cultures. Conidiophores arose singly as short unbranched aerial hyphae and ranged between 40 and 46 u in length. Conidia were 1-celled and less frequently 2-celled, hyaline and averaged 3.2 x 10.3 u (2 - 3 x 3 - 12 u). Each conidium contained one or more oil droplets. Conidial formation began as a bud pushing out from the tip of the conidiophore. Conidial chains were not formed, but conidia were held in a cluster around the tip of the short conidiophore by a gelatinous material (Plate VIa).

About five to six days after inoculation, small straw-yellow ascocarps appeared on the surface of the agar at the centre of the culture. More of these appeared concentrated in certain concentric zones and developing centrifugally. These continued to develop, becoming bright orange at maturity and reaching a size of 340 u high and 280 u wide (300 - 360 x 270 - 298 u). The neck of the



Plate VII. Three cultures showing active vegetative growth (left and right, on MEA and PDA respectively - 8 days old cultures) and zonation of perithecia (centre - 21 days old culture on PDA).

perithecium was short, measuring 60 x 110 u (58 - 62 x 100 - 116 u). Most of the pigmentation was concentrated in the ostiolar neck. Mature asci and ascospores were produced in the culture by the 12th day after inoculation. Measurements of mature asci averaged 180 x 28 u (160 - 190 x 26 - 29 u). Ascospores measured 8.6 x 10 u (8 - 9.2 x 9.4 - 10.2 u). Each ascospore typically contained a large oil droplet, was double-walled, uninucleate with no germ pores. The inner of the two walls was slightly pigmented. The ascospores were released by the deliquescence of the ascal walls. At this stage, the perithecia were hardened and brownish in colour. Agar plates inverted over a culture of mature perithecia collected no ascospores; this showed that the ascospores were not forcibly released.

DEVELOPMENT OF PERITHECIUM:

Development of the perithecium began with the appearance of coiled, short branches of vegetative hyphae scattered in the mycelium (Plate VIII). These ascogonial coils were initially single celled but later the gyres increased and septa developed towards the base of the coil (Plate IX). A young ascogonium was composed of two to six gyres, each coil being 4 - 5 microns in diameter. Though the ascogonium was often observed to be coiled around some hyphae near it, no fusion was observed to take place between these hyphae and the ascogonia.

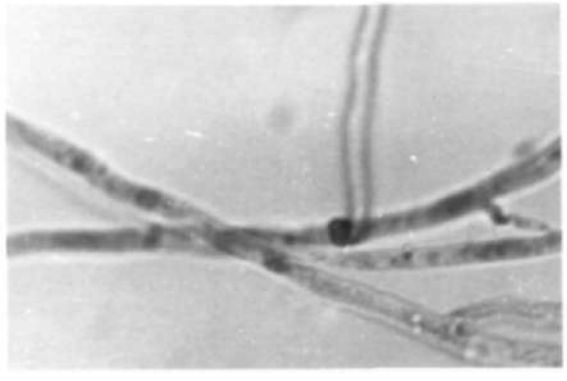


Plate VIII. An ascogonial coil-unicellular x 1200

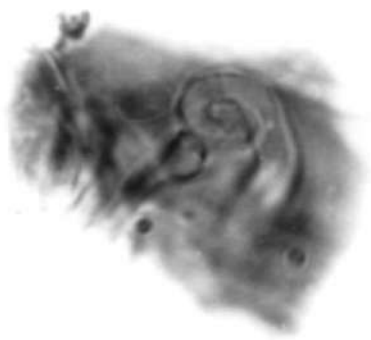


Plate IXa. Ascogonial coil with septa development. x 1500

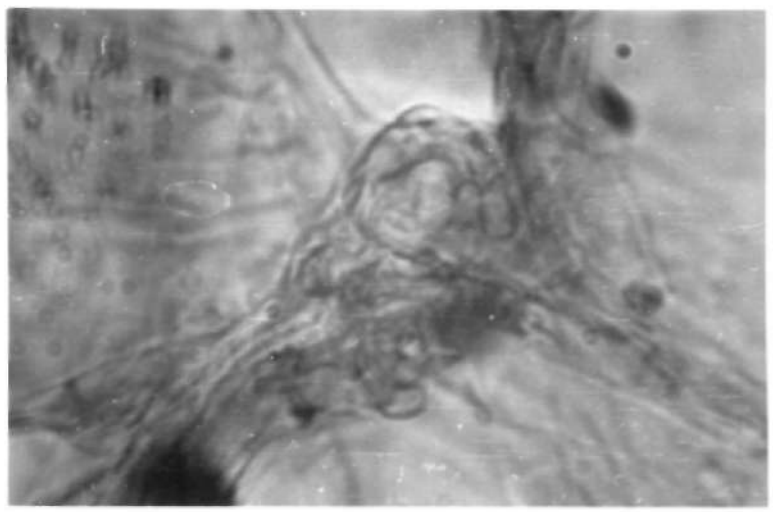


Plate IXb. Ascogonial coil surrounded by associated hyphae forming the perithecial initial. x 1500

The development of the septa in the ascogonium resulted in the cutting off of the apical swollen cell of the ascogonium. Hyphal branches grew out from the stalk cells of the ascogonium (Plate IXb). These hyphae grew and curved round the swollen apical cell to form the perithecial initial (Plate Xa). No evidence of fusion with other hyphae or spores was found. The surrounding sheath was added to centrifugally from the neighbouring hyphae which grew in and formed the outer layers. The sheath continued to increase in size and was at first prosenchymatous in appearance but soon became organised into pseudoparenchyma and formed the wall of the young perithecium. As the perithecial primordium increased in size, the centre became filled by large cells (Plate Xb) arising from the divisions of the ascogonium. This ascocarp initial at this stage stained intensely in iron haematoxylin and comprised the central ascogonium with any descendant cells and the surrounding wall.

Plates XI and XII show perithecial primordia that were pretreated with snail stomach cytase and stained by the Feulgen carmine technic. The developing ascogenous system contained monokaryotic and dikaryotic cells. Meanwhile increase in size of the young perithecium resulted from periclinal and anticlinal divisions in the primordial wall as well as from stimulus from the ascogenous system. This pressure from within was not measured.

