"STUDIES ON *ALTERNARIA CRASSA* (SACC.)RANDS AS A POSSIBLE MYCOHERBICIDE ON *DATURA STRAMONIUM* L."

BY

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Dedicated to my parents;

Mr. and Mrs. Jasper Evan Okoth

DECLARATION

I hereby declare that the contents of this Thesis are my original work and have not been presented for a degree in any other University.

SHEILA OKOTH

Date 3/11/89

This Thesis has been submitted for examination with our approval as University supervisors.

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ABSTRACT

The use of *Alternaria crassa* (Sacc.) Rands as a control agent of *Datura stramonium* L. has been studied in this work. *A. crassa* was isolated from infected seeds and leaves of *D. stramonium* asceptically on water agar (WA) at 25ºC. Cultural studies were done to determine the best media, optimum temperature, light and pH conditions necessary for maximum growth and sporulation of the fungus. The fungus grew fast and sporulated best at 29ºC, alternating light (8hr)/darkness and at a pH of 6.8. Media did not affect the rate of growth of the fungus, except host leaf decoction agar (HLDA), but influenced its sporulation. Four different media were tested and the highest sporulation was obtained on HLDA followed by potato dextrose agar (PDA), cornmeal agar (CMA) and vegetable soup agar (VSA). For mass production of inoculum (spores and mycelial fragments) liquid medium was found to be more appropriate since large amounts of spores and mycelium could be harvested. Also more than one harvest could be obtained.

Serial microtome sections of the inoculated leaves showed that the germinating conidia penetrated the leaf

in three ways: (i) by forming an appressorium followed by an infection peg which punctured the epidermal cells (ii) by forming an infection peg which penetrated the leaf between the guard cell and the epidermal cell (iii) through the open stomata. Development of the fungus in the host tissue was the same irrespective of the mode of penetration. The fungus ramified through the host tissue both inter- and intracellularly, destroying the host cells and forming necrotic spots within seven days of inoculation. Spore concentration, plant age and incubation period at 100% R.H affected infection. Thirty-three day old plants inoculated with an inoculum concentration of 10,000 conidia/ml and kept at 100% R.H for three days, had the highest infection. In the field, however, it was necessary to inoculate twice in order to avoid escapes. Within 21 days, 75.6% of the plants had died. Among the control only 0.8% died. The fungus killed the target plant but did not spread to the other crops in the garden.

The ease in sporulation of *A. crassa* within four days under inexpensive conditions, the ability of the fungus to penetrate *D. stramonium* leaves and form necrotic spots within seven days (the spots coalesced to form large necrotic areas within nine days leading to premature defoliation), the infection of *D. stramonium* leaves at any age by the fungus and the host specificity of the fungus indicated the high potentiality of *A. crassa* as a mycoherbicide on *D. stramonium*.

CHAPTER 1

INTRODUCTION AND OBJECTIVES OF THE STUDY

1:1 Introduction

The use of plant pathogens for biological control of weeds is a comparatively recent development in pest management. Only in the past three decades has serious attention been given to weed control by the use of plant pathogens such as fungi, bacteria, nematodes and viruses (Templeton,*et al* 1986; 1988, Templeton, 1987).

Walker (1982) mentioned that some 300 plants out of the 30,000 which are considered to be weeds, cause heavy losses in cultivated crops throughout the world. Seventy percent of the world's worst weeds are present in Kenya.

Datura stramonium (thornapple), a broad leaf weed common throughout the country , belongs to the family Solanaceae. It has large white, lobed, trumpet-shaped flowers , 63.5 to 76.2 mm wide, and green, oval fruits , nearly 50.8mm long, beset with spines. The seeds are kidney shaped and flattened, about 3-4 mm in their longest dimension and about 1mm thick. They are dark brown in colour and have uneven surface. One fruit may contain between 400 and 800 seeds and the number of capsules (fruits) produced by a plant ranges from 3 to 30 - a characteristic which ensures the spread of the plant. The seeds which have an average weight of 0.0056g are able to retain their viability under unfavourable conditions for long periods . The plant is reported to have been of high repute as a cough remedy in London in the 18th Century (Salisbury 1961).

The weed is an annual plant of worldwide distribution in the warmer countries. It is common in most parts of East Africa from sea level to at least 8,000 ft. It would appear, however, to be less common than formerly, but frequently reappears unexpectedly, developing from dormant seeds (Okoth *et al*, in press).

D. stramonium is principally a weed of arable crops and waste land and in particular is often one of the worst weeds in maize. Apart from their competitive effect on crops, all species are very often poisonous. Both the seeds and the leaves are poisonous and could be fatal to humans and livestock.

The fungus *Alternaria crassa* (Sacc.) Rands is known to cause leaf and pod blight of *Datura* species, whose symptoms include irregular straw coloured, zonate spots which appear first on the lower, more shaded, leaves and later spreading gradually upwards; dark sunken lesions may be formed on the pods (Ellis 1971). Heavily infested leaves are shed. The seedborne pathogen has been recorded from Cuba, Cyprus, Ethiopia, Germany, Ghana, India, Italy, Kenya, Nepal, Nigeria, Pakistan, Rhodesia, Romania, Spain, Sudan, Switzerland, Tanzania, Uganda, U.S.A. , Zambia (Halfon-Meiri, 1973).

1:2 Objectives of the study

The major objectives of the study were;

1. To isolate and culture the fungus *Alternaria crassa* from *Datura stramonium*
2. To determine the most favourable conditions for the growth and sporulation of *A. crassa*
3. To determine the effect of temperature, light and pH on the growth and sporulation of *A. crassa.*
4. To determine possibility of using *A. crassa* as a biocontrol agent on *D. stramonium.*

CHAPTER 2

REVIEW OF LITERATURE

2:1 Taxonomy

The form-genus *Alternaria* is of the Form-class Deuteromycetes : Form-order Moniliales and Form- family Dematiaceae. Members of the genus *Alternaria* produce dictyosporous conidia which are rather large and multicellular having both transverse and longitudinal septa. The conidia are formed in a blastic fashion and are usually borne acropetally in chains (catenulate) but may also occur singly at the tips of conidiophores that are virtually indistinguishable from the somatic hyphae (Alexopoulos and Mims, 1979).

In culture, *A. crassa* forms effuse, grey colonies from which conidiophores arise singly or in small groups as erect or ascending, straight or flexuous, sometimes geniculate, septate, pale or mid pale brown, up to 90um long, 7-10um thick with one or several scars. Conidia are usually solitary, occasionally in very short chains, obclavate, rostrate with the beak generally exceeding the length of the spore. Conidia are pale brown, smooth and their overall length is 120-440 (120)um. The body of the conidium is upto 140um long, with 7-10 transverse and usually several longitudinal septa.The thickness of the broadest part of spore ranges between 15-40 (22)um. The beak is pale in colour, septate, unbranched , 4-8um thick at the base, tapering to 2-2.5u (Ellis,1971).

2:2 Cultural studies

Studies on several *Alternaria* species have been done extensively but little has been done on *A. crassa* L. Considerable differences have been shown in the rates of growth and sporulation of *A. brassicicola* (Schw.) Wilt., *A. brassicae* (Berk.)Sacc., and *A. raphani* Groves and Skolko, when grown on different media. *A. raphani* and *A. brasssicae* do not sporulate as well as *A. brassicicola* on potato dextrose agar (PDA). On malt extract and standard nutrient agars, sporulation occurs in *A. brassicae* while in *A. raphani* only extreme mycelium development occurs. The cardinal temperature reported are 8, 24-28 and 30 for each fungus. *A. raphani* grows over a slightly wider temperature range than the others. The essential pH ranges reported for the three fungi are 2.9, 6.5-7.1, and slightly higher than 9.1. Spore development was plentiful under combinations of intermittent light and darkness, but inhibited in continuous darkness. Host penetration by *A. brassicae* has been reported to be stomatal or direct by *A. brassicicola* on the cruciferae (Changsri, 1963).

2:3 Biological Weed Control Methods

Mycoherbicide is the term designated to characterize fungal plant pathogens that are applied as innundative inoculum for specific, post-emergence control of their weed hosts. Their development is a biological control method that compensates for poor natural dispersal of a pathogen, relies upon high level of specificity for the target host and utilizes strong necrotrophic ability at elevated inoculum levels.

The use of plant pathogens for weed control presupposes that disease may reduce plant population levels and that disease intensity may be managed by manipulating the inciting pathogen. Mycoherbicides are developed from pathogens that normally incite disease at endemic levels in specific weed populations (Conway ,1975). These diseases are usually constrained from epidemic development by innate deficiency of the pathogen to disseminate.

Mycoherbicides kill specific weeds as effectively as chemical herbicides. Applied innundatively, using the same technologies as for chemicals, they kill weeds within three to five weeks. After weed death, the pathogens return to background levels (Templeton 1985) through natural constraints. Consequently there is little or no residual weed control from season to season after mycoherbicide application, particularly if it is an aerial pathogen. Some carry-over may occur with mycoherbicides produced from soil-borne pathogens.

Only indigenous fungi have been developed as mycoherbicides thus far. In the U.S.A., several formulations of mycoherbicides have been supplied to farmers for the control of various weeds. These include fresh spore suspensions of the persimmon wilt fungus for the control of the woody perennial - *Dyrospyros virginiana* L. in rangelands (Templeton 1987); fresh chlamydospores suspension of *Phytophthora palmivora* (Butler) Butler as "Devine^R" to control the stranglervine - *Morrenia odorata* - in citrus groves; conidial suspension of the anthracnose fungus -Collego^R- to control northern jointvetch - *Aeschynomene virginica* L. in rice and soya bean fields. It is a superior substitute for and has in fact replaced a chemical herbicide used for the control of this specific weed.

The success of mycoherbicides has stimulated increased interest in research, commercialization, and regulation of biological control agents in the U.S.A. and elswhere especially at a time when the use of chemical pesticides has been questioned on environmental grounds such as : the development of resistance in pest population shifts, toxicity to non-target organisms, harmful toxic residues in the environment and contamination of ground water. The consequence of these problems is reflected in a general reduction in the number of new chemical pesticides and increased interest in the development of alternative pest control strategies including biological pesticide.

CHAPTER 3

MATERIALS AND METHODS

3.1 Isolation of pathogen from infected tissues

Alternaria crassa, a seedborne fungus, was isolated from diseased leaf lesions and from seeds of *D. stramonium*. The leaves were collected from a garden in chiromo campus, washed in running tap water for one hour, surface-sterilized for five minutes in 0.5% sodium hypochlorite solution, plated on water agar (WA) and incubated for 20 days at 25°C.

3.2 Pathogenecity test

D. stramonium seedlings (36 days old) were germinated after treatment with sulphuric acid as described by Okoth et al (in press). The seedlings were raised singly in 13cm (diam) pots. The fungus isolated was cultured and prepared for use and inoculation done on the 20 seedlings as described in 3.4. Ten seedlings were used for histological studies. Six leaves from inoculated seedlings were cut into small pieces (approx. 1 by 1 cm discs) at 3hr intervals for upto 72hrs, each time putting the discs in vials two thirds full of Farmers fluid (mixture of absolute ethanol and glacial acetic acid in the ratio of 2:1). This fixative also decolourised the discs. Half of the total number of discs in each vial were carefully laid on a wire gauze and then thoroughly washed in running water for 30 minutes. They were transferred to 150 ml vials and put through the following treatment, each time draining off the liquid to add in the next to avoid destruction of the discs: they were dehydrated in each of 50%, 70%, 90%, absolute alcohol A and absolute alcohol B for one day in each case. The alcohol was then washed out with the following series of xylene, each treatment lasting one day; 25%, 50%, 70%, pure xylol A and pure xylol B. The discs were then infiltrated with paraplast tissue embedding medium (wax) in the following series; mixture of xylol + wax (1:1) in an oven at 59ºC, just above the melting point of the pellets, for a day and pure wax changes twice with each change lasting 12 hours.

To avoid artifacts, due to contamination, during microscopic studies, and to ensure ribbon continuity with serial sectioning, fresh pellets (unused) were used. The pellets melted between 55-57ºC. The discs were then embedded in wax, keeping the blocks under water containing ice. The blocks were then trimmed and sectioned serially (15 um thick), using a microtome. The ribbon was then broken into short pieces, using a camel brush, and floated on lukewarm water to straighten. Slides, thinly smeared with diluted office glue, (diluted with water) were used to pick the ribbons from the waterbath, and left to dry on the slides in a slide dryer for two days. The slides were then dipped in pure xylene to remove the wax, and then dipped in cotton blue in lactophenol to stain for one minute. Excess stain was removed with 70% alcohol and the slides observed under the microscope.

The other half of the discs in each vial were mounted on slides with lactophenol and observed under the microscope.

The remaining ten seedlings were left in 100% R.H. for 72 hours, uncovered and left on the greenhouse bench. The plants were observed daily for disease development.

3.3 Cultural studies

A. crassa was grown on the following four different media: Potato-dextrose agar (PDA), Vegetable soup agar (V-SA), Host-leaf decoction agar (HLDA) and Cornmeal agar (CMA). These were prepared as shown in Table 1 (Smith and Onions, 1983).

Table 1: Formulae for Media

Corn-meal Agar

(a)	Maize flour	30 g
(b)	Agar	20 g
(c)	Distilled water	1 litre
(d)	Streptomycin	0.15 g

The flour and water was placed in a saucepan and then heated till boiling and stirred continuously for 1 hour. The decoction was filtered through muslin. Agar was added and heated till dissolved then autoclaved for 15 minutes at 121°C.

Vegetable-Soup Agar

(a)	Mixed vegetable soup	200 ml
(b)	Calcium carbonate	3 g
(c)	Agar	20 g
(d)	Distilled water	800 ml
(e)	Streptomycin	0.15 g

The soup was added to lukewarm water and $CaCO_3$ and left to stand for 4 hrs. The mixture was decanted leaving the precipitate that had settled at the bottom of the flask. Agar was added to the mixture. This was heated to melt the agar and then sterilised for 20 min. at 121°C.

Host-Leaf Decoction Agar

(a)	*D. stramonium* leaves	225 g

Table 1 Contd. . .

(b)	Agar	12 g
(c)	Distilled water	1 litre
(d)	Streptomycin	0.15 g

Clean fresh *D. stramonium* leaves were chopped and macerated in a Waring blender, placed in 700 ml of water and boiled for 60 min. 600 ml of the liquid was strained using 2 layers of cheese cloth and added to 400 ml of distilled water containing 12 g of agar. This was bottled and then autoclaved.

Potato Dextrose Agar

(a)	Potatoes	200 g
(b)	Agar	20 g
(c)	Distilled water	1 litre
(d)	Streptomycin	0.15 g
(e)	Dextrose	15 g

Potatoes were cleaned, thinly peeled and cut into 12 mm cubes. Two hundred grams of the potato cubes were rinsed with distilled water and put in a saucepan containing 1 l of distilled water to cook. They were boiled till soft, filtered through a sieve and as much pulp as possible squeezed through. Twenty grams agar was added and heated till dissolved. Fifteen grams of dextrose and 0.15 g streptomycin were added. The mixture was made up to 1 l and autoclaved.

All the four media were aseptically transferred into sterilized petri dishes.

3.3:1 Effect of culture media and medium concentration on growth of *A. crassa*

The four media named above were tested. The medium concentration were made by preparing four-fold the standard formula concentration, 4X, (Fig 1) of PDA, HLDA, V-SA and CMA and diluting with distilled water to provide the following concentration ratios: 4X, 3X, 1X, 1:1, 1:2 and 1:4. Agar concentration was constant at 20g/L in all media. Eight-mm (diam) agar plugs of 4-day -old mycelium grown on PDA were used to inoculate 9-cm Petri-dishes containing the various media. Each medium had replicates of five plates each. The cultures were incubated at 25°C under constant illumination provided by three white fluorescent lamps (Model TLD 36W/54 made in Holland [] E7 Co. Phillips) located 25cm above the plates. Extra plates were put at the border to nullify the edge-effect. Colony diameters were measured daily (for 7 days) and expressed as mm radial growth per day (Ionnaidis and Main, 1973). The observations were recorded in a table and a graph drawn (Page 46, 47).

Effect of culture media and medium concentration on sporulation of *A. crassa*

Inoculum preparation and seeding was done as described above. The plates were incubated at 25°C under constant illumination provided by fluorescent lamps.By the third day, sporulation was apparent and on the fourth day, conidia were harvested from the colonies by cutting 10 plugs (8mm diam.) per plate (per replicate) and putting them in vials containig 4ml. of water. Thus each treatment had 5 vials containing 10 plugs each. Conidia were dislodged by gently srcaping-off the surface of the plugs with a bent glass rod, the vials shaken in a waterbath with a shaking device (Gallenkamp Sciex (E.A) Ltd. NRB. Kenya), for 1min. A sample of this suspension was transferred to an Improved Neubaer Haemocytometer-Spencer Bright-line, and spores counted at 400 times magnification under a microscope. The total number of of spores obtained was calculated according to the following formula (Absher, 1973);

$$Y \times 10^4 \times \text{Dilution Factor} = \text{No. of cells/ml of original suspension}$$

where, Y = No. of cell count per Y number of squares

10^4= Constant encompasing the volume and depth of the counting chamber.

Data were computed and expressed as mean number of conidia per ml$\times 10^4$ and tested for significance by analysis of variance. For microscopic studies, semi-permanent mounts were prepared in 50% glycerine:water. A Leitz Orthoplan microscope was used. Conidia characteristics, for example, length (including the beak), width, number of septa per spore were recorded and lengh/width ratios computed. Measurements of conidial size was done using a calibrated microscope.

3.3:2 Effect of incubation temperature, light and pH on growth of *A. crassa*

Inoculum preparation and seeding was done as already described above. Five incubation temperatures were used; 9,20,25,29,35 °C. Light regime treatment used were; continuous light, continuous darkness and alternating light(8hr.) /darkness. For pH studies, following pH values were used; 2.0, 3.1, 5.0, 6.8, 9 and 11. The PDA was buffered and adjusted with 1.0N HCL, and 1.0N NaOH. The average initial pH values of the samples were as indicated above. The reading of the pH was done using a pH-meter. Average final pH values after 7 days were 2.2,3.4,5.2,6.5,6.9, and 11 respectively. This was done by melting agar from two plates in each series and taking readings using the pH-meter. Colony diameter was measured daily for several days and expressed as mm radial growth per day. The data was tabulated and represented in graph form (Page 56-65).

Effect of incubation temperature,light and pH on conidial production of *A. crassa*

The different temperatures, light regime and pHs indicated in 3.4(c) were used. The method indicated in

3.4 (b) was applied. The data obtained was analysed using the analysis of variance (Parker, 1983).

3.4 Greenhouse Test

Inoculum preparation

The inoculum was prepared by cutting entire cultures from Petri plates with a sterile needle into approximately 4cm strips and placing into sterile 500ml flasks each containing 75ml of sterile distilled water. The flasks were shaken vigorously for approximately 1 min. and left to stand for 10 min. One-and one half ml of the liquid from the flasks was poured onto each of the fresh plates of HLDA and adjusted to pH 6.8. Each plate was rotated so that the added liquid completely covered the surface of the agar. The inoculated plates were incubated at 29°C with a constant light source provided by three 36-W white fluorescent tubes that were located 25cm above the cultures. After 6 days there was abundant sporulation over the entire surface of the agar. These mature spores were harvested by dislodging them with a sterile spatula and rinsing with a jet of distilled water. The cultures (harvested) were then mercerated in a Waring Blender for 30sec. Enough water was added so that the inoculum would pass readily through the atomizer.

Inoculation technique

Datura stramonium seeds were harvested from plants growing in chiromo campus fields. The seeds, known to maintain dormancy for many years in the soil (Salisbury, 1961) , were treated and induced to germinate as described by Okoth,*et al* (in press). Potted plants were each covered with a plastic bag supported with a stake at the center of the pot and fastened at the bottom with cellotape. A hole, small enough to allow only the tip of the atomizer, was made at the left upper edge of each of the bags with a pair of sterile scissors. The plants were then inoculated through this hole. Additional inoculum was atomised into the bags to obtain 100% Relative Humidity (R.H.). The holes were then sealed with cellotape and plants left on greenhouse bench at 23-25°C (Conway, 1976). Duration of the saturated atmosphere following inoculation was three days unless otherwise specified (Sciumbato & Pinkard, 1974). Control plants were atomised with sterile water only. After this exposure to high humidity for three days, the polythene bags were removed and the plants placed on the greenhouse bench.Plants were observed daily for symptoms and disease development.

Severity of leaf blight was recorded on a scale of 0 to 5 adopted from Abawi, *et al* (1977). A rating 0 referred to leaves with no symptoms, 1 indicated the prescence of only a few lesions per leaf. A score of 2,3,4 or 5 indicated that up to 10,11-30, 31-49, 50-69 or over 70% of the leaf surface was covered with lesions, respectively; no symptoms, a few scattered lesions, slight to moderate infection, moderate infection, severe infection, very severe infection respectively. The exact surface area of the lesions was calculated by placing a detached leaf under a transparency with grid drawn on it. The squares making up the grid were 1 cm^2 each. The number of whole squares covering the surface of the leaf gave the surface area of the leaf while the number of whole squares covering the lesions gave the surface area of the lesions. The percentage area covered by the lesions was calculated using the following equation:

Surface area of lesion/surface area of leaf X 100%

3.4:1 Effect of inoculum concentration on symptom development.

Thirty plates containing mature conidia were flooded with distilled water, the conidia dislodged using a sterile spatula. Spore counts were done using a haemocytometer, and dilutions made as necessary using distilled water. The following spore concentrations were obtained; 1,000, 10,000 and 50,000 conidia per ml. These were tested on 3 groups of seedlings at the 4-5 leaf stage. Each treatment had 10 replicates (pots) while each pot contained 4 plants. Control plants of equal number were treated with same amount of distilled water. The average number of lesions per replicate were computed and the results entered in a table (Page 83).

3.4:2 Effect of age of plant on symptom development.

About 14, 24, 33, 45, 56, 64, 71 and 74 day old plants with an average height of 4.2, 4.8, 5.9, 6.2, 6.9, 7.2, 10.2, 11.5 cm respectively and 2, 4, 6, 6, 8, 8, leaves respectively were used for this test. Each treatment had four (pots) replicates for inoculation and four replicates for control. Each pot contained two plants. The plants were observed daily for symptom development.

3.4:3 Effect of incubation period at 100% R.H

Four to five leaved seedlings were covered with polythene bags and then inoculated with a spore suspension containing 10,000 conidia per ml. Enough inoculum was atomized to provide a 100% R.H within the polythene bags. At intervals of 1-10 days, two pots each containing 6 seedlings had their polythene bags removed, and the pots placed on greenhouse bench. The number of lesions were counted on the twelfth day after inoculation and the results tabulated (Page 84).

3.5 Field test

The 8 by 8m field was divided into two blocks, A and B, each of 7 by 3.2m with 0.5m walkway. Seeds treated as indicated in 3.4 were planted in fifty different holes (replicates) in Block A and another fifty different holes in Block B. The 36 day old seedlings were thinned leaving five seedlings per replicate. The total number of seedlings per block was therefore 250. Along the walkway, maize seeds were sown to exclude the border effect and also to separate the two blocks.

3.5:1 Inoculum was prepared from host decoction liquid medium. This medium was prepared as indicated in Table 1 but this time agar was not added. Clean, fresh *D. stramonium* leaves were chopped and mercerated in a Waring Blender, placed in 700ml of water and boiled for 60 minutes. Approximately 600ml of the liquid was strained using 2 layers of cheese cloth, added to 400ml of distilled water and adjusted to a pH of 6.8. This liquid was poured into forty 250ml flasks. Each flask contained 25ml of the liquid. Another set of forty flasks was prepared in this manner, sealed with aluminium foil then autoclaved for 15 minutes at 121°C.

The sterile flasks were removed from the autoclave and left to cool. Entire cultures from Petri plates were cut with a sterile transfer needle into approximately 4 cm strips and asceptically placed into the flasks. The flasks were maintained at 29ºC and continuous fluorescent light. After 12 days of growth and sporulation, the fungus was harvested and inoculum prepared as already described. The same flasks were maintained at the same temperature conditions for more growth of the fungus. The liquid medium allowed for more than one harvest.

Distilled water was added to the inoculum obtained from the flasks to give a concentration of 10,000 conidia/ml This was used to inoculate plants in Block A as described in 3.4. Plants in Block B were atomized with an equal amount of distilled water. The inoculation was done in the evening to utilise the cooler night temperatures and possible high relative humidity. The results were collected by scoring randomly to get one seedling from each hole for study. The five seedlings within a hole were tagged 1, 2, 3,4,5. Casts were thrown and picked randomly to get one seedling for study. The data was entered in a table (Page 90).

The same set-up as above was repeated but this time inoculation was done twice. The second inoculation was done two days after the first one. Results were collected and analysed as indicated in 3.5 (Page 91).

3.5:2 The same set-up as in 3.5:1 was repeated, but this time the inoculated seedlings were not covered with polythene bags. Inoculation was done two times.

CHAPTER 4

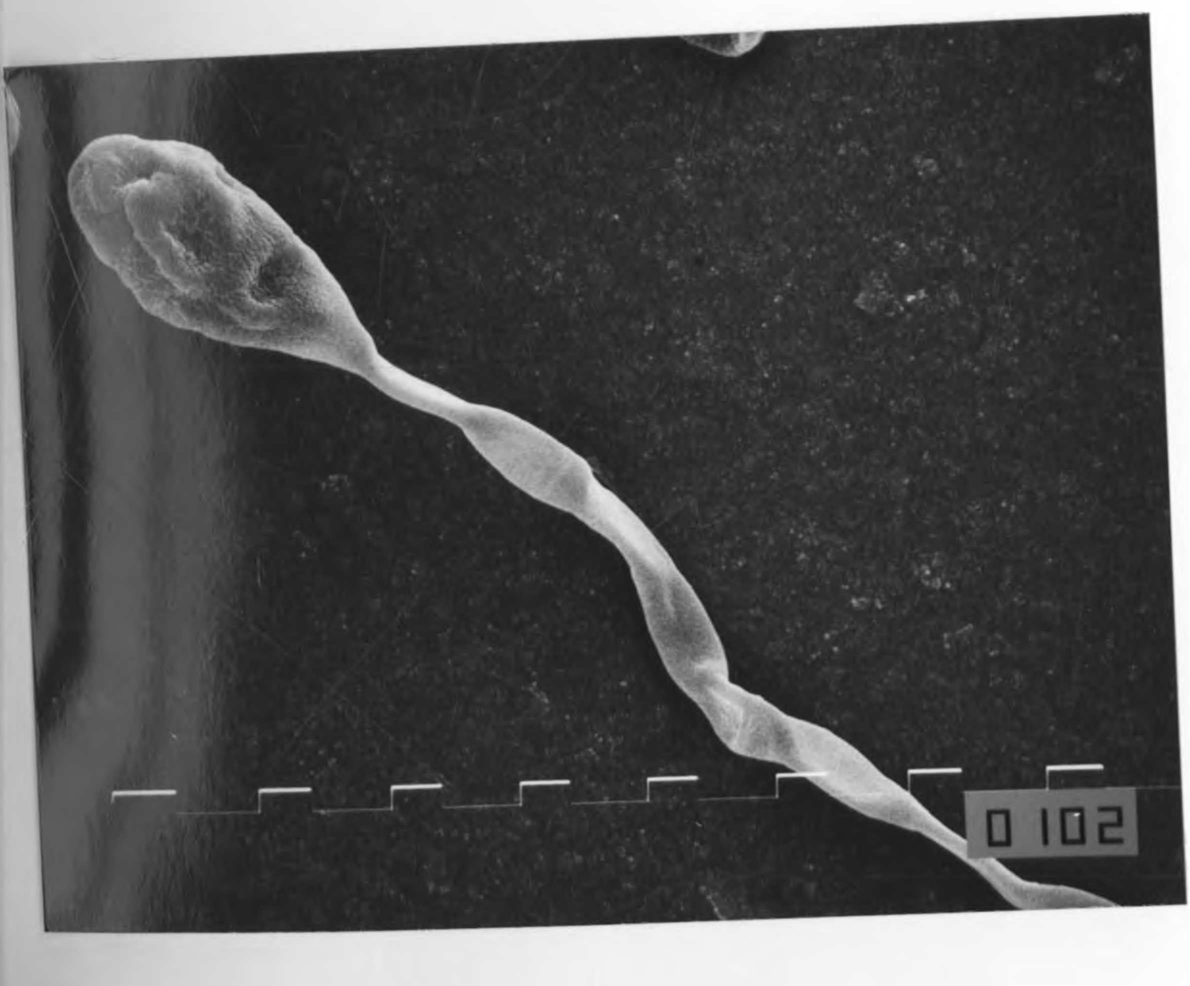
RESULTS

4.1 Isolation and identification of the pathogen

Various non-pathogenic fungal colonies identified as *Sordaria*, *Pestalotia*, *Rhizopus*, *Fusarium*, and *Aspergillus* were observed growing on plates containing diseased leaf lesion discs within 24 hours of incubation. Only one type of colony grew from seeds after 15 days. This was identified as *A. crassa* whose spores (110x12μm) had 8 transverse and 6 longitudinal septa as observed under the light microscope. The conidia produced in small chains were pale brown, smooth and pedicillate. The pedicels greately exceeded conidial lengths, Rands (1917), Ellis (1971), Plate 1a, 2a. What appeared as septa under the light microscope were seen to be depressions under the scanning electron microscope (Plate 1b and 2b). The conidia had 6-12 depressions arranged longitudinally increasing in size towards the tip. The pedicels had alternating constricted and non-consticted regions and not septa as described by Saccardo (1917). The fungus was found to be seedborne and was more easily isolated from the seeds than from the leaf lesions.

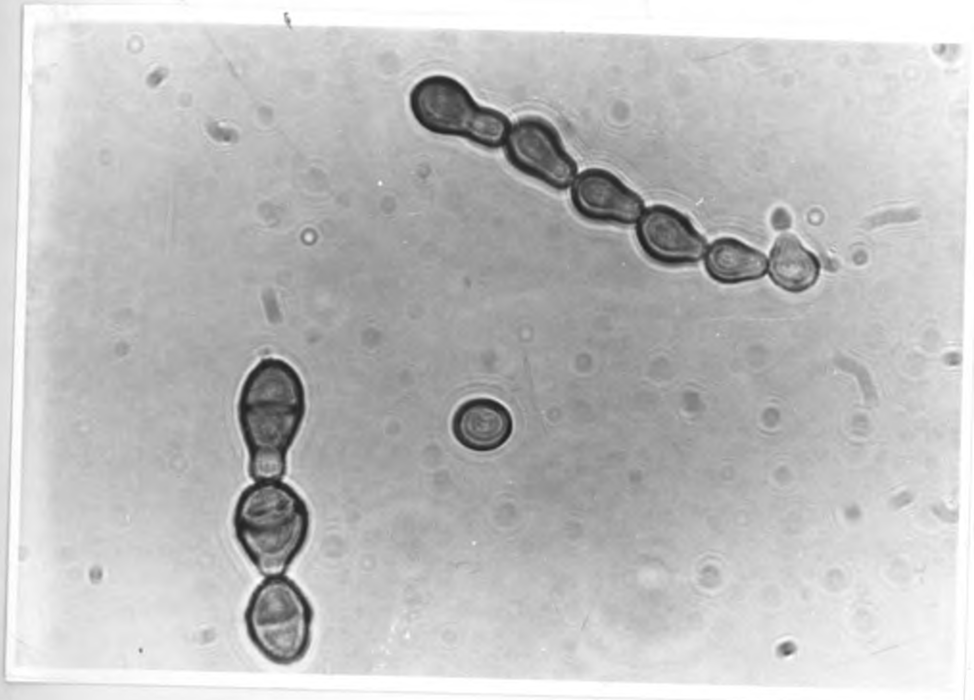
PLATE 1. Conidia of *A. crassa* (a) observed under light microscope. Note the septate pedicels. Mgx400 (b) observed under scanning electron microscope (Rod gold-coated for 10 minutes at 1 Kv and 10mA) Mgx1,700.

(a)

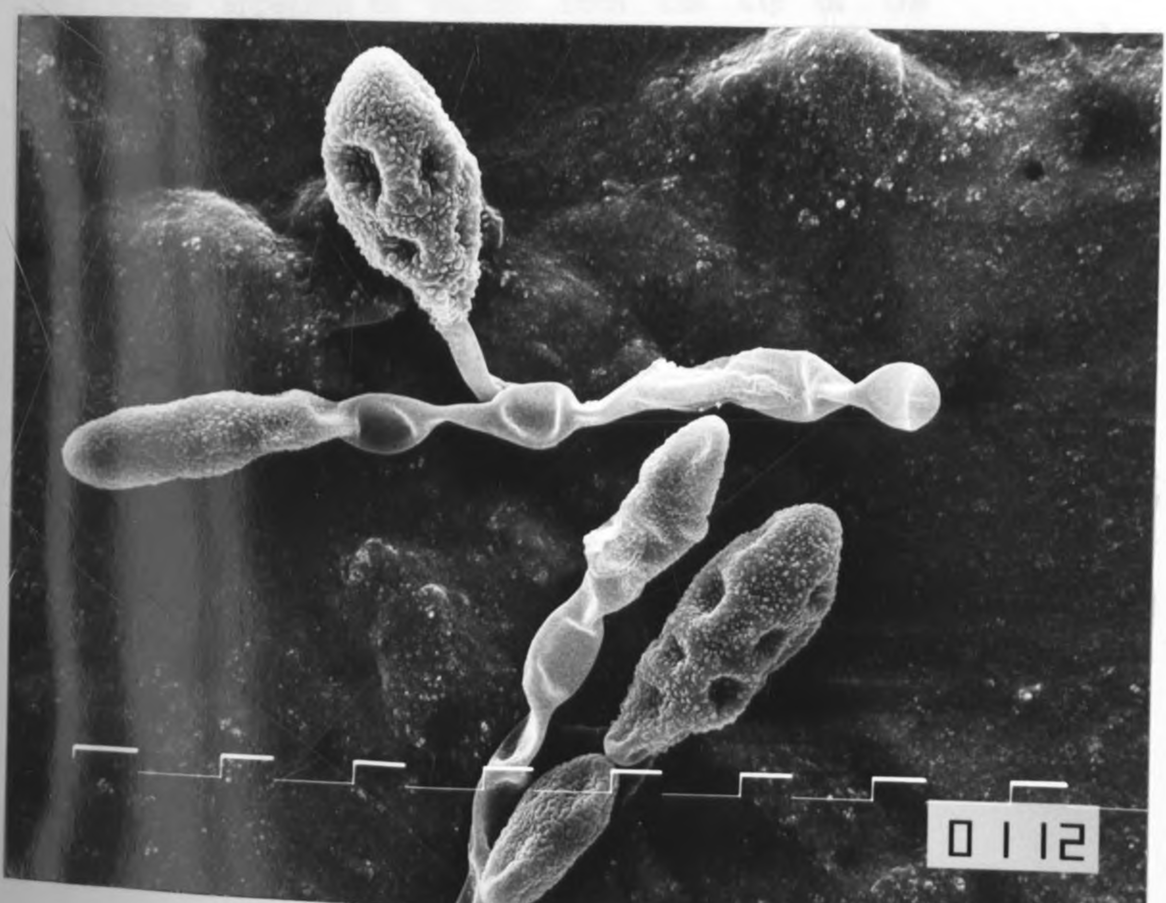


(b)

PLATE 2. Catenuate conidia of *A. crassa* (a) under light microscope, Mgx1000 (b) under scanning electron microscope (Rod gold-coated for 10 minutes at 1 Kv and 10mA) Mgx1,700.



(a)



(b)

4.2 Penetration, colonization of host tissues and symptom development in the infection process of *D. stramonium* by *A. crassa*

Four phases were distinguished in the infection process of *D. stramonium* by *A. crassa*, viz. germination, germ tube entry, inter-, and intracellular penetration followed by necrotic symptoms.

After inoculation no germ tube was observed after 6 hours. By the ninth hour, very few conidia were seen to have produced germ tubes, and by the twelfth, most of the conidia had germinated on the leaf surface whose germ tubes emerged from 1 or more cells. The germ tubes at times appeared to emerge from the tip of the pedicel (Plate 3a, b).

The germ tubes penetrated the leaves either through the stoma, directly by forming an appresorium and then puncturing host epidermal cells or through an infection peg without an appresorium. Evidence of stomatal penetration was most readily observed in cleared leaves stained with cotton blue in lactophenol. The germ tubes stained deeply compared to the conidia. Some germ tubes passed over stomata without penetrating , as was also described by Solel and Minz (1971), Plate 3a, b). The germ tubes were observed to be going for darkly stained stomata rather than the lightly stained ones. The reason for this was not immediately known.