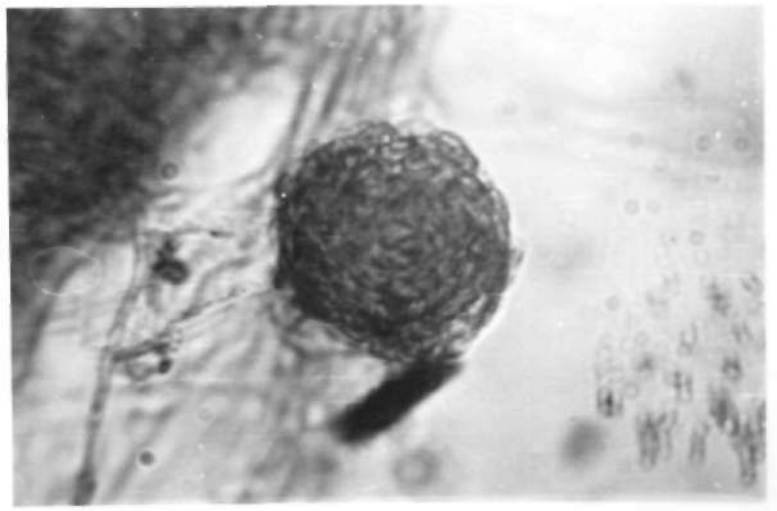


Plate Xa. Young perithecium stained in iron-haematoxylin. x1500

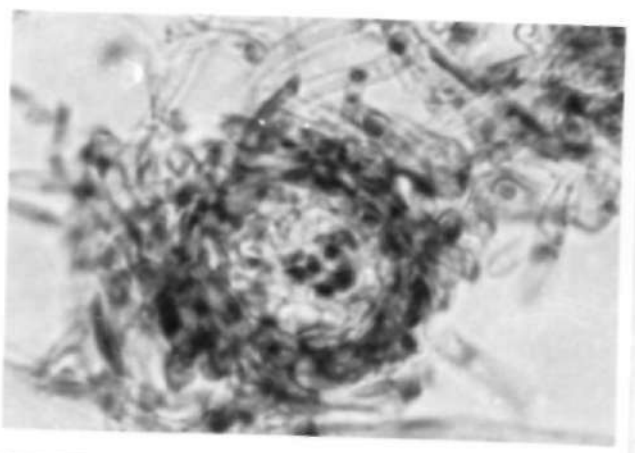
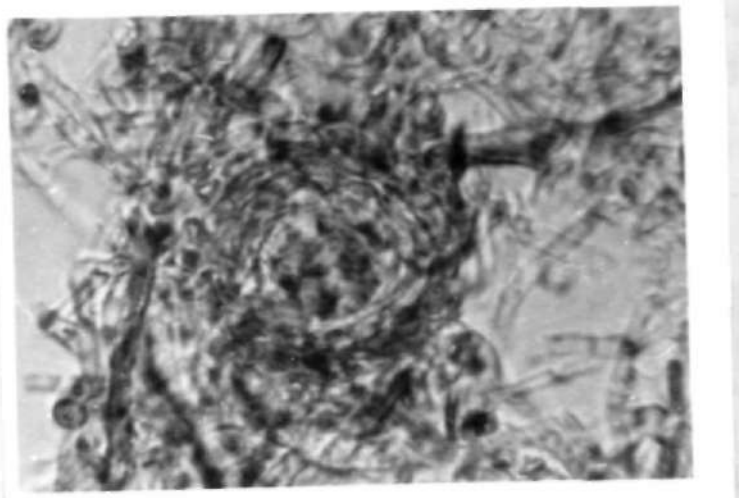


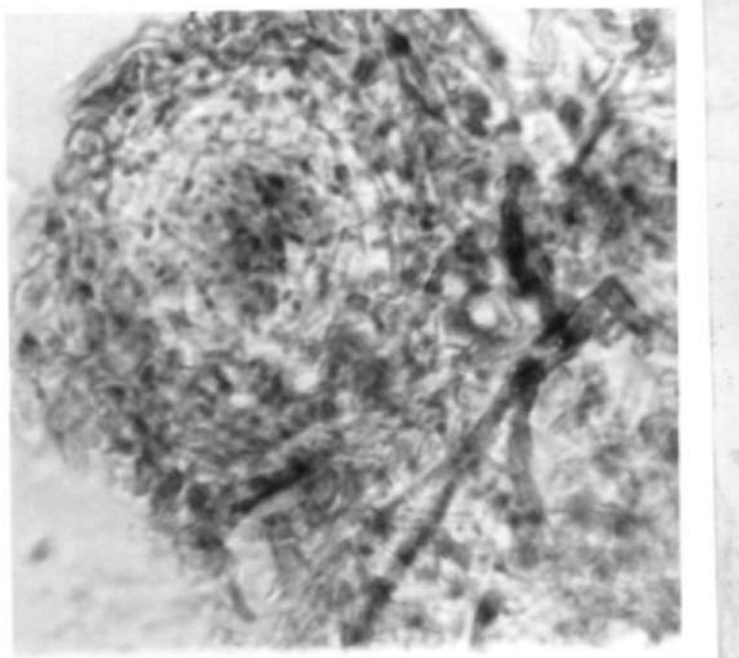
Plate Xb. Young perithecium stained with Feulgen technic after treatment with snail stomach cytase-ascogenous system developing. x1500

Plate XIa.



Growth of young perithecium with production of large central cells. x 1500

Plate XIb.