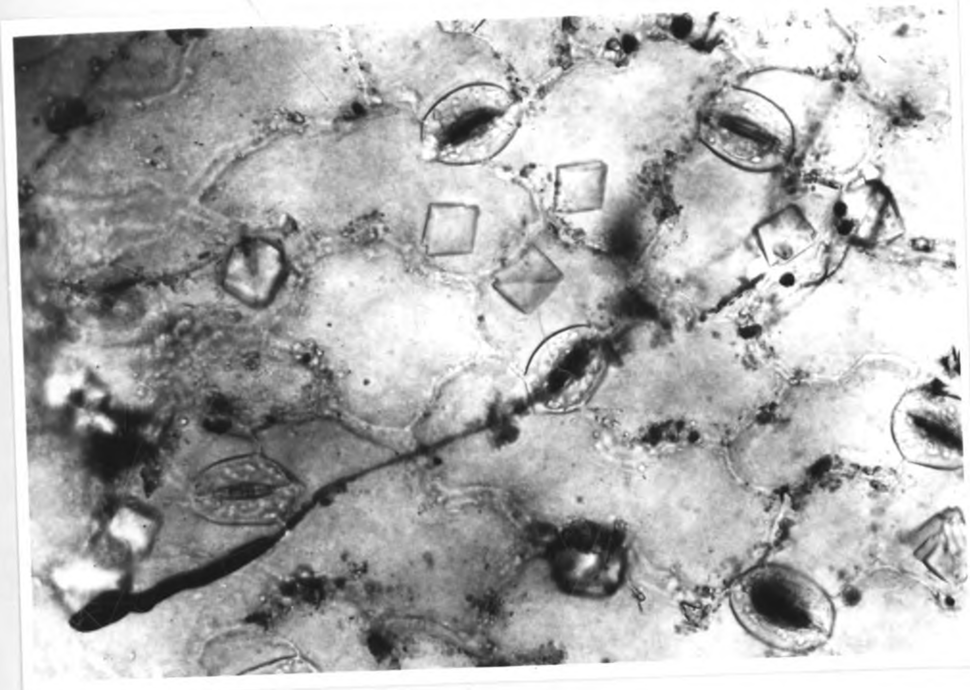
At times conidia only produced a typical infection peg which penetrated between epidermal cells, (Plate 3c). This infection peg is similar to the one formed by *Botrytis cinerea* as described by Mckeen, (1974). The infection peg caused an indentation of the epidermal wall during penetration unlike the one observed by Mckeen (loc. cit.)

Occassionally evidence of direct penetration of the upper surface of *D. stramonium* was observed in material fixed 12 and 48 hours after inoculation. A swelling of germ tube tips appressed to the leaf surface at the juncture of the epidermal cell walls and the outer guard cell was readily observed in cleared leaves fixed and stained 12 hours after inoculation (Plate 3d i). This result agrees with the finding of Diener (1955), from his studies of *Stemphylium solani* Weber on tomato leaves fixed 48 hours after inoculatiuon, which showed that the infection peg developed on the under side of the swelling, pierced an epidermal cell and rammified within the plant tissues (Plate 3d ii).

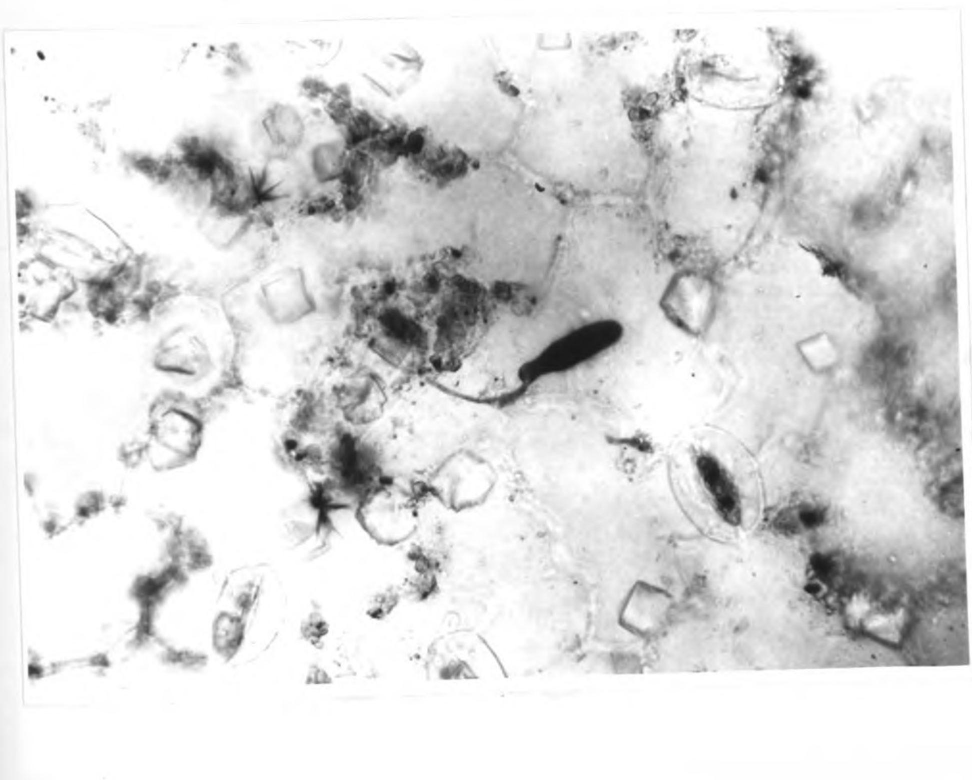
After penetration, germ tubes continued growing in the susceptible host in a similar manner following either stomatal or direct penetration. The infection hyphae branched repeatedly and ramified through the tissue

surrounding the host tissue and became intercellular. The hyphae elongated in a radial manner, proceeding intercellularly and intracellularly confirming the results of Jackson Curis R. (1959) on *A. cucumerina*. Seventy two hours after inoculation, the hyphae eventually destroyed cell contents and many cell walls, leaving small cavities and an irregular network of cell wall fragments.
Invasion of spongy parenchyma resulted in destruction and collapse of this tissue. Chloroplasts were observed irregularly scattered in the spongy mesophyll due to the destruction of the walls of the cells. Necrotic spots appeared within seven days after inoculation. These lesions enlarged due to intercellular growth of hyphae and subsequent destruction of all leaf tissues. By the ninth day, the fungus had sporulated on the surface of the lesions which often coalesced to form large necrotic areas, followed by premature defoliation.

The lesions were similar to those on leaves of *D. stramonium* growing wild in the field. These lesions were cut, surface sterilised, and plated on WA and incubated at 25°C. The fungus, A. crassa, was reisolated thus satisfying Koch s postulates for a pathogen; i.e to inoculate host plant in the greenhouse, obtain the disease symptoms originally observed in the field, and then recover the same fungus from the diseased tissue (Charudattan and Walker,1982).



(a)



(b

PLATE 3. Penetration and infection process of *A. crassa* on *D. stramonium* leaves (a) (b) Stomatal penetration from cleared leaf mounts, Mgx400. Conidia penetrating stoma 12 hours after inoculation. (c) Direct penetration from serial microtome sections, 15um, Mgx400. Conidium producing an infection peg, 48 hours after inoculation, which penetrates between two epidermal cells. (d) Direct penetration with appresorium formation 48 hours after inoculation (i) from cleared leaf mounts Mgx400 :

1 - stoma 2 - guard cell

3 - appresorium 4 - conidia

5 - epidermal cell

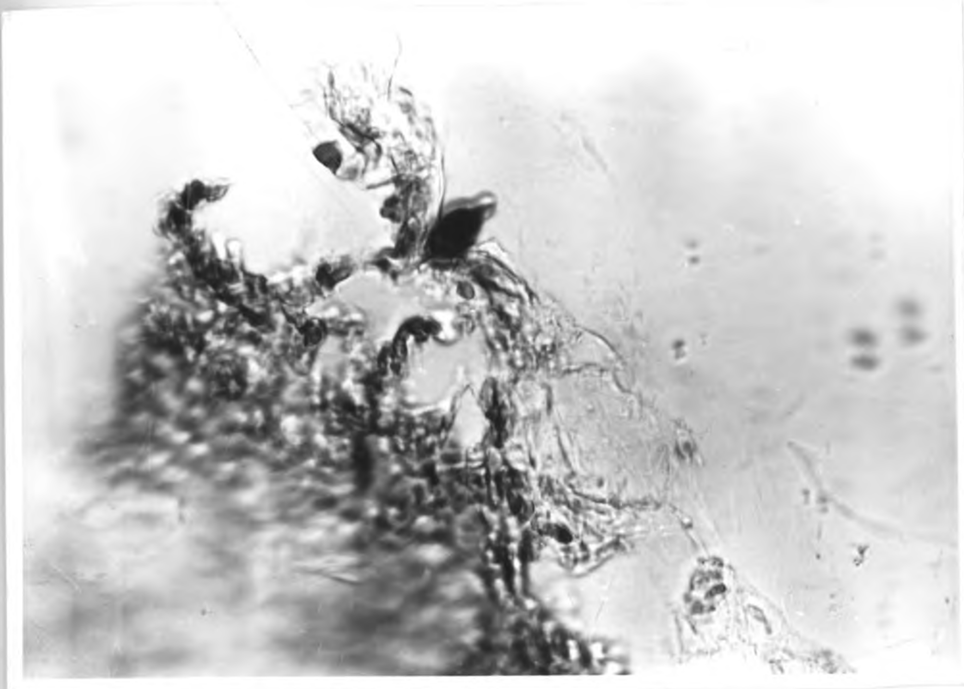
(ii) from serial microtome sections Mgx400 :

1 - appresorium 2 - infection hypha

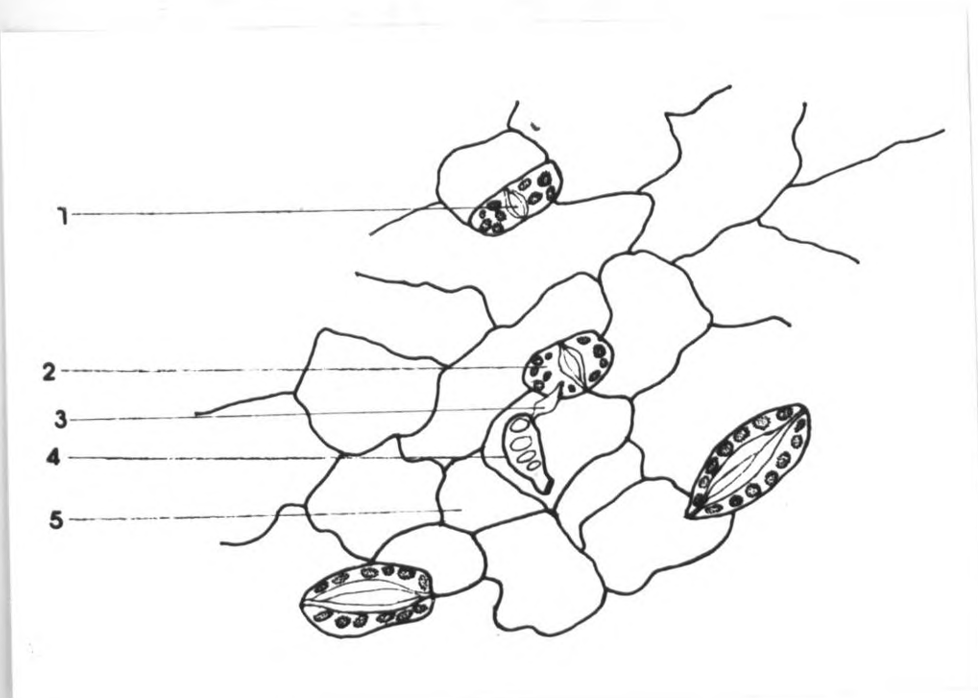
3 - epidermal cell 4 - palisade cell

5 - chloroplast 6 - intercellular hypha

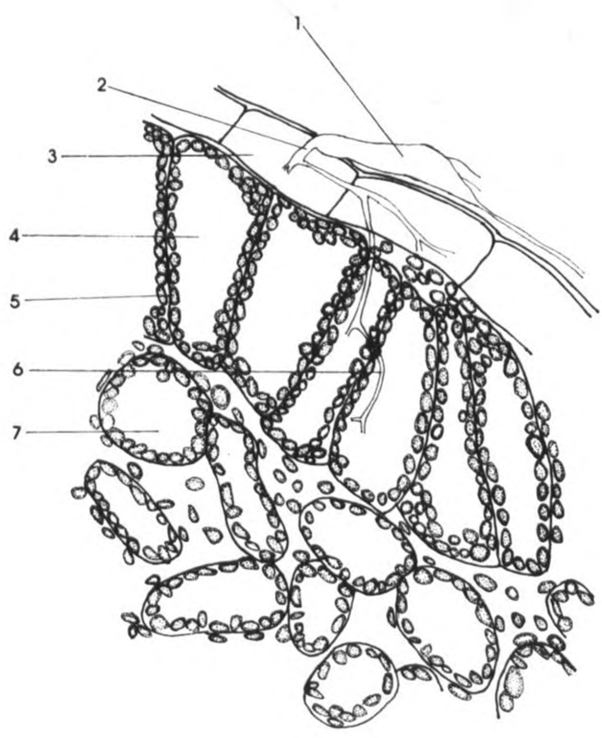
7 - spongy cell



(c)



(d)(i)



(d) (ii)

4.3 Cultural studies

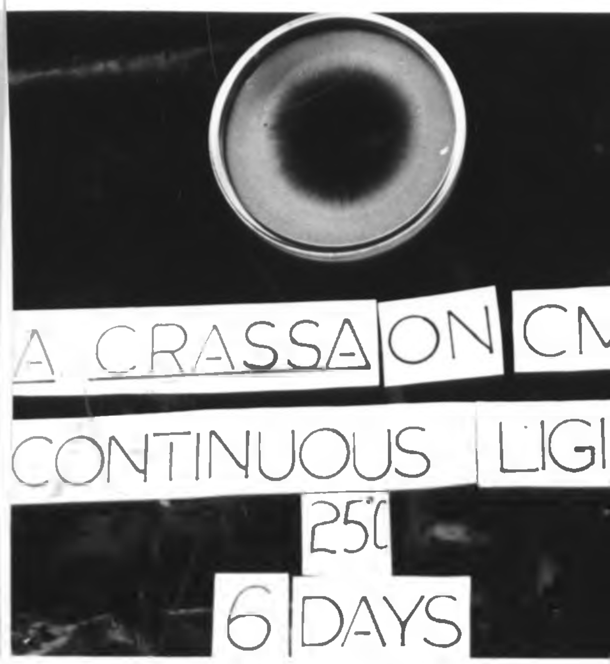
4.3:1 Effect of medium on growth and sporulation of *A. crassa*

It was observed that neither media nor medium concentration significantly affected radial growth rate of the fungus. Mycelial density increased visibly with increasing concentration for all media (Table 2, Fig.1, Plate 4). Conidial production was greatest on HLDA followed by PDA, CMA and V-SA respectively (Table 3). The difference in conidial counts was highly significant at one percent level of probability with an F-value of 11,814.208. A decraese in number of conidia occurred at either end of the concentration ranges tested for all media except V-SA indicating optimum nutritional condition for sporulation existed at intermediate concentrations (Table 5, Fig. 2). Conidial production was significantly different among individual media and media concentratrion. Conidial length was greatest on HLDA and least on V-SA whereas width was greatest on V-SA, and least on HLDA. The number of septa also varied with medium. The greatest number of septa was found on V-SA (Table 4, Plate 5).

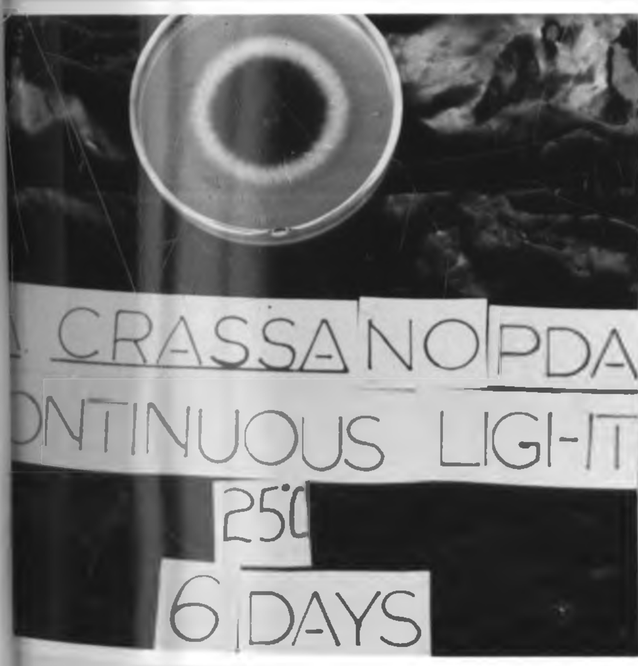
PLATE 4. *A. crassa* growing on (a) HLDA (b) CMA (c) PDA (d) V-SA, at 25°C.


A CRASSA ON
HOST DEC. MED.
CONTINUOUS LIGHT
25°C
6 DAYS

(a)


A CRASSA ON
CONTINUOUS
25°C
6 DAYS

(b)


CRASSA NO PDA
LIGHT
25°C
6 DAYS

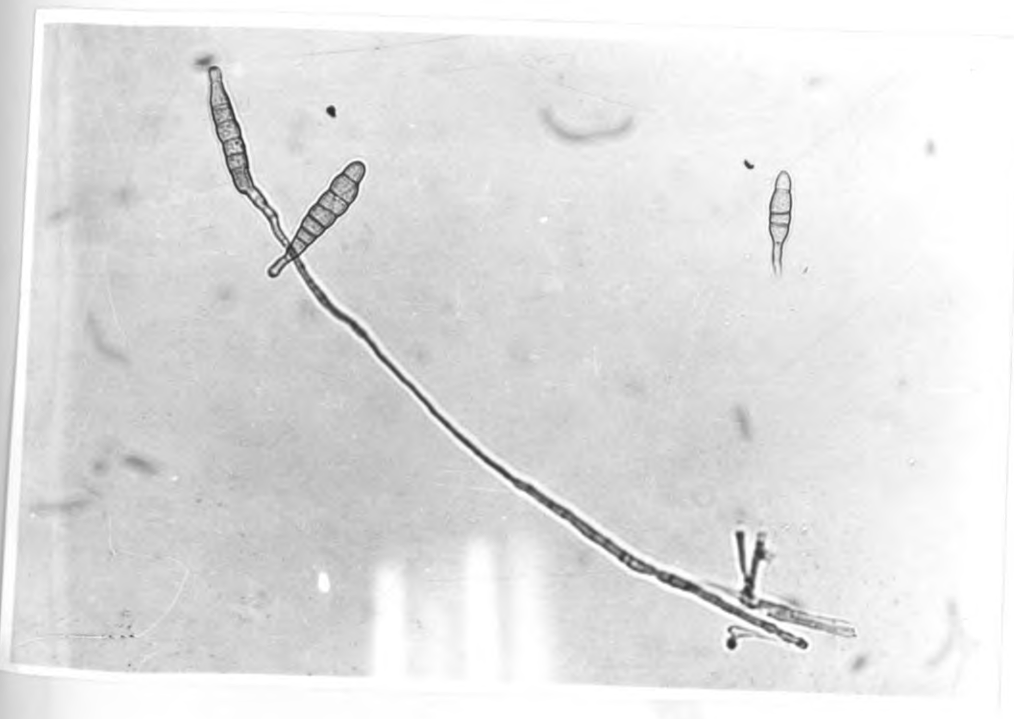
(c)


A CRASSA ON
CONTINUOUS
25°C
6 DAYS

(d)

PLATE 5. Effect of medium on conidial morphology of *A. crassa* Conidia from (a) CMA Mgx1,000 (b) HLDA Mgx250 (c) V-SA Mgx400 (d) PDA Mgx400

(a)



(b)

(c)

(d

Table 2: Effect of medium on growth of *A. crassa*

Temperature 25°C
Alternating Light (8 hr)/darkness
Radial growth measures in mm

Medium	Replicates	Day of inoculation	2	3	4	5	6	7	Mycelial
HLDA	1	0	3	14	19.5	28	35	44	xxx
	2	0	3	14	20	29	32	46	
	3	0	3	14	21	30	38	45	
	4	0	3	14	21	30.5	38.5	45	
	5	0	3	14	21	28.5	36.5	44	
	Σ	0	15	70	102.5	146	180	224	
	$\bar{X}$	0	3	14	20.5	29.2	36.	44.8	
CMA	1	0	6	17	27	40	50	57	xxx
	2	0	7	17.5	27	40	50	56	
	3	0	6	16	26	39.5	50	56.5	
	4	0	6	16.5	26	39	49	54	
	5	0	7	17.5	27	41	51	57	
	Σ	0	32	84.5	133	199.5	250	280.5	
	$\bar{X}$	0	6.4	16.9	26.6	39.9	50.0	56.1	
V-SA	1	0	7	18	28	39	47	55	xxxx
	2	0	7	18	27	36	45	54	
	3	0	7	18	28	38	47	56	
	4	0	7	18	28	38	47	56	
	5	0	7	18	27	36	45	54	
	Σ	0	35	90	138	187	231	275	
	$\bar{X}$	0	7	18	27.6	37.4	46.2	55	
PDA	1	0	6	17	27	39	48	56	x
	2	0	6	17	28	40	46	54	
	3	0	6	17	27.5	39	48	57	
	4	0	6	17	27	39	47	56	
	5	0	6	17	28	41	48	56	
	Σ	0	30	85	137.5	198	237	279	
	$\bar{X}$	0	6	17	27.5	39.6	47.4	55.8	

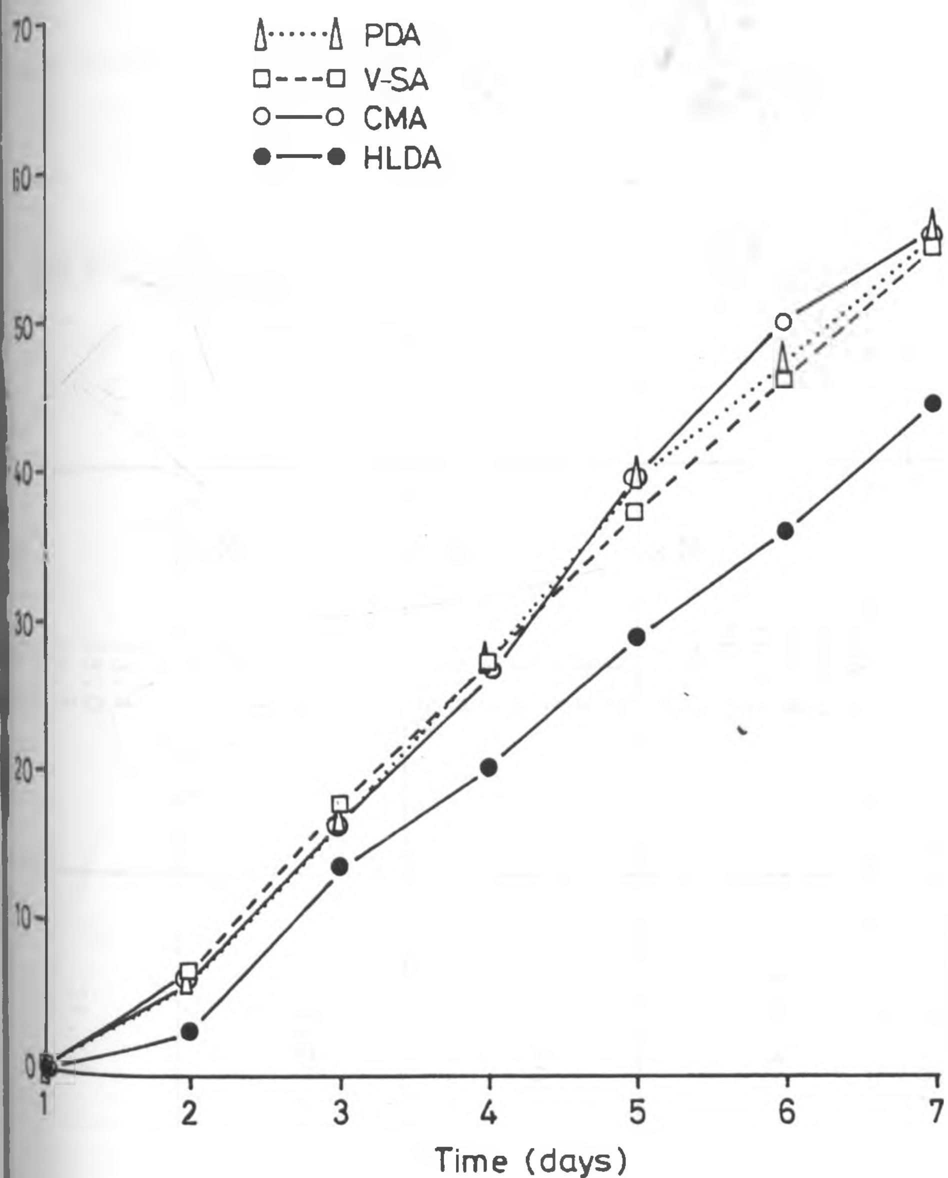


FIG. 1 EFFECT OF MEDIUM ON GROWTH OF A. CRASSA

Table 3: Effect of medium on conidial production of _A. crassa_

Medium	Replicate	Total No. of conidia/ml counted on Haemocytometer	Mean No. of conidia/ml	Average no. of conidia of the suspension ($x10^4$)
HLDA	1	981	196.2	197.88
	2	1,000	200.0	
	3	964	192.8	
	4	992	198.4	
	5	1,010	202.0	
			Σ = 989.4	
			X = 197.88	
V-SA	1	6	1.2	1.20
	2	7	1.4	
	3	6	1.2	
	4	5	1.0	
	5	6	1.2	
			Σ = 6.0	
			X = 1.20	
CMA	1	13	2.6	2.76
	2	15	3.0	
	3	15	3.0	
	4	14	2.8	
	5	12	2.4	
			Σ = 13.8	
			X = 2.76	
PDA	1	153	30.6	31.16
	2	167	33.4	
	3	160	32.0	
	4	149	29.8	
	5	150	30.0	
			Σ = 155.8	

Table 4: Effect of Medium on Conidial Morphology

Medium	Conidial dimensions*				
	Length u	Width u	L/W	No. of septa	
				Transverse	Longitudinal
HLDA	166.25	19.6	8.48	7.0	1.0
V-SA	122.5	26.25	4.67	9.0	6.1
CMA	133	22.75	5.85	7.0	2.2
PDA	154	15.75	9.78	7.0	2.0

* Data represents the mean of 25 conidia selected at random.

Table 5: Effect of medium concentration on growth and sporulation of *A. crassa*

Medium		1[a]	2	3	4	5	6	7	Conidia/ml of fluid x 10^5
	Concentration	Mean radial growth (mm)[b]							
HLDA	4x	0	4	12	21	27	36	51	32.0
	3x	0	4	12	20.7	27.2	36	49.5	34.7
	2x	0	4	12	20.3	27	36.7	49	40.2
	1x	0	4	12	20.5	27.5	36	49	38.41
	1:1	0	4	12	20.4	27.3	36.5	50.2	36.2
	1:2	0	4	12	20.5	27.2	37	50	34.0
	1:4	0	4	12	20.5	27.2	38	52	32.5
CMA	4x	0	6.9	17	29	45.9	58	67.5	0
	3x	0	6.5	18	30	46.9	61	68	0
	2x	0	6	17	30	47	60	67	0.02
	1x	0	7	17.5	30	47	59	69	0.29
	1:1	0	7	17	31	46.5	60	67.2	0.19
	1:2	0	7	17.2	31	47	60	67	0.09
	1:4	0	6.8	17	30.5	47	62	69	0.04
PDA	4x	0	4	16	26.5	37.5	49	68	3.1
	3x	0	4	16	26	37.2	48.5	68.5	3.3
	2x	0	4	16.2	27.5	37	48	67	3.3
	1x	0	4	16	26	37	48	67	3.4
	1:1	0	4	16	26	37	48	68	2.32
	1:2	0	4	16	26.2	37	48	68.2	1.90
	1:4	0	4	16	27	38	49	68.5	1.11
V-SA	4x	0	7.0	18	30	38	48	68	0.31
	3x	0	7.5	19	31	36.5	47.9	68.2	0.29
	2x	0	7.2	18	30	37	48	68	0.16
	1x	0	7.0	18	30	37	48	68	0.12
	1:1	0	7	18	30	37.5	48.5	67.5	0.1
	1:2	0	7	19	31	38	51	69	0
	1:4	0	7	19	31	39	50	68	0

[a] Day of inoculation

[b] Average of five replicates cultured at 29°C under continuous fluorescent light

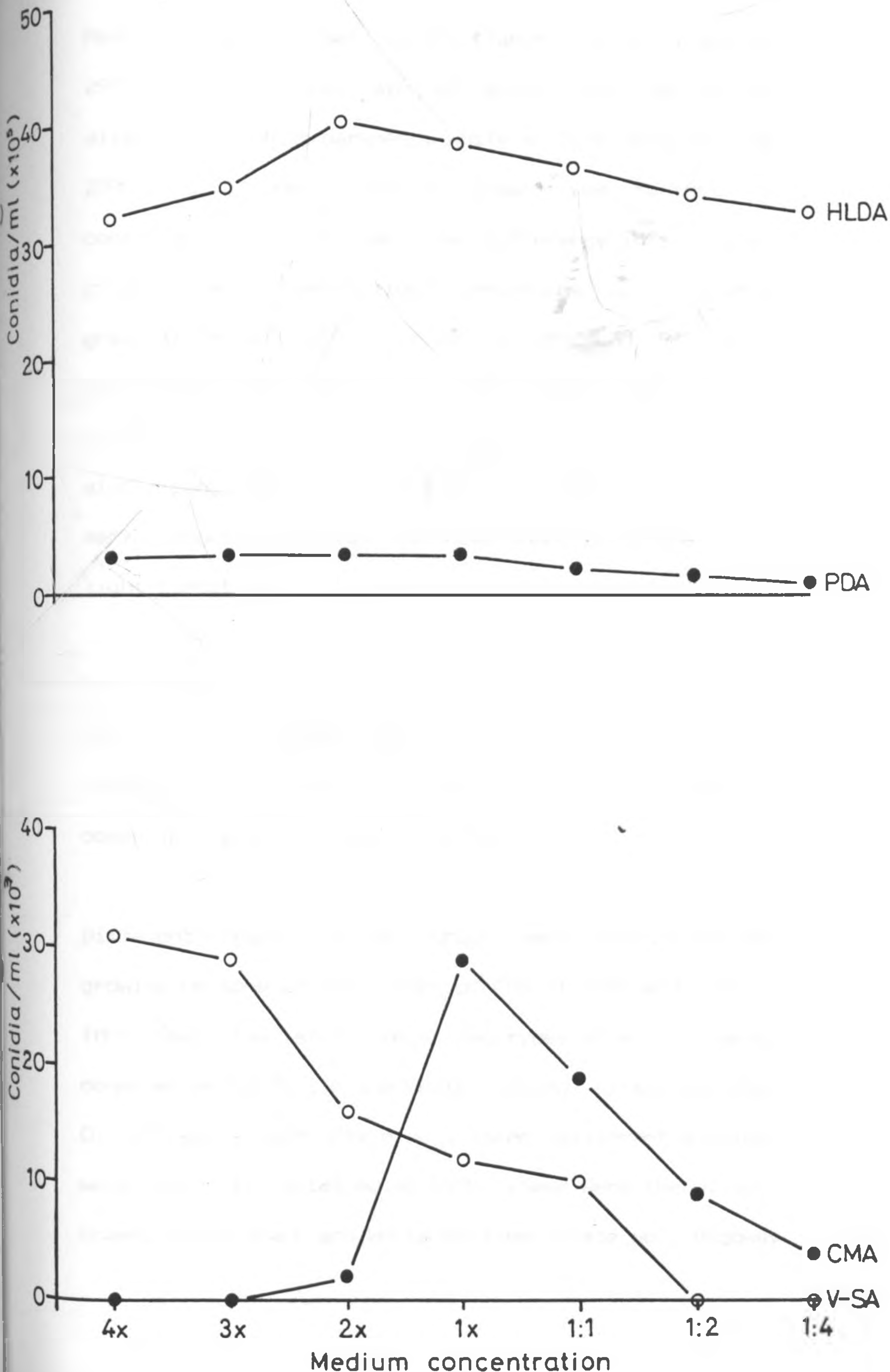


FIG. 2 EFFECT OF MEDIA AND MEDIUM CONCENTRATION ON CONIDIAL PRODUCTION

4.3:2 Effect of temperature and light on growth and and sporulation of *A. crassa*

Results indicate that at 9ºC (Table 6, Fig. 3) and at 29ºC (Fig. 6), the rate of growth was fastest in alternating light/darkness, while at 20ºC (Fig. 4) and 25ºC (Fig.5) the rate of growth was fastest in continuous light. However the difference in the rates of growth at different light conditions was not very great at 9ºC and 20ºC as it was in 25ºC and 29ºC. This means that the effect of light on the growth of A. crassa is not great at low temperatures but becomes significant at higher temperatures. There was alot of aerial growth in total darkness compared to the other light conditions.

Temperature affected rate of growth of the fungus at all light conditions. With increase in temperature, growth rate increased up to 29ºC. At 35ºC no growth was observed (Table 6, Figs. 7, 8, 9).

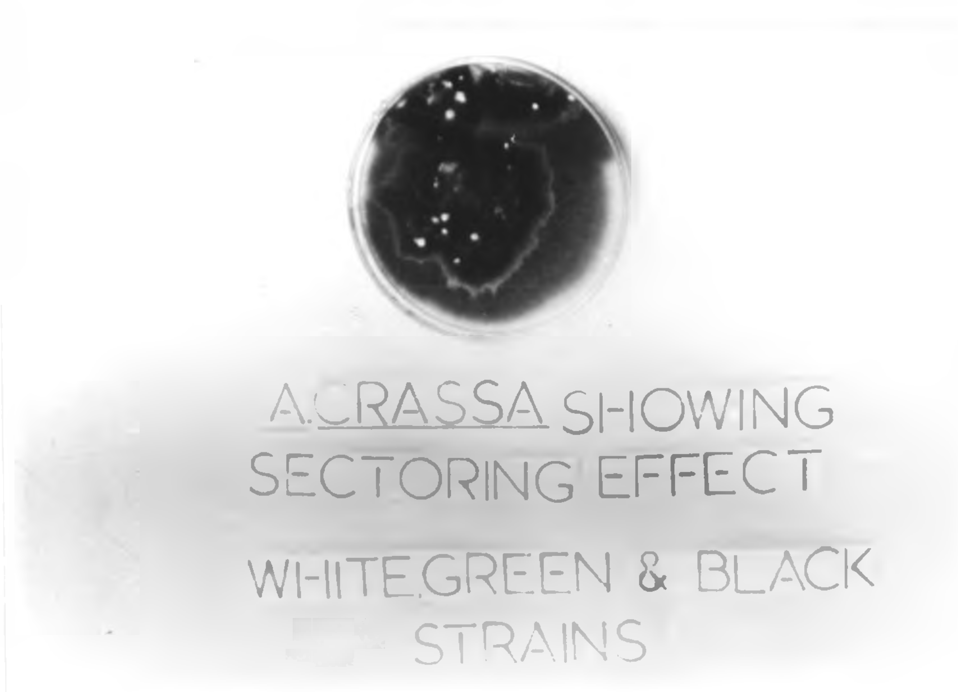
Different strains of the fungus were also observed growing on some of the plates of PDA at 25ºC and 29ºC. Throughout the study only two types of strains were observed at 25ºC, i.e the White (albino) strain and the Olive-Green strain (Plate 5a). Three different strains were frequently obtained at 29ºC. These were the Olive-Green, Sooty Black and White strains (Plate 6b), Rigdway

(1912). The black and albino strains did not produce spores and were also found to be non-pathogenic. Their rate of growth was also very slow, being slowest with the albino strain (Table 7, Fig. 10). The White strain also frequently resulted from frequent transfers and in transfers from old cultures. Temperature significantly influenced the sporulation of the fungus at each light condition tested (Tables 8, 9, 10). At 1% level of probability, the F-value for temperature influence under continuous light, total darkness and alternating light (8hr) and darkness was 23, 352.663, 9.9655 and 29.21198 respectively. In all the three light conditions tested, the highest the highest sporulation was observed at 29°C seconded by 25°C and least at 20°C. At 9°C no sporulation was observed untill after 36 days (48x104 conidia/ml) in plates kept under continuous fluorescent light). No growth occurred at 35°C.

Light also significantly affected sporulation. At 1% level of probability, the F-value for the effect of light at 20°C, 25°C and 29°C was 32.089, 73.835926 and 28,133.7 respectively. The highest conidial production occurred in plates kept at alternating light (8hrs), and darkness. In total darkness there was very little to no sporulation. (Tables 11, 12, 13).


A. CRASSA SHOWING
SECTORING EFFECT

(a)


A.CRASSA SHOWING
SECTORING EFFECT
WHITE,GREEN & BLACK
STRAINS

(b)

PLATE 6. *A. crassa* showing sectoring effect. (a) Olive-Green and White strains at 25°C and continuous fluorescent light (b) Olive-Green, Sooty Black and White strains at 29°C and continuous fluorescent light.