Growth of young perithecium with production of large central cells. x 1500

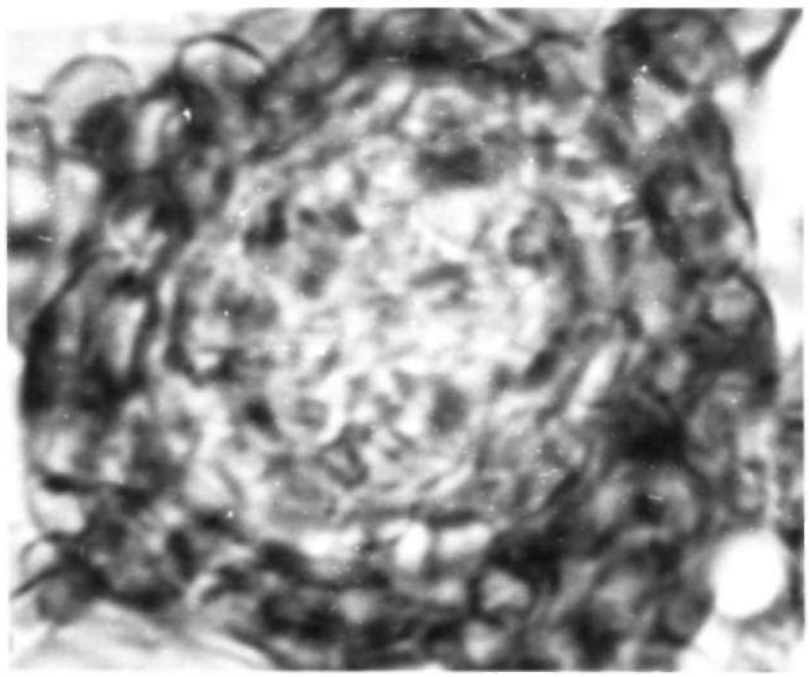


Plate XII. Ascogenous system in young perithecium. x 2000

The pseudoparenchymatous tissue constituting the wall of the young ascocarp comprised 10 - 12 layers of cells, the inner 6 - 8 layers of which were hyaline and thin walled, and the outer layers of which were thick walled and stained intensely in haematoxylin. In unstained preparation, the round young perithecium appeared cream-white in colour.

Asci started developing from the basal monokaryotic cells of the slightly convex hymenium (Plate XIII) mixed up with sterile hyphal strands. Simultaneous with the growth and development of the asci, a zone of meristematic cells appeared in the inner layers of the peridium opposite the young asci. Divisions in this zone progressed outwards to the outermost layer of the perithecium giving rise to many layers of cells (Plate XIV). Hyphal strands from the centrum proceeded to grow upwards through a schizogenous zone in the layer of young cells to the outside of the peridium. The ostiole thus formed opened into the centrum. The numerous short strands lining the ostiole with many emerging at the tip were the periphyses. Following the formation of the ostiole, there was a re-organisation of the prosenchyma around it to form a short neck measuring about 60 u in length (Plate XV).

The formation of the neck slightly preceeded the maturation of the asci. Orange coloured pigments were soon concentrated in the prosenchyma that formed the neck. This pigment exuded in a fluid to the tip of the neck with the mature ascospores and when

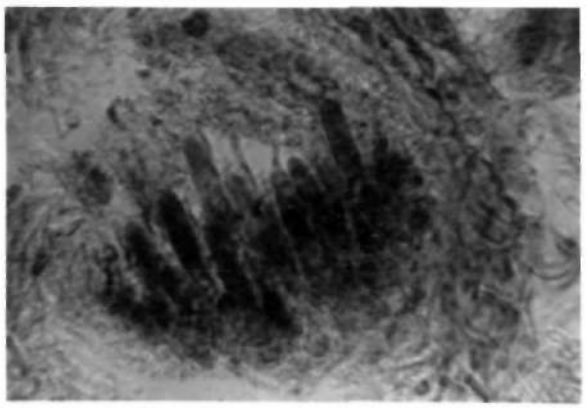


Plate XIIIa. Young asci and paraphyses borne on the hymenium in L.S. of young perithecium. x 1200

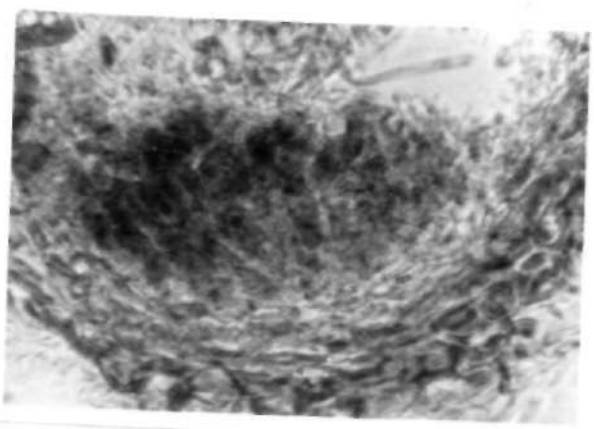


Plate XIIIb. L.S. young perithecium showing asci, paraphyses and pseudoparenchymatous peridium. x 1200

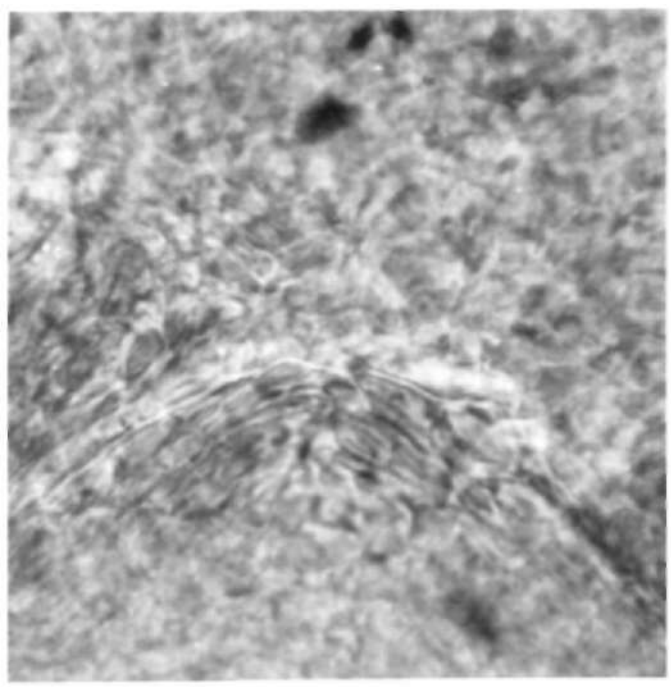


Plate XIVa. Formation of ostiole. x 1500

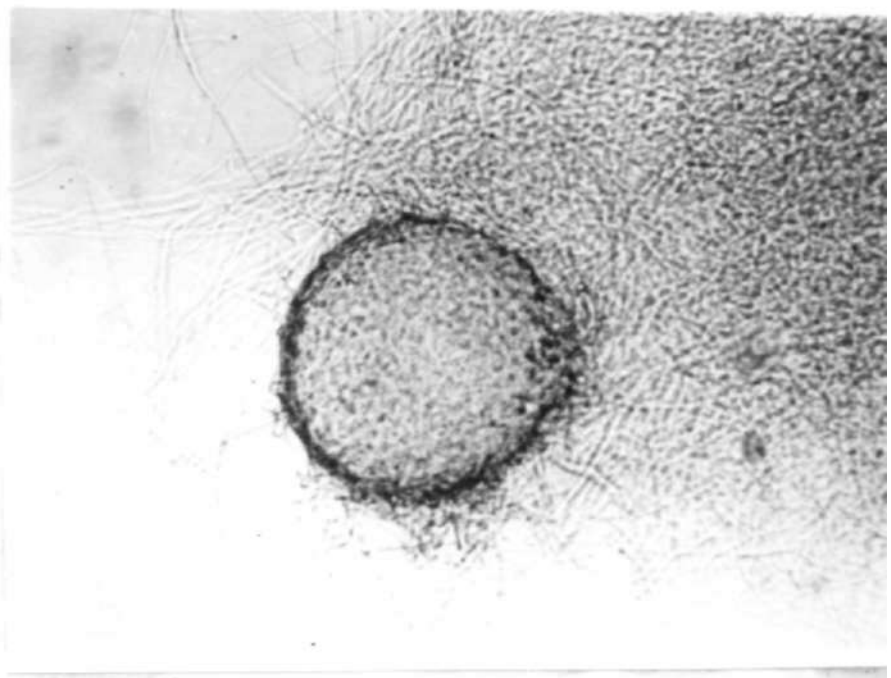


Plate XIVb. Formation of the neck. x 800

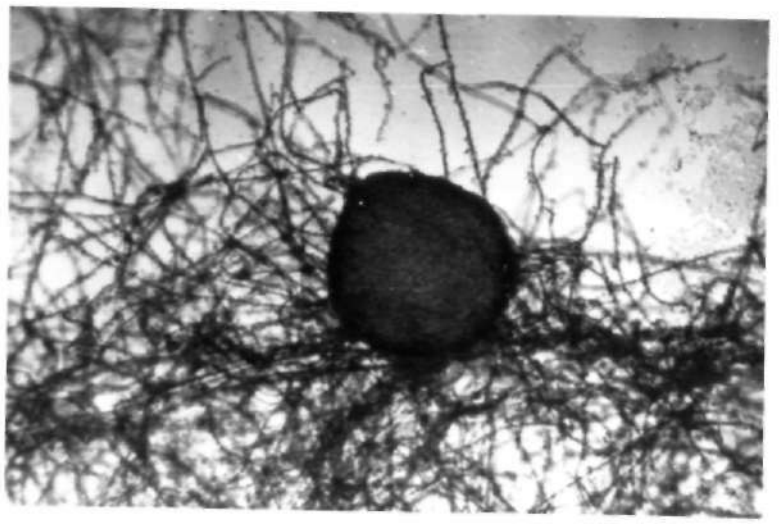


Plate XVa. Formation of a neck in a young perithecium. x 800

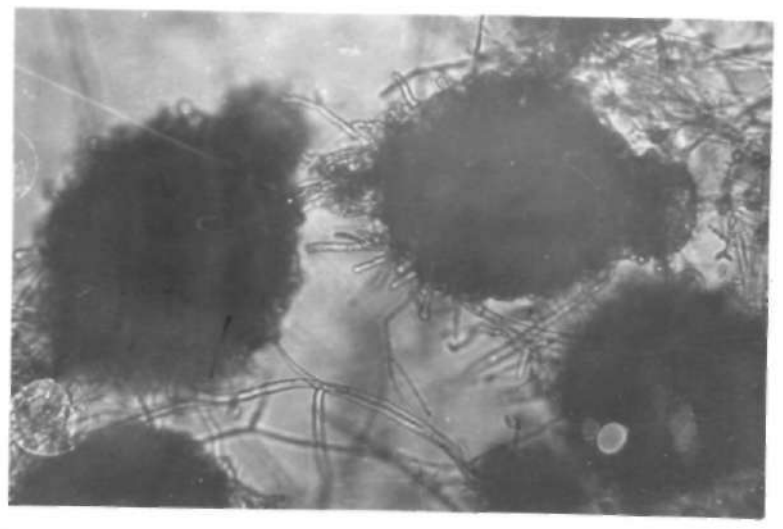


Plate XVb. True perithecia. x 500

a culture containing mature perithecia was examined, the characteristic orange coloured zonation was significant. On solid media where perithecia were produced in concentric zones, orange-coloured concentric circles of perithecia were obtained. The greater part of the inner peridial layers consisted of straw-yellow coloured cells while the outermost 2 - 3 layers were light-brown.

DEVELOPMENT OF THE ASCUS:

The ascogenous system contained both monokaryotic and dikaryotic cells (Plate XVIa). Asci developed from monokaryotic cells. Crozier formation was not observed. The monokaryotic cells (ascus mother cells) occurred basally in the centrum and grew by elongation into the centrum cavity (Plate XVIb). Meiosis occurred, forming first a binucleate ascus, then a 4-nucleate one. This was followed immediately by a third mitotic division resulting in an 8-nucleate ascus (Plate XVII). There was a laying down of cytoplasm around each of the 8 nuclei to form an 8-spored arrangement in a uniseriate ascus (Plate XVIIIa). With the delimitation of cytoplasm around the eight nuclei in the ascus, the paraphyses borne on the same hymenium deliquesced.