Table 6 : Growth of *A. crassa* at different temperature and light conditions

Condition		Day						
		1[a]	2	3	4	5	6	7
	Temp. °C	Diameter of colony[b]						
Continuous light	9	0	0	0	1	2.3	4.8	6.2
	20	0	7	12.6	19.6	23.2	33.8	41.8
	25	0	7	15	23.6	32.2	41	46.6
	29	0	4	12	20.6	27.6	36.5	48.8
	35	0	0	0	0	0	0	0
Continuous darkness	9	0	0	0	1	2.1	4.4	5.8
	20	0	7	12.7	19.1	23.0	32.7	33.3
	25	0	7	15	20	27.1	35.6	38.2
	29	0	7	16	22	29.1	37.2	45.1
	35	0	0	0	0	0	0	0
Alternating 8 hr light/ darkness	9	0	0	0	1.2	2.5	5.1	6.8
	20	0	6	11.3	18.3	25.0	30.5	41.1
	25	0	3	14	20.5	28.8	35.7	45.2
	29	0	7	16	25.5	34.0	43.0	46.9
	35	0	0	0	0	0	0	0

[a] Day of inoculation.

[b] Mean of five replicates. Measurements are in mm.

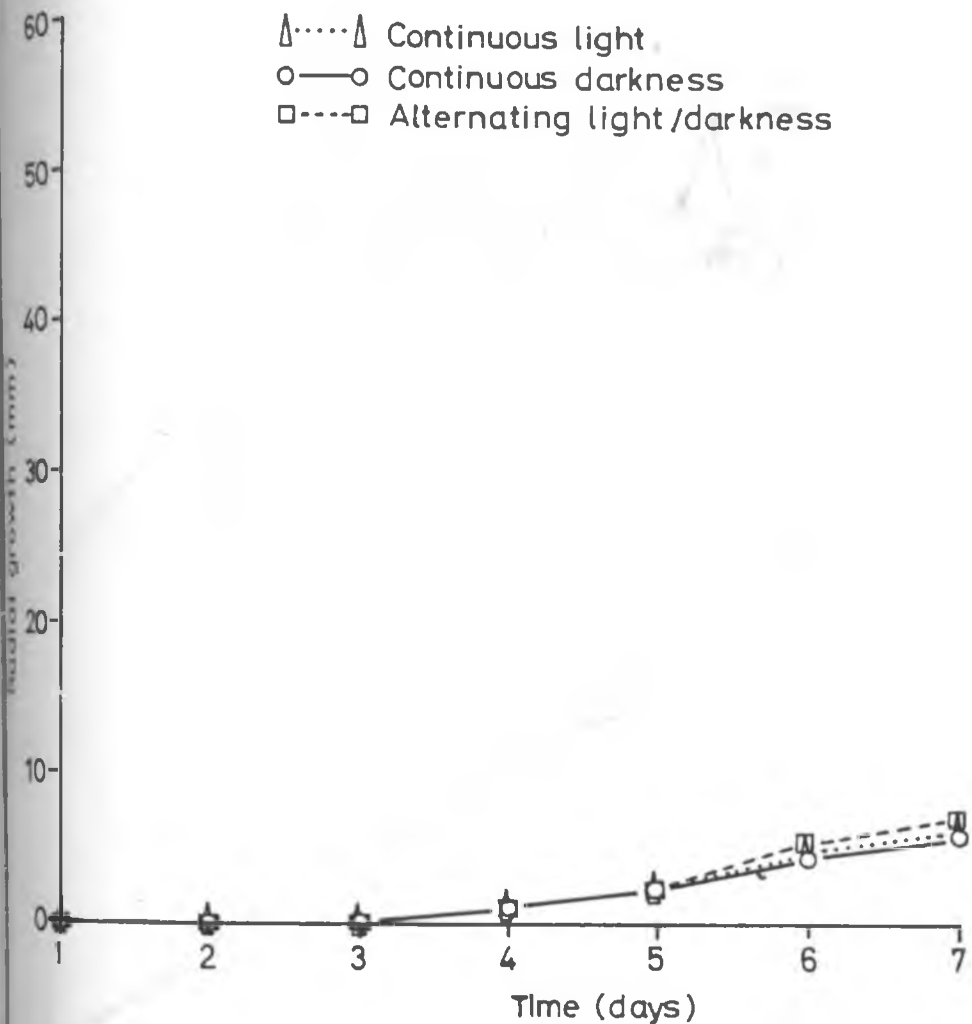


FIG. 3 EFFECT OF LIGHT ON GROWTH OF A. CRASSA AT 9 °C

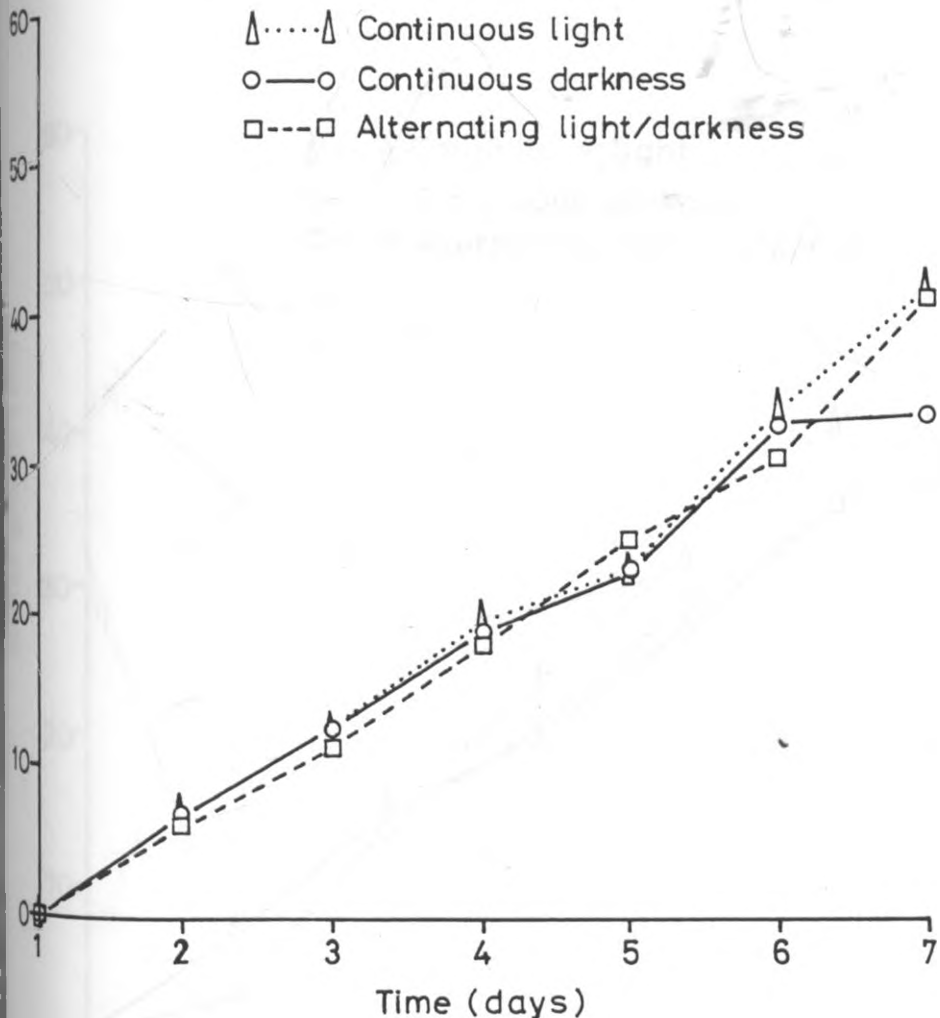


FIG. 4 EFFECT OF LIGHT ON GROWTH OF *A. CRASSA* AT 20°C

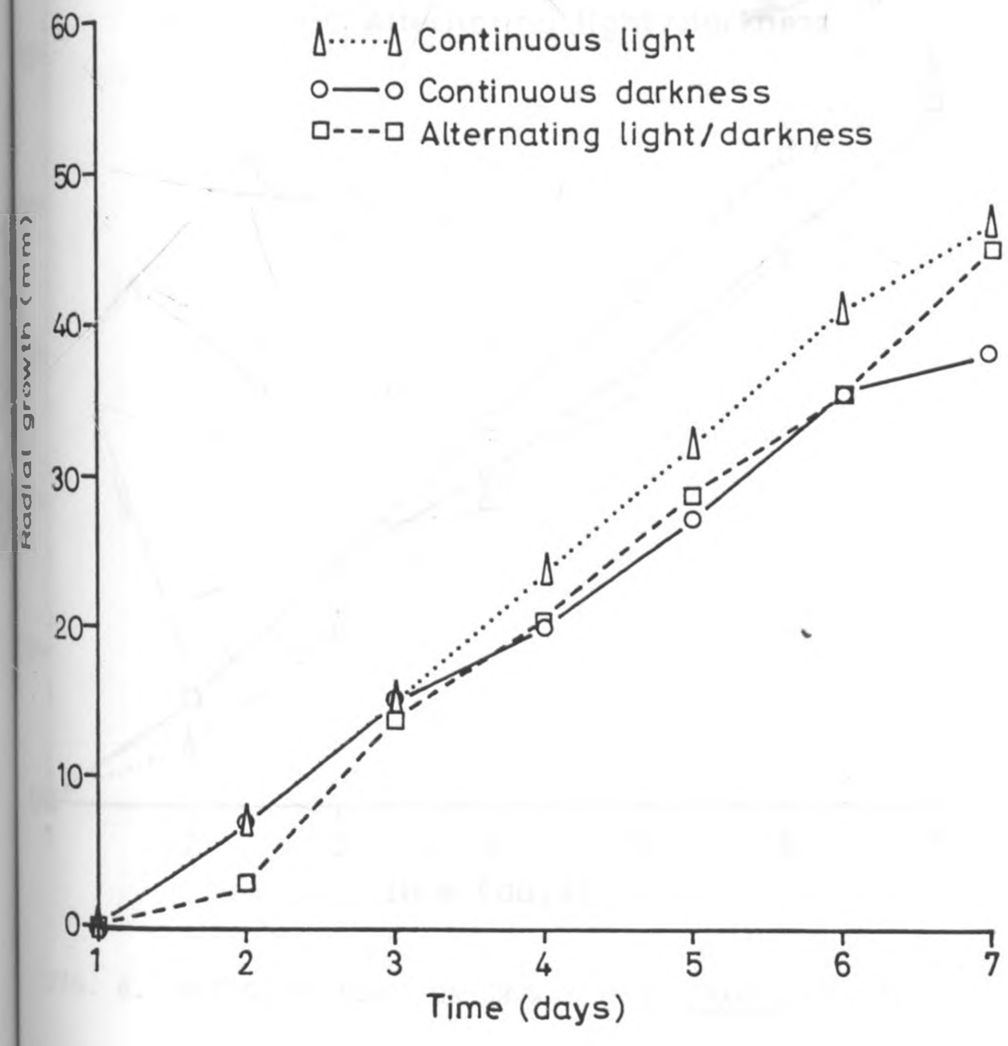


FIG. 5 EFFECT OF LIGHT ON GROWTH OF A. CRASSA AT 25°C

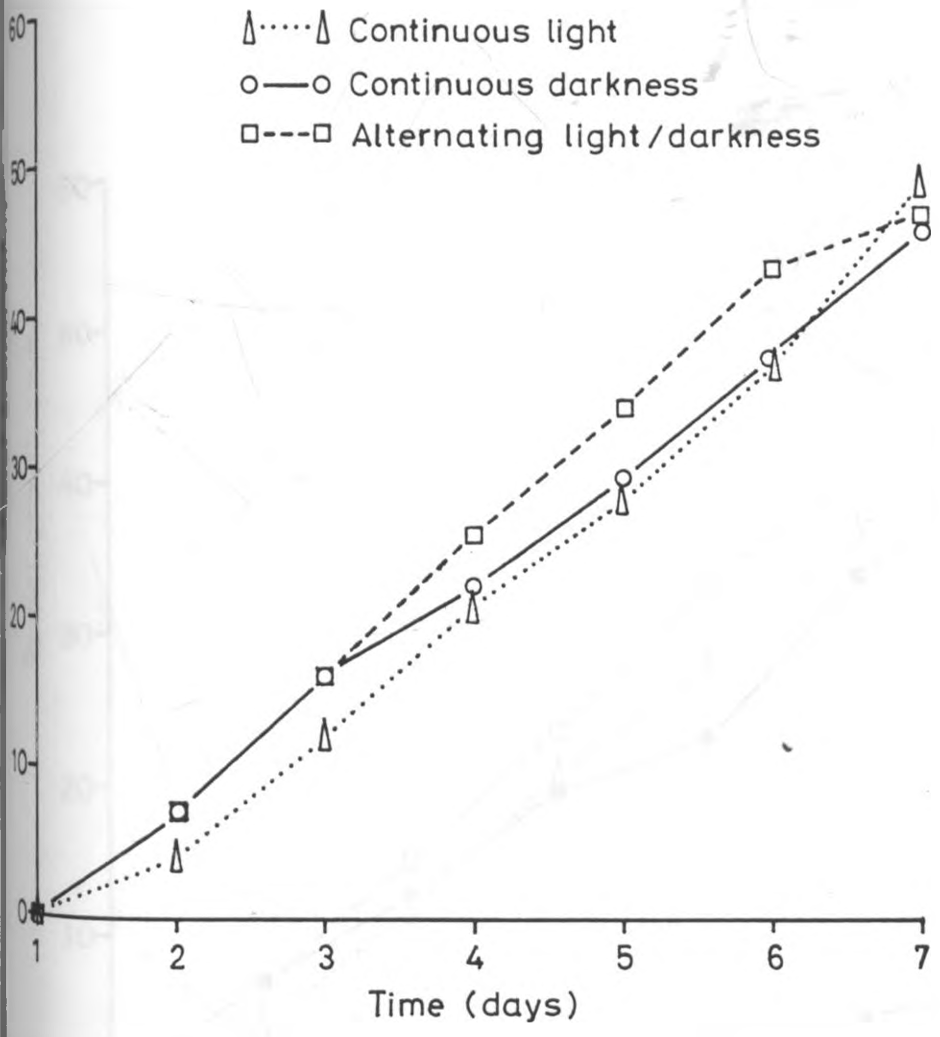


FIG. 6. EFFECT OF LIGHT ON GROWTH OF A. CRASSA AT 29°C

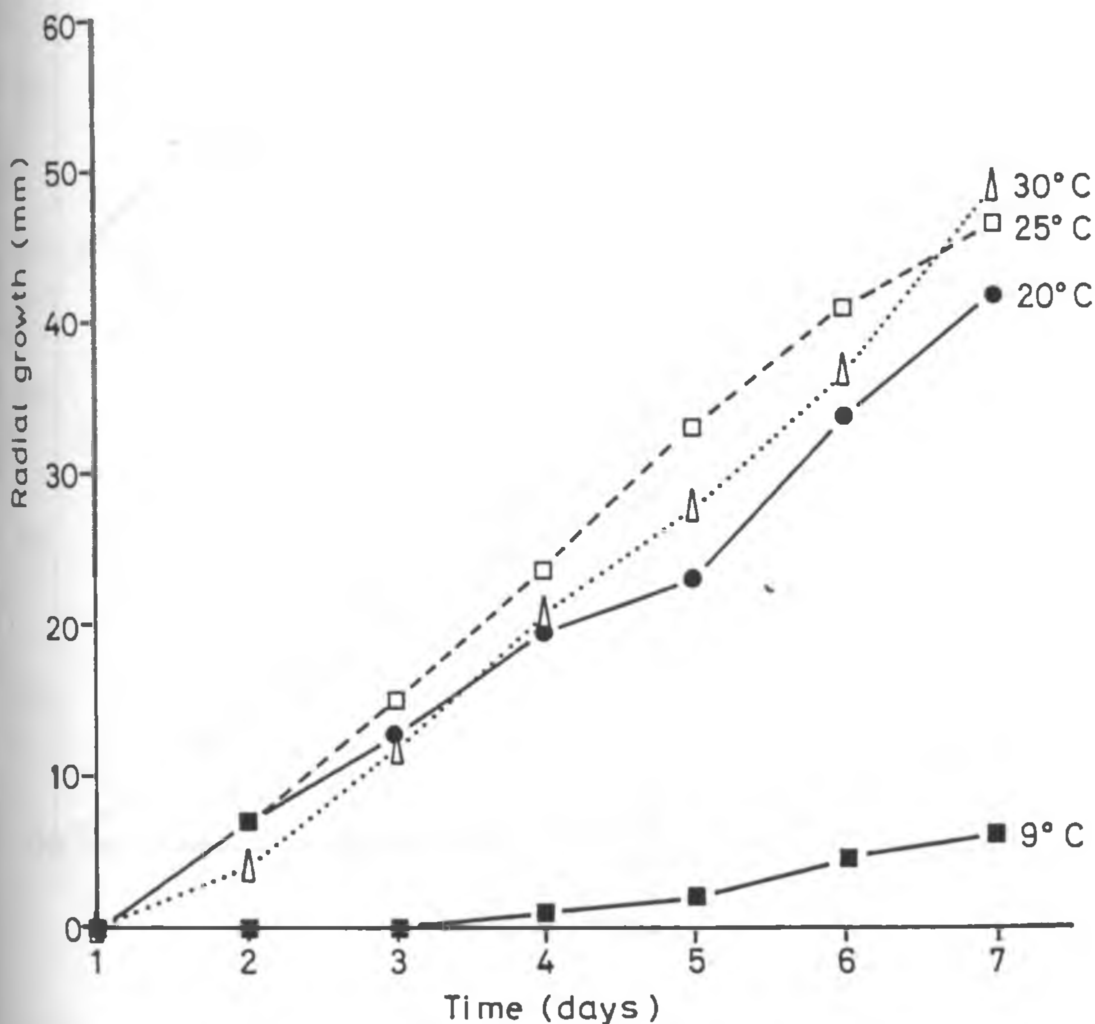


FIG. 7 EFFECT OF INCUBATION TEMPERATURE ON GROWTH OF A. CRASSA IN CONTINUOUS LIGHT CONDITIONS

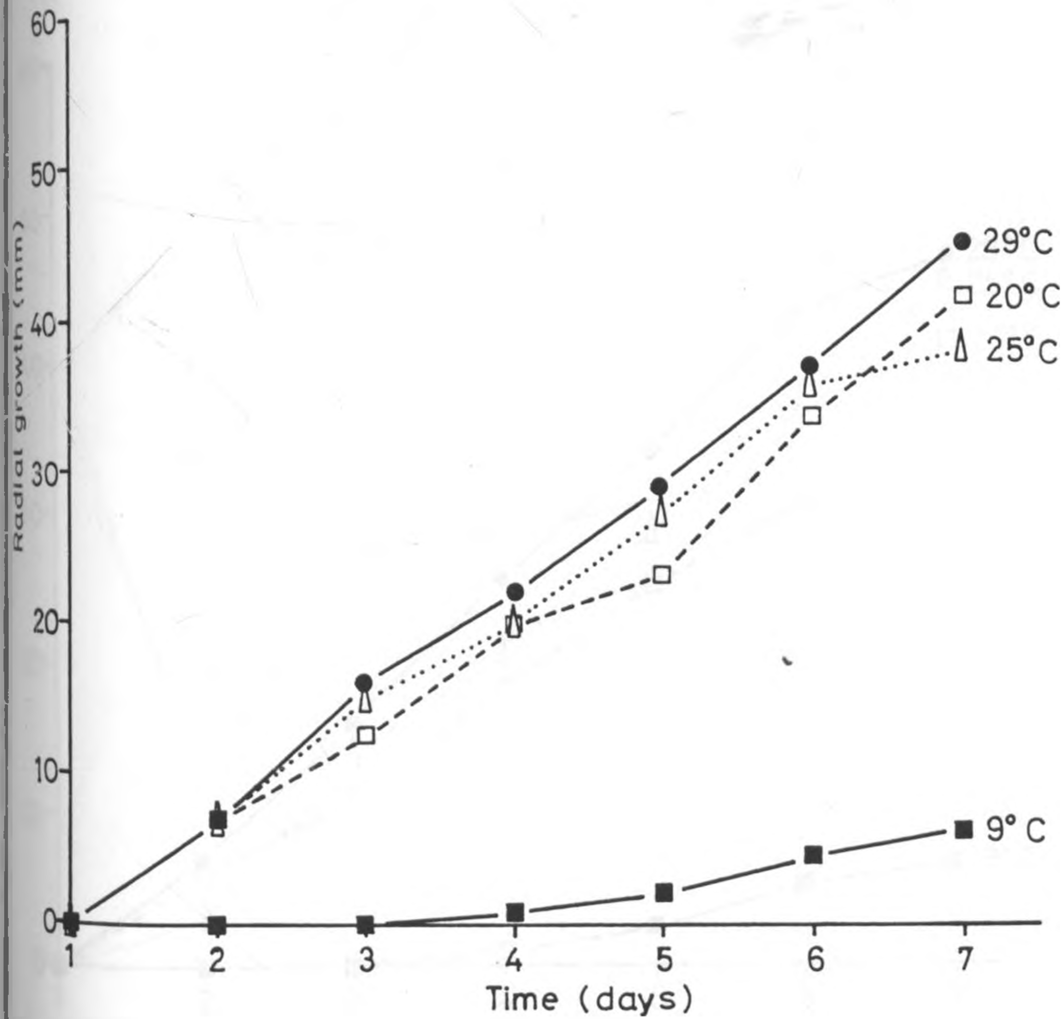


FIG. 8 EFFECT OF TEMPERATURE ON GROWTH OF A. CRASSA IN CONTINUOUS DARKNESS CONDITIONS

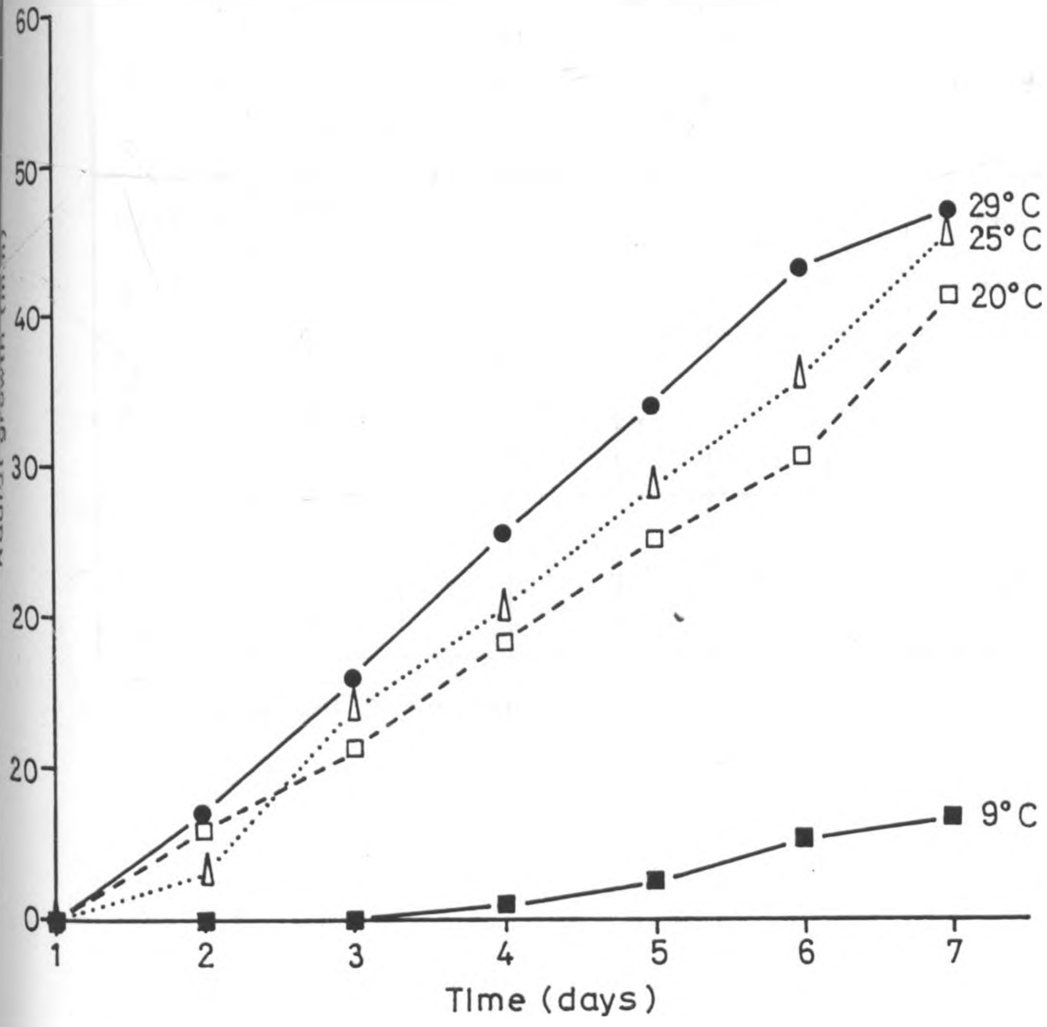


FIG. 9 EFFECT OF TEMPERATURE ON GROWTH OF A. CRASSA IN ALTERNATING (8 HR) LIGHT AND DARKNESS

Table 7: Growth of Olive Green, Sooty Black and White strains of A. crassa at 29°C continuous fluorescent light

Medium - PDA

Radial growth measured in mm

Strain	Day							Other observation
	1	2	3	4	5	6	7	
Dark green	0	4*	16.2	27.8	36	47	55	Spores produced Pathogenic
Black	0	2	3.5	10	20	23.5	25	No spores Non-pathogenic
White	0	1	1.5	5	9.5	16	19	No spores Non-pathogenic

* Average of five replicates

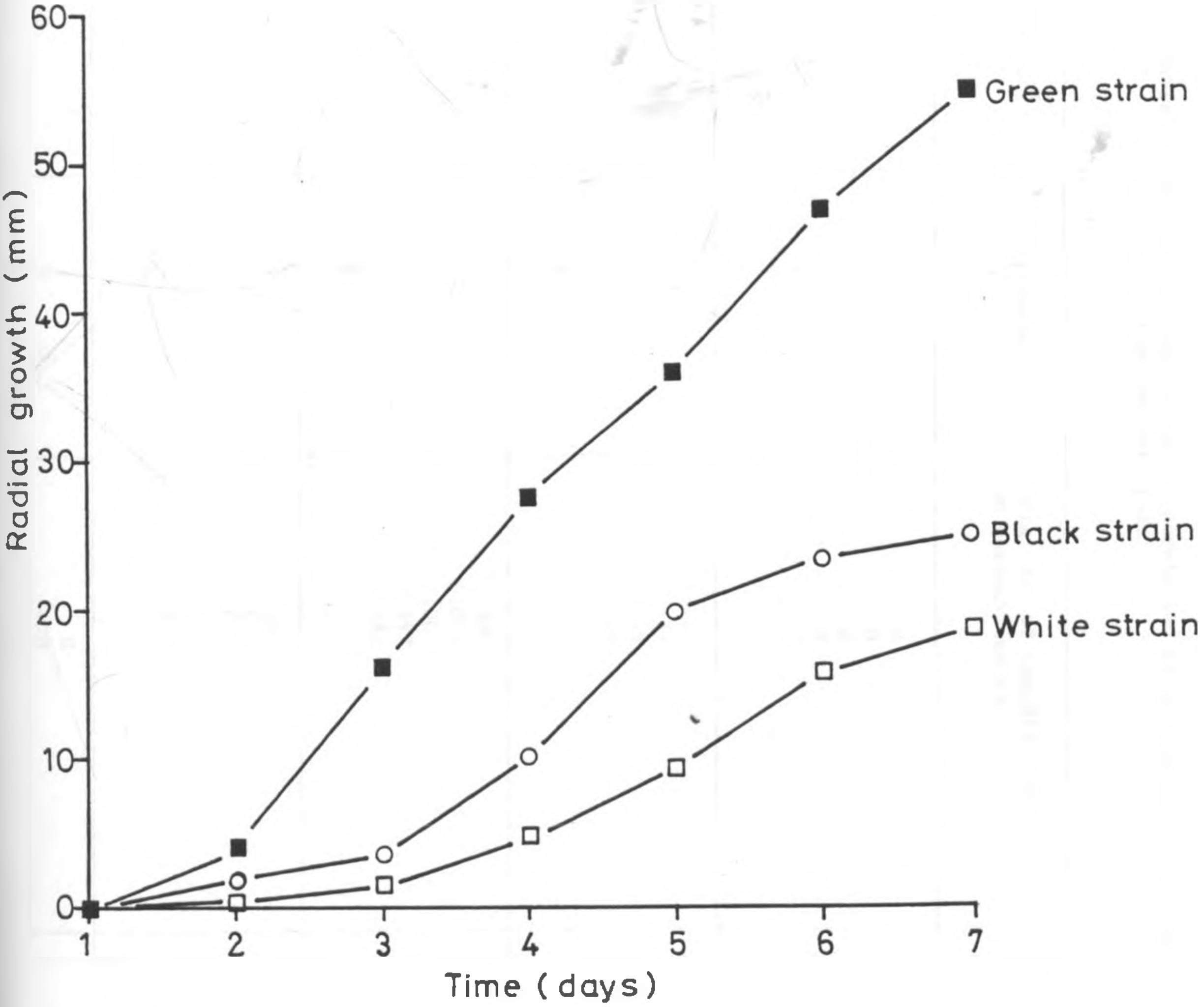


Fig. 10: Growth of Olive Green, Sooty Black and White strains of *A. crassa* at 29°C and continuous fluorescent light, on PDA

Table 8: Effect of temperature on sporulation of *A. crassa* growing on HLDA under continuous fluorescent light

Temperature °C	Replicates	Total No. of conidia counted on haemocytometer	Mean No. of conidia/ml of the suspension ($\times 10^4$)
9	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	Σ		0
	$\bar{X}$		0
20	1	40	8.0
	2	39	7.8
	3	45	9.0
	4	36	7.2
	5	41	8.2
	Σ		40.2
	$\bar{X}$		8.04
25	1	990	198.0
	2	1009	201.8
	3	1007	201.4
	4	999	199.8
	5	981	196.2
	Σ		997.2
	$\bar{X}$		199.44
29	1	1909	381.8
	2	1900	380.0
	3	1957	391.4
	4	1913	382.6
	5	1932	386.4
	Σ		1922.2
	$\bar{X}$		384.44
35	1	0	0
	2	0	0
	3	0	0
	4	0	0

Table 9: Effect of temperature on sporulation of _A_. _crassa_ growing on HLDA in total darkness

Temperature °C	Replicates	Total No. of conidia counted on Haemocytometer	Mean No. of conidia/ml of the suspension ($\times 10^4$)
9	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	Σ		0
	$\bar{X}$		0
20	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	Σ		0