Measurements of the spores in 8-spored ascus averaged 20.2 x 24 u (20 - 21.8 x 23 - 25.6 u). The delimitation of cytoplasm to form the 8-spored stage was followed immediately by two consecutive divisions in each spore to give rise to four nuclei. The cell wall was laid down around each nucleus to form four ascospores from each

Plate XVIa.
x 2000

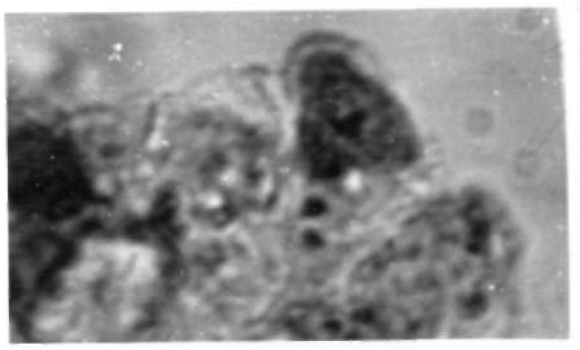
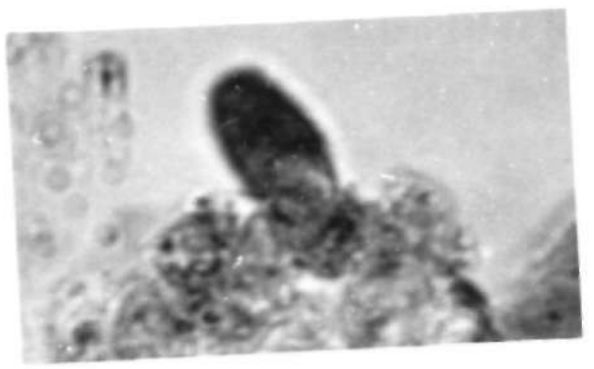


Plate XVIb.
x 2000



Squash preparations of young perithecium stained in iron-haematoxylin.

a) Ascogenous system with uninucleate cells and a binucleate cell. Young ascus with single nucleus.

b) A young ascus with several monokaryotic cells.



Plate XVII. Young asci at various stages of development in a squash preparation stained in iron-haematoxylin.

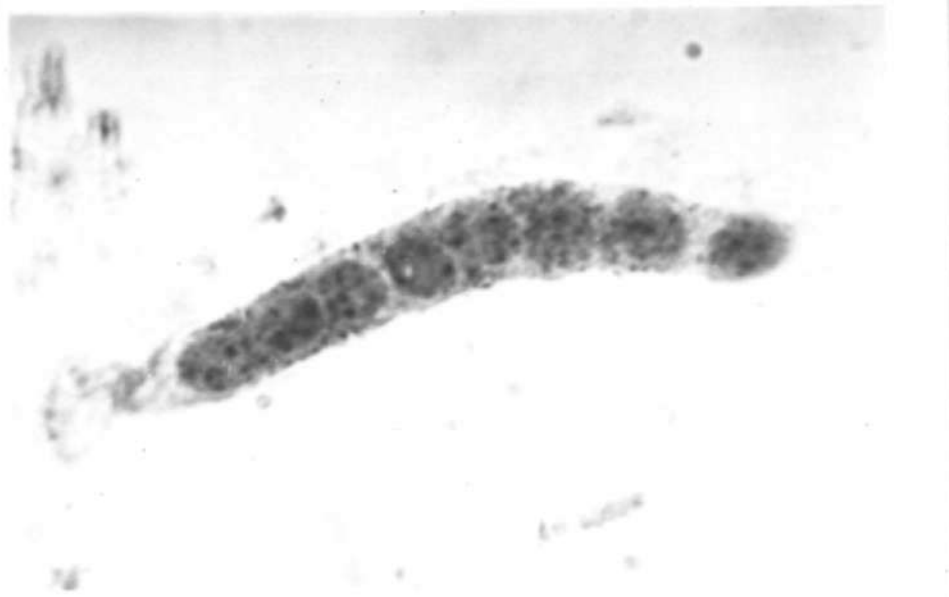


Plate XVIIIa. 8 - nucleate ascus with delimitation of large spores. x 1500



Plate XVIIIb. 2 successive divisions in each large spore resulting in the formation of a maximum of four ascospores in each large spore. x 1500

spore in the 8-spored ascus (Plate XIX). The formation of these spores following these two successive divisions synchronised with the deliquescence of the wall of the unitunicate ascus.

Maturation of the ascospores took place within the ascus with a gradual disappearance of the cytoplasm external to the spores in the ascus. Deliquescence of the inner 6 - 8 hyaline layers of the perithecial wall occurred. The ascospores set free by the deliquescence of the ascal walls filled the cavity of the perithecium thus formed (Plate XX). The breakdown of the inner perithecial walls was uneven, being confined to the sides and the base. The pseudoparenchymatous cells at the apex of the perithecium persisted with the periphyses lining the ostiole. The remaining walls of the perithecium progressively turned brown to give the characteristic brown colour of an old culture.

The ascospores exuded through the ostiole in a slimy fluid and collected at the mouth. In solid medium, the perithecium disintegrated after the spores were shed as a result of the lower thin-walled part of the perithecium being unable to support the top which collapsed into the bottom half. The spores did not germinate in the same medium in which they were produced.

The complete development of the fungus from a single spore incubated on MEA at 28^{O}C to the appearance of the first mature ascospore on the culture was completed after 9 days of inoculation.

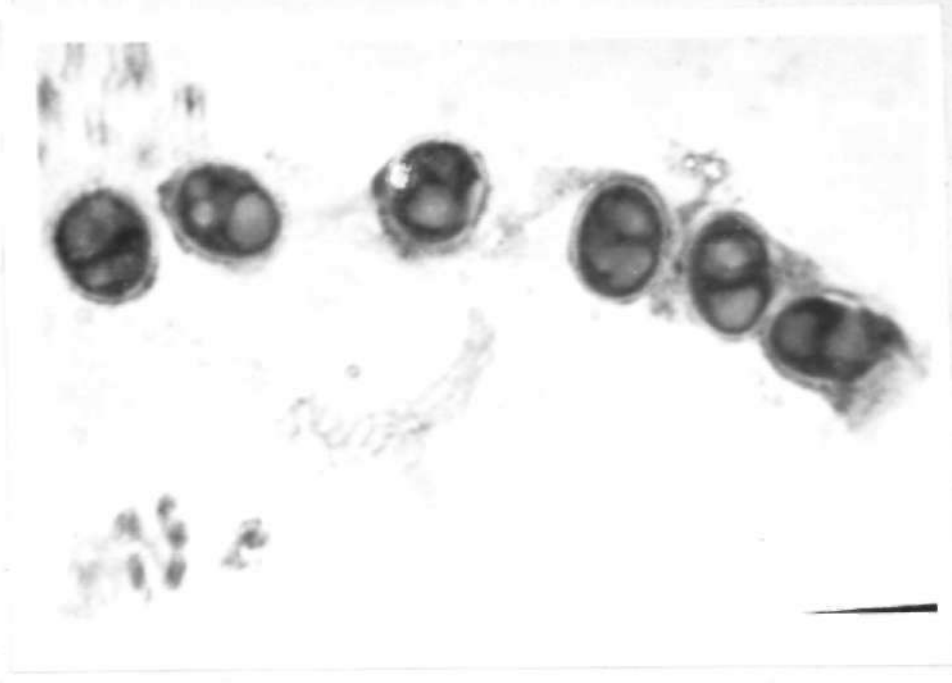


Plate XIX. Ascospore formation. x 1500



Plate XX. Mature perithecium filled with loose ascospores. x500

VI. DISCUSSION

The life cycle of fungi begins and ends in spores and can be conveniently defined as spore to spore development. It is delimited by two phases; spore germination and spore formation or sporulation separated by a rather prolonged period of mycelial growth.

A study of the initial, simplificative phase of the life cycle (spore germination) showed that the asexual spores (conidia) and the sexual spores (ascospores) differed in their environmental requirements. The conidia showed nutritional independence while the ascospores were nutritionally dependent. The existence of these two extreme types of relation to external nutrients in this fungus is significant when the roles of these spores are considered. The conidia are produced during the active vegetative growth of the fungus and are responsible for rapid spread of the organism during this phase. Its feature of nutritional independence, which is not a common feature of conidia generally, must be understood as an advantage towards fulfilling its role. The thick-walled sexually produced ascospores are specially adapted for the survival of the organism through unfavourable periods and their production occurs as a result of the arrival of unfavourable conditions in the medium (72). Ecologically, a nutritional requirement for the ascospore is without doubt, an advantage, helping to ensure that

germination would not occur in environments devoid of the essential materials needed for continued growth (23).

The inhibition of germination by the fluoride suggests that the ED-pathway is the main pathway for the glycolytic breakdown of the carbon sources in the germinating spores. This has been reported also by Blumenthal (15) for many fungi though he maintains the possibility of the existence of other, as yet undiscovered, pathways for carbohydrate metabolism in fungi. It is necessary, if at all possible, for more biochemical researches to attempt to verify these observations at more than one level.

When the spores were incubated at different hydrogen-ion concentrations, the conidia showed a double pH optimum at 3.5 and 6.0 in the nutrient medium. The fact that these conidia are capable of germinating in distilled water as well as in sugar solutions suggests that some other more complex processes other than a reflexive pH - dependent unavailability of one or more inorganic elements at the minimum occurring between the two optima are involved. Whether this minimum has a direct effect on conidial wall permeability or not, is not known.

When the fungus was grown on different carbon sources, it grew satisfactorily and sporulated on all the sources in solid medium whereas in liquid media, it grew on all the carbon sources except cellulose. With mixed carbon sources, denser growth was obtained on all the sources investigated. Even with cellulose,

large balls of mycelium were formed around the insoluble cellulose particles. These mixed carbon sources simulate the conditions that exist naturally in the soil. These results show that in a simulated natural environment, the fungus yields a weight of mycelium far in excess of growth on sole carbon sources. This result is very significant. This diversified metabolic activity will be of practical importance in a beneficial way in the disposal of carbon-containing waste products in industrial situations.

In this study of the growth of the fungus on different carbon sources, it was not possible to determine the extent of disappearance of the carbon-containing compounds except for starch in the medium. The economic co-efficient of the fungus on starch after 6 days of incubation at room temperature was found to be as low as 25%. This result agrees with the results of Perlman (79) obtained for fungi grown on dilute media. The probable soluble metabolites, the production of which is responsible for this low value are not known.

The results obtained by culturing the fungus on different nitrogen sources showed that the fungus is able to utilise both organic and inorganic nitrogen sources. That the cessation of growth on glucose-ammonium sulphate medium was not due to the exhaustion of the nitrogen source was confirmed by the test for Ammonium ion which gave a positive result. The renewed growth

after the re-adjustment of the pH from 2,8 to 6,0 in 2 weeks old cultures showed that the low pH was responsible for the stopping of growth. This increasing acidity of the medium following growth in the glucose-ammonium sulphate medium was caused by the differential utilisation of the ions present in the nitrogen source - the ammonium ions being taken up by the fungus while the sulphate ions accumulated in the medium and accounted for the lowering of pH.

Visible light is not necessary for the growth and sporulation of the fungus. The production of orange coloured pigments in the perithecium was inhibited by light only at intensities above 3,000 Lux. This poor carotenoid development at such intensities must be due to either a depression of carotenoid synthesis or to photodestruction. The exact cause is not known. UV-light also inhibited the development of pigments completely in the perithecia. The mechanism of its action is unknown.

Narrower ranges of temperature (vegetative growth, 15° - $40^{\circ}C$; sporulation, 24° - $32^{\circ}C$) and pH (vegetative growth 3 - 9.8; sporulation 5.0 - 8.4) were obtained for reproductive growth than for vegetative growth in the fungus. Because of the possible interaction with other factors, further critical investigations are necessary before any specific interpretation of these environmental factors is achieved.

In the development of the perithecium from a unisporous culture, the ascogonium was found to be a coiled vegetative hypha but no antheridium was observed. Uninucleate as well as binucleate cells were observed in the ascogenous system. The ascus was observed to arise from a uninucleate cell and no definite evidence of croziers was observed.