	$\bar{X}$		0
25	1	6	1.2
	2	6	1.2
	3	7	1.4
	4	10	2.0
	5	9	1.8
	Σ		7.6
	$\bar{X}$		1.52
29	1	12	2.4
	2	11	2.2
	3	13	2.6
	4	10	2.0
	5	9	1.8
	Σ		11
	$\bar{X}$		2.2
35	1	0	0
	2	0	0
	3	0	0

Table 10: Effect of temperature on sporulation of A. crassa growing on HLDA under alternating light (8 hr) and darkness

Temperature °C	Replicate	No. of conidia counted on Haemocytometer	No. of conidia/ml
	1	0	0
	2	0	0
9	3	0	0
	4	0	0
	5	0	0
	Σ		0
	$\bar{X}$		0
	1	54	10.8
	2	59	11.8
20	3	58	11.6
	4	49	9.8
	5	57	11.4
	Σ		55.4
	$\bar{X}$		11.08
	1	1,212	242.4
	2	1,200	240.0
25	3	1,198	239.6
	4	1,200	240.0
	5	1,900	380.0
	Σ		1342
	$\bar{X}$		2684
	1	2,420	484
	2	2,450	490
29	3	2,399	479.8
	4	2,432	486.4
	5	2,430	486
	Σ		2426.2
	$\bar{X}$		485.24

Table 11. Effect of light on sporulation of _A_. _crassa_ growing on HLDA at 20°C

Light Conditions	Replicates	No. of conidia counted on haemocytometer	No. of conidia/ml of the suspension
Continuous Light	1	40	8.0
	2	39	7.8
	3	45	9.0
	4	36	7.2
	5	41	8.2
	Σ		40.2
	$\bar{X}$		8.04
Total Darkness	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	Σ		0
	$\bar{X}$		0
Alternating Light/ darkness	1	54	10.8
	2	59	11.8
	3	58	11.6
	4	49	9.8
	5	57	11.4
	Σ		55.4
	$\bar{X}$		11.08

Table 12: Effect of light on sporulation of A. crassa growing on HLDA at 25°C

Light Conditions	Replicate	Total No. of conidia on Haemacytometer	Mean No. of conidia/ml
Continuous light	1	990	198.0
	2	1009	201.8
	3	1007	201.4
	4	999	199.8
	5	981	196.2
	Σ		997.2
	$\bar{X}$		199.44
Total Darkness	1	6	1.2
	2	6	1.2
	3	7	1.4
	4	10	2.0
	5	9	1.8
	Σ		7.6
	$\bar{X}$		1.52
Alternating light/ darkness	1	1212	242.4
	2	1200	240.0
	3	1198	239.6
	4	1200	240.0
	5	1900	380.0
	Σ		1342
	$\bar{X}$		268.4

Table 13: Effect of light on sporulation of A. crassa growing on HLDA at 29°C

Light Condition	Replicate	No. of conidia counted on Haemocytometer	No. of conidia/ml of the suspension ($\times 10^4$)
Continuous Light	1	1909	381.8
	2	1900	380.0
	3	1957	391.4
	4	1913	382.6
	5	1932	386.4
	Σ		1,922.2
	$\bar{X}$		384.44
Total Darkness	1	12	2.4
	2	11	2.2
	3	13	2.6
	4	10	2.0
	5	9	1.8
	Σ		11
	$\bar{X}$		2.2
Alternating Light/ darkness	1	2420	484
	2	2450	490
	3	2399	479.8
	4	2432	486.4
	5	2430	486
	Σ		2426.2
	$\bar{X}$		485.24

4.3:3 Effect of temperature and pH on growth and sporulation of *A. crassa*

At all the four tested ranges of pH growth was fastest at 29°C, followed by 25°C and lastly 20°C (Table 14, Fig. 11). Considering growth rate across the pH ranges and similar temperatures, growth was fastest at pH 6.8 followed by 5.0, 9.0, 3.1 respectively (Figs. 12, 13) Figure 12 shows the amount of growth on the 7th day across the pH ranges at 29°C. The optimum growth rate was at pH 6.8, declining at either end. At pH 11.0 and 2.0, there was no growth. The growth on the acid media (3.1,5.0) was relatively thin compared to that on basic medium (9.0). Conidial production increased with increase in temperature. The highest amount was observed at pH 6.8. Chlamydospores were observed at pH 3.0 (Plate 7).

PLATE 7. Chlamydospores forming on medium at pH 3.