The binucleate cells in the ascogenous system could be representing young asci but the cells were not elongated as young asci at this stage would be. These binucleate cells might be young asci prior to nuclear fusion or more probably ascogenous cells in which the formation of septa had not yet occurred after a nuclear division. Many authors (13, 42, 43, 44, 24, 28, 105) have reported similar observations in different species. In this study, it appears that the asci arose from uninucleate cells and that the binucleate cells represent a stage prior to fusion of the nuclei in the ascus mother cell. This remains to be confirmed.

The characteristics of the centrum in this species differs slightly from the Nectria type in the absence of apical paraphyses and the possession of uniformly thin-walled ascus in N. africana. More studies of the developmental morphology of the genus will be necessary before any specific statement can be made on the taxonomic relationships in the genus. The homothallism of the fungus is very simple.

IBRARY RECEIVED

SUMMARY

The results of the study of the life cycle of Neocosmospora africana from uninucleate single ascospores have shown conclusively that the fungus is homothallic.

Germination of the spores is direct, by the production of germ tube. The two types of spores generally produced by the fungus, conidia and ascospores differed in their nutritional demands for germination. Conidia germinate in the absence of nutrients provided that the spores are in contact with liquid water and other physical conditions are favourable for germination. Single-celled conidia germinate by the production of two germ tubes. Ascospores germinate under favourable environmental conditions only in the presence of nutrients by the production of single germ tube. No predetermined areas for the emergence of germ tube occur on the spore surface. In the germination of conidia, two pH optima were obtained while ascospores showed one optimum. Both conidia and ascospores are capable of germination soon after production.

Investigations of the carbon metabolism of the fungus showed that the fungus can utilise a wide range of carbon compounds ranging from monosaccharides and disaccharides to sugar alcohols and polysaccharides including cellulose. Mixed carbon sources proved to be better sources of carbon for the growth of the fungus than single carbon sources - a consequence of the operation of adaptive enzyme systems.

Of all the single carbon sources tested, mannitol proved to be the bestfor vegetative growth while L-sorbose gave the densest production of perithecia. D-glucose proved to be a good carbon source for both vegetative and reproductive growth, though not the best for any of these phases of growth. In all, no single carbon source was found to be the best for both vegetative growth and the production of perithecia.

Results of investigations of nitrogen metabolism of the fungus showed that the fungus is able to utilise ammonium nitrogen, nitrate nitrogen as well as organic nitrogen for both vegetative growth and sporulation. In the use of ammonium nitrogen as the sole nitrogen source, growth of the fungus was easily terminated as a result of pH changes despite the heavy buffering of the medium. Slight change in the morphology of the cells was obtained following increasing change in acidity of the medium with growth. The low pH obtained proved to be fungistatic and not fungicidal.

The ability of N. africana to grow and produce perithecia in a vitamin free medium showed that the fungus is auxoautotrophic. Studies of the effect of increasing concentrations of biotin and thiamine on the growth and sporulation of the fungus showed that increasing concentration of biotin up to 200 ppm remarkably depressed sporulation without decreasing vegetative growth. Increasing thiamine concentrations up to 500 ppm, neither decreased vegetative growth nor depressed sporulation.

The effects of the physical factors, light, temperature and pH on the growth and production of perithecia in the fungus were investigated. Visible light was found to be unnecessary for growth and sporulation. Production of perithecia in concentric zones was also found to be a light-independent phenomenon, though the intensity of this zonation was influenced by the periodicity of light. At very high light intensities, above 3,000 Lux, the characteristic colour of the perithecia was diminished. UV light was found to reduce vegetative growth and inhibit the development of colour in the perithecia for only very short periods of exposure to this light.

The fungus was found to grow and sporulate in the temperature ranges 15^{o}C to 39^{o}C and 24^{o}C to 33^{o}C respectively. Germination of the spores occurred in the range 23^{o}C to 40^{o}C. In all, the three different phases of growth, germination, vegetative growth and sporulation showed three slightly different optimal temperatures. A narrower optimum was obtained for the production of perithecia than for any of the other phases of growth. From the studies of the effect of pH on growth and sporulation of the fungus, a wider range of pH was found to favour vegetative growth than the production of perithecia.

Studies of the developmental morphology revealed that the ascogonium is a coiled hyphal branch. This ascogonium soon becomes surrounded by neighbouring hyphae to form the perithecial primordium.

No antheridium was observed. Uninucleate cells of the ascogenous system give rise to the asci without the formation of croziers. These unitunicate asci are borne mixed with paraphyses on a slightly convex hymenium. Two successive division occur in the 8-nucleate ascus leading to the production of a maximum of 32-spored stage in the ascus. At maturity, the ascospores are released into the perithecial cavity by the deliquescence of the ascal walls. These ascospores ooze out of the neck of the mature perithecium in a drop of liquid.

The life cycle of N. africana is simple and completely haploid with the exception of the diploid fusion nucleus of the ascus.

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APPENDIX 1b

The analysis of data of Experiment 1, as in all the other subsequent experiments, was done for each set of six observations by determining the mean ($\bar{x}$) from the relation

$$\text{Mean} = \frac{\text{Total value of all measurements}}{\text{Total number of measurements}}$$

The mean deviation of the continuous variates was also determined from the formula

$$\left(\frac{1}{N}\right) \sum_{i}^{N} = \left| x_i - \bar{x} \right| \quad \text{where}$$

N is the total number of measurements (6)

X is the continuous variate

$\bar{X}$ is the mean

and where the modulus (denoted by $||$) of $x_i - \bar{x}$, namely its numerical value ignoring the sign is employed.

Thus in the set of six observations of the percentage germination of the ascospores after 14 hours incubation in complete glucose asparagine medium:

	% Germination	Mean ($\bar{x}$)	Deviation	Mean Deviation
1st	27	26.7	0.3	0.9
2nd	26		-0.7	
3rd	25.6		-1.1	
4th	29		-2.3	
5th	26.8		-0.1	
6th	25.8		-0.9	

A P P E N D I X II

Effect of Temperature on germination of ascospores

Exp. No.	% germination at different Temperatures (°C)											
	20°C	23°C	25°C	27°C	28°C	30	32	35	37	39	40	41
I	0	5	6	21	39	53	91	97	86	45	8	0
II	0	6.6	10.3	20	33	55	82	93	71	31	10	0
III	0	3	7	23	42	51	89	97	88	38	6	0
IV	0	7.3	9	19	38	51	94	95	81	43	8	0
V	0	4	8	21	41	52	90	89	91	40	12	0
VI	0	6	8	22	40	56	88	98	80	29	6	0

A P P E N D I X III

pH	% Germination (Conidia)							% Germination (Ascospores)					
1.0	0	0	0	0	0	0		-	-	-	-	-	-
1.5	0	0	0	0	0	0		-	-	-	-	-	-
2.0	5	3	6	6	5	5		0	0	0	0	0	0
2.5	27	21	27	28	23	27		0	0	0	0	0	0
3.0	86	81	89	82	82	86		0	0	0	0	0	0
3.5	88	82	83	86	83	85		10	10	8	11	10	10.6
4.0	85	79	85	81	83	86		18	15	17	18	18	17
4.5	80	82	81	81	84	79		41	40	41	43	42	46
5.0	70	68	69	67	71	75		60	60	61	58	61	61
5.5	82	83	80	82	78	81		88	86	83	88	81	86
6.0	81	81	83	80	78	80		89	88	88	81	89	87
6.5	87	83	81	87	87	84		87	85	86	87	87	84
7.0	79	76	80	77	80	79		86	81	86	85	81	83
8.0	52	49	51	50	52	52		84	80	79	81	81	80
8.5	48	49	47	51	49	48		58	56	51	58	57	56
9.0	43	41	43	43	41	41		30	39	38	30	32	38
9.5	27	25	27	25	21	26		7	7	9	11	8	7
10.0	27	23	23	27	25	21		0	0	0	0	0	0
10.5	0	0	0	0	0.7	0		0	0	0	0	0	0

A P P E N D I X IV

Change in dry weight of mycelium every 2 days of growth

Carbon Source	2	4	6	8	10	12	14
Glucose	9	17	18.5	21	18	4	4.6
	10.7	16	21.5	19.0	5.5	10.4	1.5
	10.2	19	19	18	10.6	6.5	3
	6.8	15.3	16.5	15.5	6.7	3.5	3.8
	9.6	17.5	16.5	16.6	14	-2.5	1.6
	12	19.5	16	19.5	16.6	-4	-2
Sorbose	6.5	6	8.5	15.5	5	3.5	2.5
	5.8	9	8.6	14	11.5	2.5	0.9
	6.5	9	9.5	10.5	9.6	-1.0	1.6
	7.5	5.7	11	14.5	9	1.5	1.5
	4	4.8	9.6	15.5	10	3	-2
	5.6	6	8.5	10.5	8.5	1.5	1.0
Galactose	8	12.7	20	31.5	19.5	5.5	1.5
	9.5	11	19.5	28	18.5	9.6	2.6
	6.6	9	20.5	25.4	20.8	-4.5	1
	9	12.6	16.6	29.5	19	6	-2
	9	11.5	19.5	28.5	19.7	5.1	3.5
	10.7	10.5	19	28	19.5	4	1.5
Maltose	9.3	11.5	17.7	21.5	18.5	8	6.5
	10	9.1	15.6	19.5	16.6	10.7	5.5
	10.6	11.5	14.5	15.7	15	9	6.6
	8.5	10	15	16	16.5	8	-2.5
	9.5	11	14	20.4	17	9.8	5
	9.0	8.0	11	21	15.8	8	5.5
Sucrose	8.6	14	25.5	24	23.5	8	9.5
	9.7	12	23.5	20.5	20	10.5	-1.6
	10.8	11.2	26.4	23.5	23	6.4	6.5
	8.9	9.5	21.6	22.7	21.5	8.5	5.8
	8	13.5	24.8	20.6	22.6	-1.6	7.3
	9	14.1	24	16.5	21.5	7.3	7.1

A P P E N D I X V (See Fig. 9)

	Milligrams of mycelium after days of growth						
Vitamin level	2	4	6	8	10	12	14
With vitamins	8	8.5	19	34.6	37	46	49
	7.8	9.2	20.3	35	40.1	47.2	51
	8.3	8.2	17.8	34	38.1	48	49
	8.4	8.9	18.3	32.7	36	47	46
	6.0	8.3	18.7	35	37	45	46.2
	8.7	9.0	21.2	34	38.1	47	46
Without vitamins	6.5	12	22.5	37	40	44.4	45
	7.7	11.2	18.7	36.9	38.1	46	43
	6.9	9.8	21.6	32.4	37.8	47.1	44.2
	7.8	10.3	22.8	35.6	37	44.3	46.1
	8.1	14.0	22.0	37.8	38.9	44.0	47.1
	6.9	12.2	20.8	35	36.3	39.2	44

A P P E N D I X VI

Diameter of culture (mm) after days of incubation

Temperature of incubation	2	4	6	8	10	12	14	16
20°C	-	3	5	10	12	15	16.5	18
		2	4	9	13	14	15.5	17
		3	5	9	12	13	15	17
		3	5	8	12	14	16	18
		2.5	5	9	12	14	16	17
		3	4	9	13	14	16	17
25°C	2	8	17	36	42	48	49	50
	2	7	16	36	41	48	49.5	50
	2	7	18	34	41	47	50	51
	2	7	17	36	42	48	50	51
	1	8	18	36	41	47	50	51
	2	7	18	36	42	48	49	50
28°C	2	13	32	54	68	78	79	79
	2	14	32	54	67	78	79	80
	3	14	32	54	68	78	80	80
	2	13	32	54	67	77	79	80
	2	14	33	55	67	76	78	79
	2	14	32	54	68	78	79	80
35°C	2.0	16	35	66	78	80	80	80
	2	17	35	64	76	80	80	80
	2.5	17	34	64	78	80	80	80
	3	18	36	65	78	80	80	80
	2	16	35	65	78	80	80	80
	2	16	35	66	78	80	80	80