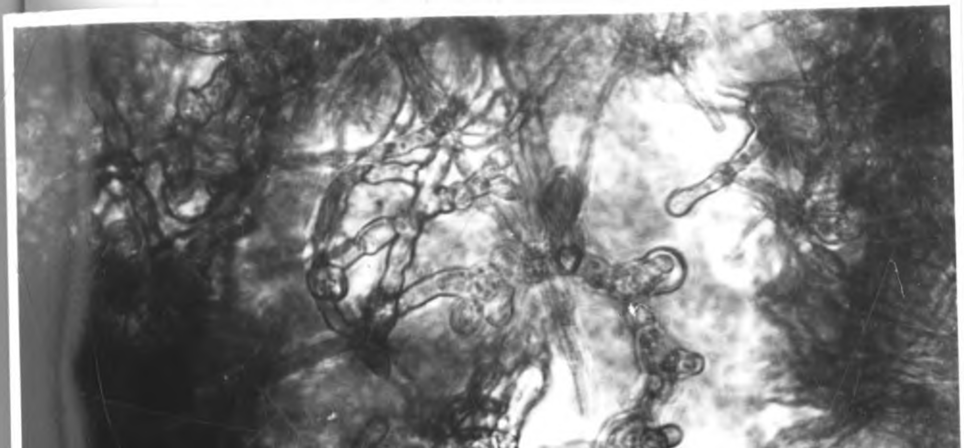
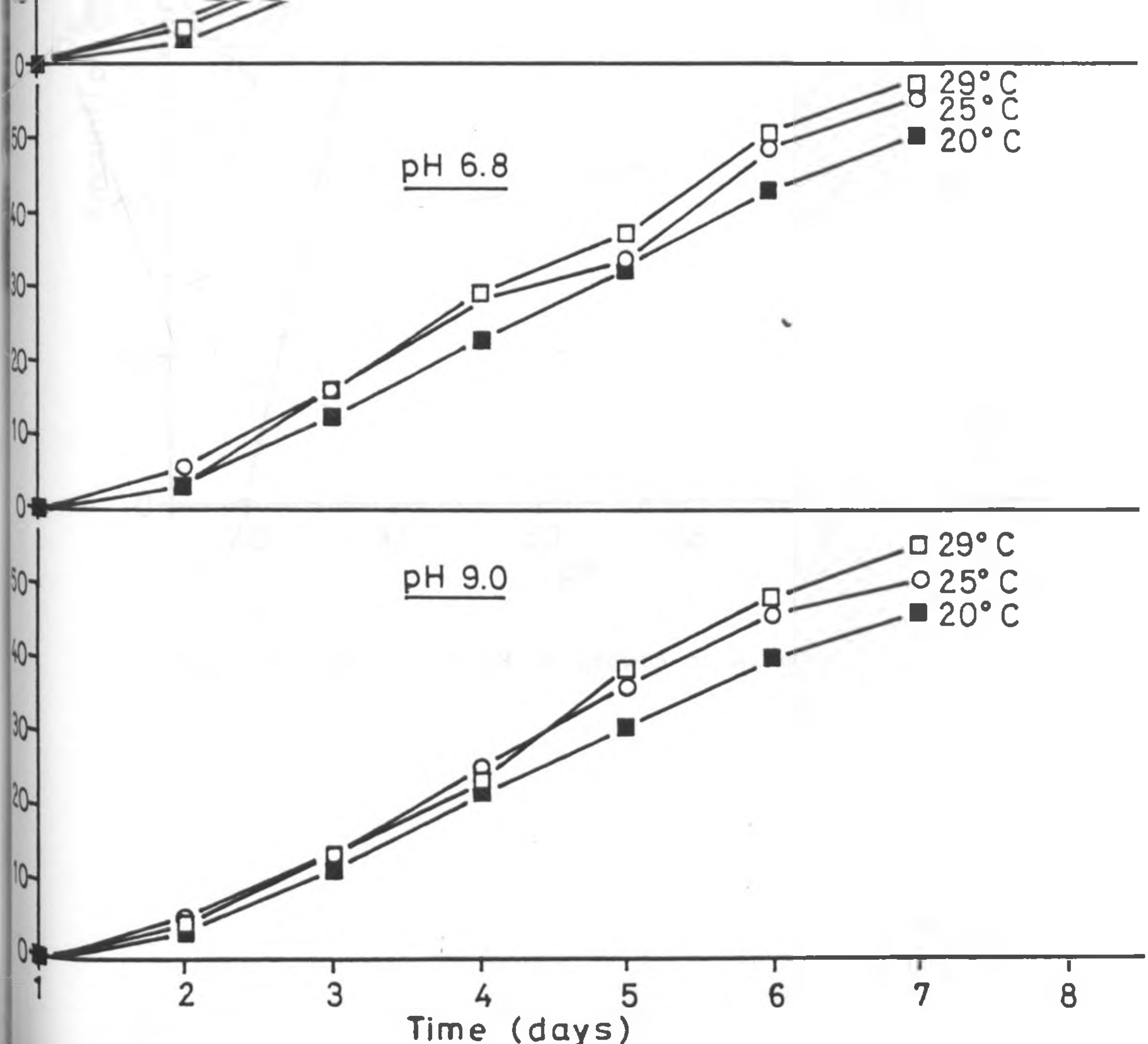


Table 14 : Effect of temperature and pH on growth and sporulation of A. crassa

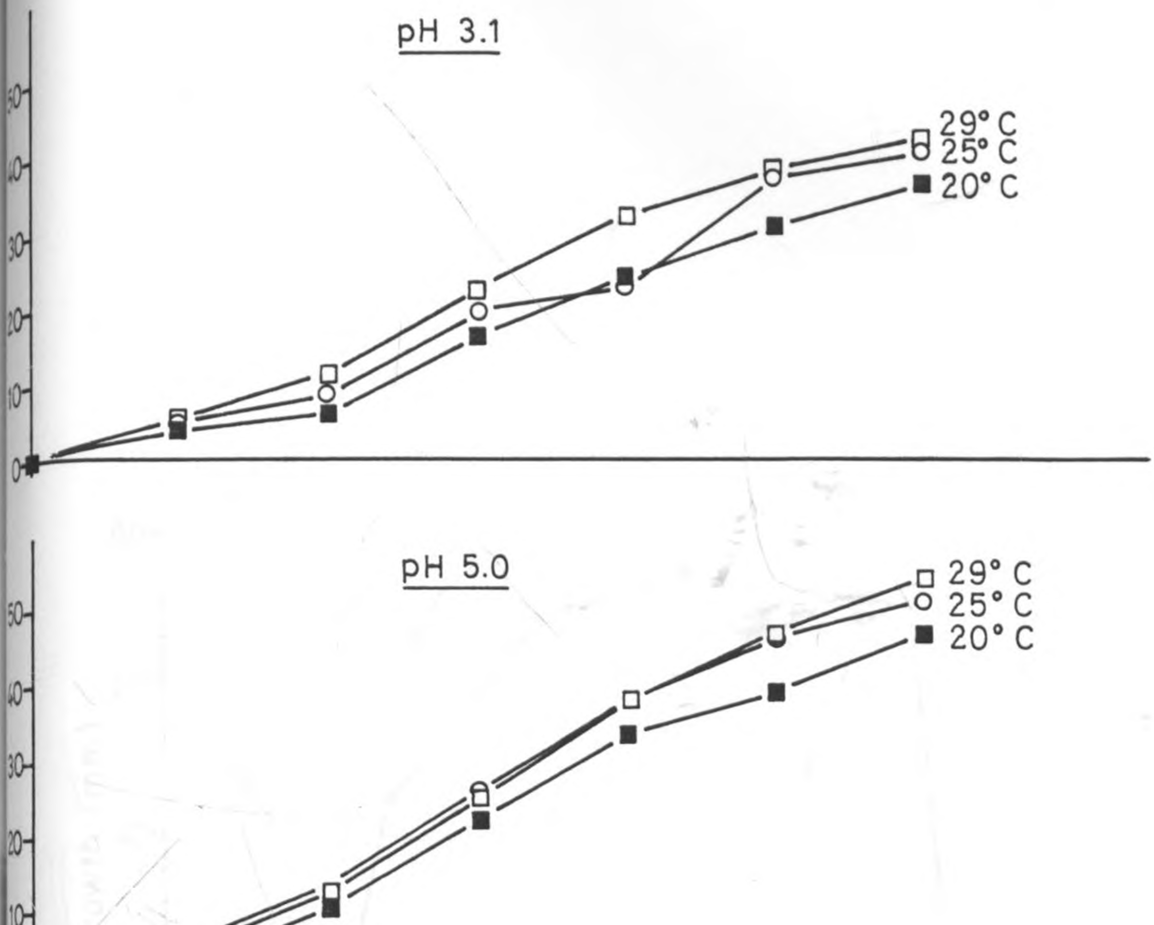
Initial pH \ DAY		1[a]	2	3	4	5	6	7	No. of conidia/ml of the suspension ($x10^4$)
	TEMP. °C	DIAMETER OF COLONY[b]							
2.0	20	No growth							
	25	No growth							
	29	No growth							
3.1	20	0	4	6	16.5	24	31	36.2	3.21
	25	0	5	8.6	19.6	32.6	37.6	40.8	3.68
	29	0	5.1	11	22.6	32	38.2	42.1	3.69
5.0	20	0	3.2	11	22.6	34.0	35.2	47	24
	25	0	6	14	26.6	38.6	46.6	51.6	29.44
	29	0	5	13.2	25.5	38.2	47	54.1	29.6
6.8	20	0	4.0	13.0	23.0	32.0	43.0	50.2	21.45
	25	0	6.2	16.2	28.2	33.6	49	55.4	32.36
	29	0	3.5	16.0	29.5	37.2	50.2	57.0	34.0
9.0	20	0	4	12	22	31	40.2	46.4	26.35
	25	0	5.8	14	25.6	36.8	46	50.2	28.92
	29	0	5	14	24	38.9	48.2	55	27.6
11	20	No growth							
	25	No growth							
	29	No growth							

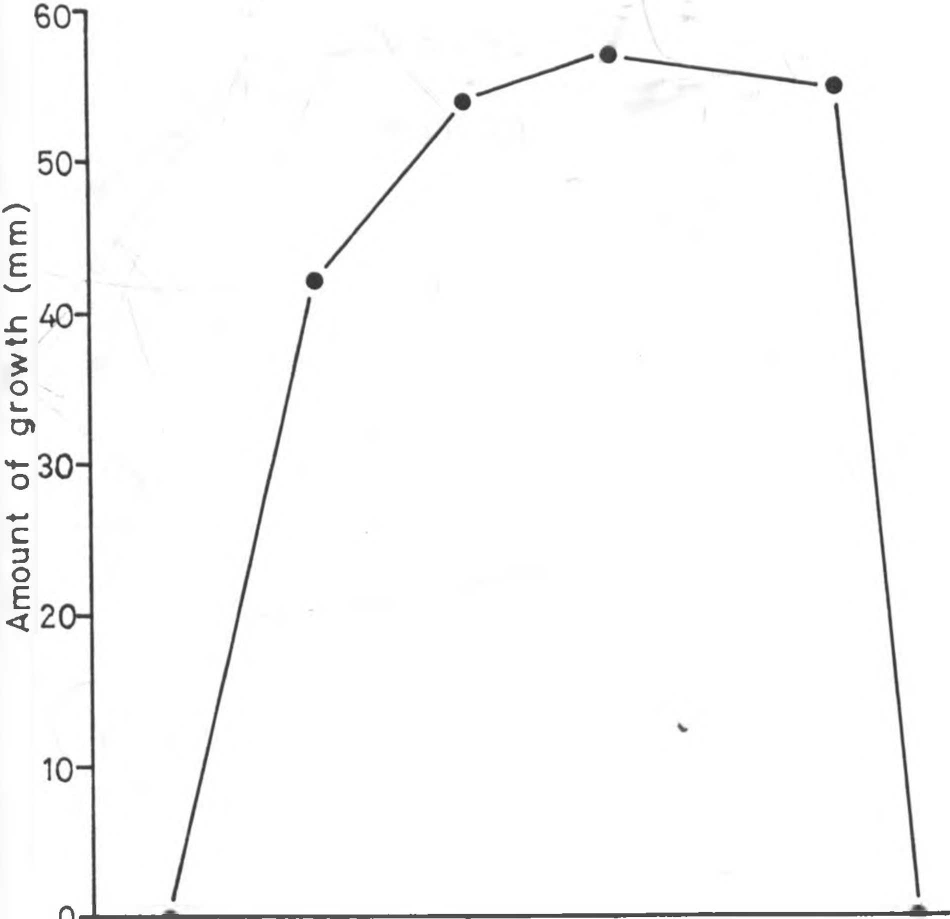
[a] Day of inoculation



pH 6.8
29° C
25° C
20° C
pH 9.0
29° C
25° C
20° C
0
10
20
30
40
50
1
2
3
4
5
6
7
8
Time (days)

Fig. 11. Effect of temperature on growth of A. crassa at various pH





Amount of growth (mm)
60
50
40
30
20
10
0

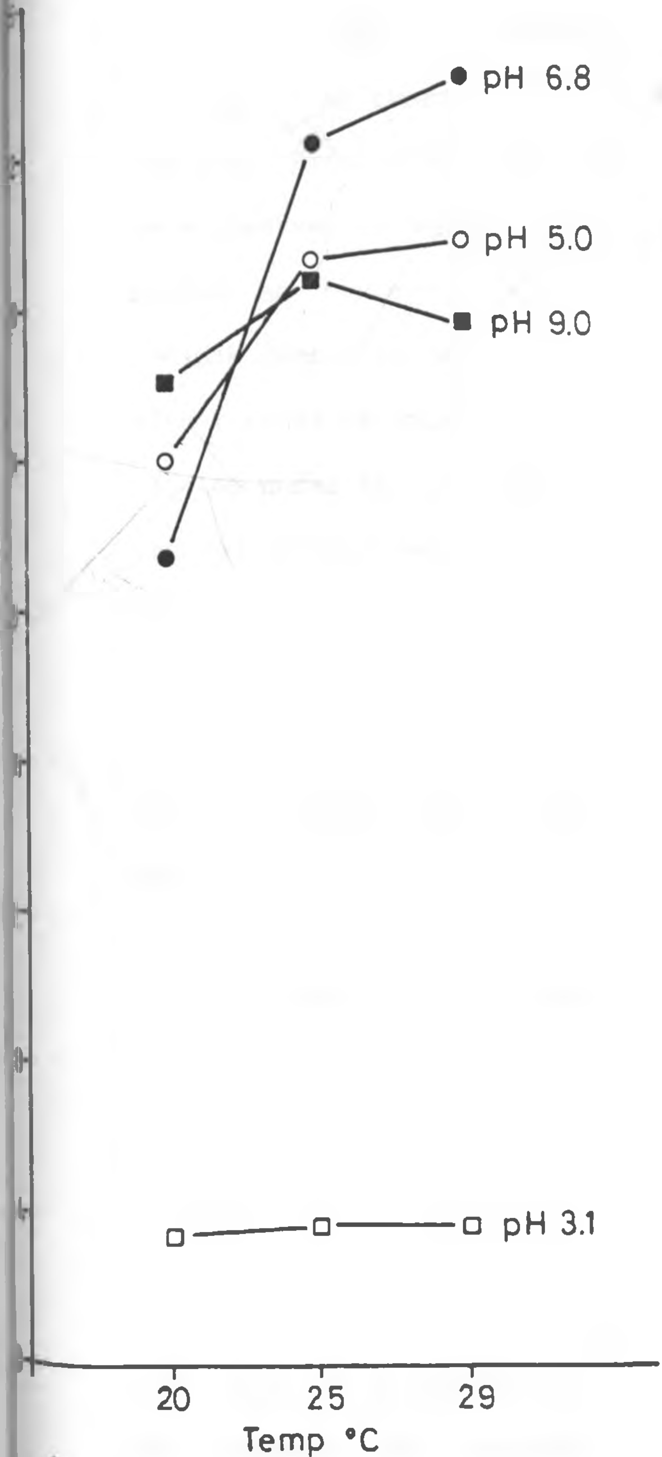


Fig. 13: Effect of temperature and pH on conidial production of *A. crassa*

4.4 Greenhouse test

4.4:1 Effect of spore concentration on symptom development

When plants were atomised with a concentration of 1,000 conidia/ml, an average of 46.8 necrotic spots developed per leaf. With 10,000 spores/ml, 55.6 spots per leaf were observed. Inoculum concentration of 50,000 conidia/ml caused an average of 10.9 lesions. The crowding of conidia seemed to cause inhibition of germination. This effect could be observed when the inoculated leaves were mounted under the microscope. In areas where spores were crowded, either very little or no germination occurred.

Symptoms which appeared on the 7th day after inoculation were in the form of irregular straw coloured, zonate spots. The lesions spread and heavily infected leaves were shed (Table 15 , Plate 8).

4.4:2 Effect of age of plant on symptom development

Results indicated that seven days after inoculation, symptoms had already appeared on all the treatments. By the ninth day disease had already spread to 69% of the surface area of the leaves aged 33 days, and they were shed. However 14 and 24 days old plants had many but small lesions which occupied 62% of their leaf surface.

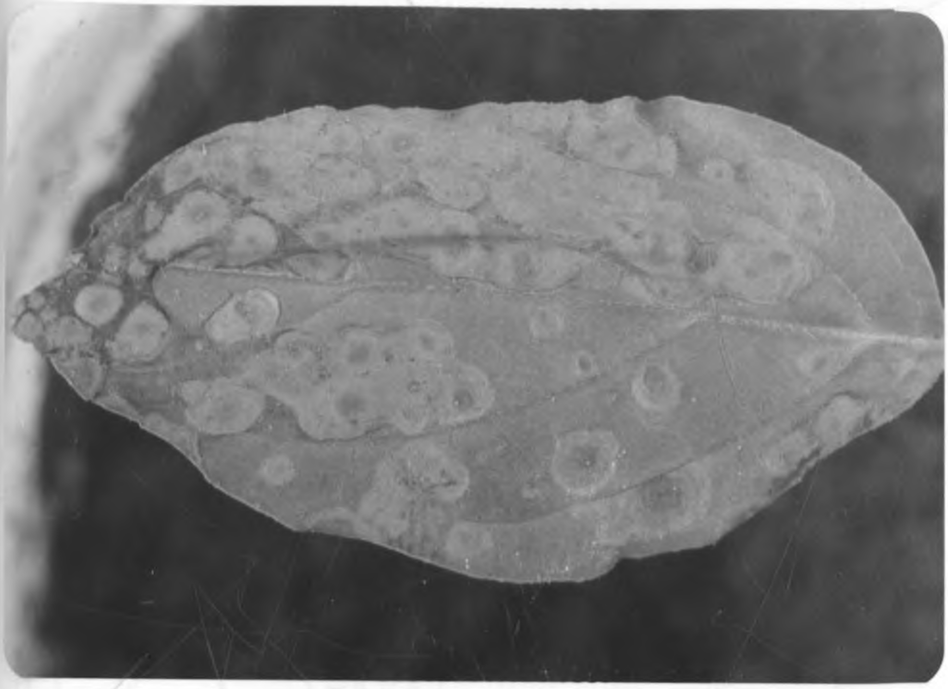
Disease had developed on 45% of the leaf surface of the plants aged 45 and 56 days and by the 16th day, they were dead.

Plants aged 71 and 74 days experienced disease infestation of upto 30% and 20% of their leaf surface respectively. By the 21st day, the leaves were being shed but the plants had not yet shown signs of dying. By the 29th day when disease had developed to 64% of their surface area, the plants died. The older plants (more than 33 days old) had larger necrotic lesions. The control plants did not show signs of infection. They had grown taller and healthier than the experimental group.

4.4:3 Effect of incubation period at 100% R.H.

Disease severity increased with extended incubation time with upto five days being the maximum (Table 16). Symptoms appeared first on plants given the longest exposure to high moisture. Beyond five days, plants were collapsing due to dampness with the leaves and stems well soaked.

PLATE 8. Effect of spore concentration on symptom development. (a) Leaf treated with 10,000 spores/ml (b) Leaf treated with 50,000 spores/ml.



(a)

(b)

Table 15: Effect of inoculum conidial concentration on symptom development

Conidial concentration	50,000/ml	10,000/m/	1,000/m/
The day symptoms first appeared	10th day after inoculation	7th day after inoculation	9th day after inoculation
Average no. of lesions/ rep. on the 12th day			
<u>Replicate</u>			
1	6	52	55
2	18	57	51
3	10	65	54
4	12	54	46
5	8	56	42
6	11	49	53
7	13	56	45
8	12	68	47
9	9	57	43
10	10	42	32
	109	556	469
$\bar{X}$	10.9	55.6	46.9
	16 days after inoculation disease had spread to an average of 45.5% of surface area of leaf	Disease had spread to 94.6% of leaf surface 11 days after inoculation	Disease had spread to 92.8% of leaf surface 12 days after inoculation
<u>Severeity of leaf blight on the 12th day</u>			
Score	3	5	5
Description	Moderate infection	Very severe infection	Very severe infection

Table 16. Effect of incubation period at 100% R.H.

Amount of time in 100% R.H.	Average number of lesions
24 hrs	No lesions
48 hrs	4
72 hrs	9
5 days	11
6 - 9 days	Defoliation

4.4 Field test

Datura stramonium plants tended to show low infection in the field, such that a second inoculation was necessary (Tables 17, 18).The second inoculation was done 48hrs after the first and the plants left under 100% R.H for 96hrs to allow enough time for the germ tubes from conidia of the second inoculation to penetrate. Necrotic spots began to appear on the leaves within seven days of inoculation in Block A. Within 16 days, leaves were dying and dropping off the plants. Plate 9 shows that the roots of the inoculated plants had stunted growth as compared to the control plants. Twenty-one days after inoculation, 189 (75.6%) plants had died.

In Block B, plants were also infected (Table 17) but at a much lower level such that new leaves were forming as the infected ones were falling off. Only 2 (0.8%) plants died.

Maize (*Zea mays* L.) plants that completely surrounded the seedlings were not affected, neither were Garden Peas (*Pisum sativum* L.), Onions (*Allium fistulosum* L.), Beans (*Phaseolus vulgaris*), Spinnach (*Beta vulgaris* L.) Potatoes (*Solanum tuberosum* L.), Sukuma wiki (*Brassica oleracea*), Cabbage (*Brassica oleracea* var. *capitata* L.) planted in the same garden, one meter away from the experimental plot affected.

Seedlings inoculated but uncovered had significantly less number of lesions than those covered immediately after inoculation.

PLATE 9. Field test (a) Infected seedlings (b) control plants (c) On the left is a healthy seedling from control plants, on the right is a retarded, heavily infected seedling from control group. Note the differences in height, leaf size and root development of the two seedlings.

(a)

(b)

(c)

Table 17

Reaction of _D_. _stramonium_ to artificial inoculation (once) in the field

Counting done 14 days after inoculation

Replications	Number of necrotic spots						
	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5		$\bar{X}$
I	24	16	22	21	19	102	20.4
II	19	24	25	23	25	116	23.2
III	26	18	28	27	23	122	24.4
IV	21	24	21	24	20	110	22.0
Control	4	0	1	0	2	7	1.4

Table 18

Reaction of D. stramonium to artificial inoculation (two times) in the field

Counting done 14 days after inoculation

Replications	Number of necrotic spots						
	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5		$\bar{X}$
I	34	37	43	30	34	178	35.6
II	36	34	36	32	31	169	33.8
III	42	33	31	35	32	173	34.6
IV	41	32	37	35	30	175	35
Control	2	1	1	0	2	6	1.2

CHAPTER 5

DISCUSSION AND CONCLUSION

The possibility of using *A. crassa* as an effective mycoherbicide can be drawn from the studies that have been done in this research.

Biological perspective

A. crassa is a host specific fungus (Ellis, 1971) that causes leaf and pod blight on *Datura* sp. It causes irregular straw coloured, zonate spots which appear first on the lower leaves and spread gradually upwards; dark sunken lesions form on the seed pods. The mycelium penetrates beneath the seed coat (where it persists over 9 months), causing pre-emergence killing and seedling blight. Infected seeds were generally grey, whereas fungus-free seeds were brown. This is in accordance with the findings of Halfon-Meiri, 1973. From the infected seeds the fungus was isolated and maintained on PDA slants. However the fungus could maintain its virulence for a a longer time when kept in tightly sealed Petri plates at room temperature. The mycelium of *A. crassa* was found to be viable (in the infected seeds) even after 9 months of storage at room temperature. It is therefore possible that the mycelium plays an important role in the (1.) survival of the fungus under less favourable conditions (Vaartnou and Tewari, 1972); (2.) dispersal of the fungus (3). maintance of the species in nature - such that the cutting down of *D. stramonium* plants does not result in elimination of the fungus.

The disease cycle of *A. crassa* is a short one and can be completed within seven days. The primary inoculum are spores which are produced in abundance on the surface of the lesions. These infectious propagules are disseminated by wind and splashing water. On landing on the host leaf, the conidia germinate when conditions are favourable. For rapid infection and disease development the fungus requires at least 72 hours of continuous high humidity. The temperature range within which the conidia germinate is wide. Warm weather and high humidity favoured rapid infection and disease development. *A. crassa* was found to be a highly virulent pathogen on *D. stramonium*. The conidia germinated on the host leaf within 12 hours of inoculation, penetrating the host (i) through the open stomata, without developing an appresorium (ii) directly by developing an appresorium (iii) directly by developing an infection peg without an appressorium.

Histological studies showed that the pathogen, *A. crassa* invaded *D. stramonium* leaves primarily through the stomata. Open stomata seemed to exert some attractive stimulus to germ tubes as described by Rathaiah (1977). This may explain why high humidity was neccecary for successful infection. At high R.H. the stomata were open and the fungus entered the stomata by the germ tube tips. Direct penetration occured by infection pegs entering between the outer guard-cell wall and the adjacent epidermal cell or by puncturing an epidermal cell wall. In some cases an appressorium was formed together with an infection peg, agreeing with the findings of Akai *et al*, 1967 as mentioned by Hill *et al*, 1980. The stimulus for the formation of appressorium was not immediately known. However since the leaves had a thick layer of wax on their surface, the hydrophobic property conferred by the wax could be attributed to this phenomenon. Hill *et al* (1980) indicated that besides the contact stimulus, which has to be considered essential, hydrophobic surfaces seemed to stimulate and accelerate appresoria formation. Various fractions from onion leaves stimulated appresoria formation of *A. porri* (Ellis) Cif. (Hill, 1980). The fungus, *A. cucumerina* (Ellis & Everh.) Elliot penetrated directly by the germination hyphae of the fungus and the terminal portions of the hyphae always enlarged prior to penetration. Jackson (1959), however, did not call these swellings appresoria. There was an indication of conidia forming an infection peg without necessarily developing an appresorium. The tip of the germ tube was held firmly against the cuticle such that procedures in preparation of the leaf for serial sectioning did not displace the conidia. The peg caused an indentation of the cuticle and epidermal cells, suggesting mechanical penetration. Wood (1960) stated that penetration of the cuticle was mechanical, and did not depend on substances produced by the hyphae of invading organisms. Marks *et al* (1965), after examining the infection of leaves of *Populus tremuloides* by *Colletotrichum gloeosporioides* stated that penetration was mechanical and that chemical erosion of the cuticle was unimportant. More recently, Goodman *et al* (1967) stated that penetration through the cell wall was regarded as a mechanical process. Brown and Harvey (1927), considered the infection peg a specialised organelle that is driven through the cuticle mechanically by growth forces, which Frey-Wyssling (1957), called "elongation growth". Conversely Dickson (1960) concluded that the infection peg is a plastic structure that is simply pressed into the host tissue by osmotic forces within the appressorium. Mckeen (1974), however, described the penetration of infection peg produced by *Botrytis cinerea* Pers. ex Pers. through *Vicia faba* as enzymatic because he observed a sharp clean pore without curled edges made through the cuticle as opposed to the indentation of the cuticle and epidermal wall that was observed with *D. stramonium*. Young (1926) stated that species of *Alternaria*, *Diplodia*, *Cephalosporium*, *Colletotrichum* caused swellings (callosites) in the cell walls of wheat coleoptiles and certain other plants, in the process of direct penetration.

The development of the fungus in the leaf tissue was the same regardless of the mode of penetration or the leaf surface invaded. Within seven days, spores were observed on the surface of the leaves, these would again be disseminated. The highly infected leaves were shed resulting in loss of plant vigor.

The infected seeds of *D. stramonium* would serve as a means of harbouring the fungus through the unfavourable climatic conditions. This interaction of the life cycles of the fungus and the host plant qualifies the fungus as a succesfull pathogen.

Leaves were susceptible to invasion at any age . However the fungus produced many small necrotic spots on young leaves compared to the conspicuous lesions on older leaves under favourable conditions. The reason for this was not immediately known. However enough inoculum, well spread on the surface, resulted in many lesions such that in both cases the plants died. Walker (1950), admits that much of the true nature of such resistance is not understood. Stakman and Harrar (1957), named three general types of resistance after entrance. The first is associated with a necrogenic effect of the pathogen and hypersensitivity of the plant infected. The second is associated with inability of the pathogen to grow normally, even in normally susceptible tissues. The third is due to mechanical barriers that limit the areas in which the pathogen can grow. Orton (1908), working on watermelon, and cowpea wilt in the Southeastern United States early in the twentieth century, pointed out several possible categories. He set up three over-all classes, i.e., (a) disease escape;

(b) disease endurance; and (c) disease resistance. Stavely and Slana (1971) while working with *A. alternata* (Fr.) Keissl. (*A. tenuis* Nees) had the same observations and attributed this effect to the potential meristematic activity of the cells of the young leaves, producing a cicatrice of host cells around the infection point which stopped the fungus from advancing to produce the large conspicuous lesions.

Technological perspective

A. crassa was observed to sporulate easily, within four days on both liquid and solid media. However, spore production on liquid media greatly simplified the task. Abundant spores were produced on the surface of the mycelial mat floating on the media. Colonies on solid media frequently formed sectors. Among the strains observed, only the dark green strain was pathogenic. Sectoring was very rarely observed in liquid media. This meant that the fungus probably maintained its genetic stability when grown in liquid media. This characteristic is important for development of a mycoherbicide. Also from the liquid media at least two harvests could be done.

The conditions favourable for maximum growth and sporulation of the fungus in culture was found to be 29°C under alternating light and darkness at pH 6.8. The rate of radial growth and sporulation was greatest at 29ºC on all the media, temperature, pH and light conditions tested. The optimum temperature for growth and sporulation of *A. crassa* is 29ºC and this was in accordance with the studies by Cochrane (1958) on various fungi. The fungus has a wide temperature range of growth and sporulation. Cochrane (1958) indicated that narrow temperature ranges are often associated with low optima (but the reverse is not true), and that most fungi are unable to grow at 35-40ºC. The rates of radial growth and sporulation of *A. crassa*increased with increase in temperature upto 29ºC. At 35ºC no growth occured, this is in accordance with the findings of Aragaki (1961); Berger and Hanson (1963). The temperature and pH ranges of sporulation were narrower than that for vegetative growth. The ranges for spore germination were similarly more narrow. Chlamydospores formed at pH 3.1 and 9.0, but spore germination was observed only between pH of 3.1 and 9.0. The same applied to temperature effects.

Mycelial fragments, conidia and chlamydospores were all found to be infective. These could be formulated as a wetable or as a suspension inoculum, Templeton, (1981). The fungus produced resting spores, chlamydospore, on acid media due to stress. These were also produced when the cultures had almost completely exhausted the medium.

Commercial perspective

Development cost of *A. crassa* as a mycoherbicide is considerably low. The fungus produces abundant conidia within four days under inexpensive conditions. To avoid escapes, inoculation in the field can be done twice. Symptoms appear within seven days and by the eighteenth day, most of the plants are dead.

From the foregoing disscussion one can envisage a time when mycological laboratories all over the world will be able to produce formulations of *A. crassa* inexpensively to supply to the farmers for the control of *D. stramonium* whenever the weed may occur.

LITERATURE CITED

Abawi, G. S., Crosier, D. C. and Cobb, A. C., 1977. Podflecking of snap beans caused by *Alternaria alternata*. Plant Dis. Reptr. 61: 901-905.

Alexopoulos, C. J. and Mims, C. W., 1979. Introductory Mycology, John Wiley International Inc. New York.

Aragaki, M., 1961. Radiation and temperature interaction on the sporulation of *Alternaria tomato*. Phytopath, 51: 803-805.

Berger, R. D. and Hanson, E. W., 1963. Relation of environmental factors to growth and sporulation of *Cercospora zebrina*. Phytopathol. 53: 286-294.

Berger, R. D. and Hanson, E. W., 1963. Pathogenecity, Host-Parasite Relationship, and Morphology of some forage legume *Cercospora* and factors related to disease disease development. Phytopathol., 53: 500-508.

Brown, W. and Harvey, C.C. 1927. Studies in the physiology of parasitism on the entrance of parasitic fungi into the host plant. Ann. Bot. 41: 643-662.

Changsri, W. and Weber, F., 1963. Three Alternaria species pathogenic on certain cultivated crucifers. Phytopathol. 53: 643-648.

Charudattan, R. and Walker, H. L., 1982. Biological control of weeds with plant pathogens. John

Wiley Edition.

Cochrane, C. W., 1958. Physiology of Fungi. John Wiley and Sons, Inc.

Conway, Kenneth, E., 1975. *Cercospora rodmani* a new pathogen of water hyacinth with biological control potential. Can. J. Bot., 54: 1079-1083.

Conway, Kenneth, E., 1976. Evaluation of *Cercospora rodmani* as a biological control of waterhyacinth. Phytopathol. 66: 914-917.

Chupp, C., 1937. The effect of temperature and moisture on vegetable disease in New York state in 1937. Plant Dis. Reptr. 21: 320-321.

Diener, U. L., 1955. Host-penetration and pathological histology in grey leaf spot of tomato. Phytopathol. 45: 654-658.

Dickson, S., 1960. The mechanical ability to breach the host barriers, Vol. 2. Pages 203-232. *In* Mckeen, Mckeen, W. E., 1974. Mode of penetration of epidermal cells of *Vicia faba* by *Botrytis cinerea*. Phytopathol. 64: 461-467.

Ellis, M. B., 1971. Dematiaceous Hyphomycetes. Commonwe. Mycol. Inst., England.

Frey-Wyssling, A., 1975. Macromolecules in cell structure. Harvard Univ. Press, Cambridge, Massachussetts. 112p *In* Mckeen, W. E., 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. Phytopathol. 64: 46-467.

Goodman, et al 1967. The biochemistry and physiology of infectious plant diseases. D. van Nosyrnd, Princeton, N. J. 354p. *In* Mckeen, W. E., 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. Phytopathol. 64: 461-467.

Halfon-Meiri, Aliza. 1973. *Alternaria crassa* a seedborne fungus of *Datura*. Plant Disease Reptr. 57: 960-963.

Hill, G. et al, 1980. Substrate surface and appressoria formation by *Botrytis cinerea*. Phytopathol. Z., 99: 186-191.

Ionnaidis, N. M. and Main, C. E., 1973. Effect of culture medium on production and pathogenecity of*Alternaria alternata* conidia. Plant Disease Reptr. 57: 39-42.

Jackson, C.R., 1959. Symptoms and host-parasite relations of the Alternaria leaf spot disease of cucurbits. Phytopathol. 49: 731-733.

Marks, G. C. et al, 1965. Direct penetration of leaves of *Populus tremuloides* by *Colletotrichum gleosporioides*. Phytopathol. 55: 408-421.

Mckeen, W. E. and Rimmer, S. R., 1973. Initial penetration process in powdery mildew infection of susceptible barley leaves. Phytopathol. 63: 1049-1053.

Mckeen, W. E., 1974. Mode of penetration of epidermal epidermal cells of *Vicia faba* by *Botrytis* *cinerea*. Phytopathol. 64: 461-467.

Okoth, S.A., Otieno, N.C. and Mibey, R.K., 1989. Germination induction of dormant seeds of *Datura stramonium* L.(in press).

Orton, W. A., 1908. The development of farm crops resistant to disease. U. S. Dept. Agr. Yearbook, 1908: 453-464, 1909. *In* Walker, J. C., 1950. Plant Pathology. McGrow-Hill Book Company, Inc. Pgs. 699

Parker, R. E., 1983. Introductory Statistics for Biology. Second Edition. Edward Arnold Ltd, 41 Bedyard square, London WCB 3DQ.

Rathaiah, Y., 1977. Stomatal tropism of *Cercospora beticola* in sugarbeet. Phytopathol. 67: 358-362.

Ridgway, R., 1912. Color standards and nomenclature. Published by the author.

Salisbury, E. 1961. Weed and Aliens. Collins Clear-type press, London and Glasgow.

Sciumbato. G. L. and Pinckard, J. A., 1974. *Alternaria macrospora* leaf spot of cotton in Louisiana in 1972. Plant Disease Reptr. 58: 201-202.

Smith, D. and Onions, A. H. S., 1983. The preservation and maintenance of living fungi. Commonwealth Mycological Institute.

Solel, Z. and Minz, G. 1971. Infection process of *Cercospora beticola* in sugarbeet in relation to susceptibity. Phytopathol. 61: 463-466.

Stakman, L. J. and Harra, G. S., 1957. Principles of of Plant Pathology. The Ronald Press Company, New York.

Stanley, J. R. and Slana, L. J. 1971. Relation of leaf age to the reaction of tobacco to *Alternaria alternata*. Phytopathol. 61: 73-78.

Templeton, G. E. 1987. Mycoherbicide - Achievements, Developments and Prospects.

Templeton, G. E., Smith, JR., R. J., and TeBeest, D. O. 1986. Progress and potential of weed control with mycoherbicides. *In* Reviews of weed Science, Vol. 2 , 1986 Weed Science Society of America.

Templeton, G. C. , Smith , Jr. , D. O. TeBeest and J. N. Beasley, 1988. Rice research overview, Mycoherbicides Arkansas Farm Research.

Templeton, G. E., 1985. Specific weed control with mycoherbicides. *In* : British crop protection conference-weeds, 1985 Pg. 601-608.

Templeton, G. E. and Trujillo, E. E., 1981. The use of plant pathogens in the biological control of weeds. *In* : CRC Handbook of Pest Management in Agriculture Vol. 11. Pg. 345-349. CRC Press Inc : Boca Raton, Florida.

Vaartnon, H. and Teeari, I., 1972. *Alternaria* on polish-type rape in Alberta. Plant Disease Reptr. 56: 633-635.

Walker, J. C., 1950. Plant Pathology. McGrow-Hill Book Company, Inc. Pg. 699.

Warner, E. E., 1936. Black rot of tomato, *Lycopersicum* *esculentum* caused by *Alternaria* sp. Phytopathol. 26: 530-549.

Wood, R. K. S. 1960. Chemical ability to breach the host barriers, Vol 2 Pg 233-272. In Mckeen, W. E. 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. Phytopathol. 64:461-467.

Young, P. A. 1926. Facultative parasitism and range of fungi. Amer. Jour. Bot. 13:502-520. In Stakman, L. J. and Harrar, G. S., 1957. Principles of Plant Pathology. The Ronald Press Company, New York.